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

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Abstract

Repetitive flash illumination of aqueous spinach chloroplast suspensions reveals the existence of two different sizes of functional photosynthetic units associated with the two different light reactions of photosynthesis. Light reaction I, associated with the formation of strongly reducing cofactors in the intact organism, was studied by means of the chloroplast-catalyzed photoreduction of cytochrome c by reduced trimethyl-p-benzoquinone ($TMQH_2$). The associated functional unit, per equivalent of cytochrome c reduced, contains about 450 chlorophyll (a + b) molecules and is comparable to that first proposed by Emerson and Arnold from similar studies of oxygen evolution by intact Chlorella cells (Emerson and Arnold, 1931, 1932). Light reaction II, associated with oxygen evolution in higher plants and algae, was examined by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) and/or ferricyanide in the Hill reaction. The associated functional photosynthetic unit contains 60-70 chlorophylls per equivalent--about 8 times smaller than the system I unit. These results are consistent with the presence of a pool of endogenous intermediate electron acceptors in the Hill reaction. There is a small difference in the results, depending

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on whether ferricyanide or DCPIP is used as the Hill oxidant.

Relatively long light flashes (6-100 msec) are employed and spectrophotometric methods are used to follow the photochemical reactions. The effects of variation of light intensity, flash duration, repetition frequency, chloroplast and substrate concentrations and phosphorylation cofactors and uncouplers support the existence of a relatively simple kinetic scheme--a rapid light activation followed by a slower first-order dark recovery--for each photoreaction. The slow dark step for the Hill reaction at room temperature has a rate constant of 30 sec^{-1} in the presence of phosphorylation cofactors or of the uncoupler methylamine.

The results are interpreted in terms of the two light reaction mechanism of photosynthesis and are shown to be in agreement with most other recent measurements of functional unit and intermediate pool sizes.

Introduction

Emerson and Arnold studied the photosynthetic evolution of oxygen by *Chlorella* in flashes of light of 10^{-5} sec duration from a neon discharge tube (Emerson and Arnold, 1931, 1932). Their results show oxygen evolution to exhibit characteristic saturation behavior as a function of flash intensity. At saturating intensities they found that one oxygen molecule was evolved in a single flash per 2500 chlorophyll molecules. This maximum efficiency of oxygen evolution per flash was found to occur when the light flashes were separated by at least 0.02 sec at 25°C. From these results they proposed the existence of a photosynthetic unit of about 2500 chlorophyll molecules with a slow dark step of 0.02 sec at room temperature.

The same size of photosynthetic unit (2000 chlorophylls) and the same limiting dark time were found for the quinone Hill reaction in *Chlorella* using the same flash length (Clendenning and Ehrmantraut, 1950).

Kohn (1936), Briggs (1941), Weller and Franck (1941), and Tamiya and Chiba (1949) found greater yields (smaller photosynthetic units) and longer dark times than those of Emerson and Arnold. These workers used flash lengths that were long ($\geq$0.6 msec) compared to the 10^{-5} sec flashes employed by Emerson and Arnold. Ehrmantraut and Rabinowitch (1952), using 10^{-4} sec flashes, reconfirmed the short flash results for overall photosynthesis and the results of Clendenning and Ehrmantraut for the quinone Hill reaction. They were the first to point out the importance of the flash length.

Gilmour, Lumry, Spikes and Eyring (1954) found that long and short flashes give different results in the ferricyanide Hill reaction of sugar beet chloroplasts. They suggested that a reservoir of photosynthetic products must be filled in order to obtain the increased yield and long dark period characteristic of long flashes, and that a flash of 10^{-5} sec is too short to fill this reservoir, regardless of intensity. Thus, they concluded that there is a pool of photo-reducing power that is operative only at high light intensity and that it is located in the Hill reaction part of photosynthesis. A comprehensive review of the early photosynthetic work performed in flashing light can be found in Chapter 34 of Rabinowitch's book, Photosynthesis (1956).

Allen and Franck (1955) explained the effect of flash duration by postulating a limiting enzyme (one for every 2000 chlorophylls). If this enzyme has a working period of approximately 10^{-4} sec then it can turn over many times during a long flash. This mechanism requires the storage of chemical energy somewhere between the first enzyme and the slow step, and so is essentially the same as that proposed by Gilmour, et al. (1954).

Kok (1956) studied photosynthetic oxygen evolution in Chlorella using flashes which varied from 0.2 msec 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 to 320 msec, had 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 partial reactions of oxygen evolution in the Hill reaction and of cytochrome c reduction by reduced trimethyl-p-benzoquinone ($TMQH_2$). The Hill reaction using 2,6-dichlorophenolindophenol (DCPIP) 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 3-(3,4-dichlorophenyl)-1,1-dimethylurea (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 the broken chloroplast preparations used in this 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,

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

(2) $\text{Reactants} + Q^* \xrightarrow{k_1} Q + \text{Products}$

where Q is a trapping site or a rate-limiting intermediate and $Q + Q^* = Q_o$ 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 ($t_f << 1/k_1$) 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_o, 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

(3) $P_f = Q_o + k_1 Q_o t_f = Q_o(1 + k_1 t_f)$.

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

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

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

(5) $$Q^* = Q_o e^{-k_1 t_d}$$

and

(6) $$Q = Q_o - Q^* = Q_o (1-e^{-k_1 d t}) .$$

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

(7) $$P_f = Q_o(1-e^{-k_1 t_d}) + Q_o k_1 t_f .$$

Equation (7) predicts that a plot of P_f vs t_f will be linear (as observed by Kok, (1956) with a slope of $k_1 Q_o$ and an intercept of $Q_o(1-e^{-k_1 t_d})$. For sufficiently long dark times, as in most of the experiments reported here, equation (7) reduces to equation (3). Thus the size of a functional unit (which we define as the number of molecules of chlorophyll <u>a</u> + <u>b</u> 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

<u>Flashing light apparatus</u>. Hill oxidant (DCPIP or ferricyanide) or cytochrome <u>c</u> 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 $\varepsilon_{580\ m\mu}$ = 19,800 l-mole^{-1}cm^{-1} at pH 7.7 for DCPIP, $\varepsilon_{420\ m\mu}$ = 1000 l-mole^{-1}cm^{-1} for ferricyanide, and $\Delta\varepsilon^{red-ox}_{549.5\ m\mu}$ = 1.9 x 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 cuvette, as described below. Light from a 1000 watt 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). 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 (λ >635 mμ) are isolated by means of a supplementary Corning 2-58 filter. The sample cuvette has aluminum foil taped to its far side to increase absorption of the exciting light by the chloroplasts, while the reference cuvette has black tape on its adjacent side so that it is not exposed to the actinic light.

