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ACTION SPECTRA AND QUANTUM YIELDS FOR NADP REDUCTION BY CHLOROPLASTS

Kenneth Sauer and John Biggins

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SUMMARY

The photoreduction of NADP by chloroplasts is observed to occur with a requirement of 1 to 3 quanta per electron transferred to NADP at wavelengths from 550 to 730 mm in a DCMU-poisoned system when reduced DCPIP is present as electron donor. In the normal reaction, with water as donor, a similar quantum requirement is observed from 550 to 620 mm, but this rises to approximately 9 quanta per electron at wavelengths longer than 700 mm. The results are interpreted as reflecting the participation of two pigment systems. Some characteristics of the absorption spectra of the two systems are discussed.

The quantum requirements at nearly all wavelengths studied were found to increase linearly with increasing incident light intensity for both reactions. We propose that an activated intermediate of the photosynthetic unit, with an estimated lifetime of 0.05 sec in the normal reduction of NADP by chloroplasts and 0.15 sec in the DCMU-poisoned system, is responsible for this intensity dependence.

Small quantasome aggregates prepared from spinach chloroplasts are shown to be nearly half as efficient as intact chloroplasts in the photoreduction of NADP using reduced DCPIP. A method is described whereby the absorption spectrum of these quantasome suspensions can be used to determine the true absorption spectrum of chloroplasts at wavelengths where light scattering is predominant.
INTRODUCTION

The normal course of photosynthetic electron transport in higher plant chloroplasts leads to the reduction of NADP to NADPH₂ with water, as substrate, becoming oxidized to molecular oxygen. Coupled with this process is the non-cyclic phosphorylation of ADP to ATP. DCMU is a potent inhibitor of photosynthesis¹, but in preparations of isolated chloroplasts this inhibition can be relieved, in part, by the addition of the couple DCPIP plus ascorbate². Under these circumstances the reduced DCPIP takes the place of water as the electron donor; NADP can then be reduced and ADP phosphorylated by chloroplasts in the light³. Oxygen is not liberated so long as DCMU is present; however, in the absence of DCMU, the oxidized form of DCPIP serves as an oxidant for the chloroplast Hill reaction, leading to the photooxidation of water to molecular oxygen. These observations led Losada, et al.³ to the conclusions that (i) there are two light reactions involved in NADP reduction where water serves as the electron donor, (ii) only one of these is involved in the photoreduction of NADP in the presence of added DCMU and reduced DCPIP, and (iii) the other light reaction alone is responsible for the Hill reaction with oxidized DCPIP.

Evidence has been presented to demonstrate that these two light reactions arise from absorption by different pigment systems in chloroplasts⁴. Several recent studies have appeared describing action spectra and absolute quantum requirements of NADP reduction by chloroplasts for the normal system (water as donor)⁵-⁷, and for the poisoned system (ascorbate/DCPIP as donor)⁵. There is a considerable measure of discordance among the various results. The present study is an attempt to
resolve some of the difficulties through the use of a different experimental approach. In particular, we have continuously monitored the concentration of NADPH$_2$ throughout the course of the reaction in order to correct for the reverse oxidation of NADPH$_2$. In addition, we have used a novel technique for determining the true absorption of chloroplasts at long wavelength where light scattering normally makes an overwhelming contribution.

MATERIALS AND METHODS

Preparation of chloroplasts and quantasomes

Chloroplasts were prepared by minor modifications of the method of Hoch and Martin$^5$. Sixty gm fresh spinach leaves were thoroughly washed and refrigerated after the removal of the mid-ribs. They were blended in 100 ml of ice-cold 0.4 M sucrose, 0.05 M Tris chloride (pH 8.0), and 0.01 M NaCl for 10 sec. The homogenate was strained through eight layers of cheesecloth and centrifuged at 1000 g for one min. The supernatant was centrifuged at 1000 g for 7 min and the pellet resuspended in 50 ml blending medium. Whole chloroplasts were sedimented by recentrifugation at 1000 g for 7 min.

