QUANTUM ACCUMULATION IN PHOTOSYNTHETIC OXYGEN EVOLUTION

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ABSTRACT Three independent methods have been used to determine the size of the quantum accumulation unit in green plant photosynthesis. This unit is defined as that group of pigment molecules within which quantal absorption acts must take place leading to the evolution of a single O_2 molecule. All three methods take advantage of the nonlinearity of oxygen yield with light dose at very low dosages. The experimental values of this unit size, based on an assumed model for the charge cooperation in O_2 evolution, ranging from 800 to 1600, suggest that there is either limited energy transfer between energy-trapping units or chemical cooperation among oxygen precursors formed in several neighboring energy-trapping units. Widely diffusible essential precursors to molecular oxygen are ruled out by these results. Inhibition studies show that O_2 evolution is blocked when 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is added to chloroplasts after two preliminary flashes and before a third flash which would have yielded O_2 in the absence of DCMU. This experiment is interpreted as evidence that the site of DCMU inhibition is on the oxidizing side of system II. Pretreatment of chloroplasts with large concentrations of Tris, previously believed to destroy O_2 evolution by blocking an essential reaction in the electron chain between water and system II, may be alternately interpreted as promoting the dark reversal of the system II light-induced electron transfer.

INTRODUCTION

Recent measurements of photosynthetic oxygen evolution in successive light flashes after previous controlled regimes of light and dark have given rise to various models for the accumulation and storage of oxidizing equivalents intermediate to oxygen evolution (Joliot et al., 1969; Renger, 1969; Kok et al., 1970; Mar and Govindjee, 1971; Rosenberg, 1969; Weiss and Sauer, 1970). Some models propose the evolution of two-electron fragments (formally equivalent to O atoms) followed by their combination in pairs to form an O_2 molecule, while others propose the accumulation of four oxidizing equivalents in a single reaction center before the release of an O_2 molecule. In the former case, the question of the range of mobility or interaction of

the O fragments is bothersome, especially since it has been shown that the relative efficiency of photochemical conversion to O_2 of partly oxidized intermediates is independent of light intensity (Kok et al., 1970).

In the present investigation, an attempt was made to estimate the size of the pigment matrix within which the quanta, which in system II produce the four equivalents corresponding to one oxygen molecule, must be absorbed. Preliminary reports of this study were presented earlier (Rosenberg, 1969). Also included in the present paper are experimental results dealing with the differential inhibition by DCMU of separate steps in the oxygen-liberating mechanism.

MATERIALS AND METHODS

Chloroplasts freshly prepared from market spinach were suspended in phosphate buffer at pH 7 containing chloride and 0.005 M $(NH_4)_2SO_4$. 0.005 M $K_3Fe(CN)_6$ was used as the electron acceptor. For all experiments the chlorophyll concentration was 0.325 mg/ml as assayed by the spectrophotometric method of Arnon (1949). Oxygen measurements were made polarographically in a meter similar to that of Fork (1963), with a DC polarizing voltage on the 25.4 mm^2 platinum cathode of -0.55 v with respect to the Ag-AgCl anode. The area of the sample was equal to that of the electrode. Details of the meter construction including a calibration to yield absolute values for O_2 will be described in a separate publication. The sample was illuminated for either continuous or flash regimes at 660 nm, isolated by an interference filter from a tungsten source. Flashes in the 1 msec range and above were secured by use of a camera shutter. Intensity was regulated by neutral density filters. Absolute intensities were measured by substituting a thermopile in the plane of the Pt electrode. The absorbance in the 0.0386 cm sample path was calculated from spectrophotometrically measured values of diluted samples in a 1 cm cuvette with the oil-paper technique (Shibata et al., 1954). The fractional absorption in the O_2 meter, integrated over the transmission band of the interference filter, was 0.530.