Fig. 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 x 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 to 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 cuvette is ca. 70 mwatts-cm^{-2} at maximum lamp voltage (referred to in the text as maximum intensity).

Reagents. Spinach [Spinacia oleracea L. var. Viroflay grown in vermiculite in the open air (Jensen and Bassham, 1966) or Spinacia oleracea var. Early Hybrid No. 7 grown in a growth chamber under the controlled conditions described previously (Sauer and Park, 1965)] was harvested four to eight 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 and 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 duPont, Wilmington. 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,* 0.05 M; sucrose, 0.35 M; and the following in μmoles/ml: cytochrome c, .050; $TMQH_2$, .055; DCMU, .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 μmoles/ml: tricine pH 7.4, 35; sucrose, 350; potassium ferricyanide, 0.26; 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. Chlorophyll a and b concentrations were measured in 80% acetone using the extinction coefficients of McKinney (1941). Chlorophyll a to 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).

Results

DCPIP Hill Reaction.

t_f, t_d, and light saturation. Fig. 2A shows a typical light saturation curve obtained when the rate of DCPIP reduction in the sample cuvette is monitored at 580 mμ as a function of exciting flash intensity.

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*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 (Sauer and Kelly, unpublished results).

From the intercept on a reciprocal plot of these data, Fig. 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).

Fig. 3 shows the effect of the dark time on DCPIP photoreduction per flash. To obtain maximum yield, 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. Fig. 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 to 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. Fig. 5A is a saturation curve in terms of DCPIP concentration, and Fig. 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 Fig. 5B.

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 Fig. 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 concentration when the latter is less than saturating. The intercept of Fig. 4 represents the amount of DCPIP photoreduced by a pool of rate-limiting intermediates, Q_o, 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 sub-millisecond 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 μmoles/ml) instead of methylamine yields a functional unit and rate constant identical to that found in the presence of methylamine.

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 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 overall rates. The higher chloroplast concentration necessitated a larger correction to light intensity saturation than was needed for the DCPIP experiments. Fig. 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 10 chloroplast preparations. The addition of a catalytic amount of DCPIP (0.006 μmoles/ml) to the ferricyanide reaction mixture containing methylamine resulted in a small, but probably real, decrease in the size of

Table I

Functional Unit Size and Dark Reaction Velocity Constant

Reaction		Number of Determinations	Functional Unit $\left(\frac{\text{Chl a + b}}{\text{electron transferred}}\right)$	Dark Reaction Velocity Constant (sec^{-1}) (22±2°C)
DCPIP Hill	+ MA	14	56 ± 10	33.3 ± 6.9
Oxidation	- MA	14	54 ± 7.4	19.5 ± 3.1
	+ ADP, Mg^{++}, Pi	1	58	32.7
Ferricyanide Hill	+ MA	10	70 ± 10	28.0 ± 8
Oxidation	- MA	6	73 ± 9	16.6 ± 3
	+ MA + cat. DCPIP	2	62 ± 10	31.8 ± 9.2
Ferricyanide Hill	+ MA	2 *	74 ± 11	29.6 ± 7.1
Oxidation	+ MA + cat. DCPIP	2 *	62 ± 10	31.8 ± 9.2
Cyt c/$TMQH_2$	+ DCMU	5	445 ± 40	13.2 ± 4

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

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 Fig. 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.

Fig. 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.

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, Fork and Amesz (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 turn-over 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 DCPIP Hill reaction involved pigment system II, as indicated by its sensitivity to DCMU and its very different action spectrum (Sauer and Park, 1965). 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 seem, 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 (8 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) indicate 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.

Kok and Cheniae (1966), in their recent review of the oxygen evolution step of photosynthesis, cite experiments on spinach chloroplasts by Forbush in their laboratory, in which a brief (4 μsec), strong flash produced only 1 equivalent of DCPIP net reduction per 1000 chlorophylls. They did observe a rapid initial transient reduction corresponding to 1 equivalent per 500 chlorophylls; however, this reversed completely in 5 msec. Whereas the net reduction was completely inhibited by DCMU, the initial transient was unaffected by this inhibitor. The assignment of the transient to reduction by photosystem I was substantiated by its absence in a Scenedesmus mutant which lacks this photosystem. In a much slower reaction, requiring several seconds for completion, the Scenedesmus mutant is capable of reducing 1 equivalent of DCPIP per 1000 chlorophylls. Kok and Cheniae tentatively proposed chlorophyll arrays of 500 and 1000 chlorophylls for the primary trapping centers of photosystems I and II, respectively.

In other experiments on the same Scenedesmus mutant in which a long (5 sec) illumination period was used, Kok, et al. (1967) observed an additional slow reduction of DCPIP occurring during the following dark period. The pool size of intermediates (secondary traps) responsible for this latent reduction corresponds to 1 equivalent per 70 chlorophylls. This number is commensurate with and is probably more comparable to our results for "long" flashes. Filling the pool of 8 intermediates by long flashes compared with 1 for short flashes would tentatively seem to be associated with a rate-limiting step having a time constant between 4 μsec and 6 msec; however, care must be used in comparing results obtained with two appreciably different organisms. It is quite

possible that the decrease in yield of O_2 per flash from Chlorella for flash lengths shorter than 2 msec observed by Kok (1956) results from this same rate-limiting step.

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. Quantum yield measurements throughout the red region of the spectrum by Sauer and Park (1965) showed the efficiency of ferricyanide reduction to be 20 to 40% lower than that for DCPIP reduction. Furthermore, Biggins and Sauer (1964) found that the addition of catalytic amounts of DCPIP to a ferricyanide Hill reaction mixture increased the efficiency of the reaction by about 20%, although the wavelength dependence of the action spectrum for the reaction did not change. As shown in Table I, we find that a similar 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 non-cyclic flow. The occurrence of the cyclic pathway may be responsible for the observation that the ferricyanide Hill reaction is coupled to photophosphorylation (Losada, et al., 1961). The addition of catalytic amounts of DCPIP, the oxidized form of which is known to uncouple phosphorylation (Gromet-Elhanan and Avron, 1964), may partially by-pass

