Functional Photosynthetic Unit Sizes for Each of the Two Light Reactions in Spinach Chloroplasts*

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ABSTRACT: From studies in flashing light of electron-transport reactions of spinach chloroplasts, we have been able to estimate the concentration, relative to chlorophyll, of the reaction centers or pools of intermediates closely associated with photosystems I and II. Relatively long flashes (6–100 msec) of saturating red light indicate a functional unit for cytochrome *c* reduction by reduced trimethyl-*p*-benzoquinone in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea corresponding to 1 equiv/445 chlorophylls for photosystem I. Similar illumination patterns for the Hill reaction using 2,6-dichlorophenolindophenol and/or ferricyanide lead to a maximum reduction of 1 equiv/55 chlorophylls for photosystem II. From other published results it appears that the large photosystem II yield results from a secondary pool of endogenous intermediate electron acceptors, and that very short (10^{-4} sec) flashes would produce evidence for a primary rate-limiting component in smaller concentration. For long flashes, the rate-limiting step for the dark reaction regenerating system I activity has a first-order rate constant of 13 sec^{-1} at room temperature. That for system II, also first order, is *ca.* 30 sec^{-1}, but is reduced to one-half this value in the absence of phosphorylation cofactors and the phosphorylation uncoupler, methylamine. Both of these rate-limiting steps appear to result from components endogenous to the broken chloroplasts.

Photosynthetic oxygen evolution by *Chlorella* and higher plant chloroplasts in short flashes of light ($\sim 10^{-5}$ sec) gives evidence of a photosynthetic unit of about 2000–2500 chlorophyll molecules which cooperates in the evolution of one molecule of oxygen during a flash (Emerson and Arnold, 1931, 1932; Arnold and Kohn, 1934). The reaction proceeds with a slow dark step of 0.02 sec at room temperature. If longer flashes are utilized (≥ 0.6 msec), smaller photosynthetic units (higher yields of oxygen) and longer dark times are observed (Kohn, 1936; Briggs, 1941; Weller and Franck, 1941; Tamiya and Chiba, 1949). Gilmour *et al.* (1954) attributed this discrepancy to a pool or reservoir of photoreducing power that is operative in the Hill reaction only at high light intensity and for long flashes.

Kok (1956) studied photosynthetic oxygen evolution in *Chlorella* using flashes which varied from 0.2 to 320 msec in length. He found that with a suitably long dark time the yield per flash as a function of flash time (t_f) was linear in the region 2–320 msec, had a positive slope, and extrapolated to a finite value at zero flash length. For flash durations of less than 2 msec, the yield per flash became progressively smaller than the values expected, falling to 70% of the extrapolated value at $t_f = 0.2$ msec. These results conform to the theories proposed above and suggest that a saturating flash of only 2-msec duration is sufficient to saturate the dark intermediates in photosynthesis.

Following the initial proposal of Hill and Bendall (1960), much evidence has accumulated to support a mechanism for higher plant photosynthesis involving two light reactions connected in series by a chain of electron-transport intermediates (Clayton, 1965). It has been possible to distinguish between the arrays of pigment molecules associated with each of the light reactions from studies of the action spectra of partial reactions driven separately by each light step (Hoch and Martin, 1963; Sauer and Biggins, 1965; Sauer and Park, 1965; Kelly and Sauer, 1965). We have undertaken to determine whether repeated pulses of high intensity light give evidence of the sizes of the functional units in spinach chloroplasts associated with the two separate light reactions. The Hill reaction using DCPIP[1] or ferricyanide as oxidant is associated primarily with pigment system II (Sauer and Park, 1965), whereas the cytochrome *c*–$TMQH_2$ photoreaction in the presence of the oxygen evolution inhibitor DCMU is driven solely by pigment system I (Vernon and Shaw, 1965; Kelly and Sauer, 1965). With steady illumination each of these partial reactions occurs with a high quantum efficiency under light-limiting conditions in broken chloroplast preparations as used in this

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[1] Abbreviations used: TMQ ($TMQH_2$), trimethyl-*p*-benzoquinone; DCPIP ($DCPIPH_2$), 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MA, methylamine; ADP, adenosine diphosphate. The abbreviations in parentheses refer to the reduced form of the compound.

study. Thus, meaningful functional sizes of the pigment array associated with each light reaction can be obtained by studying the respective partial reactions in flashing light.

Theoretical

A simple mechanism and kinetic analysis will suffice to develop the results. Consider a rapid light reaction followed by a slow dark reaction to yield stable products

$$Q \xrightarrow[\text{chloroplasts}]{h\nu} Q^* \quad (1)$$

$$\text{reactants} + Q^* \xrightarrow{k_1} Q + \text{products} \quad (2)$$

where Q is a trapping site or a rate-limiting intermediate and $Q + Q^* = Q_0$ represents the total pool of these intermediates. The concentration of the reactants is sufficiently large that reaction 2 is pseudo first order.