THE QUANTUM ACCUMULATION UNIT

We define the quantum accumulation unit as that group of pigment molecules within which all quantal absorption leading to those system II photoacts contributing to the evolution of a single O_2 molecule must occur. The unit pigment number n, for the purposes of this paper, is defined as the number of all chlorophyll molecules, in both systems I and II, divided by the total number of quantum accumulation units. If the partly oxidized intermediates leading to oxygen were diffusible, n would be a very large number; if they were stationary, n would correspond to the number of chlorophyll molecules in one to four classical energy-trapping and transferring units of the system II type plus those chlorophylls in the associated system I units.

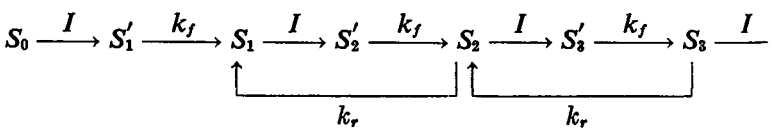
The experimental determination of n was made by three separate methods. Each method depends on the nonlinearity of oxygen yield with radiant dose for doses corresponding to the accumulation of less than 8 quanta per quantum accumulation unit within the mean lifetime of the oxygen intermediates. For each case absolute rates of quantal absorption had to be estimated and a model of the oxygen evolution process had to be assumed.

(*a*) *Method of Single Variable Nonsaturating Flashes*

Most of the flash experiments reported in the last few years in connection with oxygen-evolving mechanisms have utilized saturating flashes so short as to preclude within the period of a single flash the limiting dark reactions that must intervene between successive photochemical events in a given unit. We purposely studied oxygen evolution in nonsaturating single flashes long enough to allow both light and forward dark reactions to occur during the flash duration. Table I summarizes some of the results. The functional dependence of the oxygen yield Y on pulse length τ and on incident intensity I at low values of τ and I is of interest in testing various models. Some of the data of Table I are shown in Figs. 1 and 2 in log-log form so that the slopes give the exponential dependence of Y on τ and I respectively. The following empirical equation represents the initial slopes in these figures:

$$Y = \text{const} \times I^{4.0\pm0.2} \times \tau^{4.4\pm0.1}. \quad (1)$$

The data summarized above were fitted to the following model, similar to that of Kok et al. (1970):



$$S_4' \xrightarrow{k_f} S_0 + O_2 \quad (2)$$

TABLE I

OXYGEN YIELD PER ISOLATED FLASH IN SPINACH CHLOROPLASTS*

τ	$I \times 10^{-15}$	$Y \times 10^{-9}$ (O_2 molecules)
msec	*quanta cm^{-2} sec^{-1}*	
3.6	2.62	2
10.6	0.71	4
10.6	0.83	9
10.6	1.04	23
10.6	1.38	63
10.6	1.72	145
10.6	2.62	208
17.5	0.71	21
17.5	0.83	46
17.5	1.04	107
17.5	1.38	399
17.5	1.72	651
17.5	2.62	2083

* I is incident intensity at 660 nm; τ is flash length. Dark period before each flash was 20 min. Other details are described in Materials and Methods.

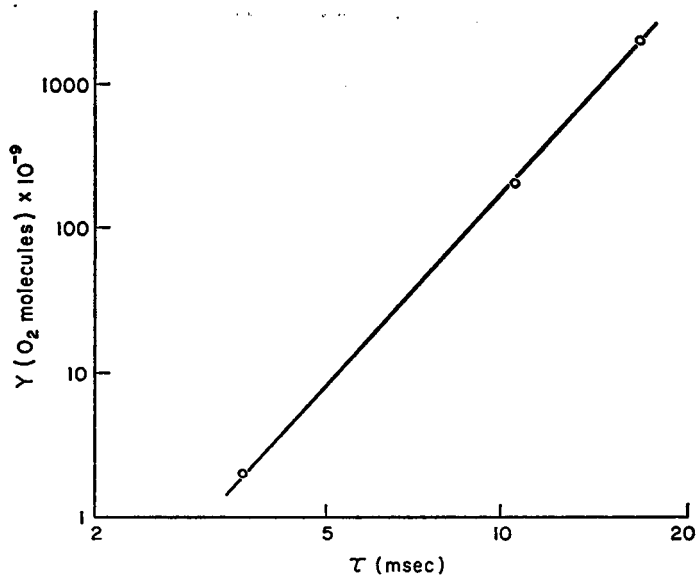


FIGURE 1 Log-log plot of flash yield vs. time. The data are from Table I for the intensity of 2.62 × 10^{15} quanta cm^{-2} sec^{-1}. The line is the least squares fit through the data points.

