The Hill Reaction of Chloroplasts. Action Spectra and Quantum Requirements*

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ABSTRACT: Chloroplasts isolated from spinach carry out the Hill reaction yielding molecular oxygen with quantum requirements as low as 2 quanta per electron transferred from water to 2,6-dichlorophenolindophenol (DCPIP) or 3 quanta per electron transferred from water to ferricyanide. Factors which favor an efficient reaction with low quantum requirements are (1) low-incident light intensity; (2) the presence of adenosine diphosphate, magnesium ion, and phosphate; (3) addition of a phosphorylation uncoupler, such as methylamine; (4) initial ferricyanide concentration of $\sim 3 \times 10^{-4}$ M; (5) excitation wavelengths between 635 and 660 mμ; and (6) the use of young (4–6 week old) spinach leaves grown under winter day length and temperature conditions.

The quantum requirement as a function of wavelength for both Hill oxidants is reasonably independent of wavelength from 635 to 660 mμ, but shows a marked increase (10-fold) starting at wavelengths greater than 690 mμ. The action spectrum of the Hill reaction is characteristic of pigment system II, including chlorophyll *b* and at least part of chlorophyll *a*. No significant enhancement was observed for DCPIP photoreduction when 650-mμ light was combined with 688-mμ light. The results are in accord with a two-pigment system of photosynthesis in which there is no transfer of electronic excitation energy from one pigment system to the other. Evidence is presented that chemical intermediates on the pathway to oxygen evolution may have lifetimes of many seconds.

Chloroplasts isolated from higher plants are capable of carrying out the photochemical evolution of oxygen from water using a variety of oxidizing agents such as ferricyanide, quinones, dyes such as 2,6-dichlorophenolindophenol (DCPIP),[1] and, under appropriate circumstances, nicotinamide–adenine dinucleotide phosphate (NADP) (Hill, 1937, 1939; Holt and French, 1948; Arnon *et al.*, 1957). This reaction, known as the Hill reaction, is of interest because it produces a substantial conversion of light energy into chemical potential and because its mechanism is believed to be related to at least part of the energy-conversion pathway of photosynthesis.

The Hill reaction can be coupled to the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (Arnon *et al.*, 1958; Avron and Jagendorf, 1959). The rate of the Hill reaction with ferricyanide as oxidant is enhanced by phosphorylation cofactors and/or by uncouplers of photophosphorylation (Arnon *et al.*, 1958; Krogmann *et al.*, 1959; Good, 1960). Using a broad band of red exciting light, Ehrmantraut and Rabinowitch (1952) observed quantum requirements for the Hill reaction corresponding to about 2.6 quanta per electron. The action spectrum of the Hill reaction corresponds closely to the absorption spectrum of isolation chloroplasts, except at wavelengths longer than 680 mμ where the quantum requirement shows a pronounced increase (Chen, 1952; Biggins and Sauer, 1964). The quantum requirement for the ferricyanide Hill reaction in monochromatic light at 675 mμ decreases sharply with decreasing light intensity and extrapolates at zero light intensity to a value of 2.0 quanta per electron transferred to the oxidant (Lumry *et al.*, 1957).

In this study the absolute quantum requirement for the Hill reaction has been studied as a function of light intensity, wavelength of excitation, presence of phosphorylation cofactors or of a phosphorylation uncoupler, and growth conditions of the plants from which chloroplasts are obtained. The intensity dependence of the quantum requirement of the Hill reaction is strongly decreased by the addition of either ADP, Mg^{2+}, and phosphate, or the phosphorylation uncoupler methylamine. Quantum requirements extrapolated to zero light intensities at any given wavelength, however, are independent of whether the phosphorylation cofactors or methylamine are present. These zero-intensity quantum requirements are very close to 2.0 quanta per electron transferred to DCPIP (3.0 quanta per electron for ferricyanide) from 635 to 660 mμ. The quantum requirements increase at wavelengths longer than 690 mμ to greater than 20 quanta per electron at 730 mμ. The action spectrum of the Hill reaction is characteristic of a pigment system containing chlorophyll *b* and part of the chlorophyll *a*. The results are consistent with a

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[1] Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; NADP, nicotinamide–adenine dinucleotide phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