In a single short flash ($t_f \ll 1/k_1$) of saturating intensity, virtually all of Q is converted to Q*. If we assume 100% efficiency for the formation of products after the light energy is trapped and an exponential decay of the excited intermediate, then the amount of product formed in a single flash (P_f) will be Q_0, if the dark time following the flash is sufficiently long for all of the Q* to return to Q.

During a long flash of duration t_f there will be a recycling of Q to form Q* so that the total conversion during one flash will be

$$P_f = Q_0 + k_1 Q_0 t_f = Q_0(1 + k_1 t_f) \quad (3)$$

In a train of light flashes separated by dark intervals of duration (t_d), the amount of Q available for excitation by a succeeding flash can be found from

$$\frac{dQ}{dt} = -\frac{dQ^*}{dt} = k_1 Q^* \quad (4)$$

where the amount of Q* and Q at t_d after the previous flash will be

$$Q^* = Q_0 e^{-k_1 t_d} \quad (5)$$

and

$$Q = Q_0 - Q^* = Q_0(1 - e^{-k_1 t_d}) \quad (6)$$

Thus the total conversion of material per flash in flashing light will be

$$P_f = Q_0(1 - e^{-k_1 t_d}) + Q_0 k_1 t_f \quad (7)$$

Equation 7 predicts that a plot of P_f *vs.* t_f will be linear (as observed by Kok, 1956), with a slope of $k_1 Q_0$ and an intercept of $Q_0(1 - e^{-k_1 t_d})$. For sufficiently long dark times, as in most of the experiments reported here, eq 7 reduces to eq 3. Thus the size of a functional unit (which we define as the number of molecules of chlorophylls a and b per electron equivalent of intermediate Q produced with saturating flashes) and a value for k_1 can be obtained from a study of flash yield as a function of flash length. It should be pointed out that such functional units measured kinetically need not correspond to actual morphologically distinct units in the chloroplast. The morphological unit may contain any integer multiple of functional units without altering the kinetic behavior.

Materials and Methods

Flashing Light Apparatus. Hill oxidant (DCPIP or ferricyanide) or cytochrome *c* photoreduction were followed spectrophotometrically in a Cary 14 spectrophotometer with Model 1462 scattered-transmission accessory as employed by Sauer and Park (1965) and Kelly and Sauer (1965). Extinction coefficients for the reagents were $\epsilon_{580\ m\mu}$ 19,800 l. mole^{-1} cm^{-1} at pH 7.7 for DCPIP, $\epsilon_{420\ m\mu}$ 1000 l. mole^{-1} cm^{-1} for ferricyanide, and $\Delta\epsilon^{redox}_{549.5\ m\mu} = 1.9 \times 10^4$ l. mole^{-1} cm^{-1} for cytochrome *c*.

The experimental apparatus is similar to that described by Sauer and Biggins (1965), but with side illumination of the reaction cuvet, as described below. Light from a 1000-W projector bulb housed in a Luxtar Model V-1000 strip film projector, with infrared wavelengths filtered out by a Corning 1-60 filter and 7 cm of water, is focused on a paddle connected to the drive of a stepping motor (Model 55-100, Cedar Engineering, Minneapolis, Minn.). The duration and frequency of the flashes produced by the stepping motor are controlled by commercial pulse generators as described by Kuntz and Calvin (1965). Red wavelengths ($\lambda > 635$ mμ) are isolated by means of a supplementary Corning 2-58 filter. The sample cuvet has aluminum foil taped to its far side to increase absorption of the exciting light by the chloroplasts, while the reference cuvet has black tape on its adjacent side so that it is not exposed to the actinic light.

Figure 1 shows traces of the light pulses, measured using a fast-response photodiode (Edgerton, Germeshausen and Grier, Cambridge, Mass., type 5D-100, response time $< 1.5 \times 10^{-8}$ sec), whose output is displayed on an oscilloscope (Tektronix 545A) and photographed. The light pulses have rise and fall times of *ca.* 2 msec. Defining the flash time (t_f) as the pulse width at half-maximum intensity, we find that reasonably square pulses are obtained in this fashion down to $t_f = 6$ msec, and thus a range of 6–100 msec is available for t_f. The time between flashes is adjustable between 6 msec and many seconds.

Variable light intensities, obtained by altering the voltage of the projector lamp, are measured on a relative scale using a silicon photocell (Hoffman CG-120). If we take the incident red light to have an average wavelength of 700 mμ, then the energy of the incident radiation at the sample cuvet is *ca.* 70 mW cm^{-2} at maximum lamp voltage (referred to in the text as maximum intensity).