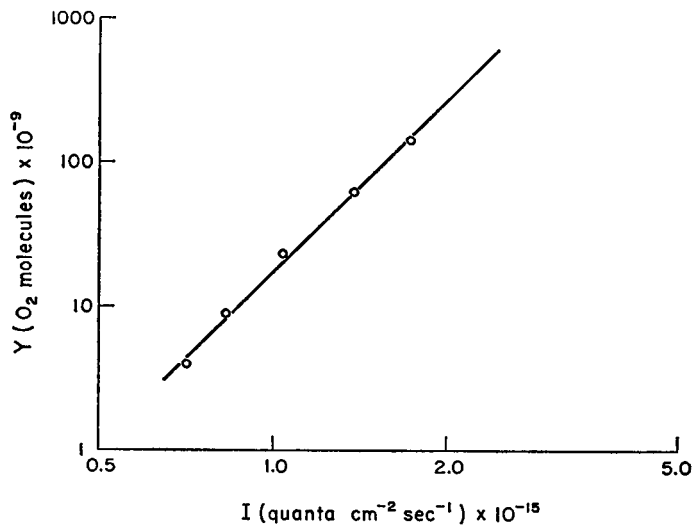


FIGURE 2 Log-log plot of flash yield vs. light intensity. The data are the first five points listed in Table I for 10.6 msec. The line is the least squares fit through the data points.

This model leads to a predicted absolute yield per flash if the starting conditions are known. If $[S_1]$, the fraction of the units in the S_1 form, is 1.0 at the beginning of the flash after 20 min dark, if flashes are so short (<0.1 sec) as to preclude the back reactions and so low in dosage as to preclude evolution of O_2 from any one

unit by more than one cycling through the S_i sequence, if $k_f\tau \ll 1$ and $i\tau \ll 1$, where i is the quantal rate of system II absorption per unit, the predicted limiting form of the yield equation is

$$y = \frac{i^3 k_f^2 \tau^5}{5!}, \tag{3}$$

where y is the number of oxygen molecules produced per unit. The proportionality between y and Y, and i and I, may be expressed by equations 4 and 5,

$$y = \frac{Yn}{clA}, \tag{4}$$

$$i = \frac{If\alpha n}{cl}, \tag{5}$$

where c is the concentration of chlorophyll molecules per cubic centimeter, I is incident quanta per square centimeter per second, l and A are the thickness and area of the sample compartment respectively, f is the fractional absorption of the incident light (0.530 in our experiments) integrated over the bandwidth, and α is the fraction of absorbed light allocated to system II. The one requisite condition for equation 3 not met in the experiments was $k_f\tau \ll 1$. Under the actual circumstances, the experimental exponent of τ is expected to be less than predicted in equation 3. If $[S_1]$ were less than 1 at the beginning of the illumination and $[S_0]$ were appreciable, the exponent of i would be greater than predicted in equation 3, approaching 4 for large values of $[S_0]$. With these reservations, the experiments may be regarded as consistent with the general features of the assumed model.

Subject to the following conditions,

$$i\tau \ll 1, \tag{6}$$

$$k_f\tau \gg 1, \tag{7}$$

$$[S_0] + [S_1] = 1, \tag{8}$$

the model predicts the following limiting form:

$$y = \frac{[S_1]_0 (i\tau)^3}{6} + \frac{(1 - 4[S_1]_0)(i\tau)^4}{24}. \tag{9}$$

The data from Table I for 10.6 and 17.5 msec flashes were plotted on Fig. 3 and the following least squares solution was obtained:

$$Y = (1.21 \pm 0.25) \times 10^{-29} \times (I\tau)^3 + (2.85 \pm 1.16) \times 10^{-43} \times (I\tau)^4. \tag{10}$$

