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THE QUANTUM REQUIREMENT OF PHOTOSYNTHESIS IN CHLORELLA

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SUMMARY

The quantum requirement of photosynthesis in *Chlorella pyrenoidosa* has been reinvestigated. Evolution of oxygen was measured electronically with an instrument which determines the paramagnetism of the gas mixture and carbon dioxide was measured by its infrared absorption. Light measurements were made with a photocell calibrated against a thermopile, using filtered sunlight as a reference beam. Consideration of possible sources of error established the reliability of quantum requirement determinations as $\pm$ 6 %. Determinations were made only with cultures which exhibited photosynthetic rates exceeding 250 μmoles O_2 evolved per mg chlorophyll per h. In 16 determinations made over a 2-fold range of photosynthetic rates, quantum requirements for O_2 varied from 8.8 to 12.4. No correction for respiration was made, and a rationale is given for not making such corrections. Quantum requirements were independent of rate of light absorption over the 2.5-fold range used in this study. A probable cause of error was discovered in previously reported quantum requirements less than 8 determined by a similar method. The measured requirements are consistent with a serial two-light reaction scheme for photoelectron transport and an additional biosynthetic requirement for ATP, generated in part by cyclic photophosphorylation.

INTRODUCTION

A scheme of electron transport in photosynthesis involving two separate light reactions for each electron[1] has received much experimental support (reviewed elsewhere[2–5]) and is today widely, though not universally, accepted. It is usually assumed that if the scheme is correct each light step requires the conversion of one light photon per electron transported, leading to a minimum quantum requirement of 8 for the four electrons which must be transported from water for each molecule of oxygen evolved.

By far the majority of investigators, using a variety of experimental techniques to investigate the quantum requirements of photosynthesis, have reported values of 8 or more light quanta absorbed per molecule of oxygen evolved[6]. In contrast, quantum requirements of 4 or less have been reported from one laboratory over a period of more than 40 years[7,8]. In the finding of these lower quantum requirements, the experimental method for measuring gas exchange was usually manometry, and

although the readings have at times been duplicated in other laboratories, the conclusions as to the quantum requirement of photosynthesis have not been confirmed (see review in ref. 6).

Part of the controversy surrounding the investigation of the quantum requirement of photosynthesis has stemmed from the interpretation of manometric data from a reaction in which oxygen is evolved as CO_2 is absorbed. This difficulty can be avoided by measuring oxygen tension in the gas phase with a paramagnetic oxygen analyzer and CO_2 tension in the gas phase with an infrared absorption analyzer. In one such study[9], however, light intensities were low and corrections for respiration were made, leading to the likelihood of energetic contribution from respiration to photosynthesis. For this reason, BASSHAM, SHIBATA AND CALVIN[10], using this method, employed light intensities at which rates of photosynthesis as high as 10 times the rates of respiration were obtained. These measurements resulted in calculated quantum requirements uncorrected for respiration of 7.5.

If the quantum requirement of true photosynthesis under steady-state conditions could be shown to be less than 8, the operation of the sequential two-light reaction mechanism for photoelectron transport in photosynthesis would require that at least one step involves the transfer of more than one electron per photon absorbed. Therefore, the quantum requirement of photosynthesis in Chlorella, using paramagnetic measurement of oxygen tension, has been reinvestigated in an effort to establish the limits of error in all aspects of the determination. In the course of this study we have found no quantum requirement less than 8.5, and have uncovered a likely cause of error in the previously reported quantum requirement of 7.5.