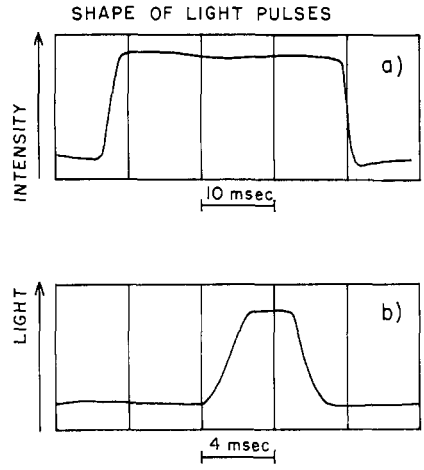


FIGURE 1: Traces of the light pulse shape. The flash duration is measured at half-maximum intensity giving (a) 32 and (b) 6 msec. Rise and decay times are about 2 msec.

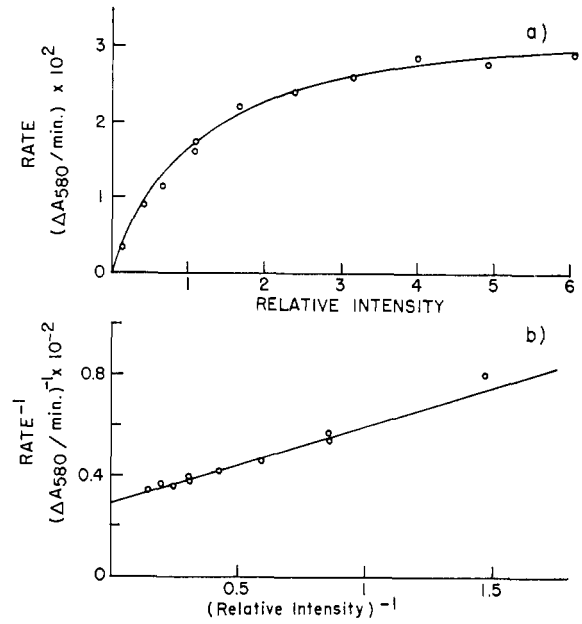


FIGURE 2: Intensity dependence studies. (a) Intensity dependence of the DCPIP Hill reaction (without methylamine) by spinach chloroplasts in flashing light. The flash duration is 19 msec, flash periodicity 2 sec, and A_{678}^{1cm} of the chloroplasts 0.240. The curve is calculated from the reciprocal plot in part b. (b) Double-reciprocal plot of the data in part a. The intercept represents the reciprocal of the maximum rate (R_∞^{-1}) which would occur at infinite light intensity.

Reagents. Spinach [*Spinacia oleracea* L. var. *Viroflay* grown in vermiculite in the open air (Jensen and Bassham, 1966) or *S. oleracea* var. *early hybrid no. 7* grown in a growth chamber under the controlled conditions described previously (Sauer and Park, 1965)] was harvested 4–8 weeks after germination. Chloroplasts were prepared as described previously (Sauer and Park, 1965) except that in the Hill reaction studies tricine buffer (Good, 1962) (General Biochemicals, Chagrin Falls, Ohio), pH 7.4 or 7.7, was used instead of phosphate in the isolation and storage of chloroplasts. $TMQH_2$ was prepared as described previously (Kelly and Sauer, 1965). DCPIP was obtained from K & K Laboratories, Jamaica, N. Y.; horse heart cytochrome *c* from Sigma Chemical Co., St. Louis; and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) from Du Pont, Wilmington, Del. Methylamine hydrochloride was dissolved in distilled water and titrated to pH 7.7 with dilute KOH.

Reaction Mixtures. The reaction mixture for cytochrome *c* photoreduction by $TMQH_2$ in the presence of spinach chloroplasts contained: potassium phosphate (pH 6.0),[2] 0.05 M; sucrose, 0.35 M; and the following in micromoles per milliliter: cytochrome *c*, 0.050; $TMQH_2$, 0.055; and DCMU, 0.020. The stock solutions of $TMQH_2$ in ethanol and DCMU in methanol were diluted 100-fold and 200-fold, respectively, in the final reaction mixture.

For the ferricyanide Hill reaction, the standard reaction mixture consisted of the following in micromoles per milliliter: tricine (pH 7.4), 35; sucrose, 350; potassium ferricyanide, 0.26; and potassium ferrocyanide, 0.26. For the DCPIP Hill reaction, different amounts of DCPIP were used in place of the ferricyanide and ferrocyanide. In those samples so indicated, methylamine was added to a final concentration of 10.0 μmoles/ml.

Chlorophyll Concentrations. Chlorophylls a and b concentrations were measured in 80% acetone using the extinction coefficients of Mackinney (1941). Chlorophyll a:b ratios varied from 2.78 to 3.25 for the spinach used. Sufficient chloroplast preparation was added in the dark at the start of each measurement to give an absorbance at 678 mμ of 0.2–0.5 (1-cm path length).

