

The Photosynthetic Unit in *Chlorella* Measured by Repetitive Short Flashes¹

Received for publication March 11, 1971

JACK MYERS AND JO-RUTH GRAHAM

Departments of Botany and Zoology, The University of Texas, Austin, Texas 78712

ABSTRACT

Apparent size of the photosynthetic unit in *Chlorella pyrenoidosa* was estimated by the method of Emerson and Arnold: rate of oxygen evolution was measured under repetitive saturating flashes of about 10-microsecond duration separated by dark periods of 0.033 to 0.100 second. Cells used were taken from six steady state cultures maintained at different light intensities. Cell characteristics included a variation in chlorophyll content from 1 to 5%. Apparent size of the photosynthetic unit varied systematically with chlorophyll content in the range of 1560 to 2350 chlorophylls per O₂ per flash. Values for unit size showed no unusual statistical distribution and were not changed significantly by addition of low level background light at 645 or 705 nanometers. Maximal rate of unit turnover, calculated from light-saturated rate and unit size, varied inversely with chlorophyll content in the range of 70 to 180 per second.

In 1932 Emerson and Arnold (4, 5) introduced the classical technique of measuring photosynthesis under repetitive short flashes (10 μ sec). With a sufficient dark time between saturating flashes they observed a maximal flash yield of about 1 O₂ per 2000 chlorophylls ($a + b$). The actual results varied from 2000 to 3100 (average 2480) but did not vary systematically with chlorophyll content of the *Chlorella* cells used. However, accuracy of the value obtained was not then in question beyond the first significant figure.

Immediate impact of the Emerson and Arnold experiments was that notions of simple solution photochemistry would have to be abandoned, that some large number of chlorophyll molecules must cooperate in the evolution of one O₂, that there was necessary the then novel photophysical concept of a chlorophyll unit (2), subsequently called the photosynthetic unit. Following work was concerned with reality, generality, and interpretation of the phenomenon. In addition to the review by Rabinowitch (19), the instructive and definitive experiments of Kok (12) should be noted.

Innovations of the Joliot's made possible measurement of oxygen yield from a single flash (6-8). The problem of dark inactivation (8, 9) was circumvented by use of preilluminating or background light. The Joliot's used the single flash yield to estimate the number of operable units or, in current terminology, the

number of "open" reaction centers for system 2. They sought precision of measurement for the fraction of reaction centers open rather than for absolute number of centers (or units). However, the maximal attainable flash yield turned out to be essentially identical to the Emerson and Arnold value obtained with repetitive flashes.

The Emerson and Arnold number is so widely quoted and used in calculations that attempts toward more accurate measurement are appropriate. Questions arise about proper method of measurement, possible variation in unit size, and the statistical meaning of unit size, however accurately it is measured (22). The present report is directed simply toward repetition of the Emerson and Arnold experiments with study of effects of systematic variation in chlorophyll concentration. Since chlorophyll was varied by light intensity under steady state growth conditions of *Chlorella pyrenoidosa*, the descriptive data obtained are useful also for other purposes. Hence this report also extends previous work of our laboratory (14, 15). A partial treatment of those data related to productivity of algal cultures has been presented elsewhere (16).

MATERIALS AND METHODS

Plant Material. The alga used was *Chlorella pyrenoidosa* carried continuously in this laboratory since it was received from Robert Emerson. It is the same alga used by Emerson and Arnold (4, 5). The alga was grown in the 6-mm thick layer of an annular chamber at constant cell concentration (1.6-3 μ l of cells per ml in different cultures) maintained by dilution under photometric control (17). Illumination was provided by tubular 40-w tungsten lamps held parallel to the chamber, and light intensity for different cultures was determined by number of lamps (two to eight), distance (3-28 cm), and addition or omission of cylindrical reflectors behind the lamps. Since illumination was multidirectional, effective light intensity is not given but is evident in the dependent function of specific growth rate. Specific growth rate $\mu = (\ln V/V_0)/t$ where V is the volume of suspension and t is measured in days. Input medium contained (mg/l): 2500 MgSO₄·7H₂O, 1250 KNO₃, 1250 KH₂PO₄, 50 CaCl₂, 165 sodium citrate, and microelements at 0.5 B, 0.5 Mn, 0.05 Zn, 0.02 Cu, 0.01 Mo, 0.01 V, 5.0 Fe at pH 5.2. At steady state in the chambers at 26 C and aeration with 4% CO₂ in air the pH was 6.2 to 6.8, depending on cell concentration maintained.