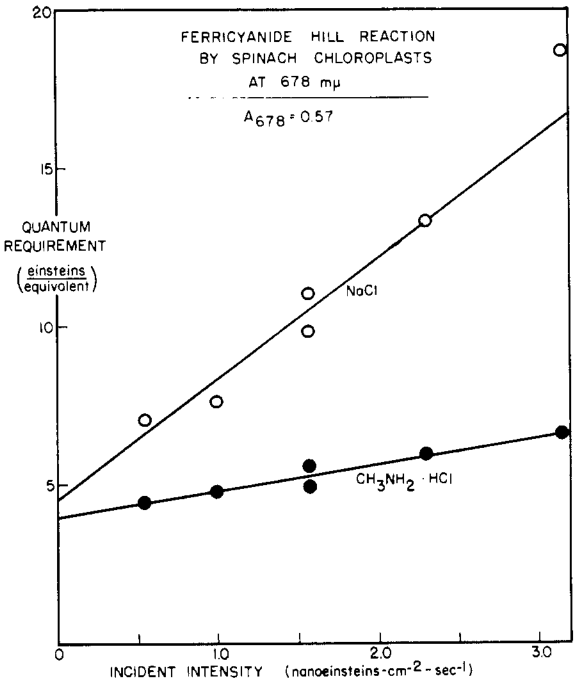

FIGURE 1: The dependence of quantum requirement on incident light intensity at 678 mμ for the ferricyanide Hill reaction by spinach chloroplasts with and without added methylamine. Chloroplasts sufficient to give an absorbance of 0.57 at 678 mμ were added to the standard reaction mixture.

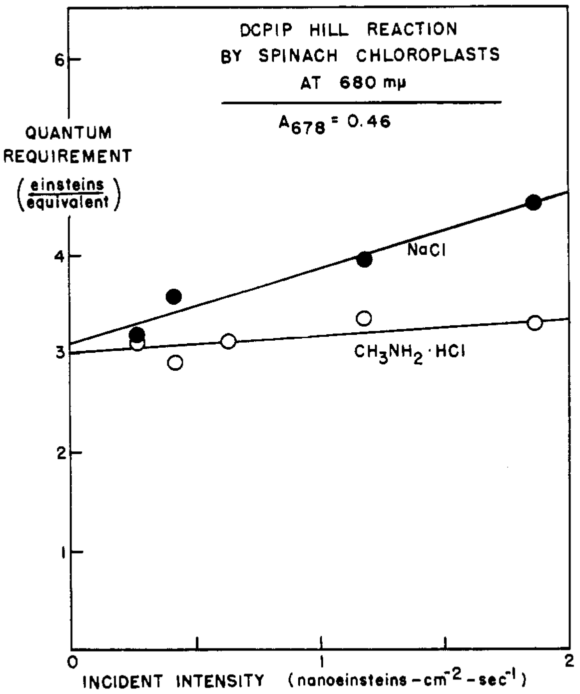

FIGURE 2: The dependence of quantum requirement on incident light intensity at 680 mμ for the DCPIP Hill reaction by spinach chloroplasts with and without added methylamine. Chloroplasts sufficient to give an absorbance of 0.46 at 680 mμ were added to the standard reaction mixture.

two-pigment system mechanism of photosynthesis in which there is no interaction between the pigment systems at the level of electronic excitation. Evidence is presented that intermediates on the pathway to oxygen evolution may have lifetimes of many seconds.

Experimental Procedures

Preparation of Chloroplasts. Chloroplasts prepared from spinach obtained from several sources were compared. For most of the work reported here, leaves of *Spinacia oleracea* var. Bloomsdale were obtained from plants grown from seed in a growth chamber. Plants were grown in soil with a 10-hr photoperiod with 80°F day temperature and 55°F night temperature. Plants were harvested between 4 and 8 weeks following germination. Typically, 10 g of leaves was homogenized for 20 sec in 50 ml of 0.5 M sucrose, 0.1 M (K)PO_4, pH 7.4, buffer in a micro Waring Blendor attachment. The resultant homogenate was strained through 8 layers of cheesecloth and centrifuged according to Park and Pon (1961) to isolate once-washed chloroplasts. These chloroplasts were suspended in 3–5 ml of 0.5 M sucrose, 0.05 M (K)PO_4, pH 7.4, and stored at 0° until used. In some cases commercial spinach, fully grown, was used for the sample material. These instances are noted in the text.

Reagents. Stock solutions of ferricyanide, ferrocyanide, and DCPIP (K & K Laboratories, Jamaica, N. Y.) were always prepared freshly on the day of the experiment. The stock DCPIP solution was prepared in 0.05 M (K)PO_4 buffer at pH 7.45, and the concentration was confirmed by absorbance measurements after the undissolved dye had been removed by centrifugation. The extinction coefficients of Armstrong (1964) were used. ADP was obtained from the Pabst Brewing Co., Milwaukee, Wis., and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) from duPont de Nemours & Co., Wilmington, Del. Methylamine hydrochloride was dissolved in distilled water and titrated to pH 7.4 with dilute NaOH.