Results

DCPIP Hill Reaction. t_f, t_d, AND LIGHT SATURATION. Figure 2a shows a typical light saturation curve obtained when the rate of DCPIP reduction in the sample cuvet is monitored at 580 mμ as a function of exciting flash intensity. From the intercept on a reciprocal plot of these data, Figure 2b, the maximum rate (R_∞) which would occur at infinite intensity can be calculated. From such plots, values of R_∞ can be obtained even for small t_f (where saturation is not attained with the maximum intensity used).

Figure 3 shows the effect of the dark time on DCPIP photoreduction per flash. To obtain maximum yield,

[2] A more acidic pH is used here than was employed by Kelly and Sauer (1965) because the dark reoxidation noted previously has been studied more thoroughly and found to be dependent upon the hydroxide ion concentration to the first power (K. Sauer and J. Kelly, unpublished results).

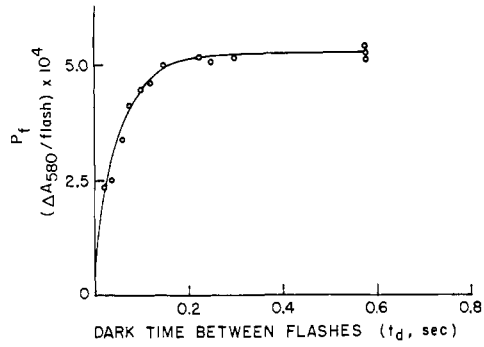


FIGURE 3: Flash yield dependence on dark time between flashes for the DCPIP Hill reaction (without methylamine) by spinach chloroplasts. The reaction is run with t_f = 19 msec at maximum obtainable intensity (90% of saturation) and with the A_{678}^{1cm} 0.270 for the chloroplast suspension. The curve is calculated from eq 7 using experimentally derived parameters.

a dark time of at least 0.2 sec is required. In most of the studies described below a dark time of 2.0 sec was used. Figure 4 shows the effect of the flash length on the reduction per flash. When the short flash results are corrected to light saturation by means of reciprocal plots (filled circles) the yield per flash is observed to be linear in flash duration for the range 6–100 msec.

CONCENTRATION DEPENDENCE. The rate of DCPIP photoreduction is linearly dependent upon chloroplast concentration, as was observed for the Hill reaction under continuous illumination. It was found, however, that a somewhat higher DCPIP concentration than that used by Sauer and Park (1965) in their steady illumination studies is necessary to obtain the maximum yield per flash. Figure 5a is a saturation curve in terms of DCPIP concentration, and Figure 5b is its reciprocal plot. This shows that a concentration of 6×10^{-5} M of DCPIP in the reaction mixture gives about 80% of the maximum attainable rate. Since DCPIP absorbs in the red region of the spectrum, a high concentration of DCPIP reduces the effective intensity available to the chloroplasts. Also the precision of the spectrophotometric analytical technique employed decreases when the DCPIP concentration is much greater than 5×10^{-5} M. For these reasons, experiments were performed at concentrations between 2 and 4×10^{-5} M DCPIP and corrected to saturating concentration by means of Figure 5b.

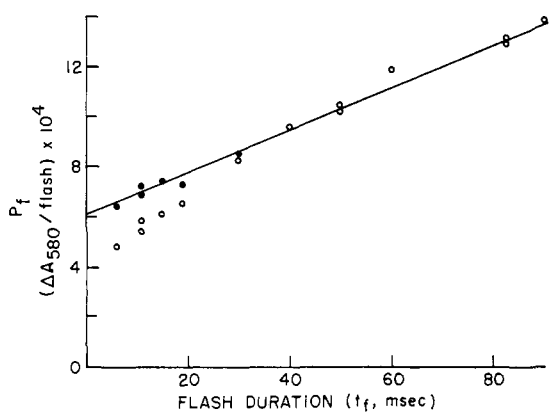


FIGURE 4: Flash yield dependence on flash duration for the DCPIP Hill reaction (without methylamine) by spinach chloroplasts. The flashes are at maximum obtainable intensity with a repetition period of 2.0 sec, chloroplast A_{678}^{1cm} 0.270, and [DCPIP] = 3.82×10^{-5} M. The filled points are points corrected to light intensity saturation by means of reciprocal plots such as in Figure 3.