Cellular Characteristics. Cell number was obtained by hemacyt counting with about 1000 cells counted for each sample. Packed cell volume was obtained by centrifuging a measured aliquot of cell suspension at 2500 g for 1 hr in tubes with lower section of precision bore capillary and calibrated with mercury. Cell dry weight was obtained by centrifuging, washing once in water, and drying at 60 C *in vacuo* to dryness and then at 105 C in air to constant weight. Chlorophyll was extracted in minimal

¹ This study was supported by Grant GM 11300 from the National Institutes of Health.

volume of boiling methanol, diluted to much larger volume in 80% acetone, and estimated as described by Arnon (3).

Oxygen Exchange. Oxygen exchange was measured in a Beckman oxygen macroelectrode covered with a 1-mil Teflon membrane. The electrode was polarized at 0.6 v, and its current was amplified by a Keithley model 417 picoammeter and recorded at a sensitivity of about $0.02 \mu\text{l O}_2/\text{ml}$ per chart division. Calibration checks were made occasionally at zero oxygen (about 0.02×10^{-9} amp) and periodically at air-saturated water (about 3.5×10^{-9} amp). The electrode was inserted into the side of a 1.5-ml glass cuvette, 1.0×1.0 cm in cross section enclosed in a water jacket of 2.5×2.5 cm and provided by temperature control at 25 ± 0.02 C. Magnetic stirring was provided by a glass-covered bar driven by a synchronous motor.

Although a single sample could be maintained in the cuvette and subjected to a series of illuminations (e.g., at different light intensities), we found that reproducibility in rate of oxygen evolution was poor and subject to effects of previous treatments. A larger sample of cell suspension could be removed from the growth chamber and held in darkness or very low light; sequential measurements on replicate aliquots showed slowly drifting rates of oxygen evolution. Finally, we resorted to a procedure in which each measured sample was withdrawn from the growth chamber, diluted with growth medium (saturated with 4% CO_2) to give 0.6 to $1.0 \mu\text{l}$ of cells per ml, and transferred to the cuvette. (We are grateful to Dr. Ivan Setlik who suggested to us that such a method would be necessary.) Since cellular characteristics and rates of oxygen exchange are measured on separate samples taken from the chamber at different times, precision of measurements suffers in becoming dependent on maintenance of constant cell concentration (about $\pm 2\%$) by photometric control. A merit of the procedure is that each measurement describes cells growing under a stated condition since it is made within 10 min of removal from the chamber and without change in suspending fluid.

Illumination. Repetitive flashes were provided by a small "end window" xenon flash tube FX-101 (E.G. and G., Boston, Mass.) of 22 mm diameter. It was mounted in front of its trigger transformer and enclosed in a 25-mm metal tube allowing for forced air cooling and positioning of filters. Standard filtering was provided by a Wratten No. 12 plus 2 mm Plexiglas, removing wavelengths < 500 nm. Attenuation of flash intensity was obtained by addition of Wratten No. 96 neutral filters. Without the yellow filter we sometimes observed decaying rates of oxygen evolution with time.

The tube was flashed by discharge from a 2-microfarad capacitor which was charged to 500 v at all flash rates used (10, 12, 15, 20, or 30/sec). Flash rate was established by a timing device which triggered a flash at a chosen fraction of the 60 Hz line frequency. Flash rate was usually set at 20/sec and was limited to 30/sec by average power limitations of the FX-101.