EXPERIMENTAL

Photosynthesis and gas measurement

The gas handling system for circulating air and CO_2 through the suspension of *Chlorella pyrenoidosa* and through instruments for measuring CO_2 and O_2 tension was similar to that described previously[10]. A small diaphragm pump was used for recirculating the gas, provision being made for pressure equilibration and for introduction of the various gas mixtures. The effective volumes for O_2 and CO_2 were determined by measuring changes in the readings of the instrument resulting from the addition to the system of loops of known volume containing known gases. The effective volume for O_2 was 10 ml, and for CO_2 was 150 ml. Measurements of photosynthetic rate were made using levels of CO_2 between 2 % and 3.5 % in air. Measurement of O_2 partial pressure was made with an Arnold O. Beckman (Model F-3) O_2 analyzer. Measurement of CO_2 partial pressure was made with a CO_2 analyzer (LIRA Model 200, Mine Safety Appliances Co.). Output signals from both instruments were recorded by a Leeds and Northrup Multipoint recorder.

Photosynthesis cell

The cell used for photosynthesis is shown in cross-section in Fig. 1. The inside thickness of the cell was 5 mm, while the area was 8.8 cm × 8.8 cm. The cell was illuminated by a 500-W incandescent tungsten lamp from which the light passed through two infrared-absorbing filters in rapidly circulating tap water and then through a set of blocking filters and interference filters attached to the cell itself.

These filters and the wall of the algae chamber were cooled by running water. The plastic edges of the cell were beveled in the direction of light propagation to facilitate measurement of scattered light. An aluminum mask surrounding the window through which light from the lamp enters the algae cell reflected back-scattered light near the edges to the forward direction. As in the previous study[10], it was determined that back-scattering in the direction of the lamp was negligible.

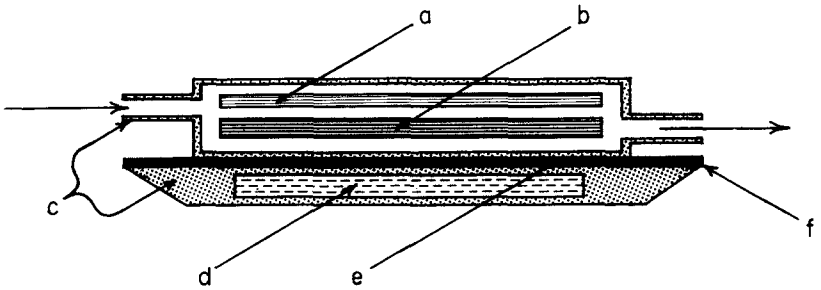

Fig. 1. Photosynthesis cell for quantum requirement measurements. a, blocking filter (B-2X); b, interference filter (B-3X); c, translucent plastic cell walls; d, suspension of Chlorella or clear nutrient solution; e, translucent plastic sheet for light diffusion; f, reflecting aluminum surface. Cross-sectional view from the top.

Light source

Light from the incandescent lamp passed through first two infrared absorbing filters (Corning 1-69) and then through an infrared blocking filter (Baird Atomic B-2X), and an interference filter centered at 627 mμ (Baird Atomic B-3X). To obtain the 10 cm × 10 cm filter area, four 5 cm × 5 cm filters were placed together with the intervening strips carefully masked to prevent the passage of unfiltered light. A transmittance curve for the combination of the three filters is shown in Fig. 2.

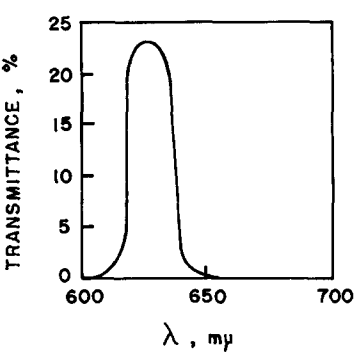

Fig. 2. Transmittance curve for the combination of infrared absorption filter (Corning 169), infrared blocking filter (B-2X), and interference filter (B-3X). No transmittance was detected at shorter wavelengths in the visible region or in the region 700–1000 mμ.