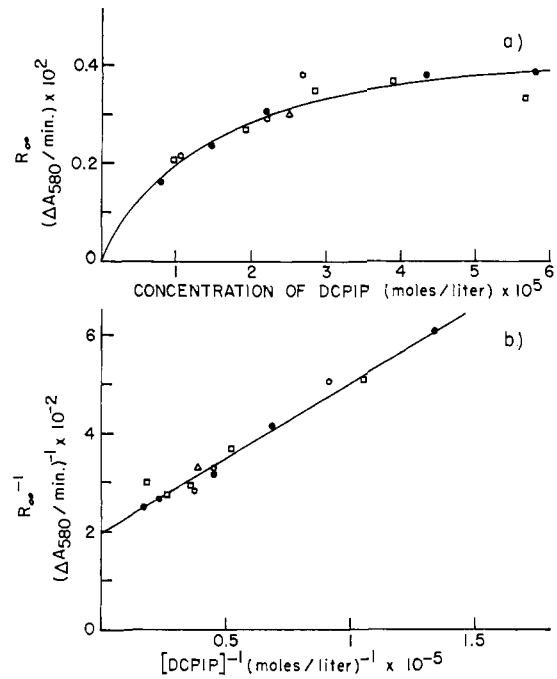


FIGURE 5: R_∞ dependence on DPIP and its reciprocal plot. (a) Maximum flash rate (R_∞) dependence on DCPIP concentration for the DCPIP Hill reaction ±MA by spinach chloroplasts. The flash duration is 50 msec, flash periodicity 2 sec, and A_{678}^{1cm} 0.270 for the chloroplasts. The different symbols denote separate chloroplast preparations. The curve is calculated from the reciprocal plot in part b. (b) Reciprocal plot of the data in part a. The intercept represents the reciprocal of the rate under saturating light conditions and where the DCPIP concentration is no longer limiting.

DCPIP FUNCTIONAL UNIT. When the relatively long flashes used here completely saturate the dark intermediates of the DCPIP Hill reaction, then the slope of the curve in Figure 4 represents the rate of photoreduction at saturation under continuous illumination. This rate is both temperature dependent (Clendenning and Ehrmantraut, 1950), as it depends on the rates of dark enzymatic reactions, and dependent on DCPIP con-

TABLE I: Functional Unit Size and Dark Reaction Velocity Constant.

Reaction		Determinations	Functional Unit Chlorophyll (a + b) / electron transferred	Dark Reaction Velocity Constant (sec^{-1}) (22 ± 2°)
DCPIP reduction	+MA	14	56 ± 10	33.3 ± 6.9
	−MA	14	54 ± 7.4	19.5 ± 3.1
	+ADP, Mg^{2+}, P_i	1	58	32.7
Ferricyanide reduction	+MA	10	70 ± 10	28.0 ± 8
	−MA	6	73 ± 9	16.6 ± 3
	+MA + catalytic DCPIP	2	62 ± 10	31.8 ± 9.2
Ferricyanide reduction	+MA	2[a]	74 ± 11	29.6 ± 7.1
	+MA + catalytic DCPIP	2[a]	62 ± 10	31.8 ± 9.2
Cytochrome *c*–$TMQH_2$	+DCMU	5	445 ± 40	13.2 ± 4

[a] The effect of catalytic amounts of DCPIP on a single chloroplast preparation.

centration when the latter is less than saturating. The intercept of Figure 4 represents the amount of DCPIP photoreduced by a pool of rate-limiting intermediates, Q_0, under the conditions where each of these intermediates has been activated once (eq 3). It should be noted that this intercept is obtained by extrapolation of results using relatively long flashes, and it may not be the same as would be observed for submillisecond flashes. By converting the intercept into molecules of DCPIP reduced (after correcting to saturation for DCPIP concentration) and dividing by the total number of chlorophyll molecules present in an equal volume of the reaction mixture, an experimental value for the size of the functional unit of the DCPIP Hill reaction can be obtained.

Table I presents a summary of calculations of the size of the functional unit and of the dark reaction velocity constant in the presence and absence of methylamine. Methylamine affects the value of k_1 but not the size of the functional unit. Addition of phosphorylation cofactors (ADP, 1.0; $MgCl_2$, 7.5; and potassium phosphate (pH 7.7), 50; all in micromoles per milliliter) instead of methylamine yields a functional unit and rate constant identical with that found in the presence of methylamine.

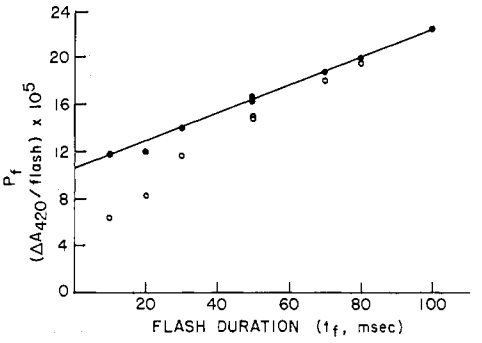


FIGURE 6: Flash yield dependence on flash duration for the ferricyanide Hill reaction (with methylamine) by spinach chloroplasts. The flash periodicity is 0.4 sec, using maximum intensity and with chloroplast A_{678}^{1cm} 0.420. The filled circles are calculated from experimental values by correcting to light intensity saturation using reciprocal plots such as in Figure 3.