Our flash energy of 0.25 joule is small compared with the 4.5 joules of Emerson and Arnold (4) or the 12 joules of Schmid and Gaffron (21). However, this is offset by the small size of the flash tube and measuring cuvette, their close proximity (1 cm), and the low absorbance of the algal suspension. The time-averaged irradiance incident on the cuvette was measured by a large area (1-cm^2 diaphragm) Cambridge thermopile with long time constant. The energy per flash was $2400 \text{ ergs}/\text{cm}^2$ and independent of flash rate from 10 to 30/sec. Our measurements of flash duration, 13 and $22 \mu\text{sec}$ to $\frac{1}{3}$ and $\frac{1}{10}$, respectively, of peak height, are longer than the manufacturer's specification of $3 \mu\text{sec}$ to $\frac{1}{3}$ peak height. Our measured value is probably too long by virtue of capacitance in the measuring circuit.

Light intensity curves and saturated rates of oxygen evolution were measured at 620 nm provided by a projection system through 8 cm of water, a blocked interference filter (Baird, 13-nm half-width), and wire screens for intensity control. Low level back-

ground illumination was selected at 645 nm (half-width 13 nm) or at 705 nm (half-width 16 nm) by other blocked interference filters. Irradiance was measured by a Cambridge large area thermopile calibrated against standard lamps.

RESULTS

Cell Characteristics. Characteristics of the cells used are given in Figure 1 and Table I. Figure 1 presents one of several closely agreeing irradiance curves obtained for cultures at each of the six light intensities used for growth. Each culture is described by its specific growth rate, μ . The lower and separate curve, taken from Phillips and Myers (18), shows μ as a function of relative light intensity but with no intention of comparing irradiance curves for μ and P . Sets of measurements of flash yield (see below) always were accompanied by one or more measurements of the light-saturated rate. The complete range of light-saturated rates is shown at the right-hand end of each curve together with average values which are cited also in Table I. It is evident that the light-saturated rate of photosynthesis per chlorophyll is varied by a factor of almost four times by the light intensity used for growth.

Data of the first three columns of Table I show the same trends in cell density, cell size, and total chlorophyll content expected from previous work (14). Variation in the chlorophyll a/b ratio is significant but smaller than that reported for another species of *Chlorella* (20). Dark respiration, measured before and immediately after the light-saturated photosynthesis also showed expected variation. Initial rates best describe the cells used; final

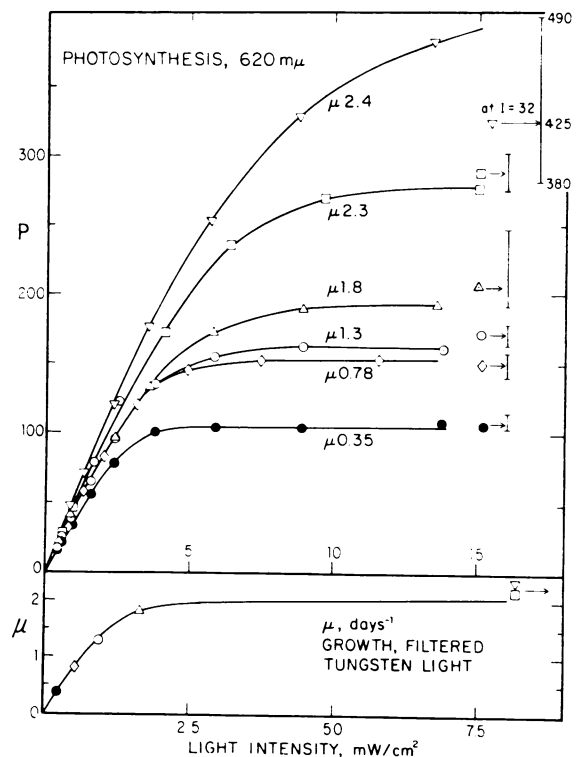


FIG. 1. Light intensity curves of oxygen evolution for cells from each of six different cultures. Each curve is labeled by the specific growth rate, μ , at which the culture was managed by choice of light intensity for growth. Rate of oxygen evolution, P , is in moles O_2 (mole chl) $^{-1}$ hr $^{-1}$. At the right-hand end of each curve a vertical bar indicates the total range of values of the light-saturated rate taken from single measurements in other sets of experiments, and an arrow shows the average of all measured values. The lower and separate curve taken from Reference 18 describes the six cultures upon the light intensity curve for specific growth rate, μ .