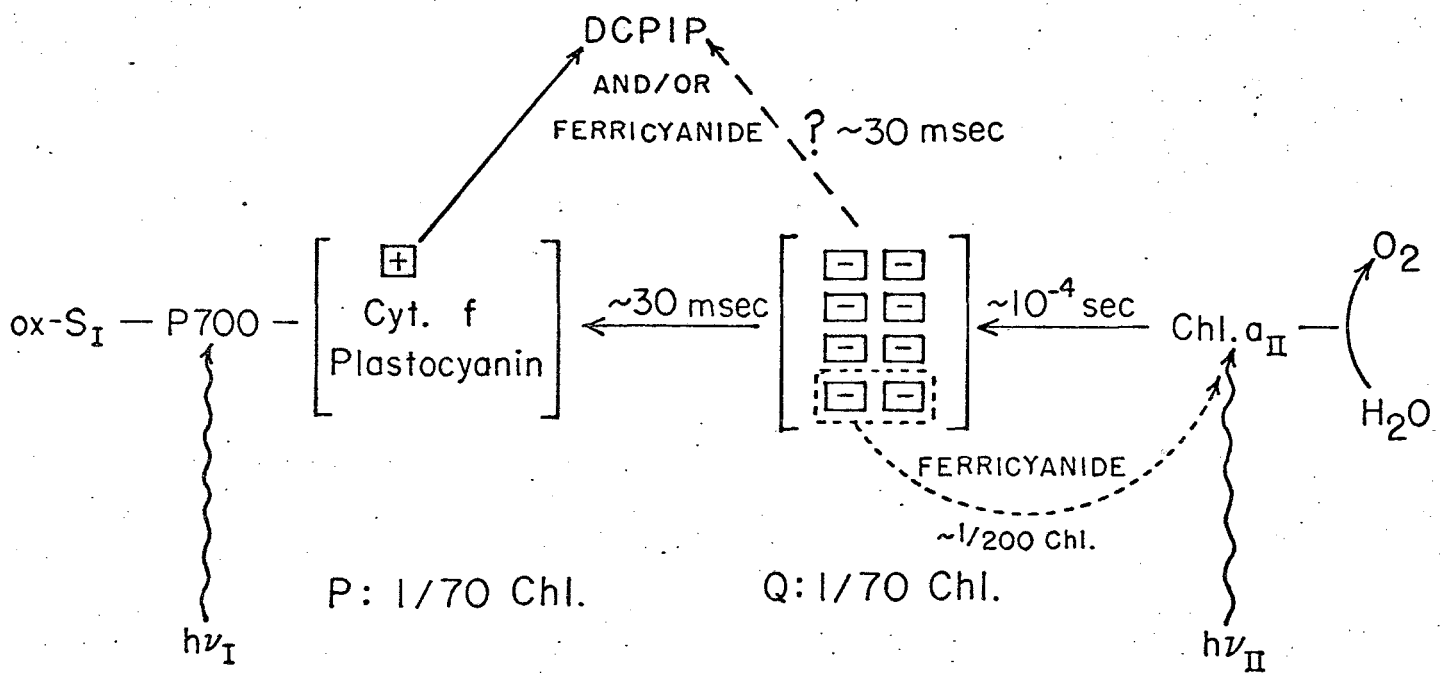
the cyclic pathway and thus enhance the efficiency of the ferricyanide Hill reaction, concommitantly reducing the apparent size of the functional unit. The studies to date leave unanswered the question of whether system I or system II pigments are responsible for the cyclic flow.

For the non-cyclic electron flow leading to oxygen evolution, the best evidence at present suggests that both DCPIP and ferricyanide Hill oxidants utilize only system II. Experiments designed to detect the presence of an Emerson enhancement stimulated by supplementary far-red light, indicative of cooperative action between system I and system II, have demonstrated the absence of such an effect both for the DCPIP (Sauer and Park, 1965) and for the ferricyanide (Avron, 1966) Hill reactions. The work of Forbush on the Scenedesmus mutant, cited above, further supports the conclusion that system I activity is not required for the net reduction of DCPIP (Kok, et al., 1967).

The pool sizes of electron transport intermediates between system I and system II have been measured recently by several kinetic methods. Witt, et al. (1966), from studies of light-induced absorption changes of endogenous components of Chlorella, concluded that there exists a pool of ca. 12 equivalents per P700, capable of receiving electrons from system II. From the wavelength dependence of the absorption changes these intermediates appear to be plastoquinone. An additional pool of electron acceptors, containing 6 equivalents per P700 and lying closer to system I, rapidly reoxidizes half the molecules in the first pool. The acceptors associated with system II can again be reduced if the flash is sufficiently long (> 10^{-2} sec). The removal

of the electrons from these intermediates by the system I photoreaction is relatively slow (> 0.12 sec). It would appear that, if all of these intermediates were to operate between light reaction II and the site of reduction of DCPIP or ferricyanide, the functional unit observed for the Hill reaction would be 18 times smaller than that for system I. Our observed ratio of 8 is significantly smaller than this, suggesting that the exogenous oxidants such as DCPIP and ferricyanide receive electrons from only a portion of the combined pools of Witt, et al. Although this qualitative conclusion is probably correct, precise comparisons of pool sizes reported by different experimenters may be misleading. For example, the calculations based on observed light-induced absorption changes by Witt, et al. (1966) and by Anderson, Fork and Amesz (1966) invoke assumed values for the extinction coefficients of P700 that differ by 20%. The close agreement of our value for the functional unit for cytochrome c reduction to the chlorophyll/P700 ratio of Anderson, Fork and Amesz causes us to favor these workers' results; however, this introduces additional assumptions of identity which are presently unproven.

The results reported in this paper appear to be largely consistent with those from the laboratories of Kok, Witt and Joliot (vide infra), 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 Chl 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


DCPIP
AND/OR
FERRICYANIDE
? ~30 msec
ox-S_I — P700 —
Cyt. f
Plastocyanin
~30 msec
~10^-4 sec
Chl. a_II
O2
H2O
FERRICYANIDE
~1/200 Chl.
P: 1/70 Chl.
Q: 1/70 Chl.
hν_I
hν_II

XBL 679-6196

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 Chl) of Malkin and Kok (1966), the A_2 (1/70 Chl) of Joliot (1965), which are perhaps identical to the electron acceptors associated with system I (plastocyanin, cytochrome f and P700) of Witt, et al. (1966)].*

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 Fig. 3. When the same data are plotted in semi-logarithmic fashion, as in Fig. 8, they are found to result from a single first-order process. The rate constant for this dark decay process at room temperature (22 ± 2°C) is ca. 32 sec^{-1} in the presence of methylamine or phosphorylation cofactors, but it is about half this value when these compounds are missing (e.g., for the experiment described in Figs. 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

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*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; and Kok, 1956) present evidence that there are more Q than Chl a_{II}.

step at or near the site of coupling to non-cyclic 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.

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 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 μmoles/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.