Ferricyanide Hill Reaction. RESULTS. The effect of the concentration of ferricyanide–ferrocyanide (equimolar) on the rate of the Hill reaction in flashing light (t_f = 100 msec, t_d = 300 msec) is essentially the same as that observed by Sauer and Park (1965). An optimum concentration is *ca.* 2.5 × 10^{-4} M, with rates about 15% less at either one-half or twice this concentration. Because of the low molar extinction coefficient of ferricyanide, high chloroplast concentrations and short times between flashes are used in order to provide large changes in optical density with time. The dependence on dark time between flashes is similar to that observed for DCPIP, but t_d = 0.300 sec is routinely used in the ferricyanide studies in order to give greater over-all rates. The higher chloroplast concentration necessitated a larger correction to light intensity saturation than was needed for the DCPIP experiments. Figure 6 illustrates this correction and shows that the ferricyanide Hill reaction exhibits a linear relationship between yield per flash and t_f over the millisecond region, as was observed for DCPIP. Table I includes a summary of functional unit size and k_1 values for the ferricyanide Hill reaction measured using ten chloroplast preparations. The addition of a catalytic amount of DCPIP (0.006 μmole/ml) to the ferricyanide reaction mixture containing methylamine resulted in a small, but probably real, decrease in the size of the functional unit and an increase in the dark reaction velocity constant to the value obtained for the DCPIP Hill reaction.

CYTOCHROME *c* PHOTOREDUCTION WITH $TMQH_2$. Saturation curves similar to that of Figure 2b occur for cytochrome *c* photoreduction, but a substantially

lower light intensity is sufficient to reach saturation. This suggests that a larger pool of chlorophyll molecules is associated with each rate-limiting electron-transfer site for this reaction. The dark reaction reaches completion in about 0.5 sec, and intervals of 2 sec between saturating flashes give the maximum yield per flash. A twofold variation of the cytochrome *c* or $TMQH_2$ concentrations in either direction gives the same results, indicating that the concentrations generally employed are saturating.

Figure 7 shows the variation of cytochrome *c* reduction per flash as a function of flash length, and again the dependence is approximately linear in t_f for the long-flash region that we examined. For this reaction, saturation could be obtained for the shortest light pulses used. The size of the functional unit and the dark reaction velocity constant are summarized in Table I.

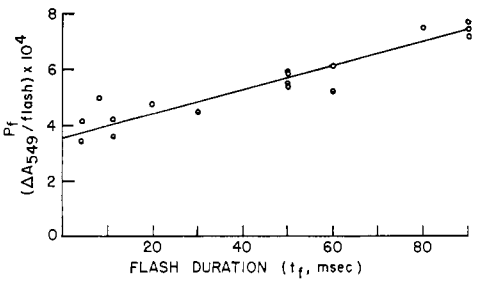


FIGURE 7: Flash yield dependence on flash duration for cytochrome *c* photoreduction with $TMQH_2$ in the presence of spinach chloroplasts and DCMU. The flash periodicity is 2.0 sec with maximum intensity and with chloroplast A_{678}^{1cm} 0.489.

Discussion

The chloroplast-catalyzed photoreduction of cytochrome *c* is thought to reflect solely system I activity, on the basis both of its insensitivity to DCMU poisoning (Vernon and Shaw, 1965) and of its characteristic action spectrum (Kelly and Sauer, 1965). With this assumption, the flashing light studies summarized in Table I indicate that the system I functional unit contains 445 chlorophyll molecules. This number is quite similar to the ratio of chlorophyll to P700, the presumed reaction site of system I, as estimated for spinach chloroplasts by Kok and Hoch (1961). Subsequent measurements by Anderson *et al.* (1966) of the chlorophyll (a + b): P700 ratio, also for spinach chloroplasts, yielded a value of 440. The agreement of our size of the functional unit for the cytochrome *c* reduction by spinach chloroplasts suggests that system I is limited in efficiency by the turnover of one trapping site (P700 or some stoichiometrically equivalent molecule) per 440 chlorophyll molecules. The result also provides further support for the assignment of cytochrome *c* reduction as a system I reaction.

The Hill reaction using either DCPIP or ferricyanide involves only pigment system II, as indicated by its sensitivity to DCMU, its very different action spectrum (Sauer and Park, 1965), and the absence of Emerson enhancement for these reactions (Sauer and Park, 1965; Avron, 1966). For this reaction we find a functional unit containing 55 ± 10 chlorophyll (a + b) molecules for light flashes from 6 to 100 msec in duration. There seems, therefore, to be about 8 system II functional units for each P700 in chloroplasts. If we assume that *only* pigment system II mediates DCPIP photoreduction, then these results can be explained in two ways: either system II has many excitation energy trapping sites (eight for each P700 of system I), or there is only a small number of system II trapping sites (perhaps stoichiometric with those of system I) which transfer energy to a pool of intermediates, as suggested initially by Gilmour *et al.* (1954). The flashing light experiments of Kok (1956) indicated that, for shorter saturating flashes than we used, a smaller pool of intermediates becomes rate limiting and a larger system II functional unit is obtained.