Table I. Cell Characteristics and Results with Flashing Light for Six Cultures Used

Cell Characteristics									Flashing Light		
a	b	c	d	e	f	g	h	i	j	k	l
Growth rate, μ	Cell density ¹	Cell size ²	Chlorophyll		Respiration		Photosynthesis saturated rate		Measured P_{chl} ⁶	U Chl/O ₂ ⁷	Turnover rate ⁸
			$a + b$	$\frac{a}{a + b}$	Initial Q_{O_2} ³	Final Q_{O_2} ⁴	Q_{O_2}	P_{chl} ⁵			
$days^{-1}$	g/ml	$\times 10^{12} ml$	%								U/sec
2.4	0.242	75	1.14	0.84	13.8	13.3	121	425	46.2	1560 \pm 100 (16)	180
2.3	0.256	92	1.53	0.81	11.9	11.3	110	288	38.3	1880 \pm 80 (6)	150
1.8	0.252	49	3.10	0.80	11.2	13.0	158	205	35.5	2030 \pm 100 (12)	120
1.3	0.215	34	3.92	0.79	8.7	11.4	165	169	34.5	2090 \pm 90 (18)	100
0.78	0.197	32	4.52	0.78	5.9	8.8	164	146	31.3	2300 \pm 140 (8)	90
0.35	0.179	30	5.19	0.77	4.6	8.0	143	110	30.8	2350 \pm 100 (12)	70

¹ Cell density = cell dry weight/packed cell volume.

² Cell size = packed cell volume/cell number.

³ Q_{O_2} = μl O₂/mg cells·hr; initial is rate observed in period 3–6 min after removal from culture.

⁴ Final is rate observed in period 0.5 to 4 min after period of saturating light.

⁵ P_{chl} = moles O₂/moles total chl·hr.

⁶ Actually measured rate under 20 flashes/sec.

⁷ U is apparent size of a photosynthetic unit; number of observations in parentheses.

⁸ Maximal rate of turnover of U calculated from columns i and k.

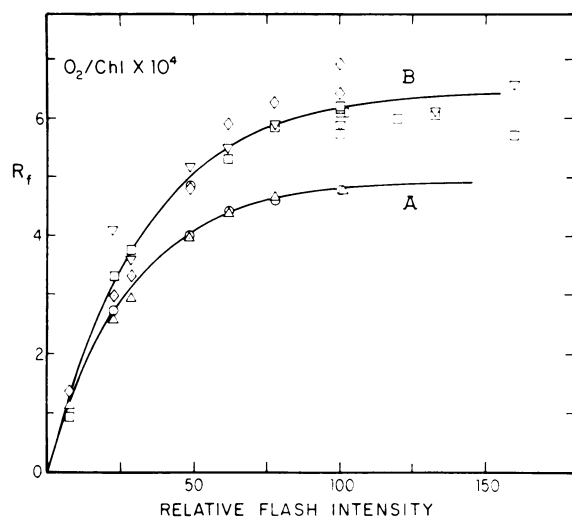


FIG. 2. Flash yield, R_f , versus relative flash energy. A: Two sets of measurements at cell concentration 2.1 $\mu l/ml$ on cells grown at μ 1.8; B: three sets of measurements at cell concentration 1.3 $\mu l/ml$ on cells grown at μ 2.4. The flash rate was 20/sec. Relative flash energy 100 was that used in all other experiments reported. Lower energies were obtained by attenuation with neutral filters; energies above 100 were obtained by increased voltage supply to the flash tube, addition of a backing mirror, or both. The character of solid curves shown is discussed in the text.

rates were numerically added to the observed light rates to obtain the apparent rates of photosynthesis which are cited.