Calibration of photocell for light measurement

The thermopile (Charles M. Reeder Co. RBL-500) was calibrated against two standard lamps supplied by the U.S. National Bureau of Standards, according to procedures recommended by literature supplied with these standard lamps. The method consists of measuring the output voltage from the thermopile resulting from

light received by the thermopile from the standard lamp at a distance of 200 cm in a black box. The mean value of 15 measurements was 29.86 ± 0.30 ergs·(sec·cm^2·μV)$^{-1}$ (area of detecting surface 2.5 cm^2). It is important to note that the accurate calibration of the thermopile requires that the light falling on its detecting surface be uniform. A narrow beam of light reaching only part of this surface will not necessarily cause the same output as the same amount of light uniformly distributed over the entire surface because the sensitivity of the detecting surface may not be uniform.

Since the light transmitted through the algae is scattered, it is necessary to use a photocell for the actual light measurement. The response of the photocell with wavelength is not constant, and it is essential that in the calibration of the photocell against the thermopile the light source should be parallel, uniform, yet of the same wavelength quality as that used in the illumination of the algae. Also, the intensity of the light should be comparable to that which is measured during the experiment, to avoid any error due to non-linearity of response *versus* intensity.

The beam from a monochromator is commonly used as a reference light source for the calibration of the photocell against a calibration thermopile. However, with monochromators available to us, it was found that only the center of the beam provided the necessary uniformity of intensity and wavelength. Therefore, during the calibration, the thermopile had to be placed far enough from the monochromator so that is detecting surface (25 mm × 10 mm) was illuminated only by the central portion of the monochromator beam. When this was done, the intensity of the light beam was so low that noise from the environment became a problem. Furthermore, the light intensity provided by the monochromator under these conditions, as compared with the light to be measured during the quantum requirement determination, was so low as to raise the question of whether the sensitivity of the photocell would be linear over such a long range (two orders of magnitude). Consequently, another method was found for providing the monochromatic light beam for calibration of the photocell against the thermopile.

The best available light source for this purpose proved to be the sun. In order to eliminate scattered light from the sky, a long black box was built (15 cm × 15 cm × 150 cm). The box was equipped with a shutter at the front and two windows (15 mm × 25 mm) 65 cm apart, so that the sunlight beam passing through the windows was large enough to cover the entire detecting surface of either the thermopile (25 mm × 10 mm) or the photocell (15 mm × 10 mm), which were alternately positioned behind the second window. An infrared absorption filter was placed on the front window and an interference filter and blocking filter were placed at the back window. No special cooling was provided, but during operation the shutter was opened for only a few seconds to permit taking of the reading. Since the readings from the detectors returned to the same base line when the shutter was again closed, it could be assumed that heating of the filters had not led to problems of re-radiation.

The black box was mounted on a wooden stand which permitted rotation in two directions. A tube 2.5 m long and 2 cm in diameter was attached to the box parallel with its axis and served as a sighting tube to direct the box towards the sun. The axis of the box was adjusted until a clear circular image of the tube appeared on the white cardboad at the back of the tube. The shutter of the box was then opened briefly, and the signal from the thermopile was recorded. This was repeated several times. Since only a few seconds were required, the movement of the sun did not affect the

precision of the measurement. The thermopile was then replaced by the photocell, and the position of the box was adjusted for another set of measurements. This cycle of operation was repeated several times on a clear, cloudless day until it was assured that there was no drift in the intensity of the calibrating source. A series of ten measurements during two cycles gave a ratio of thermopile signal to photocell signal of 0.0895 $\pm$ 0.0002. Actually the ratio obtained by using the monochromator as a light source was found to be 0.090 $\pm$ 0.001. Thus, the non-linearity in the ratio sensitivities over two orders of magnitude of incident energy was shown not to be a serious problem. The incident intensity obtained during the calibration with the sun was comparable to that which was to be measured during the actual experiment for determining the quantum requirement of photosynthesis.

Algal culture

C. pyrenoidosa was grown in a shaking flask in a water bath maintained at 20°. The culture was illuminated by six 90-W fluorescent lamps (about 3000 ft-candles). A gas mixture of 96 % air and 4 % carbon dioxide was bubbled through the culture. A modification of Myer's medium[11] was used as the nutrient solution. (Under these conditions the fresh weight of the algae doubled in 12–16 h.) During the experimental period, half of the total culture was taken out from the flask every day, and the same amount of new nutrient medium (800 ml) added.

