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FLASH KINETICS AND LIGHT INTENSITY DEPENDENCE OF OXYGEN EVOLUTION IN THE BLUE-GREEN ALGA *ANACYSTIS NIDULANS*

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SUMMARY

Patterns of oxygen evolution in flashing light for the blue-green alga *Anacystis nidulans* are compared with those for broken spinach chloroplasts and whole cells of the green alga *Chlorella pyrenoidosa*. The oscillations of oxygen yield with flash number that occur in both *Anacystis* and *Chlorella*, display a greater degree of damping than do those of isolated spinach chloroplasts. The increase in damping results from a two- to threefold increase in the fraction (α) of reaction centers "missed" by a flash. The increase in α cannot be explained by non-saturating flash intensities or by the dark reduction of the oxidized intermediates formed by the flash. Anaerobic conditions markedly increase α in *Anacystis* and *Chlorella* but have no effect on α in broken spinach chloroplasts. The results signify that the mechanism of charge separation and water oxidation involved in all three organisms is the same, but that the pool of secondary electron acceptors between Photosystem II and Photosystem I is more reduced in the dark, in the algal cells, than in the isolated spinach chloroplasts.

Oxygen evolution in flashing light for *Anacystis* and *Chlorella* show light saturation curves for the oxygen yield of the third flash (Y_3) that differ markedly from those of the steady-state flashes (Y_s). In experiments in which all flashes are uniformly attenuated, Y_3 requires nearly twice as much light as Y_s to reach half-saturation. Under these conditions Y_3 has a sigmoidal dependence on intensity, while that of Y_s is hyperbolic. These differences depend on the number of flashes attenuated. When any one of the first three flashes is attenuated, the variation of Y_3 with intensity resembles that of Y_s. When two of the first three flashes are attenuated, Y_3 is intermediate in shape between the two extremes. A quantitative interpretation of these results based on the model of Kok et al. (Kok, B., Forbush, B. and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475, and Forbush, B., Kok, B. and McGloin, M. P. (1971) Photochem. Photobiol. 14, 307–321) fits the experimental data.

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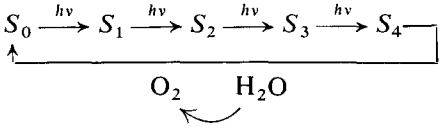
INTRODUCTION

Short saturating flashes of light have been an important tool in the investigation of photosynthetic oxygen evolution [1–4]. As a result of such studies, Kok et al. [5, 6] proposed a model for photosynthetic oxygen evolution involving a linear, four-quantum process. Each reaction center acts independently to accumulate oxidizing equivalents with successive photons absorbed until a total of four are stored. Oxygen is evolved in a rapid dark reaction between water and the four-electron oxidized state, S_4:



During an interval of several minutes in the dark, S_2 and S_3 become reduced to S_1, which is stable in the dark. S_0 is only produced in the reaction between S_4 and water. A certain small (approx. 10 %) fraction of the reaction centers, α, is not affected even by the saturating flashes (misses). Another small fraction (β) is activated twice by a single flash (double hits).

Some modifications of this model have been suggested by Joliot and Joliot [7], Radmer and Kok [8], Joliot et al. [9] and Bouges-Bocquet [10]. Weiss et al. [11] concluded that the observation that the oxygen yield of the third flash in a series required more light for saturation than did later flash yields could not be explained by the model by Kok et al. [5, 6].

We find that the patterns of oxygen evolution in broken spinach chloroplasts and in whole cells of *Anacystis nidulans* or *Chlorella pyrenoidosa* are essentially the same. We have confirmed that the oxygen yield of the third flash requires more light to reach saturation than do steady-state flash yields and provide an explanation for this behavior in terms of the model of Kok et al. [5, 6].