Flash Intensity and Rate. Attenuation by neutral filters gave curves such as Figure 2 for flash yield vs. relative flash energy. The curves drawn to fit the data describe the killing-type relation observed by Kohn (11): $\log(1 - M) = -AE$, where M is the fraction of maximal yield observed at flash energy E and A has the meaning of an absorption coefficient for a photosynthetic unit. Our only concern of close approach to flash saturation was with high light-grown cells (curve B). Even then we could not observe

significant increase in yield by increases in voltage to the flash tube and use of a backing mirror to obtain still higher energy. Hence, we judge that our flash energy was sufficient to obtain 95% or more of maximal flash yield at our standard flash energy which is scaled at 100 in Figure 2.

Variation in flash rate gave no change in flash yield in our range of 10 to 30/sec. The only useful information obtained is that at our standard 20/sec flash rate the 0.05-sec dark time did not limit the flash yield. An equivalent statement is that the measured rates of oxygen evolution in flashing light (Table I, column j) are low enough to fall on the linear segments of the irradiance curves of Figure 1.

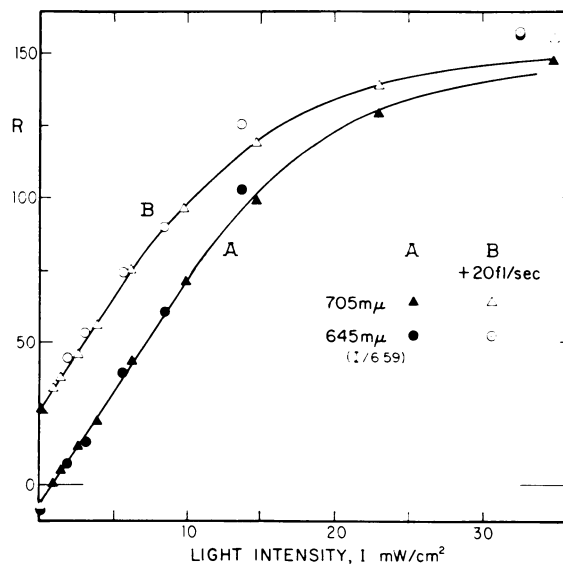


FIG. 3. Effect of background light. A: Measured rate of oxygen evolution, R , in moles O₂ (mole chl)⁻¹ hr⁻¹ (not corrected for respiration), versus light intensity, I , of 705 nm or light intensity, $I/6.59$, of 645 nm; B: net rate observed at various intensities plus 20 flash/sec. Cells used were grown at μ 0.78 and used at a concentration of 2.1 $\mu l/ml$.

Flash Yields. In the context of the Emerson and Arnold experiments we express the reciprocal of flash yield, *i.e.*, chlorophylls/ O_2 , U , with the meaning of apparent size of the photosynthetic unit as measured in oxygens. The data are given in column k of Table I together with the number of determinations and the standard deviations. Since we also have the light-saturated rate of oxygen evolution (P_{chl} , column i), we can estimate the maximal turnover rate for the photosynthetic unit, U/sec (column l).

Effects of Background Light on Flash Yields. The work of Schmid and Gaffron (21, 22) suggests that background light can change the apparent size of the photosynthetic unit. Our efforts with light 1 (705 nm) and light 2 (645 nm) backgrounds have been entirely negative in showing any significant effects on flash yields in *Chlorella*. Our most complete set of experiments is shown in Figure 3. Curve A shows the initial portion of the light intensity curve at 705 nm and at 645 nm with intensities adjusted to provide best fit of the two curves. For each point on curve A there is a point at a corresponding intensity on curve B showing the rate observed with added flashing light. Curves A and B are parallel all the way to zero intensity. We prefer to consider as not significant the small increases observed in 645 nm and interpret the experiment as showing negligible effect of any low intensity of 645 or 705 nm.