Further information about the pools of intermediates associated with photosystem II has been reported by Joliot (1961). For dark adapted

Chlorella pyrenoidosa a burst of oxygen corresponding to one O_2 per 140 chlorophylls (1 equivalent per 35 chlorophylls) occurs during the first second of illumination by intense light. He attributes this burst to the accumulation during the prior dark period of a pool of an oxidized compound, designated A, of equivalent size. Joliot's compound A closely resembles the Q of Kok in its properties. For very short flashes (0.1 msec) of saturating light, on the other hand, the oxygen produced per flash is only 1/10 of this amount. This originates from a second, smaller pool of intermediates (1 equivalent per 350 chlorophylls) which he designates compound E and which may be Chl a_{II} in the notation of Witt. For such short flashes it is necessary to initiate the process (in Joliot's scheme, to generate the active form of E) by a prior flash which produces no oxygen or by a steady background illumination of low intensity. During longer periods of illumination the active form of E can be regenerated during the light pulse. From studies of the yield per flash as a function of flash duration in the range 10^{-4} to 0.2 sec the regeneration of A appears to be diphasic, with rate constants of about 70 and 7 sec^{-1}, respectively (Joliot, 1965). This is interpreted as giving evidence of the existence of two kinetically distinguishable components, A_1 and A_2, in the pool A. In Chlorella the amount of A_1, the rapid component, appears to be one-quarter of the total pool of A, which latter is estimated to constitute 1 equivalent per 25 chlorophylls from these experiments. Following the cessation of illumination there is a slow (40 sec) phase of extra oxygen uptake (apart from that attributable to respiration) associated with the formation of a reduced product, PH, by system I and estimated to correspond to 1 equivalent per 140 chlorophylls.

When isolated chloroplasts, instead of whole Chlorella cells, were examined by de Kouchkovsky and Joliot (1967) evidence was again found for two intermediate compounds in photosystem II. The one corresponding to A was present at a concentration of 1 equivalent per 70 chlorophylls and that corresponding to E at 1 per 2800 chlorophylls. 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. Evidence of PH, associated with photoreaction I, was also obtained; however, its concentration was not estimated.

The fluorescence induction studies of Malkin and Kok (1966) and their detailed kinetic analysis by Malkin (1966) provide another measure of pool sizes. The complementary relationship between fluorescence and photochemistry in the utilization of absorbed light quanta permits the estimation of pool sizes of intermediates from the kinetics of the fluorescence increase upon illumination. In this manner Malkin and Kok determined the presence of two intermediates, designated P and Q, associated with photosystem II in isolated chloroplasts. Upon illumination with saturating light the pool of Q (1 equivalent per 70 chlorophylls) rapidly becomes reduced. This is followed by a thermal reaction (k_1 = 30-40 sec^{-1}) in which electrons are transferred from reduced Q to the pool of P (also 1 equivalent per 70 chlorophylls). Each of these pools is identical in size to the pool of A observed by de Kouchkovsky and Joliot (1967) from oxygen burst measurements on isolated chloroplasts and is very close to that calculated from our results on Hill oxidant utilization. It is reasonable to conclude that the same pools of photosystem II intermediates are involved in each of these studies.

The rate constant for the dark decay process in the Hill reaction at room temperature as measured here is ca. 30 sec^{-1} in the presence of the cofactors of phosphorylation or of the phosphorylation uncoupler methylamine. This rate-limiting regeneration step is responsible for the dependence of the yield per flash on the duration of the dark interval between flashes, as shown in Figs. 3 and 8. 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 per 60 to 70 chlorophylls) in isolated chloroplasts. The oxygen burst studies on chloroplasts by de Kouchkovsky and Joliot (1967) also demonstrated a small pool of intermediates (1 per 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.

Conclusion

Using partial reactions associated with photosystems I and II, respectively, we have been able to estimate the concentration, relative to chlorophyll, of the reaction centers or pools of intermediates closely associated with them in broken spinach chloroplasts. Relatively long flashes (6-100 msec) of saturating red light produce a maximum yield of photoreactions corresponding to 1 equivalent per 445 chlorophylls for system I and 1 equivalent per 55 chlorophylls for system II. From other published results it appears that the larger system II unit results from a secondary pool of intermediates and that very short flashes (10^{-4} sec) would produce evidence for a primary rate-limiting component in smaller concentration. 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 msec, but is reduced to 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.

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FIGURE CAPTIONS

Fig. 1. Traces of the light pulse shape. The flash duration is measured at half maximum intensity giving A) 32 msec and B) 6 msec. Rise and decay times are about 2 msec.

Fig. 2A. Intensity dependence of the DCPIP Hill reaction (without methylamine) by spinach chloroplasts in flashing light. The flash duration is 19 msec, flash frequency 2 sec, and $A_{678}^{1\ cm}$ of the chloroplasts 0.240. The curve is calculated from the reciprocal plot in Fig. 2B.

Fig. 2B. Double reciprocal plot of the data in Fig. 2A. The intercept represents the reciprocal of the maximum rate (R_{∞}^{-1}) which would occur at infinite light intensity.

Fig. 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 $A_{678}^{1\ cm}$ = 0.270 for the chloroplast suspension. The curve is calculated from equation 7 using experimentally derived parameters.

Fig. 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 frequency of 2.0 sec, chloroplast $A_{678}^{1\ cm}$ = 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 Fig. 3.

FIGURE CAPTIONS (Cont.)