Reaction Mixtures. For the ferricyanide Hill reaction, the standard reaction mixture contained, in μmoles/ml: potassium phosphate, pH 7.45, 35; sucrose, 350; potassium ferricyanide, 0.26; potassium ferrocyanide, 0.26; methylamine hydrochloride or sodum chloride, 10.0. For the indophenol Hill reaction, 0.03 μmole/ml of DCPIP was used in place of the ferricyanide and ferrocyanide. Sufficient chloroplast preparation was added in the dark at the start of each measurement to give an absorbance of 0.3–0.7 at 678 mμ (1-cm path). Each experiment was performed using 2 ml of reaction mixture which was divided between the sample cuvet and the reference cuvet in the spectrophotometer. Variations of these conditions are noted in the text.

Apparatus. Experiments were carried out using a

Cary Model 14 spectrophotometer modified so that the absorbance of the Hill oxidant could be monitored continuously while the sample was being irradiated from the side with longer wavelength light, as described elsewhere (Sauer and Biggins, 1965). Ferricyanide concentration was monitored with the Cary 14 at 420 mμ (ϵ_{420} 1000 l. mole^{-1} cm^{-1}) using a broad-band interference filter in front of the photomultiplier, and DCPIP concentration was monitored at 580 mμ (ϵ_{580} 19,800 l. mole^{-1} cm^{-1} at pH 7.4) using a Corning 4-96 filter.

As in the previous study, the cuvets used had four clear sides and a rectangular internal cross section 3 mm × 10 mm. The monochromatic measuring beam passed through the 10-mm path length, and the actinic light, incident at right angles on the sample cuvet, traversed a 3-mm light path in the reaction mixture. Light intensity was measured with a calibrated silicon solar cell (Hoffman, Type 120 CG) placed at the site of the cuvet; corrections for reflection losses were applied. The inner filter effect was taken into account in calculating quantum requirements for the DCPIP Hill reaction (Biggins and Sauer, 1964).

Results

Light Intensity, Methylamine, and Phosphorylation Cofactors. The ferricyanide Hill reaction shows a linear increase in quantum requirement with increasing incident light intensity, as was first shown by Lumry *et al.* (1957). The dependence is very similar to that reported previously for NADP reduction (Sauer and Biggins, 1965). The presence of methylamine, a phosphorylation uncoupler, at concentrations of 10^{-3} M or of ADP, Mg^{2+}, and inorganic phosphorus (P$_i$), or of both together, largely removes the intensity dependence. As seen in Figure 1, the presence of methylamine (or of phosphorylation cofactors, not shown) has no effect on the quantum requirement extrapolated to zero light intensity. In other words, light saturation of the Hill reaction begins at much higher intensities when phosphorylation either occurs rapidly or is uncoupled.

Figure 2 shows that a similar effect of methylamine occurs for the indophenol Hill reaction. In the absence of the uncoupler, the intensity dependence of the quantum requirement is less pronounced than with ferricyanide. This may reflect the fact that oxidized indophenol itself is a strong phosphorylation uncoupler (Gromet-Elhanan and Avron, 1963, 1964; Shen *et al.*, 1963). The addition of 1.9 × 10^{-5} M DCMU, a substance known to inhibit oxygen evolution, increased the quantum requirement for ferricyanide reduction over 100-fold at 678 mμ, both in the presence and absence of methylamine.

Comparison of measurements made using a number of chloroplast samples prepared by identical procedures either from growth-chamber spinach or from commercial spinach showed the latter to be much less reproducible than the former. Preparations from commercial spinach frequently exhibited quantum requirements three or four times as great as those observed uniformly with growth-chamber spinach. For commercial spinach no consistent correlations appeared with season, condition of the leaves, etc. The effects of light intensity and phosphorylation uncouplers occurred with commercial spinach as well as with growth chamber spinach.