Quantasome aggregates were prepared by lysing the whole chloroplasts in 40 ml $10^{-2}$ M tris-Cl, pH 8. The suspension was agitated gently for 10 min and centrifuged at 10000 g, 10 min. The green sediment was resuspended in 15 ml $10^{-2}$ M tris-Cl, pH 8, and sonicated for 90 sec by means of a Raytheon 100W, 9 Kc magnetostriiction oscillator operated at full voltage. The sonicate was then ultracentrifuged at 105000 g for 20 min and the supernatant retained as our preparation of quantasome aggregates. Typically these particles
contain an average of 8 quantosomes as seen in the electron microscope.

**Enzyme preparations**

PPNR was prepared from spinach according to the procedure of San Pietro and Lang through the 75% acetone precipitation step. The protein was redissolved, centrifuged, dialyzed against $5 \times 10^{-3}$ M Tris chloride (pH 8.0), and further purified through the first DEAE-cellulose column treatment of Hill and Bendall's method. This partially-purified preparation contained chloroplast ferredoxin and ferredoxin-NADP reductase. The preparation was assayed in the photoreduction of NADP by chloroplasts using white light and thereafter used in saturating concentrations in the quantum requirement determinations.

**Reagents**

NADP and Trizma base were obtained from the Sigma Chemical Co., St. Louis; ADP from the Pabst Brewing Co., Milwaukee; DCMU from duPont de Nemours, Wilmington; and DCPIP from K and K Laboratories, Jamaica, New York.

**Reaction mixtures**

The reaction mixtures contained the following in umoles/ml: potassium phosphate, pH 7.5, 50; NADP, 0.5; ADP, 1.0; MgCl$_2$, 7.5 and a saturating amount of PPNR. In DCMU-poisoned preparations the above reaction mixture was used with the following in umoles/ml: DCMU, 0.01; sodium ascorbate, 5.0; and DCPIP, 0.04.

**Apparatus**

Experiments were carried out using a Cary Model 14 spectrophotometer modified in such a way that the absorbance of the reaction mixture at 339 nm could be monitored continuously while the sample was being irradiated from the side with longer wavelength light. A diagram of the
apparatus is shown in Fig. 1. A Cary Model 1482 Scattered Transmission Accessory was modified so that the exciting light could be directed through a set of filters in the side wall to irradiate the sample cuvette in the spectrophotometer beam from one side. Light from a tungsten projection lamp (General Electric DFX, 1000 watt), powered with a Variac, was collimated using a spherical mirror and passed through 4 cm of water and a Corning 1-59 infrared-absorbing glass. One or more cut-off filters (CF 1) and an interference filter (IF 1) were placed in a port in the side of the Scattered Transmission Accessory and a lens inside served to concentrate the beam incident on the sample cuvette. An opaque barrier prevented exciting light from reaching the reference cuvette, which contained a portion of the same reaction mixture as that in the sample cuvette. All samples were irradiated at room temperature.

For a second set of experiments a grating monochromator (Bausch and Lomb, 500 mm) was used to provide actinic light in place of the arrangement involving interference filters described above. Suitable cut-off filters were used with the monochromator to reduce stray light at shorter wavelengths. Results obtained using the two activation systems were in good agreement at all wavelengths studied. With the interference filters the maximum intensity available was more than ten times greater than from the monochromator at an equivalent bandwidth, however.

The cuvettes used were constructed with four clear sides and a rectangular internal cross-section 3 mm x 10 mm. The spectrophotometer measuring beam traversed the long dimension, as shown in Fig. 1. It was necessary to place a set of complementary filters, CF 2 and IF 2, in front of the photomultiplier (Dumont 7664) in order to prevent stray
exciting light or chloroplast fluorescence from interfering with the absorption measurements. For this purpose, an interference filter (Baird Associates), transmitting in its third order in the ultraviolet, was coupled with a Corning ultraviolet-transmitting filter (7-60).

The excitation wavelength interference filters (IF 1) were of the blocked, narrow-band type (Baird Associates, Type B-1) with half bandwidths about 10 μm. The supplementary cut-off filters were selected to reduce the transmitted intensity of shorter wavelengths to a very low level. The Corning supplementary filters used at each wavelength studied were: (i) 3-68 at 550 and 600 μm; (ii) 2-58 at 649, 660, and 679 μm; (iii) 2-64 at 688 μm; (iv) 2-64 plus 4-77 at 703, 709, and 720 μm; and (v) 2-64 plus 7-59 at 730 μm.