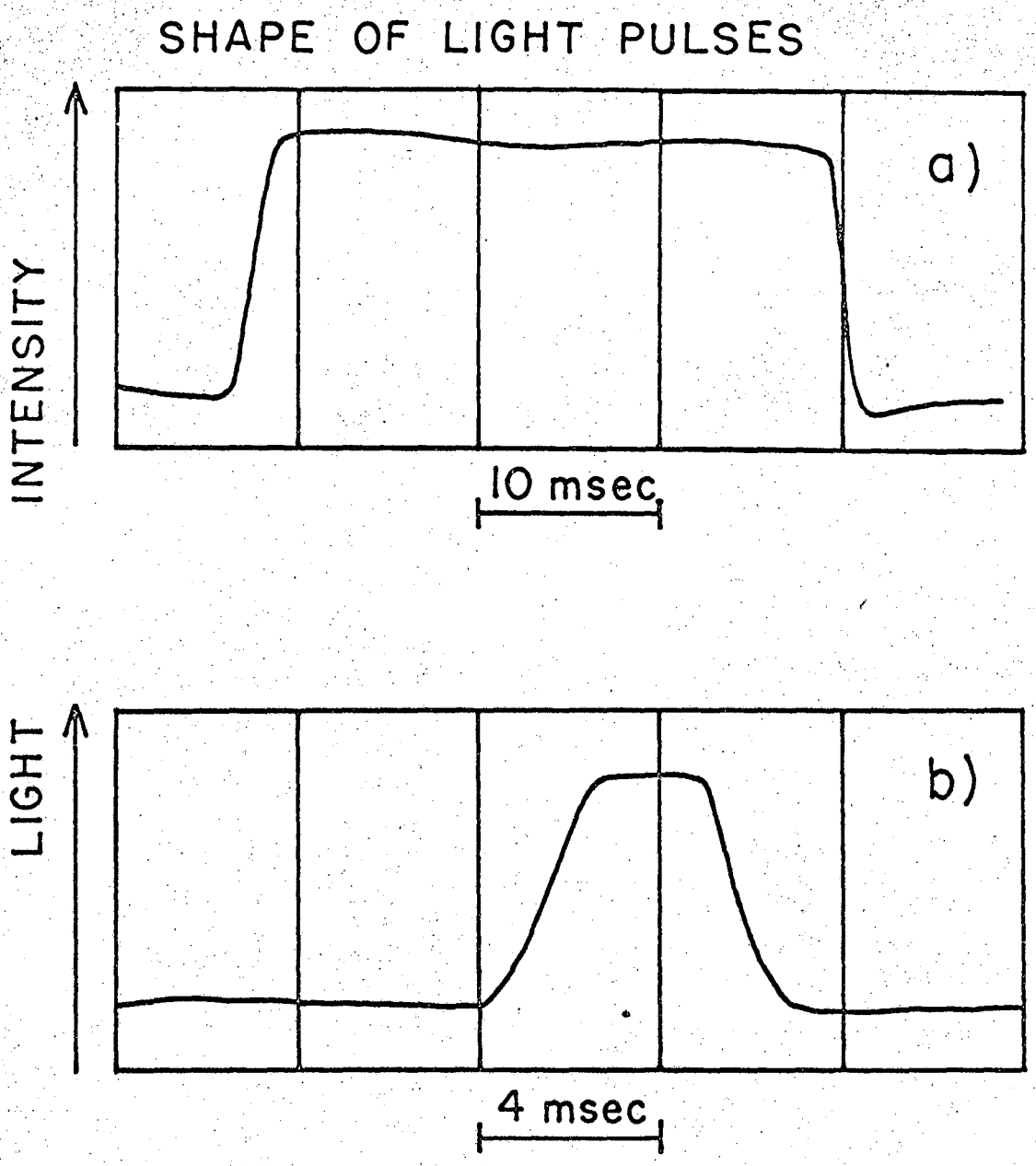
Fig. 5A. Maximum flash rate [R] dependence on DCPIP concentration for the DCPIP Hill reaction ± MA by spinach chloroplasts. The flash duration is 50 msec, flash frequency 2 sec, and $A_{678}^{1\ cm}$ = 0.270 for the chloroplasts. The different symbols denote separate chloroplast preparations. The curve is calculated from the reciprocal plot in Fig. 5B.

Fig. 5B. Reciprocal plot of the data in Fig. 5A. The intercept represents the reciprocal of the rate under saturating light conditions and where the DCPIP concentration is no longer limiting.

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

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

Fig. 8. Semi-logarithmic plot of P_f as a function of dark time between flashes. The data is the same as that for Fig. 3 and the graph indicates that the process involved is first-order.