In principle, the matching of the empirical equation 10 to the predicted equation 9 by means of the scaling equations 4 and 5 could yield values for both n and $[S_1]_0$. In practice, the model should not be stretched too much because of the large error

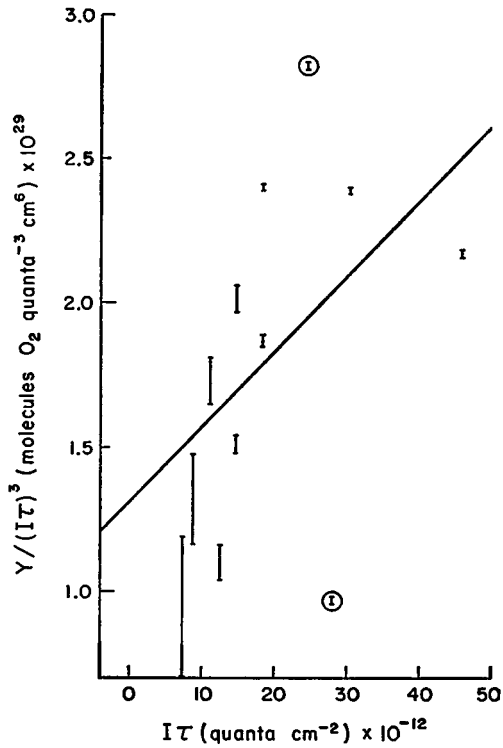


FIGURE 3 Experimental basis of equation 10. The data are based on the 10.6 and 17.5 msec values listed in Table I. The line is the least squares fit through all plotted points except the two enclosed in circles.

in the quartic term of equation 10 and because of the failure to satisfy equation 6. (ir, the mean number of quanta absorbed per accumulation unit during the period of the flash, ranges in value from 0.2 to 2 for the crucial data. The minimum k_f value of 2×10^8 sec^{-1} given by Kok et al. [1970] assures that the condition of equation 7 is met.)

Instead, we accept a value of $[S_1]_0 = 0.75$ from Kok et al. (1970) and use just the cubic term in equation 10, for which the lower values of ir are more responsible. If α is taken as 0.5 (Myers, 1963), we compute:

$$n = 1200 \pm 140. \tag{11}$$

(*b*) *Method of Steady-State Rates at Low Intensities*

The scheme of equation 2 predicts that the rate of steady-state oxygen evolution per unit, v, at very low intensity should show the following behavior:

$$4v/i = [1 + (r/2) + (r^2/4)]^{-1} = \frac{\text{Quantum yield at low intensity}}{\text{Limiting quantum yield at moderate intensity}}, \tag{12}$$

where

$$r = k_r/i. \tag{13}$$

The quantity (v/i) is proportional to the quantum yield and thus to the macroscopic ratio (V/I), where V is the observed rate of oxygen evolution. Table II shows that the empirical relative quantum yield indeed falls off at very low intensities below the limiting value observed at moderate intensities. The combination of equations 5 and 13 leads to a value of n once the magnitude of k_r is known. We found in separate experiments that the yield of a second strong 1.2 msec flash decreased exponentially with the time interval separating the first and second flashes over the range 1–9 sec, and similarly the yield of a third such flash decayed exponentially with the interval between the second and third flashes over the range 1–10 sec, for a fixed 4 sec interval between the first two flashes. The two semilog decay plots (not shown) had identical slopes, and this fact suggests that the two decay processes in equation 2 have the same rate constant k_r, the value of which was determined from the slopes as 0.18 sec^{-1}. The estimate of n by this method is 1800 $\pm$200.