MATERIALS AND METHODS

The blue-green alga *Anacystis nidulans* and the green alga *Chlorella pyrenoidosa* were grown at 22 °C in aerated one liter batch cultures in shakers exposed to continuous illumination. Three times a week about 90 % of the culture volume was replaced by sterile growth medium (Kratz and Myers [12] medium C for *Anacystis* and a modified Myers [13] medium lacking sodium citrate but containing 10^{-5} M $Ca(NO_3)_2$ for *Chlorella*). Algae were pelleted by low speed centrifugation (10 min at $3000 \times g$) and resuspended in a buffered electrolyte solution consisting of 0.1 M KCl, 0.01 M K_2HPO_4, pH 7.6. Spinach chloroplasts were prepared as described by Sun and Sauer [14].

In the experiments reported here, the bare platinum electrode, xenon flash lamp and attendant amplification and recording devices previously described by Weiss and Sauer [4] and Babcock and Sauer [15] were used. The circulation electrolyte was 0.1 M KCl, 0.01 M phosphate buffer, pH 7.6. For aerobic experiments, the electrolyte was continuously aerated by bubbling with 4 % CO_2 in air. For an-

aerobic experiments, the electrolyte was bubbled with nitrogen. The amount of oxygen evolved by a flash was determined to be proportional to the maximum amplitude of the electrode current following the flash, as described by Duysens [16]. Flashes were attenuated by the use of calibrated metal-film neutral density filters (Balzers) used singly or in combination to give the desired transmittance.

RESULTS

Oxygen evolution by Anacystis, Chlorella and spinach chloroplasts in flashing light

The pattern of oxygen evolution in flashing light following a dark period is shown in Fig. 1 for *Anacystis nidulans*, *Chlorella Pyrenoidosa*, and broken spinach chloroplasts. The damped oscillation in the oxygen response of the chloroplasts is similar to that described by Kok et al. [5].

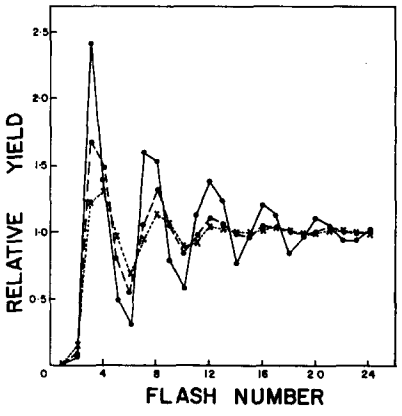


Fig. 1. Oxygen evolution by dark adapted, broken spinach chloroplasts (●—●); *Chlorella pyrenoidosa*, (○- -○) and *Anacystis nidulans*, (×- - -×) in flashing light. The length of the flashes was 10 μs. Time between flashes was 0.5 s. Oxygen yields were normalized with respect to the average of the last 5 flashes. Dark times preceding the flashes were 5 min for the chloroplasts and *Chlorella* and 3 min for *Anacystis*. Reaction mixtures were as follows; chloroplasts (300 μg Chl-ml^{-1}), NADP ($1.5 \cdot 10^{-4}$ M), ferredoxin (0.5 μg-ml^{-1}), sucrose (0.4 M), NaCl (0.1 M), tris buffer (0.5 M, pH 7.6). Algae; KCl (0.1 M), K_2HPO_4 (0.01 M, pH 7.6).

While *Anacystis* and *Chlorella* cells exhibit the same basic response, they differ from spinach chloroplasts in one important respect: both of the algae show a greater degree of damping. This is demonstrated by three observations. Relative to the chloroplasts, the oxygen yield of the third flash (Y_3) in the whole cells is diminished while that of the fourth flash (Y_4) is increased. In *Anacystis*, Y_4 is actually larger than Y_3. The number and magnitude of oscillations before achieving a steady-state (constant oxygen yield per flash, Y_s) are smaller in the algal cells. Finally, the sum of the oxygen yields of the first four flashes is from 16 to 36 % lower in the algae than in the isolated chloroplasts. All three of these effects are expected consequences of increased damping.