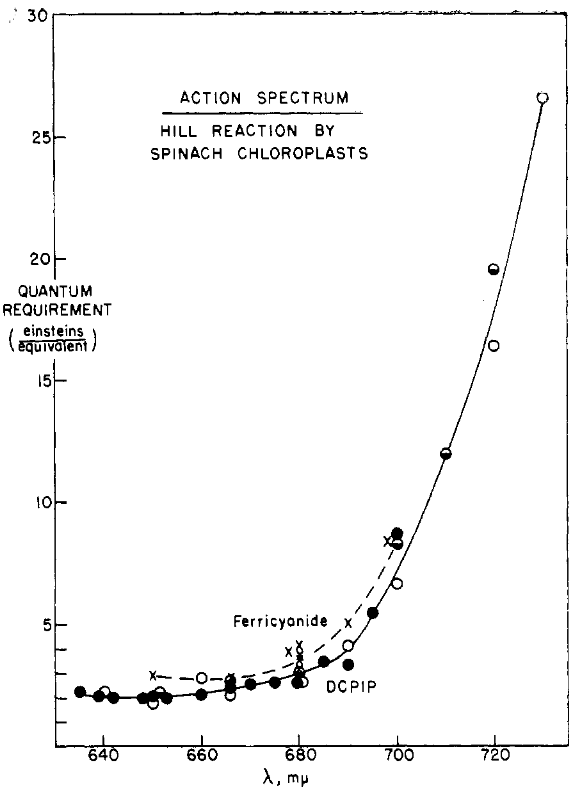

FIGURE 3: Action spectra for the Hill reaction by isolated spinach chloroplasts in the presence of methylamine using DCPIP (three different chloroplast preparations, ○, ●, ◒) or ferricyanide (×) as the oxidant. The quantum requirements are values obtained from extrapolations to zero light intensity at each wavelength.

Initial Concentrations of Ferricyanide and Ferrocyanide. Lumry and Spikes (1957) showed that the rate of the ferricyanide Hill reaction is dependent on the initial concentration of ferricyanide. We found, in the presence of methylamine, an optimum ferricyanide concentration around 2.6 × 10^{-4} M. At half this initial concentration the rate was about 15% less, and at 2.5 or 5 times greater concentration it was about 30% less.

Brewer and Jagendorf (1965) have observed a preservative effect resulting from the use of equimolar ferrocyanide/ferricyanide in the initial reaction mixture. We did not observe any noticeable improvement in quantum requirement resulting from the addition of ferrocyanide initially; however, there did seem to be much less scatter in the results when ferrocyanide was present. For this reason we included it in the reaction mixture for all the ferricyanide Hill reaction studies.

The rates of the ferricyanide Hill reaction were generally measured during the first 60% of conversion

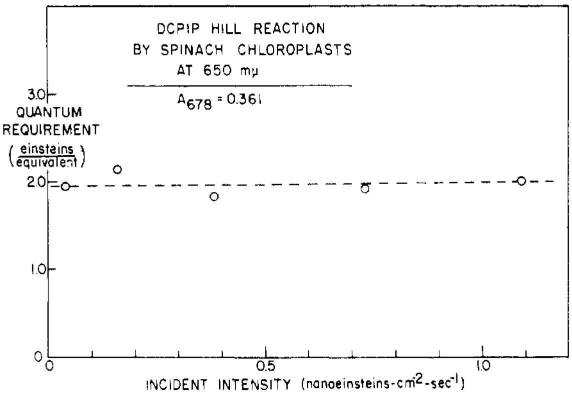

FIGURE 4: The dependence of quantum requirement on incident light intensity at 650 mμ for the DCPIP Hill reaction by spinach chloroplasts with added methylamine. Chloroplasts sufficient to give an absorbance of 0.361 and 678 mμ were added to the standard reaction mixture.

of the available oxidant. In a test experiment at a single light intensity, the rate of the reaction decreased about 15% during 90% conversion of the ferricyanide. For both the Hill oxidants studied, a slow back reaction was observed in the dark following illumination, particularly after a large fraction of the available oxidant had been reduced. Experiments were designed so that the back reaction was always significantly less in magnitude than the photoreduction, and appropriate corrections to the forward rate were made. The rate of the back reaction varied erratically from one chloroplast preparation to another, and often for successive experiments with a single preparation. Frequently no back reaction at all was observed.

Action Spectra. As in the previous study (Sauer and Biggins, 1965), quantum requirements measured over a 10-fold range of incident monochromatic light intensity were extrapolated to zero light intensity. These zero-intensity quantum requirements are plotted against wavelength of the exciting light in Figure 3 for ferricyanide and for indophenol as the Hill oxidant. Results from three different preparations are shown; in each case methylamine was present. A quantum requirement of 2.0 quanta per electron transferred to DCPIP was observed repeatedly in the wavelength region 640–660 mμ. This requirement increased to 3.0 near 680 mμ and then increased sharply at still longer wavelengths. The same general behavior was observed for ferricyanide reduction by spinach chloroplasts; however, the quantum requirements were uniformly 20 to 40% higher. Actinic light with a band width of 7 mμ, obtained from a Bausch and Lomb 500-mm grating monochromator, was used for most of the results reported in Figure 3. In one experiment, the effect of band width was studied at 690 mμ. This is at the steepest region of the absorption spectrum, where the consequences of a broad band width should be most pronounced. The measured quantum requirements differed by only 10% when a 10-mμ band width was replaced by one of 17.5 mμ.