(c) *Method of Transient Rates in Continuous Light*

Joliot (1966) showed that the initial rate of oxygen evolution in very weak continuous light rises from an initial value of zero to a steady-state value over the course of a transient which can be observed. Similar phenomena have been reported by Kok et al. (1970). We have observed, as have Kok et al. (1970), that the rate-time curve at zero time after a long dark period has zero slope (Fig. 4). Sahu (1971) has shown that this condition can be observed in chain models similar to equation 2 only if at least three successive light reactions are needed, starting from the resting state in the dark, to generate an oxygen molecule. The time to reach half the maximum rate

TABLE II

STEADY-STATE OXYGEN YIELD IN SPINACH CHLOROPLASTS*

$I \times 10^{-13}$	V	$\frac{V}{I} \times 10^{13}$
quanta cm^{-2} sec^{-1}	*arbitrary units*, sec^{-1}	
0.182	3.5	19.2
0.348	14.5	41.7
0.66	37	56.0
1.33	77	57.9
1.99	117	58.8
2.66	158	59.5
3.66	216	59.0

* Experimental details are described in Materials and Methods.

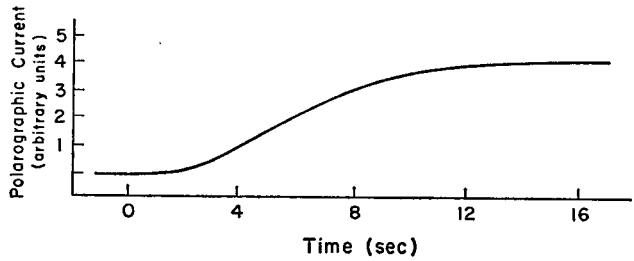


FIGURE 4 Oxygen rate transient in spinach chloroplasts at low light. Dark time before illumination was 20 min. Light was turned on at zero time. The curve is a tracing of the O_2 meter chart recording. Incident intensity at 660 nm was 12 ergs cm^{-2} sec^{-1}. Other experimental details are described in Materials and Methods.

may be taken as a characteristic of such a transient curve. Numerical integration of the differential equations based on equation 2 leads to a correlation of half-time as a function of r. In Fig. 4, the rate reaches half its maximum in 6.7 sec. If 0.8 sec is allowed for instrumental lag (an approximate value determined from the shape of the O_2 evolution curve in a short saturating flash), the following set of parameters is obtained, consistent with each other and with the starting conditions evaluated in section *a* ($[S_0]_0 = 0.25$; $[S_1]_0 = 0.75$):

$$\begin{aligned} r &= 1.84, \\ i &= 0.098 \text{ sec}^{-1}, \\ n &= 780 \pm 120. \end{aligned} \tag{14}$$

(*d*) *Summary*

The three independent methods have given n values in the range 800–1600 chlorophylls. The indicated error limits are based on the scatter in the fit of the data to the respective models. Additional error is expected because of faults in the models and because of failure to allow for deviations from perfect quantum efficiency in the chloroplasts. Even if the numerical values are not too accurate, two facts stand out from the order of magnitude calculations:

(*a*) The values all seem to be higher than the number of chlorophylls in the classical energy-trapping photosynthetic unit. This suggests that either some limited excitation energy is transferred among units or that several units cooperate in the charge accumulation leading to oxygen evolution (Izawa and Good, 1965). The uncertainties of the determinations are such that quantitative model proposals for superunits cannot be made on the basis of our data.

(*b*) The fact that the n values are as low as they are provides independent evidence that freely diffusible oxidized intermediates, which would allow extensive charge cooperation over more than 10 energy-trapping units, cannot be involved in oxygen evolution.

DIFFERENTIAL INHIBITION BY DCMU

An attempt was made to determine whether the DCMU-sensitive site is essential in the coupling of oxygen evolution to the final photochemical stages in the quantum accumulation process. In separate experiments, DCMU was added before the second or third in a series of 1.2 msec flashes after a 15 min dark period. The final concentration of the inhibitor in each case was 5×10^{-6} M, corresponding to 95% inhibition of the ferricyanide Hill reaction in continuous illumination at moderate intensity.