Using the model of Kok et al. [5], with $S_0 = 1.0$, $S_1 = 3.0$ and $S_2 = S_3 = 0$ as starting conditions, we could obtain a good fit to the data for the isolated chloro-

plasts with $\alpha = 0.10$ and $\beta = 0.015$. With the same starting conditions, we were able to achieve fair fits ($\pm 15\,\%$ maximum) to the data for *Chlorella* with $\alpha = 0.22$ and $\beta = 0.14$ and for *Anacystis* with $\alpha = 0.32$ and $\beta = 0.033$. We could also obtain a good fit to the experimental data for *Anacystis* with $S_0 = S_1 = 2.0$ and $S_2 = S_3 = 0$ as initial conditions and setting $\alpha = 0.20$ and $\beta = 0.06$. This choice of parameters corresponds to a situation in which there is significant reduction of S_1 in the dark [9, 10]. Good fits could not be obtained by further increasing S_0 at the expense of S_1. Thus, using the model of Kok et al., we could account to a large extent for the differences seen in Fig. 1 as resulting from a 2- to 3-fold increase in the value of the miss parameter α in the algae relative to the isolated chloroplasts. The variations in β are relatively small and contribute little to the differences in Fig. 1.

Experiments in which we varied the time between the second and third flashes (Δt_{2-3}) of a saturating series showed that the oxygen yield of the third flash was constant for values of Δt_{2-3} between 0.01 and 1 s. For times longer than 1 s, Y_3 decreased. Since the time between the flashes in the experiments shown in Fig. 1 was 0.5 s, the larger value of α seen in the algae cannot be due to reduction of the S states in the interval between flashes.

Recently, evidence has been presented suggesting that in algae the fraction of misses in saturating flashes can be influenced by the redox state of the A-pool, which is in turn affected by the amount of oxygen in the environment [8, 17, 18]. Fig. 2 shows the pattern of oxygen evolution in flashing light for *Anacystis* under

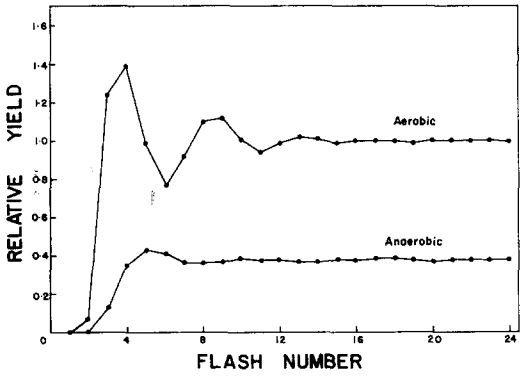


Fig. 2. Oxygen evolution by dark adapted *Anacystis* cells in flashing light under aerobic, (●—●) and anaerobic, (○—○), conditions. Anaerobic conditions were maintained by bubbling the electrolyte flowing past the cells with nitrogen. Both experiments shown were performed on the same sample. The aerobic experiment was performed first and was separated from the anaerobic experiment by 30 min of darkness. Both curves are normalized by division by the average of the final 5 flash yields of the aerobic experiment.

aerobic (closed circles) and anaerobic (open circles) conditions. The anaerobic response shows a much greater degree of damping than does the aerobic response. The flash yields are depressed by anaerobiosis, and oscillations are almost absent. This effect is completely reversible upon restoration of aerobic conditions. Similar results were obtained for *Chlorella*. Isolated chloroplasts, however, showed

no important differences in their responses to flashing light between anaerobic and aerobic conditions.

The effects of non-saturating flashes on oxygen evolution

Weiss et al. [11] observed that, in *Chlorella* cells subjected to short flashes of light, considerably more light was required to saturate the oxygen yield of the third flash than was needed to saturate the steady-state yield.