Light intensity measurements

Measurements of light intensity were based on secondary standard lamps obtained from the National Bureau of Standards, Washington, D. C. A Reeder thermopile (C. M. Reeder Co., Detroit), calibrated using the standard lamp, was used to determine the wavelength dependence of the sensitivity of a silicon solar cell (Hoffman, Type 120 CG), using the Bausch and Lomb 500 mm grating monochromator and a tungsten source. Incident intensities for the illumination experiments were measured at the site where the sample cuvette is normally placed in the apparatus. Corrections were applied to the results for the measured reflection loss (5%) at incidence on the cuvette sides.

RESULTS

Absorption spectrum of chloroplasts

An essential prerequisite to the calculation of quantum requirements of photochemical reactions is an accurate measure of the number of quanta
absorbed. In the case of chloroplast suspensions this is difficult, especially at wavelengths longer than 690 μm, owing to the substantial light scattering exhibited. Use of the measured transmission of the sample in the calculations may lead to large errors on this account.

The technique we used for estimating absorbed intensities is based on a study of the spectral properties of chloroplast and quantasome preparations. Figure 2 presents a part of the absorption spectrum for a single preparation of spinach chloroplasts measured using three different techniques: direct absorption using a Cary 14 Spectrophotometer with a normal cell compartment, the opal glass technique of Shibata, Benson, and Calvin, and the scattered-transmission method with a red sensitive photomultiplier (Dumont 6911). The latter two spectra are nearly identical except that the opal glass technique is not reliable beyond 700 μm. A sample of quantasomes prepared from another portion of the same chloroplast preparation was also measured using the scattered-transmission method. The long wavelength tail of the red absorption band of a concentrated suspension of quantasomes is shown in Fig. 3 along with the spectrum in the same region of a concentrated suspension of the chloroplasts. The latter has been normalized so that the absorbances at 678 μm are each 10.0. The effect of light scattering on the chloroplast spectrum at these long wavelengths is such that direct measurements, even using the scattered-transmission technique, would not give a good measure of the true absorption of the preparation.

The spectrum of the small quantasome aggregates can be satisfactorily corrected for light scattering. The correction is small in this case, and it can be executed accurately based on turbidity measurements from 750 to 900 μm, where there is no absorption. Some measured
optical density values from 760 to 900 mu are plotted versus the reciprocal fourth power of the wavelength in Fig. 4. The points fall on a good straight line passing through the origin, as is expected for light scattering from small particles (Rayleigh scattering). Extrapolation of this line to shorter wavelengths permits the calculation of the contribution of scattering to the measured spectrum at any wavelength and, by difference, the true absorption spectrum of the quantasome preparation (dashed curve in Fig. 3). The fact that the scattering correction is small in the case of the quantasome preparation permits the computation of a reliable absorption spectrum by this method. This is less true for chloroplast suspensions, where the particles are large and the turbidity of the preparations is greater than the true absorbance at wavelengths longer than 700 mu.

We now make the assumption that the true absorption spectrum of chloroplasts is identical to that of quantasomes prepared from them, after normalization based on the measured values at the maximum at 678 mu. Some arguments supporting the validity of this assumption are presented by Sauer and Park. Thus, from a single scattered-transmission measurement of chloroplast absorption at 678 mu, where the contribution from light scattering is relatively minimal, and from the normalized quantasome absorption spectrum, we compute the absorption of the chloroplast suspensions at wavelengths where the direct measurement is impossible by any technique short of using an ideal integrating sphere.

Photoreduction of NADP by chloroplasts

Our initial method of studying the photoreduction of NADP by
chloroplasts was similar to that of Black, et al. and Hoch and Martin. Samples were illuminated for a fixed time period, the chloroplasts were removed by centrifugation, and the 339 nm absorbances of the supernatants recorded. In our hands this technique proved unreliable, owing to the very active first-order reoxidation of NADPH₂. We subsequently showed that catalytic activity for this reoxidation was associated with our PPNR preparations and with the chloroplast stroma, and that it could not be removed by short term ultracentrifugation of the samples. We found it necessary, therefore, to adopt the continuous spectrophotometric measurement of NADPH₂ concentration. In this way true initial velocities for the photoreduction uncomplicated by the reoxidation could be obtained.