We find the ferricyanide Hill reaction of spinach chloroplasts to exhibit a functional unit of 70 ± 10 chlorophyll molecules for long flashes. This unit is about 30% larger than that for the DCPIP Hill reaction, and the difference appears to be significant. The inefficiency of the ferricyanide Hill reaction as compared to the DCPIP Hill reaction has been observed before in quantum yield measurements (Biggins and Sauer, 1964; Sauer and Park, 1965). As shown in Table I, we find that the addition of catalytic amounts of DCPIP gives nearly a 20% decrease in the functional unit for the ferricyanide Hill reaction in the presence of methylamine. These results appear to be entirely consistent with one another. They may be accounted for by assuming that ferricyanide participates in a cyclic electron flow, resulting in no net ferricyanide reduction, in competition with the noncyclic flow.

The pool sizes of electron-transport intermediates between systems I and II have been measured recently by several kinetic methods. Table II presents a summary of these studies; however, care must be used in comparing results from appreciably different organisms.

The results reported in this paper appear to be largely consistent with those from the laboratories of Kok, Witt, and Joliot, apart from differences attributable to the photosynthetic organisms examined. The scheme presented below combines the notations of Witt and of Kok and is used to relate our studies to theirs. Light absorbed by photosystem II very rapidly (10^{-4} sec) converts chlorophyll a_{II} or a closely associated compound (Joliot's E or Duysens' Q) to an activated (reduced) state (Joliot, 1961; Duysens and Sweers, 1963). This reduced primary compound rapidly transfers an electron to a molecule of Q (Kok's notation and the one adopted here), and during a long flash (2 msec) this process is repeated until all of the pool of Q is reduced. During this interval roughly half the electrons acquired by Q are passed on to a third pool of intermediates [the P (1/70 chlorophyll) of Malkin and Kok (1966), the A_2 (1/70 chlorophyll) of Joliot (1965), which are perhaps identical with the components

TABLE II: Summary of Recent System II Intermediate Pool Size Studies.

Reference	Method	Material	Author's Symbol	Equiv of Electron Acceptor/ Chlorophyll (a + b)
Joliot (1961)	O_2 burst after dark adaptation	*Chlorella*	A	1/35
	O_2 burst after dark adaptation, short flash of light (10^{-4} sec)	*Chlorella*	E	1/350
Duysens and Sweens (1963)	Fluorescence quenching	*Chlorella*	Q	1/300
Joliot (1965)	Kinetics of O_2 evolution	*Chlorella*	A_1[a]	1/100
			A_2[a]	1/35
Witt *et al.* (1966)	Light-induced absorption changes of endogenous quinones	*Chlorella*		~1/35[b]
Malkin and Kok (1966)	Fluorescence induction	Spinach chloroplasts	Q[c]	1/70
Malkin (1966)			P[c]	1/70
Kok *et al.* (1967)	Dark yield of DCPIP reduction on cessation of saturating illumination	*Scenedesmus* mutant lacking P700		1/70
de Kouchkovsky and Joliot (1967)	Kinetics of O_2 evolution	*Zea mays* chloroplasts	A	1/70
			E[d]	1/2800

[a] Evidence suggested that the earlier (Joliot, 1961) pool A had two kinetically distinguishable components: A_1 (regeneration rate constant 70 sec^{-1}) and A_2 (7 sec^{-1}). [b] This value depends upon assumptions about the values of extinction coefficients of P700 and endogenous quinones. [c] The thermal reaction in which electrons are transferred from reduced Q to the pool of P has a rate constant of 30–40 sec^{-1}. [d] The low concentration of E, relative to that in *Chlorella*, was attributed to inactivation of some of the system II reaction centers during chloroplast isolation.

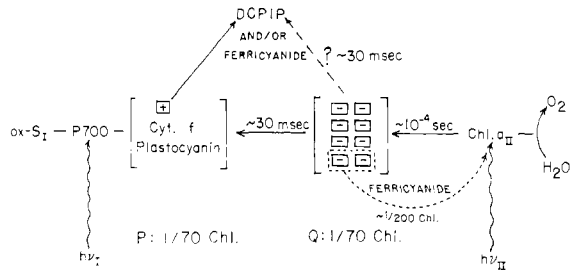
which donate electrons to system I (plastocyanin, cytochrome *f*, and P700) of Witt *et al.* (1966)] (Scheme I).[3]

In our studies, where DCPIP and/or ferricyanide are present in the reaction mixture, the remaining pool of reduced Q then proceeds to reduce these terminal acceptors. This hypothesis is supported by kinetic analysis of the dependence of the yield per flash on the duration of the dark interval between flashes, as shown in Figure 3. When the same data are plotted in semilogarithmic fashion, as in Figure 8, they are found to result from a single first-order process. The rate constant for this dark decay process at room temperature ($22 \pm 2°$) is *ca.* 32 sec^{-1} in the presence of methylamine or phosphorylation cofactors, but it is about one-half this value when these compounds are missing (*e.g.*, for the experiment described in Figures 3 and 8). The dependence of the rate constant on the presence of phosphorylation cofactors or an uncoupler suggests that it is measuring the rate-limiting step at or near the site of coupling to noncyclic phosphorylation in the Hill reaction.