We confirmed these findings for *Chlorella* and observed a similar effect in *Anacystis*. Fig. 3 shows light saturation curves for the steady-state flash yields (solid circles) and the yield of the third flash (open circles) in *Anacystis nidulans*. The flash

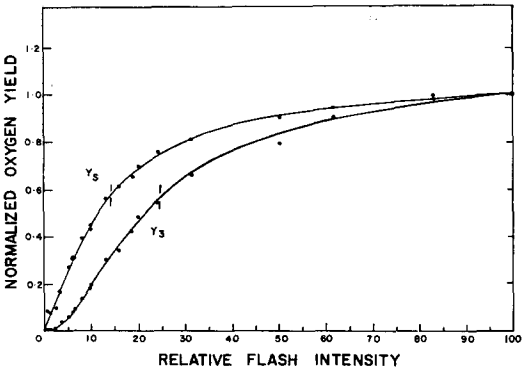


Fig. 3. Light saturation curves for the steady-state (● — ●) flash yields and the yields of the third flash, (○ — ○), in dark adapted *Anacystis* cells when all flashes are attenuated. After 3 min of darkness *Anacystis* cells were subjected to a train of (10 μs) flashes. The intensity of the flashes was reduced by the use of calibrated Balzers neutral density filters. The oxygen yield of the third flash or of the steady state is shown as a functi ⊟ of the relative flash intensity.

yields at infinite intensity were obtained by extrapolation using a double reciprocal plot. These extrapolated values were used to calculate the flash intensity which half-saturated the flash yields. The small vertical lines in Fig. 3 indicate half-saturation values. The half-saturation intensity for steady-state flashes (Y_s) is only 0.56 of that required to half-saturate the oxygen yield of the third flash (Y_3). A further difference is seen in the shape of the curves. At low light intensities, Y_3 is a sigmoidal function of light intensity while Y_s is linear. This sigmoidal behavior of the Y_3 vs I curve was seen in all experiments.

Fig. 4 shows similar data for *Chlorella*. When all flashes are attenuated, the Y_3 vs I curve (crosses) differs markedly from the Y_s curve (closed circles) in both half-saturation intensity and shape. Fig. 4 also shows that the extent of the differences between the Y_3 and Y_s saturation curves depends strongly on the number of flashes attenuated. If only one of the first three flashes is attenuated (open circles), the saturation curve for Y_3 is almost identical to that of Y_s. This is true no matter which of the first three flashes is attenuated. When any two of the first three flashes are attenuated (triangles), the resulting saturation curve for Y_3 is intermediate between the two extremes.

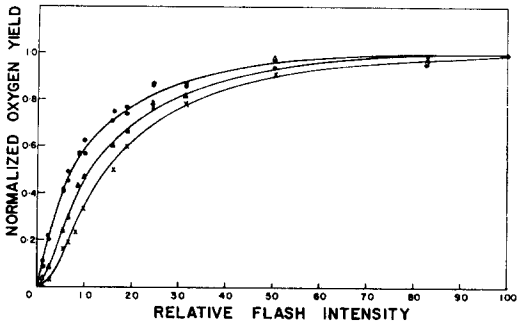


Fig. 4. Light saturation curves for various flash yields in dark adapted *Chlorella* cells. The flashes attenuated were; all flashes, steady-state, (●—●); third flash only, (○—○); second and third flashes only, (△—△) and all three of the first three flashes, (×—×). Samples kept in darkness for 5 min before exposure to the train of flashes.

DISCUSSION

The responses of *Anacystis* and *Chlorella* to non-saturating flashes of light can be understood in terms of the model of Kok et al. [5, 6]. According to this model, the average oxidation state of the Photosystem II reaction centers at the start of a series of flashes following a long dark period is significantly different from that during the steady state. At the start of a series of flashes, all of the reaction centers are in the two lowest oxidation states (S_0 and S_1) owing to the reduction of the higher oxidation states in the dark. By the time the steady state is achieved, the four possible oxidation states (S_0, S_1, S_2, S_3) are equally populated. These different conditions result in significant differences in the flash saturation curves.