An experimental problem arose because 10–15 min are required for the establishment of a steady base line in the O_2 meter readout after a sample is placed in the apparatus or after the sample, already in place, is mechanically disturbed by addition of a new reagent. The back reactions in equation 2, however, prevent the retention of appreciable amounts of the oxidized intermediates S_2 and S_3 for more than about 15–20 sec. To work within the shorter time span, the cellulose acetate membrane normally covering the sample in the O_2 meter was dispensed with so that there was neither a steady base line nor was there the geometric sample condition for which the meter was quantitatively calibrated. Nevertheless, qualitative evidence for oxygen evolution was observed under the nonsteady-state conditions in the controls in which a microdrop of buffer was added to the sample just before a flash. In Fig. 5 *a*

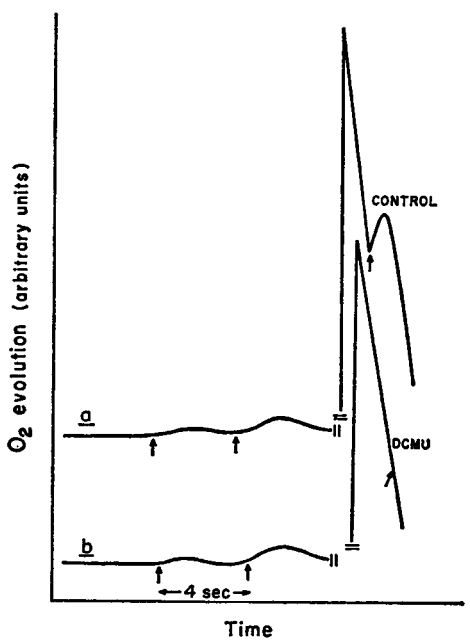


FIGURE 5 Effect of DCMU on O_2 evolution in spinach chloroplasts. Chlorophyll, 0.325 mg/ml; $Fe(CN)_6^{3-}$, 5×10^{-3} M; DCMU after addition, 5×10^{-6} M. Arrows mark the application of 1.2 msec light flashes. Additions after second flash: (*a*) buffer, (*b*) DCMU. Final concentration of ethanol in the DCMU experiment was 0.05%; ethanol was not used in the control.

a tracing of the readout is given for the control, in which two flashes were given under steady-state conditions, plain buffer was added, then a third flash was applied about 10 sec after the second flash. Although the current at the platinum electrode showed a momentary spike followed by a quickly falling phase, the O_2 evolution caused by the third flash can definitely be seen superimposed on the background. The magnitude of the spike, greater than that due to the second flash, shows that much of the effect is due to the photoprocessing in the third flash of the S_3 made in the second flash. In Fig. 5 *b*, on the other hand, representing the same procedure except that a microdrop of DCMU was added after the second flash, no spike due to the third flash could be seen superimposed on the background. The same result was obtained in over a dozen such experiments: an O_2 spike was never seen in a second or third flash if DCMU was added just before that flash, but a spike was always seen in the corresponding controls. We conclude that S_3 cannot generate O_2 in the light in the presence of DCMU.

If the site of DCMU inhibition were on the system I side of Q (Fig. 6 *a*), as proposed by Duysens and Sweers (1963) and by Yamashita and Butler (1968), we would expect some O_2 evolution in the presence of DCMU when DCMU is added before the third flash, since the electron transport chain between system I and Q should not need to operate in order to transfer one last electron to Q in the light. Our contrary finding is strongly suggestive that the DCMU-sensitive site is in the chain transporting electrons from water to Y (referred to as Z by other authors) on the oxidizing side of system II (Fig. 6 *b*). To accept this view we must provide an alternate explanation for the high fluorescence observed under DCMU inhibition. Duysens and Sweers (1963) and others postulated that oxidized Q is the fluorescence quencher of system II and that Q must therefore remain reduced in the presence of DCMU. An alternative explanation of the same facts (Franck and Rosenberg, 1964) is that the photochemical act of system II, rather than energy transfer to Q, is itself the quenching process, in preparation for which Q must be in the oxidized state