A typical trace of the time course of the photoproduction of NADPH₂ is shown in Fig. 5. The following characteristic features of the trace were generally observed in determinations summarized in this report:

(i) A stable level of absorbance at 339 nm in the dark, (ii) an initial rapid rise during which about 0.2% of the available NADP is reduced, (iii) a period during which a slower, but constant, rate of formation of NADPH₂ occurs, (iv) a gradual decrease in this reaction velocity after about 5% reduction of the initial NADP, (v) a brief rapid decrease in absorbance upon cessation of illumination followed by (vi) a slower decrease of the absorbance which continued for a long period of time.

Stage (ii) was not observed if a second successive period of illumination was given to the same sample, even after a dark interval of several minutes.

The steady initial velocity chosen for the reaction was the limiting
slope of the nearly linear portion [stage (iii)] following the transient initial rapid rise. The location of this limiting tangent could, in general, be determined unambiguously from a region in which there was no pronounced curvature of the trace. After this linear portion, the reoxidation becomes sufficiently great even in the light to cause a distinct decrease in the net rate of \( \text{NADPH}_2 \) formation.

In order to make certain that other changes in absorbance at 339 \( \mu \text{m} \) were not influencing the magnitude of our results, we scanned a number of the difference spectra from 300 to 550 \( \mu \text{m} \) before and after periods of illumination. The spectra consisted invariably of a simple absorption peak at 339 \( \mu \text{m} \), and there was no evidence of significant bleaching of the photosynthetic pigments or changes in turbidity of the illuminated sample. In separate experiments we determined that the presence of chloroplasts at the highest concentrations used did not significantly affect the extinction coefficient of \( \text{NADPH}_2 \) at 339 \( \mu \text{m} \) in the difference spectra measured using the scattered-transmission technique.

**Quantum requirements and action spectra**

Quantum requirements for \( \text{NADP} \) reduction for each experiment were calculated from the steady initial reaction velocities and the calculated absorbed intensities. Experiments were carried out alternately for the normal and the DCMU-poisoned systems with otherwise identical reaction conditions. At each wavelength the incident intensity was varied over at least a ten-fold range by adjusting the lamp voltage.

Figures 6 and 7 show examples of the dependence of the quantum requirement on incident intensity. At all wavelengths studied and for
both reaction systems the quantum requirement increases linearly with increasing intensity. The positive intercepts, determined from extrapolations to zero intensity, are plotted as a function of actinic wavelength in Fig. 8. These data are mean values resulting from up to five separate experiments at each wavelength. Some 40 chloroplast preparations and three PP"RN preparations are involved. These quantum requirements are also presented in Table I, along with some values in the same units which we have calculated from data and curves given in the recent literature.

Both the normal and the DCMU-poisoned reactions have quantum requirements in the range 1.5 to 3.0 einsteins/equivalent of NADP reduced between 648 and 679 μm. At wavelengths longer than 679 μm the normal reaction shows a pronounced increase in quantum requirement characteristic of the first Emerson effect, whereas the requirement for the poisoned reaction appears to decrease to about one-half its value at shorter wavelengths. We took particular care to insure that the normal reaction does indeed proceed at 720 and 730 μm with requirements of no more than about 9 quanta per electron. No effective stray light of wavelengths shorter than 700 μm was present in the actinic beam, as demonstrated by the introduction of additional supplementary cut-off filters. By closing the spectrophotometer slit for a period of time during the progress of experiments at these long actinic wavelengths, we showed that the 339 μm analyzing beam was ineffective in sensitizing the reduction of NADP. Furthermore, the reduction of NADP in the normal system at wavelengths greater than 700 μm was completely inhibited by the addition of DCMU.
Dependence on light intensity

The quantum requirement for NADP reduction by chloroplasts increases linearly with increasing incident light intensity for both reactions studied and at all wavelengths from 550 to 730 nm, except for the normal reaction at the longest wavelengths, as noted below. This behavior has been reported previously by Hoch and Martin\(^5\), and a similar dependence was observed for the Hill reaction using ferricyanide\(^{16,17}\). Lunry and Rieske postulated several mechanisms for the interpretation of these results\(^{18}\). The following mechanism is one of the simpler ones they proposed and it appears to be sufficient to explain our observations:

\[
\begin{align*}
\text{\(C\)} & \xrightarrow{\text{\(I\)}} \text{\(C^*\)} & \quad \text{(1)} \\
\text{\(C^*\)} & \xrightarrow{\text{(1-p)}} \text{\(C\)} & \quad \text{(2)} \\
\text{\(C^* + T\)} & \xrightarrow{\text{\(P\)}} \text{\(C + T^*\)} & \quad \text{(3)} \\
\text{\(T^* + R\)} & \xrightarrow{\text{k_1}} \text{\(T + P\)} & \quad \text{(4)}
\end{align*}
\]