DISCUSSION

Aware of the observations of Schmid and Gaffron (21, 22), we sought but did not find any large variations of the photosynthetic unit within any one culture. The greatest ratio between maximal and minimal values within any one culture was 1.22, and there was no statistically unusual distribution of values. However, our data have no bearing on the question of uniformity of unit size within the cells of any one culture.

Our values for the apparent size of the photosynthetic unit are somewhat lower than those of Emerson and Arnold. Of our total 72 measurements as a whole the lowest and highest values were 1360 and 2540. Our comparison of culture conditions reveals obvious, though not simple, relations between culture growth rate, chlorophyll content, and apparent size of the photosynthetic unit. A similar trend was not observed by Emerson and Arnold. Their cultures were harvested at different times from batch cultures grown over neon or mercury discharge tubes, and all factors responsible for chlorophyll concentration, including possible iron and trace element deficiencies, are not known. For the usual laboratory batch culture, with less than one doubling per day at harvest, our results predict a unit size of about 2300. Hence, we consider our results a remarkably close confirmation of the Emerson and Arnold value obtained 39 years ago with far more difficult technical problems.

Our values for the maximal turnover rate of a photosynthetic unit are grouped around the widely used approximation of 100/sec (13) but show a consistent variation. When proceeding from lower to higher light intensities for growth (or from higher to lower chlorophyll content), the light-saturated rate (P_{chl}) increases by a factor of 3.9. Of this 1.5 X lies in reduction in unit size and 2.6 X in unit turnover rate.

At first we were surprised by the lack of effect of background light at 645 or 705 nm on the repetitive flash yield. The single flash yield observed by Joliot (8) was dependent on wavelength of background light with a spectrum closely analogous to an enhancement spectrum and a 2.5 X difference between 645 and 705 nm. However, such variation was observed by a method which recorded oxygen evolution with a time resolution of about 0.1 sec. The oxygen produced in the first 0.1 sec after a flash was sensitive to wavelength of background; but total oxygen produced in the 10 sec after a flash was virtually independent of wavelength of background (Fig. 8 in Ref. 8). Our method of measurement has very

poor time resolution and merely integrates with time the total oxygen produced by many flashes together with that produced by any background light. From such measurements one cannot resolve two effects which must be operating: (a) effect of background light on flash yield and (b) effect of flashes on yield from the background.

The differences in effect of background light on the single flash *versus* the repetitive flash yield raise two problems which we cannot now resolve. First there is the question of whether all reaction centers become completely relaxed or "open" during the dark periods between repetitive flashes. Secondly, there is a related question of whether there are equal numbers of reaction centers for the two light reactions. Use of repetitive flashes involves a quasi-steady state measurement, and there is no reason *a priori* to decide whether the method counts reaction centers for photoreaction 1 or photoreaction 2.

In comparison of data from single *versus* repetitive flash yields there is also a second difference which appears to be an important anomaly. The relation between flash yield and flash energy (E) observed by Arnold (1) and by Kohn (11) can be written $\log q = -AE$, in which q is the fraction of unreacted reaction centers. This is equivalent to a killing curve by which the fraction of units surviving is titrated by a dosage of incident quanta. The term A (if measured in absolute dimensions) would have the meaning of the constant optical cross section of each unit. A different relation is predicted by the findings of the Joliot (6, 7) and Joliot, Joliot, and Kok (10). They have shown that steady state rate of oxygen evolution (V) is less than first order with respect to the fraction (q) of open reaction centers for system 2. They considered this as evidence for transfer of excitation energy between system 2 units. If the reaction center of one unit is closed, there is a probability (a) that excitation energy in that unit may reach the center (open or closed) of an adjacent unit. Their data fit the relation $V/V_{max} = q/[1 - a(1 - q)]$, where a has a value of 0.5 to 0.6. This implies that a reaction center does not have a constant optical cross section but one which increases by a factor of 2 to 2.5 as q approaches 0.

Our data (as Fig. 2) seem to fit best the relation described by Arnold (1) and Kohn (11) and appear incompatible with the idea of a variable cross section. We are studying further the apparent anomaly in search of explanation.