The observation of a simple first-order behavior points to a single pool of electron-transport intermediates as the origin of the terminal step in the reduction of the Hill reagents. No evidence is seen of the biphasic character observed by Joliot (1965) for the oxygen burst for *Chlorella*, where only endogenous oxidants associated with photosystem II are involved. On the basis of this observation, and noting that our pool size of intermediates is about one-half that observed by Joliot (compound A), we propose that DCPIP and/or ferricyanide react with only a single, small pool of photosystem II intermediates (1/60–70 chlorophylls) in isolated chloroplasts. The oxygen burst

[3] Another possible scheme (not presented here) is that there are as many system II traps as Q molecules and that many system II units funnel their electrons to the same P700. The kinetic analyses of Malkin (1966) favored this proposal, but it is felt that experiments using very short flashes (Clendenning and Ehrmantraut, 1950; Gilmour *et al.*, 1954; Kok, 1956) present evidence that there are more Q than chlorophyll a_{II}.

SCHEME I



studies on chloroplasts by de Kouchkovsky and Joliot (1967) also demonstrated a small pool of intermediates (1/70 chlorophylls). They did not report on the kinetics of the decay for the chloroplast system, however.

It is possible that the terminal reduction occurs through the transfer of electrons through a second pool (Joliot's A_2 or Kok's P) of endogenous molecules, but the associated steps leading to substrate reduction must all be rapid. The close similarity of the rate constant of 30–40 sec^{-1} at room temperature for the transfer from reduced Q to P, as measured by Malkin and Kok (1966), to our values for the DCPIP and ferricyanide Hill reaction rate-limiting steps makes this hypothesis appealing. This pathway appears in our kinetic scheme by way of coupling through the pool of electron acceptors associated with system I, although other formulations are possible. The transfer of electrons to the terminal Hill acceptor, if sufficiently rapid, would prevent the pool of P from filling and, therefore, in our kinetic analysis we would observe only the pool of reduced intermediates prior to the rate-limiting step.

A scheme sufficient to account for the cytochrome *c* reduction by $TMQH_2$ can be constructed in much simpler fashion. In this case the component responsible for the rate-limiting step for saturating light flashes is stoichiometric with P700 and may be identical with it. A second reasonable candidate for this component is cytochrome *f* (Boardman and Anderson, 1967). Since the cytochrome *c* reduction reaction does not require added ferrodoxin or the $NADP^+$–ferredoxin reductase, both of which are lost during the chloroplast isolation procedure, neither of these can be rate limiting for this reaction. In all probability we are measuring directly the ultimate size of photosystem I, which is a particularly nice feature of using this reaction. It will be interesting to learn whether this size is affected by the use of very brief flashes. Although cytochrome *c* is a large molecule and might be expected to have restricted access to the sites of photochemical activity in the chloroplast lamellae, relatively low concentrations of cytochrome *c* (0.025 μmole/ml) appear to be saturating. This saturation may result from a prior binding of the oxidized cytochrome *c* at a site close to the system I reaction center during the 75-msec interval required to reactivate the light reaction. The binding is not the rate-limiting step, however.

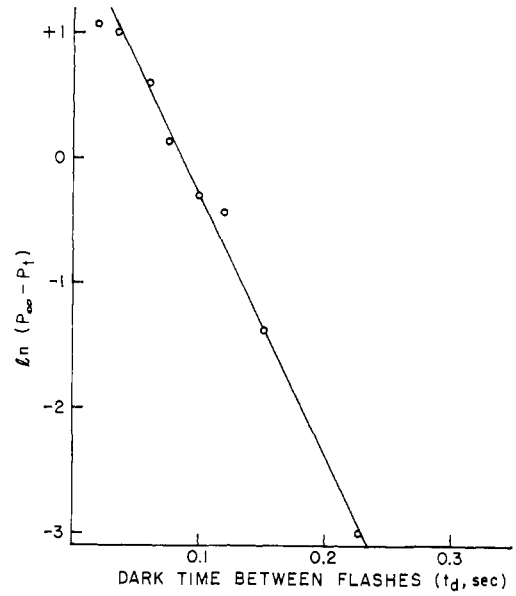


FIGURE 8: Semilogarithmic plot of P_f as a function of dark time between flashes. The data are the same as that for Figure 3 and the graph indicates that the process involved is first order. $k_1 = 21$ sec^{-1}.

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