If α is the average fraction of reaction centers not advanced by a flash, then $(1-\alpha)S_n$ will be the number of reaction centers advanced from oxidation state S_n to state S_{n+1} by a flash. (This ignores double hits, which are small relative to the misses, see Results.) A saturation curve of Y_s is essentially a measurement of how $(1-\alpha)S_3$ changes as the flash intensity changes. At this point it becomes important to distinguish between two kinds of misses. There is that fraction of reaction centers which is missed even at infinite light intensity (α_0) and a variable fraction (α_v) the extent of which depends on light intensity. α_0 is the fraction of reaction centers which, at the time of the flash, are closed or are in some way unable to use the light energy supplied by the flash. α_v, on the other hand, is the fraction of reaction centers which do not receive any excitation energy from the flash.

In these terms;

$$Y_s = (1-\alpha_v)(1-\alpha_0)S_3 \tag{1}$$

The saturation curve for Y_3 is slightly more complex. Since the initial state is S_1 rather than S_3, three flashes must be given before oxygen is evolved. Both α_0 and α_v occur on each flash. Thus

$$Y_3 = (1-\alpha_v)^3(1-\alpha_0)^3S_1 \tag{2}$$

Since $(1-\alpha_v)^3$ decreases more rapidly than $(1-\alpha_v)$ as α_v increases, the apparent half-

saturation intensity for Y_3 would be expected to be larger than that for Y_s. Furthermore, the Y_3 saturation curve would be expected to show a non-linear response to light intensity since it has a cubic dependence on α_v. An interpretation of half-saturation values can be made if the relationship between $(1-\alpha_v)$ and the flash intensity, I, is known.

In this model, Y_s is a linear function of $(1-\alpha_v)$. Since α_0 is assumed to be a constant in these experiments, in the steady state the term $(1-\alpha_0)S_3$ is also a constant. Because of this, $(1-\alpha_v)$ is proportional to Y_s, and any equation describing Y_s as a function of I may also be used to describe $(1-\alpha_v)$ as a function of I.

An equation which we have found empirically to fit the Y_s vs I curve is

$$Y = \frac{kI}{i+I} \tag{3}$$

where I is the flash intensity, i is the intensity which gives half-saturation and k is a constant of proportionality. We use the proportionality between Y_s and $(1-\alpha_v)$ described in Eqn (1) and write the steady-state saturation curve as

$$Y_s = (1-\alpha_0)S_3\left[\frac{I}{i+I}\right] \tag{4}$$

The data presented in Fig. 3 have been normalized so that the flash yield at full intensity is unity. The normalized flash yield for the steady state ($\bar{Y}_s$) can be calculated by dividing Eqn (4) by the constant $(1-\alpha_0)S_3$ to give

$$\bar{Y}_s = \frac{I}{i+I} \tag{5}$$

In a similar fashion, Y_3 and $\bar{Y}_3$ are determined to be

$$Y_3 = (1-\alpha_0)^3 S_1\left[\frac{I}{i+I}\right]^3 \tag{6}$$

and

$$\bar{Y}_3 = \left[\frac{I}{i+I}\right]^3 \tag{7}$$

This is true when all of the first three flashes are attenuated. In general, when n of the first three flashes are attenuated, the yield of the third flash will be

$$Y_3 = (1-\alpha_0)^3 S_1\left[\frac{I}{i+I}\right]^n \tag{8}$$

The variation of the shape of the Y_3 saturation curve with the number of attenuated flashes is easily understood in terms of Eq. (8). When only one flash is attenuated, the equation reduces to Eqn (4) with the resulting hyperbolic shape.