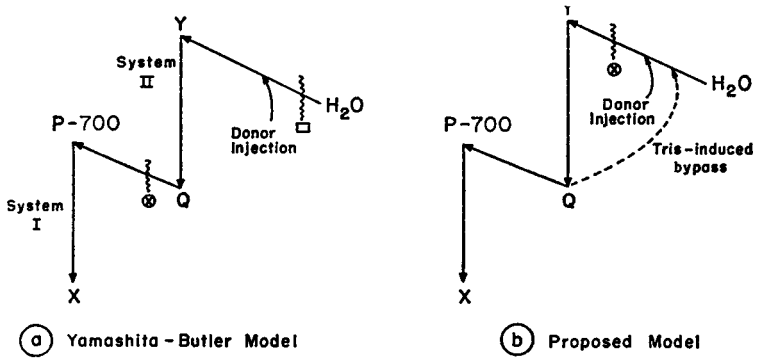


FIGURE 6 Alternate models for inhibition. ⊗, DCMU block; □, block in Tris-washed chloroplasts.

and Y must be in the reduced state. Arguing against this interpretation, Yamashita and Butler (1968) cite their observed low fluorescence in Tris-washed chloroplasts, when O_2 evolution cannot occur and when *Y* would appear forced to remain in the oxidized state. An alternative explanation of the Tris-induced damage consistent with the fluorescence data is that some structural change accelerates an electron bypass or a back reaction between reduced *Q* and oxidized *Y*. According to this picture (Fig. 6 *b*), fluorescence would remain low because the primary system II reaction could still occur as a result of the back reaction, even though O_2 evolution is impeded. The function of phenylenediamine and other electron donors which allow the Tris-washed chloroplasts to sustain the Hill reaction without O_2 evolution might then be assumed to inject electrons somewhere within the bypass. If DCMU inhibits within the bypass route between *Y* and the site of electron injection, it would prevent the cycle from operating in the light and would lead to a high fluorescence, as Yamashita and Butler (1968) indeed observed. The finding by Govindjee et al. (1970) that absorbance changes of chlorophyll a_{II} are still observed in Tris-washed chloroplasts is also consistent with this view of Tris washing.

The postulated accelerated bypass in Tris-washed preparations is not intended to represent the only mechanism for reaction of Y^+ with Q^-. Slower routes are known to exist in undamaged preparations and can persist even in the presence of DCMU (Bennoun, 1970; Wolford, 1968). Alternate mechanisms for the back reaction could easily differ in their chemiluminescent properties as well. Thus, the findings by Mohanty et al.[1] that the fluorescence transient in Tris-washed chloroplasts treated with DCMU is restored after several minutes of darkness and that the afterglow in Tris-washed preparations is no greater than in normal samples should not be taken as evidence contradictory to our general scheme.

Similarly, multiple points of electron entry by exogenous donors must exist (Cheniae and Martin, 1970). The entry point suggested in Fig. 6 *b* might be representative for phenylenediamine and ascorbic acid, while NH_2OH can probably provide electrons directly to Y^+ (Bennoun, 1970; Bennoun and Joliot, 1969; Mohanty et al., 1971). Some difficulties still exist in reconciling observations on the kinetics of NH_2OH oxidation in similar preparations (Bennoun and Joliot, 1969) with our general scheme. The multiple functions of NH_2OH, including both donation of electrons and interaction with system II–bound manganese (Cheniae and Martin, 1970), and the uncertainty of identification of the polarographically active postulated NH_2OH oxidation product may complicate the interpretation of NH_2OH experiments.

Referees have suggested an alternate explanation of our DCMU effect, namely an acceleration of the back reactions in equation 2, perhaps a result of the 0.05% ethanol present in the DCMU experiment. Unfortunately, we did not perform the

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[1] Mohanty, P., B. Z. Braun, and Govindjee. Personal communication.

control experiment with the same small amount of ethanol, nor did our experimental method allow a direct test of the hypothesis by repeating the experiment at reduced dark time after addition of DCMU.

Some of this material was abstracted from the dissertation of Saura C. Sahu submitted to the University of Pittsburgh in partial fulfillment of the requirements for the Ph.D. degree awarded in 1971.

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