XBL 679-6175

Fig. 1

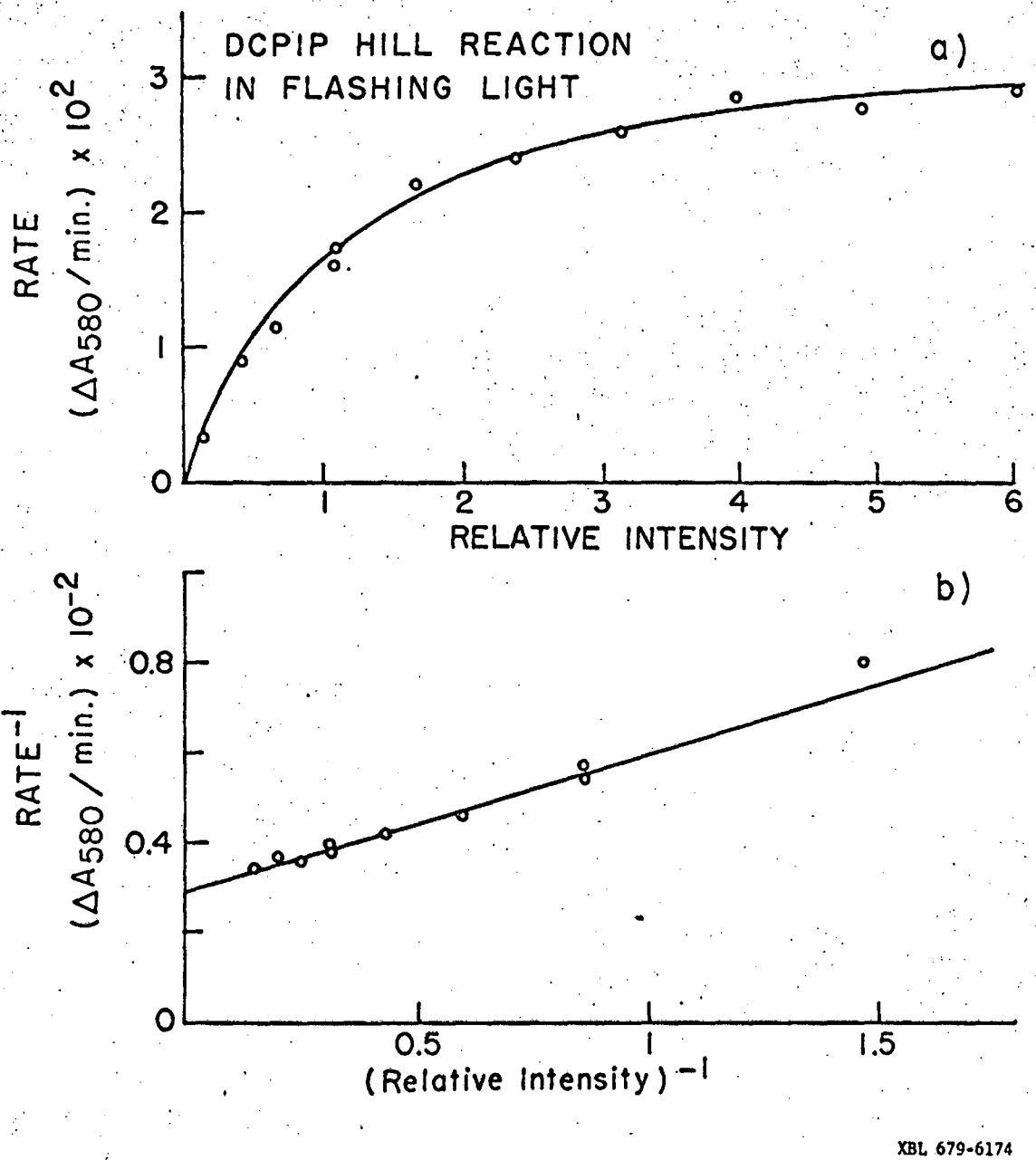
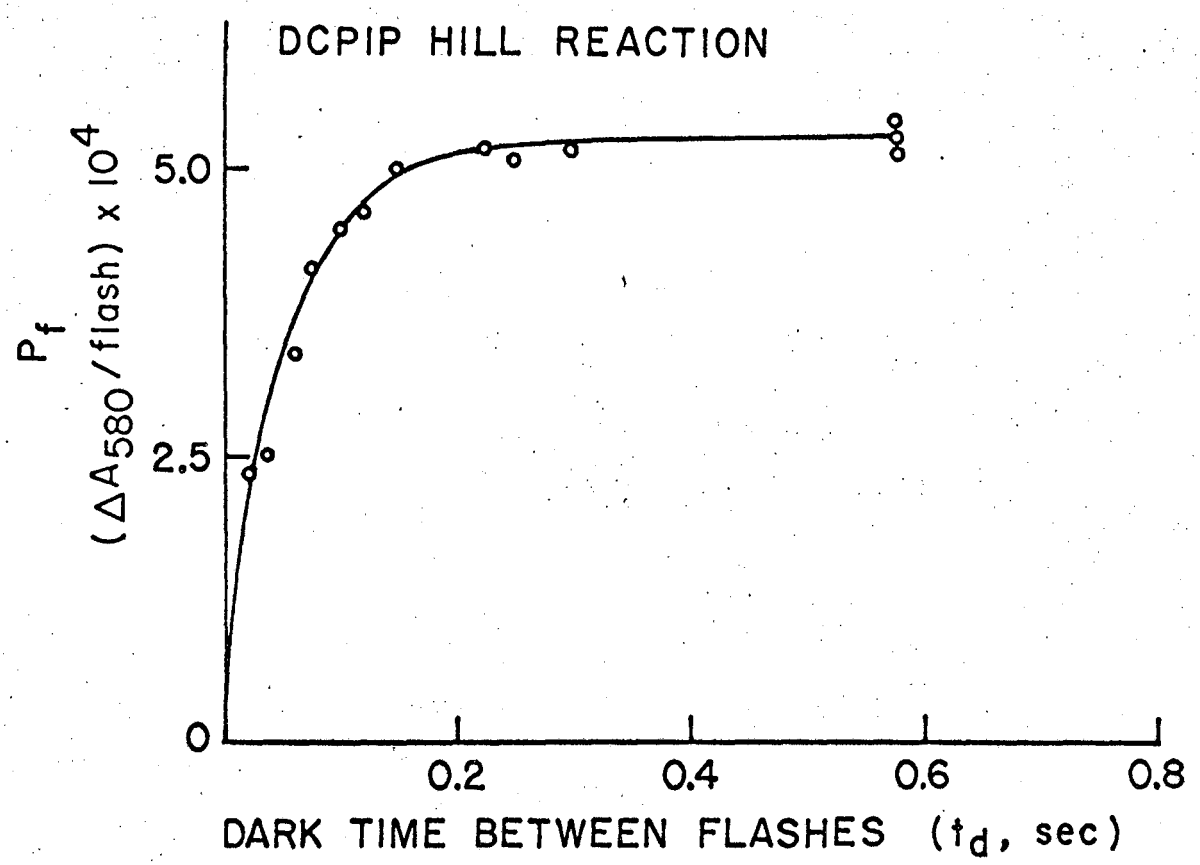
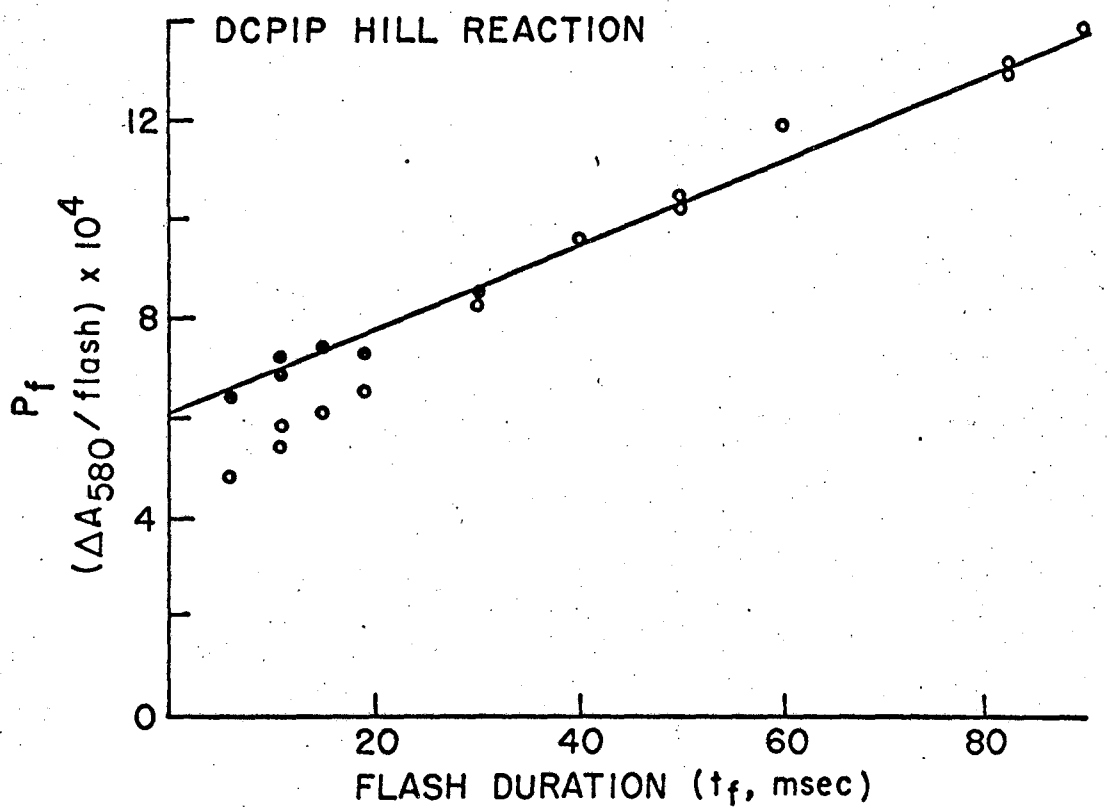

DCPIP HILL REACTION
IN FLASHING LIGHT
a)
RATE
(ΔA580/min.) x 10^2
3
2
1
0
1
2
3
4
5
6
RELATIVE INTENSITY
b)
RATE^-1
(ΔA580/min.) x 10^-2
0.8
0.4
0
0.5
1
1.5
(Relative Intensity)^-1
XBL 679-6174