Fig. 5 shows fits obtained for the data shown in Fig. 4. Good fits were obtained using Eqn (8). The values of i giving the best fits were; $i = 0.070 \pm 0.002$ for the steady state and single flash attenuation curves (top), $i = 0.050 \pm 0.002$ for the

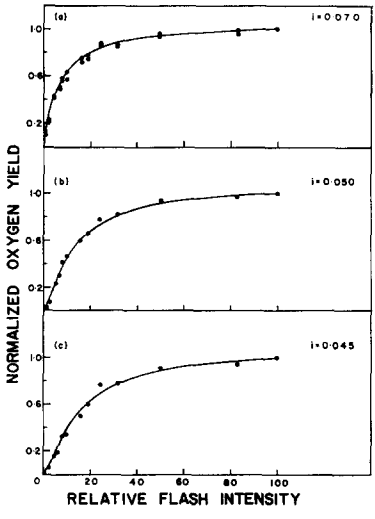


Fig. 5. Fits to experimental data from *Chlorella* using Eqn (8) (see text). The value of i and n and the flashes attenuated are: (a) $i = 0.070$, $n = 1$, ●—●, steady-state flashes; ○—○, third flash only; (b) $i = 0.050$, $n = 2$, ●—●, second and third flashes only; (c) $i = 0.045$, $n = 3$, ●—●, all of the first three flashes attenuated. All theoretical curves are normalized at $I = 100\,\%$.

case in which two of the first three flashes were attenuated (middle), and $i = 0.045 \pm 0.002$ when all of the first three flashes were attenuated (bottom). Good fits could also be obtained to the data for *Anacystis* shown in Fig. 3.

Sigmoidal behavior in flash light-saturation curves has been described by Diner and Mauzerall [18] for steady-state flash yields at low oxygen tension. This behavior is due to increased reduction of the A-pool, however, and is distinct from the effect described above. It is of further interest to note that our model predicts a sigmoidal behavior for the light saturation curve of the third flash which is independent of the redox state of the A-pool and involves the use of only one photon per reaction center per flash.

From the results presented in Fig. 1, it is clear that the method of charge storage in Photosystem II in isolated spinach chloroplasts, and whole cells of *Chlorella* and *Anacystis* is basically the same. Sofrová et al. [20] have recently reported evidence from experiments with inhibitors that supports this view. *Anacystis* and *Chlorella* show from two to three times more reaction centers missed by a flash than do chloroplasts. The increase in α is not due to insufficient flash intensity nor is it due to the reduction of oxidized intermediates in the dark time between flashes, and probably relates more to the functioning of the primary photochemistry of Photosystem II than to events between the reaction center and the site of oxygen evolution. (*Anacystis* may also show a different $S_0 : S_1$ ratio after a dark period, which would indicate different redox conditions on the oxidizing side of System II.)

Radmer and Kok [8] proposed that the misses which occur during saturating flashes are due to reaction centers which are unable to respond to a flash because the primary acceptor (Q) is reduced. They also suggested that Q is in equilibrium with

the large pool of secondary electron acceptors between Photosystem II and Photosystem I (the *A*-pool). Diner and Mauzerall [18] and Velthuys and Amesz [19] presented evidence supporting this view. Diner and Mauzerall also showed that anaerobic conditions result in an increased reduction of the *A*-pool. We find that both *Chlorella* and *Anacystis* exhibit a significantly greater degree of misses under conditions of low oxygen tension. This seems to indicate the presence of an equilibrium between Q and the *A*-pool in these organisms. If this is the case and if the equilibrium between Q and A is fast relative to the time between flashes, then the larger value of α seen in *Anacystis* and *Chlorella* indicates that the *A*-pool is more reduced in these organisms than in isolated chloroplasts. Diner and Mauzerall [18] have proposed a model in which the redox poise of the *A*-pool is controlled by the opposing action of oxygen and some endogenous reductant. The insensitivity of isolated spinach chloroplasts to oxygen tension suggests that they may lack the endogenous reductant.

The similarity in charge storage mechanisms seen in *Anacystis*, *Chlorella*, and isolated spinach chloroplasts suggests that the same mechanism is used to oxidize water in all of them. If these organisms are indeed typical representatives of their taxonomic groups, it appears that this mechanism has remained essentially unchanged through the billions of years of evolution since they shared a presumably common ancestor.

ACKNOWLEDGMENTS

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