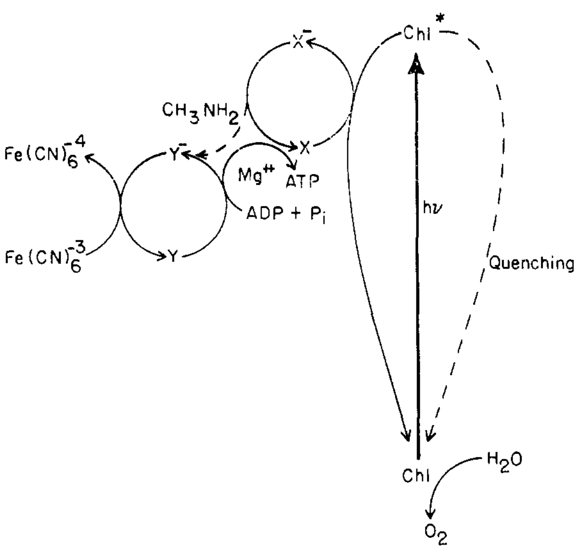

FIGURE 5: Proposed mechanism to account for the photochemical kinetics of the Hill reaction.

Two-Wavelength Studies. Experiments were carried out to determine whether the DCPIP Hill reaction of chloroplasts exhibits two-wavelength enhancement effects of the type described by Emerson *et al.* (1957). Preliminary studies were carried out using 650- and 688-mμ wavelengths. The two beams were combined using a half-silvered mirror and were incident on the reaction mixture from the same direction. First one wavelength was presented to the sample, then the second was added, and finally the first was removed. In other experiments the order was reversed. If the three rates are designated R_1 at 688 mμ, R_2 at 650 mμ, and R_{12} for both wavelengths, then a sensitive measure of enhancement is given by the ratio $r = (R_{12} - R_2)/R_1$. Our observations averaged $r = 1.03 \pm 0.08$ for a series of nine experiments. No significant effect resulted from a 3-fold variation of the intensity of either beam. The intensities chosen were such that the ratio R_2/R_1 varied from 0.05 to 1.1. Our preliminary conclusion is that, within the experimental uncertainties, there is no Emerson enhancement for the reduction of DCPIP by spinach chloroplasts.

Very Low Light Intensities. In several experiments we found that the quantum requirements for the indophenol Hill reaction remained constant, even at an incident light intensity of less than 0.1 neinstein cm^{-2} sec^{-1}. Results of such an experiment at 650 mμ are shown in Figure 4. At the lowest intensity studied, 0.040 neinstein cm^{-2} sec^{-1}, a quantum requirement of 1.94 quanta per electron was obtained. The dark rates, measured immediately before and after the photoreaction, were less than 10% of the rate in the light in this case. The conditions of the experiment were such that 31.6% of the incident light was absorbed in the 3-mm illumination pathway, 8.0% by the chloroplasts and 23.6% by the indophenol. Using the extinction coefficients of McKinney (1941) and assuming a chlorophyll *a*/chlorophyll