Preparation of algae for quantum requirement measurement

The density of the algae in the growing flask was found to be 0.4 cm^3 packed volume per l of suspension. These algae were harvested within 0.5 h prior to the experiment and centrifuged from the suspending medium. The algae were then resuspended in fresh, modified Myer's medium[11], which has a pH of 5.3. These suspensions were made up to 0.03–0.6 cm^3 packed algae per 100 ml of suspension as indicated in RESULTS.

25 ml of this algae suspension were placed in the photosynthesis cell for the quantum requirement measurement. The mixture of CO_2 and air was bubbled through the algae, and the algae were illuminated and allowed to photosynthesize with white light (interference filter removed) for 15 min prior to the quantum efficiency measurement. During this time the rate of photosynthesis was determined and quantum requirement measurements were made only if the rate was in excess of 35 μmoles of oxygen evolved per min per cm^3 of wet-packed algae. Since the chlorophyll content of the algae was typically around 8.4 mg of chlorophyll per cm^3 wet-packed algae, this corresponds to a rate of 250 μmoles of O_2 evolved per mg chlorophyll per h. This is also about the maximum rate for young healthy leaves of spinach[12], one of the most actively photosynthesizing green plants which have been investigated.

Besides providing a determination of the health of the algae, the period of photosynthesis with white light served to overcome the induction period for photosynthesis following the period of exposure to low light conditions during harvesting. At the end of this 15-min period, the interference filter sets were put in place and the measurement of rate of oxygen evolution and of CO_2 uptake and light transmitted through the algae was commenced. The actual rate of gas exchanged could be determined with reasonable accuracy after about 4 min of photosynthesis, but the experiment was usually extended for 30 min to assure greater accuracy and to guarantee that

the rate was constant with time. Following this period of 30 min, the light was in some cases turned off and the dark respiration was determined, although it is not used in our calculation.

Measurement of light absorption

The light coming through the cell was measured with the algae suspension in the cell and with only nutrient medium in the cell. The difference gives the light absorption by the *C. pyrenoidosa*.

For these measurements the light coming through the cell was carefully determined by measuring the energy received from each 10 mm × 15 mm area (the area of the photocell) without any overlap or missed areas. This was accomplished by using a series of plastic rulers with widths of 15, 30, and 45 mm and of the same thickness as the holder of the photocell. The photocell was positioned on top of the ruler in accordance with marks which were made every 10 mm. The entire surface of the plastic cell containing the algae, plus the beveled edges, was carefully mapped this way. The total of all of the readings thus gave the total light energy passing through the cell.

Because of the nature of the measurements, it was assumed that all light passing through the cell was determined, regardless of possible scattering in transmission through the plastic. To test this assumption, a sheet of plastic of the same thickness as the front wall of the algae cell was masked in the same shape as the window to the algae cell, and was placed in the same position as that occupied by the front wall of the algae cell during the photosynthesis experiments. Light emerging from this plastic was carefully measured and was found to be the same (within experimental error) as the light coming through the complete algae cell when it was filled with nutrient medium.

Sensitivities and calculations

The sensitivity of the thermopile was determined to be 29.86 ± 0.30 ergs·(sec·cm^2·μV)$^{-1}$. The sensitivity of the photocell was calculated to be $(29.86 \pm 0.30) \times (0.0895) = 2.6725$ ergs·(sec·cm^2·μV)$^{-1}$. Since the area of the photocell is 1.5 cm^2, the sensitivity of the photocell as a whole was 2.6725 ergs·(sec·cm^2·μV)$^{-1}$ × 1.5 cm^2 = 4.0087 ergs/sec·mV. From the transmittance curve (Fig. 2) it was determined that the weighted average of the wavelengths of the incident beam was 627.2 mμ. From this, the average energy of 1 Einstein is calculated to be $1.9127 \cdot 10^6$ ergs. The sensitivity of the photocell is then determined to be 0.1258 ± 0.0016 μEinstein/min·mV. A typical energy absorption calculation is the following:

The power transmitted through the cell filled with 25 ml of suspension medium was 383.12 mV as measured by the photocell. This corresponds to (0.1258 μEinstein/min·mV) × 383.12 mV) = 48.2 μEinstein/min. The power transmitted through the cell filled with 25 ml of 0.6 % Chlorella suspension was 202.42 mV as measured by the photocell. This corresponds to (0.1258 μEinstein/min·mV) × (202.42 mV) = 25.46 μEinstein/min. Therefore, the power absorbed by the Chlorella was 48.20—25.46 = 22.74 μEinstein/min.

Rate of oxygen evolution

The rate of oxygen evolution corresponding to the above rate of energy absorption was found to be 0.0451 % per min. Therefore, the rate of oxygen evolution was

$0.0451 \cdot 10^{-2} \times 120$ ml $= 54.06 \cdot 10^{-3}$ ml/min. Since 1 μmole of oxygen at 20° and 1 atm is 24.5 μl, the rate of oxygen evolution in terms of μmoles/min is (54.06/24.5) = 2.207 μmoles/min.

Quantum requirement for oxygen evolution

Since quantum requirement is defined as the number of photons required to evolve 1 molecule of oxygen from water, it is obtained by dividing the rate of energy absorption by the rate of oxygen evolution.

Q.R. = (rate of energy absorption)/(rate of oxygen absorption) = (22.74 μEinstein/min)/(2.207 μmoles/min) = 10.30 μEinstein/μmole.

RESULTS

Accuracy of measurements

The uncertainty in the light measurement due to the calibration of sensitivity of the photocell is estimated to be 0.1258 $\pm$ 0.0016 μEinstein/min·mV, or $\pm$ 1.2 %. The measurement of light energy transmitted through the algae cell when it contains only nutrient medium was found to vary less than 1 % in measurements made before and after the photosynthesis experiment. In a typical experiment, about half as much light would be transmitted through the algae suspension. In this case, there would be still a 1 % possible error in measured light, leading to an error of $\pm$ 0.5 % of the light energy coming through the cell without algae. Consequently, this gives a maximum error of $\pm$ 1.5 % of the light transmitted through the cell without algae or $\pm$ 3 % of the light energy calculated to be absorbed by the algae. The combined uncertainty in the measurement of photosynthetic rate all comes from the uncertainty in the determination of the oxygen volume of the system which is at most 2 %. Therefore, the maximum error of the quantum requirement measurement is estimated at $\pm$ 6 %.

Quantum requirement measurements

The results of a number of quantum requirement measurements made under the conditions described are summarized in Table I. Although the respiration rates found in the darkness following a period of photosynthesis are shown, it is emphasized that no correction for respiration has been made and that the quantum requirements shown in the 6th and 7th columns of the table are based solely on gas exchange rate in the light absorption. The 2nd column shows the incident light intensity which varies over a 2-fold range of intensity, while the 1st column shows the algae density which also was varied over a two-fold range. The 3rd column shows that the rate of energy absorption varied over a three-fold range. The relative narrowness of this range is dictated by the fact that the only valid region for studying quantum requirements is between those intensities where the rate of photosynthesis is already several times dark respiration and those intensities at which further increase in light does not cause a proportional increase in photosynthetic rate (approaching saturation). The values of the quantum requirement for oxygen evolution range from 9.0 up to 14.0, with the lower values generally being found with the lower density of algae (44–51 % absorption). There is considerable variation in the photosynthetic quotient with the value of the ratio CO_2/O_2 generally decreasing at higher light intensities.