LITERATURE CITED

1. ARNOLD, W. 1935. Kinetics of photosynthesis in *Chlorella*. Cold Spring Harbor Symp. Quant. Biol. 3: 124-127.
2. ARNOLD, W. AND H. I. KOHN. 1934. The chlorophyll unit in photosynthesis. J. Gen. Physiol. 18: 109-112.
3. ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Plant Physiol. 24: 1-15.
4. EMERSON, R. AND W. ARNOLD. 1932. A separation of the reactions in photosynthesis by means of intermittent light. J. Gen. Physiol. 15: 391-420.
5. EMERSON, R. AND W. ARNOLD. 1932. The photochemical reaction in photosynthesis. J. Gen. Physiol. 16: 191-205.
6. JOLIOT, A. 1965. Etude cinétique des relations entre vitesse d'émission et concentration de l'oxygène photosynthétique chez *Chlorella pyrenoidosa*. Physiol. Vég. 3: 329-344.
7. JOLIOT, A. AND P. JOLIOT. 1964. Etude cinétique de la réaction photochimique libérant l'oxygène au cours de la photosynthèse. C. R. Hebd. Seances Acad. Sci. (Paris) 258: 4622-4625.
8. JOLIOT, P. 1965. Cinétique des réactions liées à l'émission d'oxygène photosynthétique. Biochim. Biophys. Acta 102: 116-134.
9. JOLIOT, P. 1968. Kinetic studies of photosystem II in photosynthesis. Photochem. Photobiol. 8: 451-463.
10. JOLIOT, P., A. JOLIOT, AND B. KOK. 1968. Analysis of the interactions between the two photosystems in isolated chloroplasts. Biochim. Biophys. Acta 153: 635-652.
11. KOHN, H. I. 1936. Number of chlorophyll molecules acting as an absorbing unit in photosynthesis. Nature 137: 706.
12. KOK, B. 1956. Photosynthesis in flashing light. Biochim. Biophys. Acta 21: 245-258.
13. KOK, B. AND G. M. CHENIAE. 1966. Kinetics and intermediates of the oxygen evolution step in photosynthesis. In: D. R. Sanadi, ed., Current Topics in Bioenergetics. Academic Press, New York. pp. 1-47.

14. MYERS, J. 1946. Influence of light intensity on cellular characteristics of *Chlorella*. *J. Gen. Physiol.* 29: 419-427.
15. MYERS, J. 1946. Influence of light intensity on photosynthetic characteristics of *Chlorella*. *J. Gen. Physiol.* 29: 429-440.
16. MYERS, J. 1970. Genetic and adaptive physiological characteristics observed in the *Chlorellas*. In: Prediction and Measurement of Photosynthetic Productivity (Proc. IBP/PP Technical Meeting, Trebon). Centre for Agricultural Publishing, Wageningen. pp. 447-454.
17. MYERS, J. AND L. B. CLARK. 1944. An apparatus for the continuous culture of *Chlorella*. *J. Gen. Physiol.* 28: 103-112.
18. PHILLIPS, J. N., JR. AND J. MYERS. 1954. Growth rate of *Chlorella* in flashing light. *Plant Physiol.* 29: 152-161.
19. RABINOWITCH, E. I. 1956. In: Photosynthesis, Vol. II, Part 2. Interscience Publishers, New York. pp. 1433-1483.
20. REGER, B. J. AND R. W. KRAUSS. 1970. The photosynthetic response to a shift in chlorophyll *a* to chlorophyll *b* ratio in *Chlorella*. *Plant Physiol.* 46: 568-575.
21. SCHMID, G. H. AND H. GAFFRON. 1968. Photosynthetic units. *J. Gen. Physiol.* 52: 212-239.
22. SCHMID, G. H. AND H. GAFFRON. 1969. Photosynthetic units in higher plants. In: H. Metzner, ed., Progress in Photosynthetic Research. International Union of Biological Sciences, Tübingen. pp. 857-870.