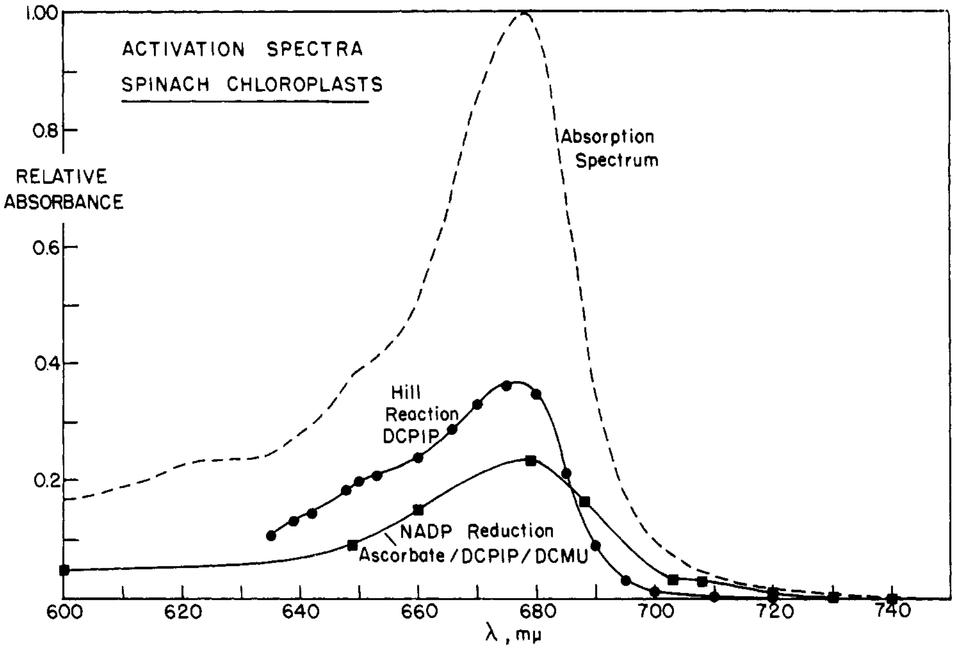

FIGURE 6: Absorption spectra of pigments responsible for the DCPIP Hill reaction (●) and for the reduction of NADP by ascorbate and $DCPIPH_2$ in the presence of DCMU (■) by spinach chloroplasts. Dashed curve gives the normalized absorption spectrum of spinach chloroplasts, corrected for light scattering (Sauer and Biggins, 1965). Points shown for the two reactions are computed by multiplying the quantum yield in equivalents per einstein absorbed (extrapolated to zero intensity; from Figure 3 and from Sauer and Biggins (1965), respectively) by the chloroplast absorbance at the corresponding wavelength.

b ratio of 160/70 (Park and Biggins, 1964), we calculate that each chlorophyll (*a* or *b*) molecule absorbed a light quantum on the average once every 630 sec. If one assumes that there are 230 chlorophyll (*a* and *b*) molecules in a photosynthetic unit (Park and Biggins, 1964), then each unit absorbed a quantum every 2.7 sec. Since four electrons are required from water for each oxygen molecule produced, and since the measured quantum requirement per electron transferred was about 2, this means that about 22 sec would be required to produce an oxygen molecule at each photosynthetic unit without measurable loss in efficiency. The presence of very long-lived intermediates is suggested by these calculations, unless the "photosynthetic units" in turn act cooperatively, or the unit is much larger than that above.

Discussion

The occurrence of an increase of the quantum requirement for a photochemical reaction with increasing incident light intensity is indicative of a rate-limiting dark reaction. Our study suggests that the rate-limiting step in the Hill reaction is closely associated with the site at which phosphorylation is coupled to the electron transport chain. A simplified scheme illustrating these interactions is given in Figure 5. Photoexcitation of chlorophyll molecules in pigment system II, following the notation of Duysens *et al.* (1961) and Duysens and Amesz (1962), normally leads to the reduction of substance X to X^-. A rate-limiting reaction between X^- and Y coupled to the phosphorylation step regenerates oxidized X in a form such that it can be reactivated by the next light act. The reduced product Y^- is ultimately oxidized by the terminal electron acceptor, *e.g.*, ferricyanide, possibly with the mediation of pigment system I. At high light intensities the limited pool of substance X empties, and the repeatedly excited pigment molecules are quenched by some dissipative process, leading to an increase in the observed quantum requirement for the Hill reaction. The rate-limiting step becomes more rapid when the phosphorylation cofactors, ADP, Mg^{2+}, and phosphate, are present, or when the phosphorylation uncoupler, methylamine, is added. Methylamine presumably produces a short circuit for electrons at the site of coupling to phosphorylation. The intensity dependence of the quantum requirement is less pronounced when DCPIP rather than ferricyanide is used as the oxidant. This is consistent with the known uncoupling action of the oxidized dye.

A calculated absorption spectrum for the pigment system responsible for DCPIP reduction is given in Figure 6. The spectrum is obtained by multiplying the quantum yield at each wavelength by the total pigment absorption (dashed curve). Some justification of this approach is presented in view of a recent publication of action spectra of the ferricyanide Hill reaction by Horio and San Pietro (1964). We believe that the approach of those authors, in comparing percentage activity spectra with absorption spectra in order to determine the relative photochemical effectiveness of various absorption components, is a misleading one. The shape of a per cent activity spectrum in principle changes drastically with chloroplast concentration, whereas the shape of the absorption spectrum does not. Action spectra of the type reported by Horio and San Pietro should properly be compared with a plot of the fraction of incident light absorbed *vs.* wavelength for the particular concentration and optical path length employed. The absorption leading to DCPIP reduction (Figure 6) peaks at about 675 mμ, demonstrating that chlorophyll *a* participates strongly in activating this reaction. The shoulder at 650 mμ suggests the participation of chlorophyll *b* as well. Absorption leading to reduction of NADP by ascorbate/$DCPIPH_2$ in the presence of DCMU is relatively much higher at wavelengths longer than 690 mμ (Hoch and Martin, 1963; Sauer and Biggins, 1965). The absorption spectrum of the pigment system responsible for this reaction (system I absorption) based on the relatively few data available is also shown in Figure 6. The system I absorption exhibits a maximum near 680 mμ and does not show an obvious shoulder at 650 mμ. It is worthy of note that the curves are similar in shape and that their maxima are only slightly displaced from one another.