Fig. 2



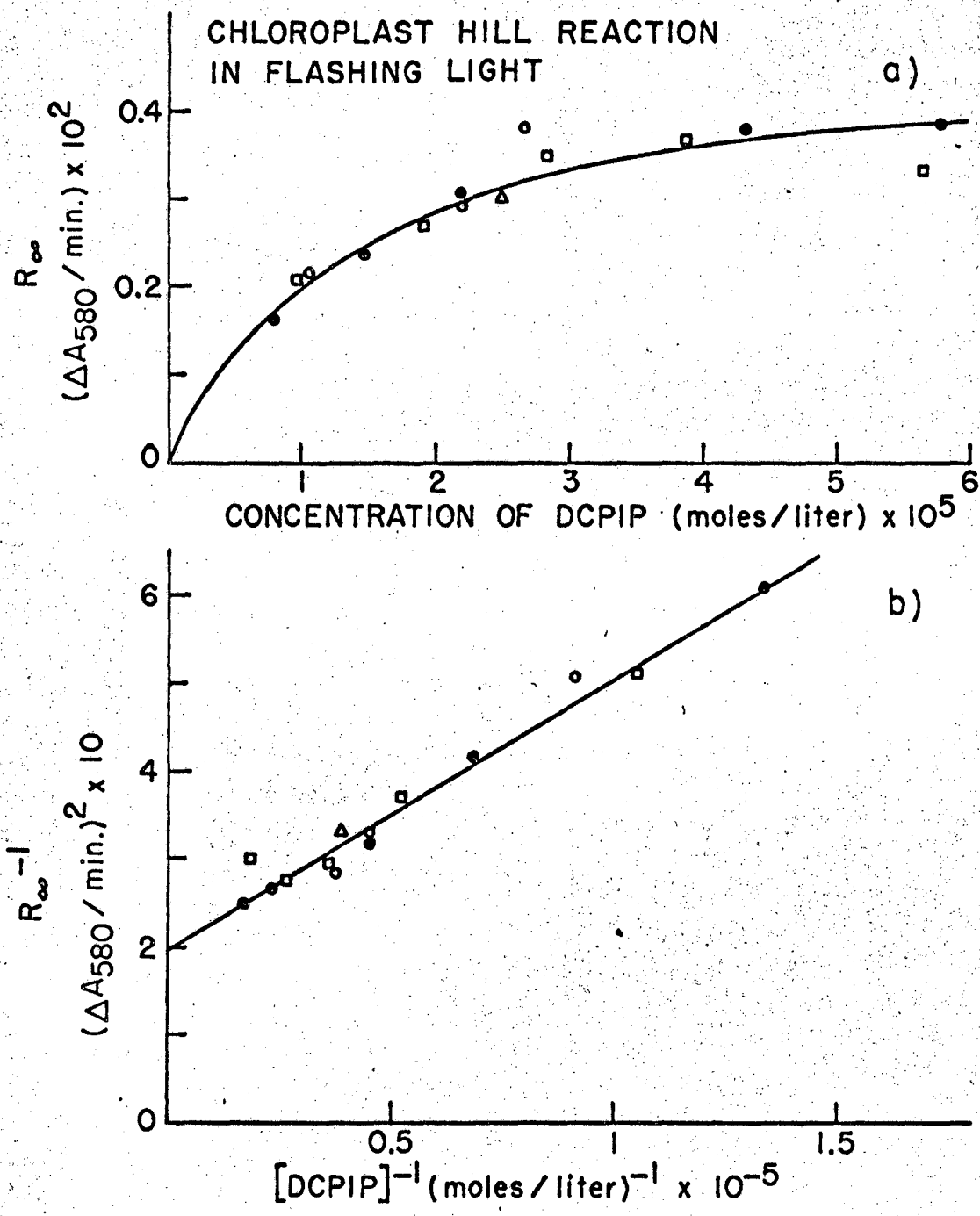
XBL 679-6179

Fig. 3



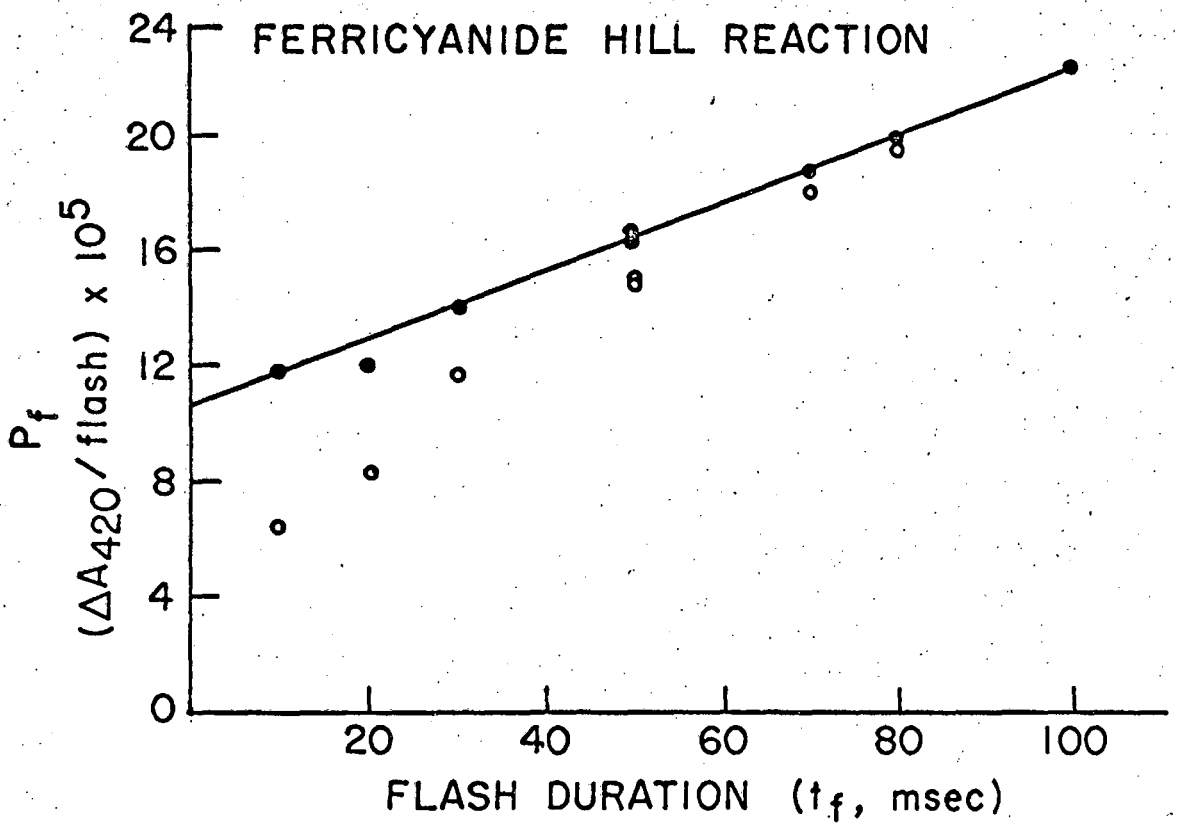
XBL 679-6173

Fig. 4



XBL 679-6172

Fig. 5



XBL 679-6170