In this scheme light intensity \(I\) absorbed by pigment molecules \(C\) (presumably chlorophyll in the wavelength range studied) excites them to reactive species \(C^*\) with a primary quantum efficiency \(\phi\). At very low light intensities some fraction \(p\) of \(C^*\) transfers its excitation to a trapping site or reactive intermediate, \(T\), according to reaction (3). The remainder of \(C^*\) is deactivated according to reaction (2) with an intrinsic probability (1-p) and will not lead to the formation of products. The excited trapping sites \(T^*\), once formed, inevitably lead to the conversion of reactants (NADP and water or ascorbate) to products (NADPH\(_2\) and oxygen or dehydroascorbate) by a series of reactions summarized...
in equation (4). At higher light intensities the steady-state concentration of \( T \) will be reduced from the value \( T_0 \) present in the dark or at low limiting light intensities, and the probability for reaction (3) will decrease from \( p \) to \( p(T/T_0) \).

Using the steady-state approximation for the concentration of the excited species \( C^* \) and \( T^* \), one readily obtains for the reaction velocity the result

\[
V = \frac{k_1 P^* T_0 I}{k_1 T_0 + P^* I}
\]

(5)

where

\[
V = \frac{dP}{dt} = k_1 RT^* = k_1 T^*
\]

The pseudo first-order rate constant, \( k_1 \), will be constant only during the early stages of the reaction before the concentration of reactant \( R \) has decreased appreciably. The quantum requirement, \( Q \), is then given by

\[
Q = \frac{I}{V} = \frac{1}{P^*} + \frac{I}{k_1 T_0} = Q_0 + \frac{I}{k_D}
\]

(6)

Equation (6) predicts a linear relationship between the quantum requirement and the absorbed intensity with a positive slope. The intercept, \( Q_0 \), is the intrinsic quantum requirement for the overall reaction at zero intensity, and the slope, \( 1/k_D \), is a measure of the rate parameter for reaction (4).

The slopes of plots of quantum requirement of NADP reduction versus absorbed light intensity for both the normal and the DCMU-poisoned system appear to be independent of wavelength; however, the data for the normal system at wavelengths beyond 708 nm scatter too much to give reliable values (e.g., see Fig. 6). Average values for the pseudo zero-order rate constant \( k_D \) were calculated from all results regardless of
of wavelength. The values obtained were $1.6 \pm 0.7 \times 10^{-7}$ equivalents-liter$^{-1}$-sec$^{-1}$ for the DCMU-poisoned system and $4.8 \pm 1.7 \times 10^{-7}$ for the normal system. This parameter characterizes the rate of reaction (4) in the mechanism presented above and presumably includes a dependence on the concentration of one or more endogenous chloroplast cofactors. It is significant that this parameter indicates that reaction (4) is three times faster in the normal system than in the DCMU-poisoned system.

A study was made to determine whether the concentration of the DCPIP/ascorbate couple is limiting for the DCMU-poisoned system. For this purpose it is sufficient to study the dependence of quantum yield of NADP reduction on DCPIP concentration. Figure 9 summarizes the results of such an experiment carried out using 670 nm illumination at two different incident intensities. Sufficient chloroplasts were present to give an absorbance of 0.80 (1 cm path). Concentrations of all other components were the same as those normally used, with the exception of DCPIP, which was varied. At the higher light intensity a typical saturation curve is obtained. If indophenol is included specifically as a limiting reagent (first-order) in reaction (4) it can readily be shown that the quantum requirement, $Q$, should be linearly related to the reciprocal of DCPIP concentration. The data are in good agreement with such a prediction. For a plot of quantum yield versus DCPIP concentration the theory predicts a hyperbolic relationship. The curves shown in Fig. 8 are drawn on this basis for comparison with the data. The saturation values of quantum yield with increasing dye concentration, obtained from reciprocal plots, are 0.263 and 0.378 at the higher and lower intensities, respectively. The concentration of DCPIP normally used throughout this study (0.04 moles/ml) gives over 90% of the saturation quantum yield at
each intensity. This experiment shows that no increase in DCPIP concentration will increase the quantum efficiency of the photoreduction to anywhere near the level attainable by decreasing the incident light intensity. The DCPIP concentration (and, by inference, the ascorbate concentration) is therefore not a limiting factor in the kinetic scheme under the conditions studied.