In higher plants one cannot learn from action spectra alone whether the Hill reaction with a given oxidant is driven only by system II, or whether participation by system I is involved as well. If both pigment systems must be activated, then relatively high quantum requirements would be expected at long wavelengths where system II absorbs weakly. There is no corresponding region in higher plants where system I absorbs weakly. The simplest mechanism involving both pigment systems requires a minimum of 2 quanta per electron transferred, whereas system II alone could operate with a requirement of only 1. As no experimental values significantly less than 2.0 quanta per electron have been reported for the Hill reaction, either mechanism is possible. One consequence of the participation of both pigment systems in a given reaction is the presence of enhancement effects, such as have been shown for over-all photosynthesis (Emerson *et al.*, 1957). In the case of the Hill reaction there is a decided conflict in the literature. The Emerson enhancement has been reported by two groups: in one case for the Hill reaction using benzoquinone as oxidant with intact *Chlorella* (Govindjee *et al.*, 1960; Govindjee and Rabinowitch, 1961) and in the second for the ferricyanide Hill reaction with isolated chloroplasts (Bishop and Whittingham, 1963). On the other hand, two other groups find enhancement to be absent for the Hill reaction, under conditions where enhancement could be observed for over-all photosynthetic oxygen evolution, for either *Chlorella* or plant chloroplasts using ferricyanide or benzoquinone as oxidants (Mayne and Brown, 1963; Gibbs *et al.*, 1963). Furthermore, Fork (1963) has shown that far-red enhancement observed for transient endogenous oxygen evolution in the absence of ferricyanide can no longer be observed when substrate levels of ferricyanide are added to the same photosynthetic system. Our results must also be counted as negative evidence on the occurrence of enhancement in the reduction of DCPIP by chloroplasts. The results are, however, preliminary and need to be extended to a wider variety of conditions and to be compared with situations where enhancement can be clearly demonstrated.

The reason ferricyanide reduction has a higher quantum requirement than does DCPIP reduction at all wavelengths studied may be that system I participates in the former case and not in the latter. Alternatively, there may be a cyclic process present in the ferricyanide Hill reaction by which ferrocyanide is partially reoxidized to ferricyanide in the light. Either of these possibilities would increase the quantum requirement over that of a simple system II reaction.

The spectrum calculated for the DCPIP Hill reaction (Figure 6) is probably a good representation of the system II absorption spectrum, regardless of whether system I participates in this reaction as well. If both light reactions are required, then the limitation in efficiency is set at each wavelength by whichever pigment system absorbs less. At long wavelengths this is clearly system II, and at short wavelengths the observation of a quantum requirement of 2 for DCPIP reduction demands that the two pigment systems absorb approximately equally.

At no wavelength do the calculated absorptions shown in Figure 6 for the two pigment systems add up to a value greater than the total absorption. This finding is consistent with the conclusion of Hoch and Martin (1963) that there is no electronic energy transfer from either pigment system to the other. This conclusion implies that the two systems are isolated from one another and act independently, except at the chemical level of electron-transport reactions. If there is the possibility for efficient transfer of electronic excitation energy, one might expect quantum requirements approaching unity for either the Hill reaction or the ascorbate/$DCPIPH_2$ reduction of NADP at wavelengths below 680 mμ where both pigment systems absorb appreciably. The kind of evidence available is such that it can always be argued that the photoreactions are occurring at less than half their optimal efficiency owing to losses in activity of the isolated chloroplasts. It is then difficult to explain why the NADP reduction by ascorbate/$DCPIPH_2$ is efficient at long but not at short wavelengths.

Long-lived intermediates have been sought in photosynthesis on numerous occasions. There is much evidence from intermittent illumination experiments (Emerson and Arnold, 1931, 1932; Kok, 1956), alternating wavelength studies (Blinks, 1957; Myers and French, 1960), and delayed fluorescence measurements (Kautsky *et al.*, 1960). It is much more difficult to demonstrate that these phenomena reflect substances which participate directly in electron transport related to photosynthesis. In our studies the high sensitvity of our method of following the chloroplast-sensitized photoreduction of DCPIP permits measurements to be carried out at light intensities considerably below those normally used. The observation that the quantum requirement of DCPIP reduction is constant down to these very low intensities has a direct bearing on this problem. The production of one molecule of oxygen from water requires the transfer of four electrons from water to the acceptor DCPIP, and each molecule of DCPIP must receive two electrons to become reduced to the stable product $DCPIPH_2$. It is impossible energetically for the entire reaction to be carried out as a consequence of the absorption of a single quantum of red light; however, a mechanism involving 2 quanta per O_2 produced (*i.e.*, 2 electrons transferred per quantum absorbed) is energetically feasible. It would require the formation of at least one metastable intermediate in the process. Our experiments show that this postulated intermediate would have a lifetime of at least 11 sec at the lowest incident intensity studied, if the approximately 230 chlorophyll molecules contained within a single quantasome act as a discrete unit. If, as is generally assumed, a minimum of 4 quanta are necessary to produce an O_2 molecule, then this lower limit is increased to 22 sec and at least three distinguishable intermediate states are involved.