Fig. 6

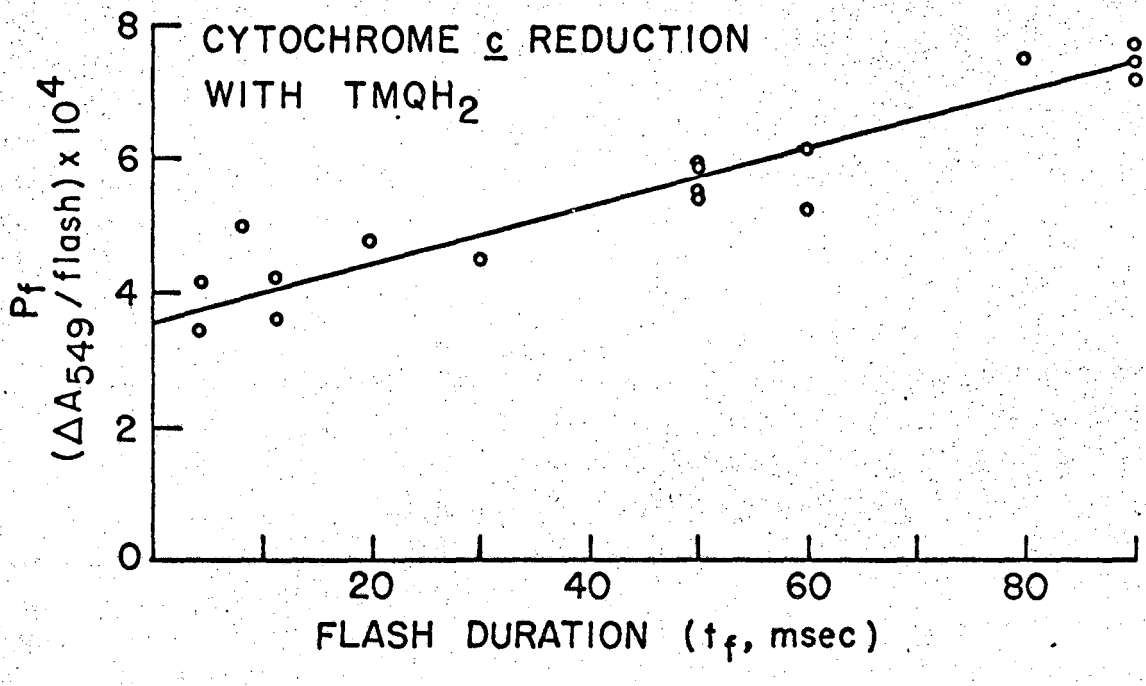


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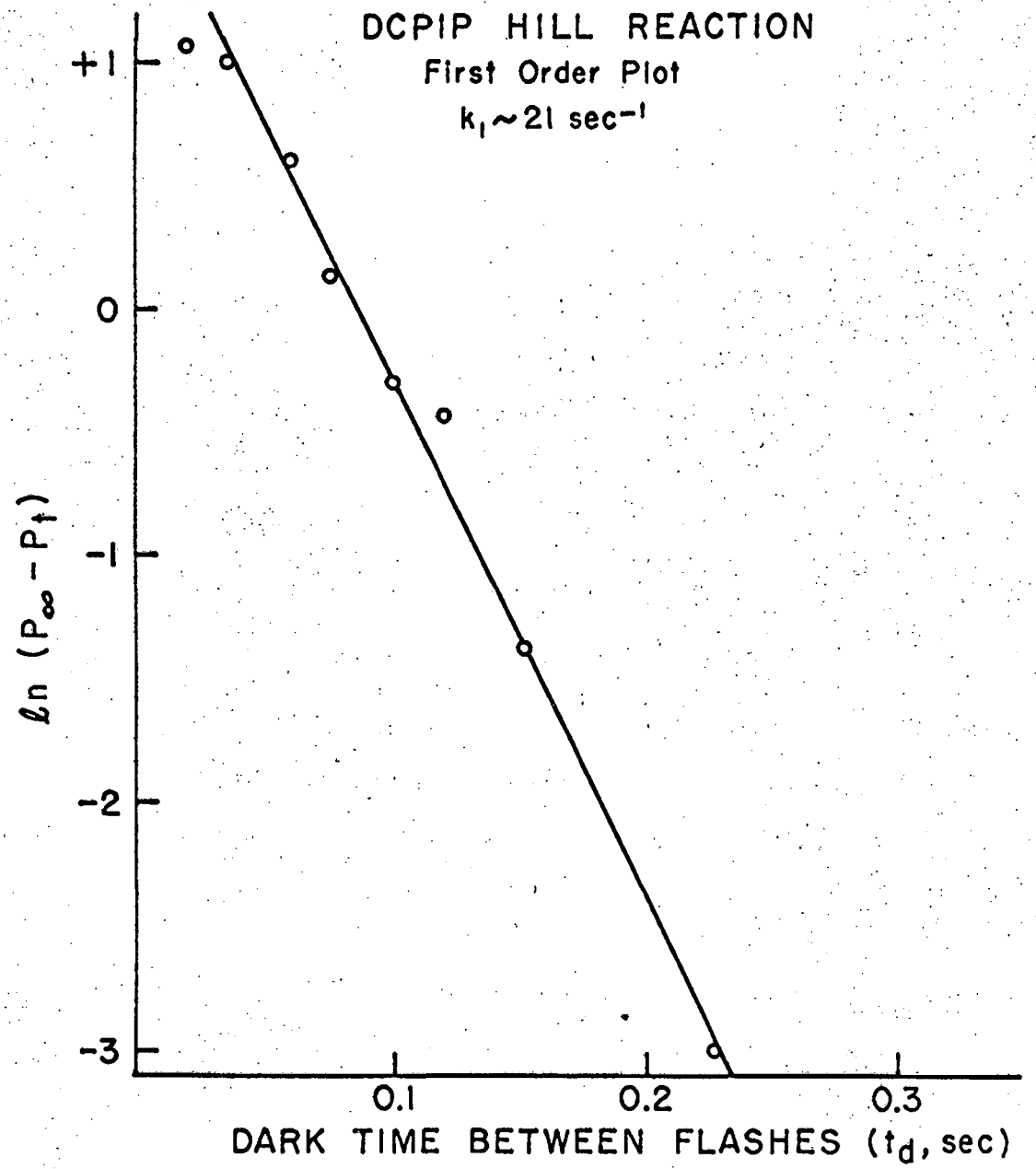
Fig. 7



XBL 679-6180

Fig. 8