Photoreduction of NADP by quantasomes

In addition to the ability of quantasome preparations to carry out the Hill reaction and to support CO₂ fixation, we have now observed a relatively high level of activity for NADP reduction in the presence of added PPNR. Best results were obtained using the ascorbate/DCPIP couple as reductant in the DCMU-poisoned reaction, and in the absence of ADP and MgCl₂. Under these conditions the quantum requirements at zero incident intensity were 6.2 at 649 and 678 μm and 4.0 at 703 and 720 μm.

The intensity parameter k₀ could be measured reliably only at 649 and 678 μm and values of 1.3 x 10⁻⁷ equivalents-liters⁻¹-sec⁻¹ were obtained in each case. These results show that the quantasome preparations are almost half as active as chloroplasts in their ability to photoreduce NADP. We feel that the demonstration of such a high level of activity increases the plausibility of the important assumption underlying our quantum requirement calculations for the chloroplast system—namely, that the true absorption spectra of chloroplasts and quantasomes are essentially identical in the long wavelength region.

DISCUSSION

Three recent publications present action spectra for the absolute quantum requirement of NADP reduction by chloroplasts with water as
reductant$^5-7$. In addition, results for the DCMU-poisoned system using ascorbate/DCPIP as reductant are presented by Hoch and Martin$^5$. Several values giving the wavelength dependence for the poisoned system have been reported on a relative basis by Arnon, et al.$^4$. Some pertinent results extracted from these studies are put in the common units and summarized along with our results in Table I. There is a substantial lack of agreement among the various workers. For example, for the normal reaction at about 700 nm, Black, et al.$^6$ observe a quantum requirement of 14 einsteins/equivalent; Hoch and Martin$^4$ report 2.9 to 3.3, and Govindjee, et al.$^5$ report 6.3, and we observe 4.0.

Black, et al. used absorption values measured by the opal glass technique. It would appear from the previous discussion of the large contribution of scattered light to such measurements that the large quantum requirements they report for the normal reaction at long wavelengths may be appreciably overestimated on this basis. This factor may also account for their rise in quantum requirement around 550 nm where the chloroplast scattering is, again, relatively great. Our studies show a quantum requirement at 550 nm which is slightly lower, if anything, than those at longer wavelengths.

In general, our results agree rather well with those of Hoch and Martin$^5$ for both the normal and poisoned systems. Their study is based on absorption measurements using an integrating sphere spectrophotometer, which should markedly reduce the light scattering interference at long wavelengths. Although they were aware of the dark reoxidation of NADPH$_2$, it is not apparent from their paper that any correction for it was attempted. This factor could be expected to contribute to the discrepancies
which remain between their data and ours. These differences are greatest for the DCMU-poisoned system, where we find the reoxidation to be more pronounced. This would have the effect of making their quantum requirements appear to be too large. They are some 50% greater than our corresponding values.

The values obtained by Govindjee, et al. are substantially greater throughout this wavelength region for the normal system. This discrepancy is puzzling since their measurements were carried out using the same techniques and with the same reaction mixture as were those reported by Hoch and Martin.

At wavelengths less than 660 µm our measurements indicate quantum requirements for both the normal and the poisoned reactions of 1.5 to 3 einsteins/equivalent and not strongly wavelength dependent. Hoch and Martin and Black, et al. report little change in the quantum requirements from 640 to 678 µm. The relative quantum requirements obtained by Arnon, et al. for the DCMU-poisoned system exhibit a maximum at 661 µm and lower values at both longer and shorter wavelengths. This behavior is different from each of the other previous studies and from ours. Arnon, et al. did not extrapolate their measurements to zero light intensity, and insufficient details of their experiments are reported in the publication for us to attempt to resolve the discrepancies.