In Table II are shown the results of quantum requirement measurements which

TABLE I

RESULTS OF QUANTUM REQUIREMENT MEASUREMENTS

Density of algae (%)	*Incident light intensity* (*μEinstein/min*)	*Absorption* (*μEinstein/min*)	*Rate of*		*Quantum requirement for*		*Respiration* (*μmoles O_2/min*)
			O_2 evolution (*μmoles/min*)	*CO_2 absorption* (*μmoles/min*)	*O_2 evolution*	*CO_2 absorption*	
0.3	24.8	8.0	0.74	0.75	10.8	10.7	
	31.8	10.8	1.10	1.09	9.8	9.9	
	36.7	13.5	1.25	1.15	10.8	11.7	0.22*
	42.2	14.4	1.60	1.23	9.0	11.7	0.06**
0.4	33.0	11.5	1.19	1.26	9.6	9.1	
	40.6	17.7	1.82	1.57	9.7	11.3	
	49.4	24.2	2.07	1.65	11.7	14.7	0.28*
	55.5	25.1	2.10	1.84	11.9	13.6	
0.6	39.1	20.0	16.10	13.8	12.4	16.1	
	48.2	22.7	22.25	17.7	10.3	13.2	0.11**

* Respiration during first 4 min after photosynthesis.
** Respiration between 8 min and 30 min.

TABLE II

SUMMARY OF THE RESULTS OF QUANTUM REQUIREMENT MEASUREMENTS

See text for explanation of correction.

Density of algae (%)	*Incident light intensity* (*μEinstein/min*)		*Absorption* (*μEinstein/min*)		*Rate of*		*Quantum requirement for*			
					O_2 evolution (*μmoles/min*)	*CO_2 absorption* (*μmoles/min*)	*O_2 evolution*		*CO_2 absorption*	
	Before correction	*After correction*	*Before correction*	*After correction*			*Before correction*	*After correction*	*Before correction*	*After correction*
0.35	32.73	42.50	10.64	13.85	1.29	1.07	8.24	10.70	10.0	13.0
	40.03	58.60	15.19	19.70	1.62	1.24	9.41	12.25	12.3	16.0
0.4	30.92	40.20	10.81	14.10	1.60	1.53	6.77	8.82	7.07	9.20
	42.40	55.20	18.30	23.80	2.33	1.97	7.87	10.25	9.28	12.05
0.5	20.22	26.00	10.67	13.90	1.49	1.14	7.14	9.30	9.36	12.20
	48.85	63.51	24.60	32.00	3.43	2.54	7.05	9.16	9.53	12.40

was were made earlier and in which the photocell was calibrated by a method similar to that described by BASSHAM, SHIBATA AND CALVIN[10]. In that method the thermopile was calibrated first against the standard lamp as is usually done, but then a piece of opal glass was placed in front of the standard lamp as a diffuser and a new sensitivity was determined. It was then assumed that as long as the opal glass was in place, causing diffusion of the incident light, the thermopile could be used for direct measurement of scattered light. The correctness of this method rests on the assumption that the opal glass will uniformly scatter light to the same degree irrespective of its incident scattered or parallel characteristics. Unfortunately, with the opal glass available to us in the present study, which we believed to be similar to that used in the earlier study[10], it was found that this assumption was not completely valid. Thus, when the photocell was calibrated against the opal glass-equipped thermopile, it was found that a 30 % lower sensitivity was obtained as compared with calibration against the thermopile with no diffuser. Thus the values of the quantum requirement obtained using the low sensitivity had to be corrected upward as indicated in Table II. It seems likely that the values reported by BASSHAM, SHIBATA AND CALVIN also should be corrected upward by about 30 %, in which case the reported value of 7.5 would become 9.8.