Two alternative explanations of these low-intensity experiments need to be considered. In the first place, if there is efficient electronic energy transfer from the pigment molecules of one photosynthetic unit to those of neighboring units, then of course the number of absorbing molecules feeding energy into a single photochemically active site will be greatly increased and the necessary minimum lifetime of the intermediates will be correspondingly decreased. Such long-range energy transfer would seem unlikely if it can be conclusively shown that there is no electronic-energy transfer between the two-pigment systems within a single photosynthetic unit, as discussed above. The second case that requires consideration is the possibility that the photosynthetic units can cooperate at the chemical level. This would occur if the intermediates produced are sufficiently mobile to migrate rapidly from one unit to another, or if coupling factors are available which can transport the chemical potential from one site to another. Migration of this type would serve to reduce appreciably the calculated minimum possible lifetime of the proposed intermediates. One test of whether such migration occurs can be made by examining the dependence of the quantum requirement at low light intensities for suspensions of small lamellar fragments, where the average path between photosynthetic units is drastically increased. Comparison of the calculated minimum lifetimes from such experiments with diffusion times in solution should produce an answer to the question of whether migration of active intermediates from one photosynthetic unit to another does occur.

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Action Spectrum and Quantum Requirements for the Photoreduction of Cytochrome *c* with Spinach Chloroplasts*

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ABSTRACT: The photoreduction of cytochrome *c* in the presence of intact chloroplasts occurs with a high quantum efficiency, using reduced trimethyl-*p*-benzoquinone ($TMQH_2$) as reductant and in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This reaction has a requirement of 2 quanta absorbed per electron transferred to cytochrome *c* for exciting light in the wavelength region from 620 to 680 mμ; the quantum requirement then falls to 1 quantum per electron at wavelengths greater than 700 mμ. These results confirm the conclusion of Vernon and Shaw [Vernon, L. P., and Shaw, E. R. (1965), *Biochemistry 4*, 132] that this oxidation–reduction reaction is mediated by chloroplast pigment system I in the presence of DCMU. The quantum requirement of unity observed at long wavelengths shows that the reaction probably occurs with the maximum efficiency obtainable. The evaluation of the action spectrum for cytochrome *c* reduction together with that for the chloroplast Hill reaction [Sauer, K., and Park, R. B. (1965), *Biochemistry 4*, 2791], which is probably photocatalyzed by pigment system II, strongly suggests that there is no appreciable transfer of electronic excitation energy between the two pigment systems in spinach chloroplasts.

The two light reactions apparently interact only at the chemical level of photosynthetic electron transport. A model is presented which rationalizes this conclusion by the physical separation of the two pigment systems on opposite sides of the chloroplast lamellar unit.

The recent studies of Vernon and Shaw (1965) demonstrated that the photoreduction of cytochrome *c* by whole chloroplasts is stimulated by the addition of various hydroquinones, including reduced trimethyl-*p*-benzoquinone ($TMQH_2$).[1] The stimulation is only partially decreased in the presence of DCMU, a potent inhibitor of oxygen evolution by chloroplasts. This finding suggested to Vernon and Shaw that these hydroquinones serve as electron donors for the long wavelength pigment system I of chloroplasts.

The present investigation seeks to determine by means of its action spectrum whether this photoreduction is a system I reaction. The evidence strongly suggests that such is the case, and the data are used to derive the spectral absorption of pigment system I. The action spectrum for cytochrome *c* reduction by chloroplasts is found to be similar to that reported previously for the photoreduction of NADP with $DCPIPH_2$ and ascorbate in the presence of DCMU, a known system I reaction (Hoch and Martin, 1963; Sauer and Biggins, 1965). Furthermore, the consideration of the action spectrum for cytochrome *c* reduction together with that for the chloroplast Hill reaction (Sauer and Park, 1965) provides strong evidence to support the hypothesis that there is no transfer of electronic excitation energy between the two pigment systems in spinach chloroplasts.

Materials and Methods

Chloroplasts were prepared either from fully grown commercial spinach leaves or from 6–8 week-old plants grown from seed in a growth chamber, as described

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[1] Abbreviations used in this work: $TMQH_2$, reduced trimethyl-*p*-benzoquinone; NADP ($NADPH_2$), nicotinamide-adenine dinucleotide phosphate; DCPIP ($DCPIPH_2$), 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.