DISCUSSION

The reliability of measurements of the quantum requirement of photosynthesis depends on accurate determination of the rate of light absorption and on accurate determination of the evolution of oxygen and uptake of carbon dioxide. The use of non-manometric techniques of determining the gas exchange by means of instruments which independently measure the changes in oxygen and CO_2 tension are well established and reliable. Provided care is taken in the calibration of the instruments and determination of the effective volume of the system, measurements with an accuracy of $\pm$ 2 % can be obtained. However, the use of these instruments entails the requirement for photosynthesis by a substantial amount of plant material, and this imposes new problems of arranging a light source with a narrow wavelength band and measured intensity over a relatively large area to provide rates of photosynthesis which exceed several-fold rates of dark respiration. At the same time, many problems arise with respect to accurate determination of the absorbed light energy, since both the cell and the plants tend to diffuse and scatter the supplied light. Finally, the measurement of the scattered light itself presents some problem since the primary detector, the thermopile used for the measurement of light energy, cannot be used directly to measure scattered light. By using a secondary detecting device, the surface of which can be placed directly in contact with the area over which light flux must be measured, and by carefully calibrating the secondary detector against the thermopile, and by accurate mapping of all of the light flux passing through the cell containing the algae, it is possible to determine the absorbed light with an error of not greater than 4 %.

Equally serious for considerations of the theoretical minimum quantum requirement of photosynthesis is the requirement that the most active and efficient possible plants be selected. This requirement can at least be approached by the selection of *C. pyrenoidosa* cultured in such a way that their rate of oxygen evolution per mg chlorophyll is close to the maximum that has been reported for any green plants. After all

this has been done, we find the minimum measurable requirement for oxygen evolution to be around 9, similar to what many other workers have reported in the past.

In Fig. 3, all quantum requirements from Tables I and II have been plotted *versus* rate of light energy absorption per cm^3 of algae. There appears to be no dependence on rate of light absorption over the range used in our experiments. Thus we can rule out the possibility that partial self-shading at the lower light intensities caused higher quantum requirement. In fact, the independence of quantum requirement *versus* rate of light absorption also argues against the need for any respiration correction, since such a correction would be more important at the lower light absorption rate as compared with the higher light absorption rate.

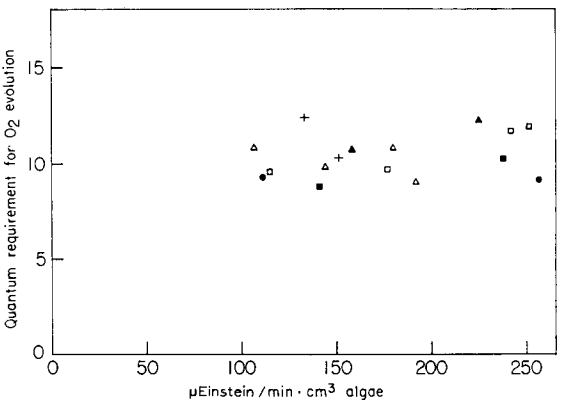

Fig. 3. Rate of oxygen evolution *vs.* rate of light energy absorption for several experimental conditions. The rates of oxygen evolution and of light absorption have been calculated per cm^3 of packed *Chlorella pyrenoidosa* to permit comparison of all experimentally determined values. Density of algae: △, 0.3 % (Table I); □, 0.4 % (Table I); +, 0.6 % (Table I); ▲, 0.35 % (Table II); ■, 0.4 % (Table II); ●, 0.5 % (Table II).

Probably, light respiration in *C. pyrenoidosa* is insignificant compared with the rate of photosynthesis. Tracer studies with ^{32}P and ^{14}C during photosynthesis and in darkness with *C. pyrenoidosa*[13] indicated that there is a switch from photosynthetic metabolism to dark metabolism. Glycolysis and oxidative pentose phosphate cycle are apparently inactive in the light but become active in the dark, using the same metabolic pools as were used in photosynthesis in the light.

It appears that ATP can diffuse rapidly in and out of chloroplasts. Thus ATP is available to the entire plant cell from photosynthesis in the light and from respiration in the dark. The requirement for ATP for non-photosynthetic processes as well as synthesis inside the chloroplast is probably met in part by cyclic photophosphorylation. Depending on such additional requirements for ATP and on the quantum efficiency of cyclic photophosphorylation, the total quantum requirement of photosynthesis will be increased above the theoretical 8 predicted by the two light steps, one photon per electron scheme.

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