**Mechanism involving two pigment systems**

Our observations of the dependence of quantum requirement for NADP reduction on wavelength for these two reaction systems can be accounted for nicely in terms of the two-pigment system mechanism proposed for higher plant photosynthetic energy conversion\(^1\)\(^-\)\(^3\). We follow the notation
of Duyssens, et al.\textsuperscript{21} in designating the long wavelength absorbing pigments as system I and the shorter wavelength pigments as system II. There is much evidence to support a current formulation of the mechanism of photosynthetic energy conversion in which two photo-absorbing steps act in series to give the reduction of NADP using water as reductant and leading to the evolution of molecular oxygen. In this mechanism pigment system I mediates the transfer of electrons from an electron transport intermediate (cytochrome f ?) to NADP via ferredoxin, and pigment system II mediates the transfer of electrons from water to an oxidized intermediate (plastocyanin ?) leading to oxygen production. The reduced plastocyanin would then react through an electron transport chain with oxidized cytochrome f in a dark reaction which can couple to a phosphorylation step. Both pigment systems must be activated for the normal reaction to proceed efficiently. With added DCMU the second light reaction is blocked and electrons provided by cofactors such as reduced DCPIP can be transferred to NADP using system I alone.

The mechanism described above would require a minimum of two quanta absorbed per electron transferred to NADP for the normal reaction and one quantum per electron for the poisoned system. At wavelengths where one of the pigment systems absorbs relatively weakly, higher quantum requirements would be expected.

Our results can be accounted for in the framework of this mechanism by assuming that, at wavelengths longer than 700 \textmu m, pigment system I accounts for approximately 80\% of the total absorption and system II accounts for 20\%. At wavelengths shorter than 680 \textmu m each pigment system is responsible for about half the total absorption. Furthermore, we
assume that electronic excitation energy cannot be transferred as such from one pigment system to the other. These assumptions lead to the following conclusions: (i) for the normal reaction at short wavelengths the optimum quantum requirement of 2 would be obtained since both pigment systems are being activated at equal rates; (ii) at longer wavelengths the normal reaction would have a quantum requirement four times greater, since the low absorbance of pigment system II at these wavelengths limits the overall efficiency with which electrons can be obtained from water; (iii) for the DCMU-poisoned reaction a quantum requirement of 2 is observed at short wavelengths, since only half the quanta absorbed are used to excite system I; and (iv) at longer wavelengths, where system I accounts for 80% of the absorption, this quantum requirement drops nearly to 1. These conclusions are in good agreement with the data of Table I and, if the hypotheses are correct, they lead to the observation that at low limiting light intensities these photoreductions of NADP by chloroplasts are operating very near optimum values.

It would be tempting to interpret the initial rapid transient increase in absorbance at 339 μm (stage ii, Fig. 5) as representing an even more efficient production of NADPH₂ during the very first stages of the reaction. Such a conclusion is not yet warranted, however. The effect is so small that we cannot convincingly demonstrate that this earliest absorption change does indeed represent NADPH₂ formation or even reflects a band which exhibits a maximum at 339 μm. The effect is somewhat variable and is frequently absent or barely observable at the lowest light intensities. It may, therefore, reflect the initial approach to the steady-state condition, during which the concentration of T is decreasing from its initial value T₀ and during which a faster
reaction velocity would be expected. This simple explanation will not account for the rapid transient decrease in absorbance at 339 
when the light is turned off, however. The two effects together could be accounted for by postulating a small activated (bound) fraction of NADP which gets converted to NADPH₂ very efficiently at the onset of illumination and is regenerated at the expense of bound NADPH₂ when the light is turned off. Further studies are called for in order to elucidate the cause of these interesting transients.

**Kinetic limitations at high light intensities**

An analysis of the dependence of the quantum requirement for NADP reduction on the incident light intensity suggests that at higher intensities an increasing fraction of the absorbed radiation is diverted from the photochemical process. The simplest explanation implicates an intermediate or trapping site present in relatively low concentration in the system. Electronic excitation energy resulting from the absorbed light is transferred to this intermediate or trap and results in its activation. Apparently the return of this species to its unexcited state is a relatively slow reaction in comparison with the rate of absorption of quanta at high intensities, and a low steady-state concentration of unexcited intermediates results. Under these circumstances the excess absorbed quanta are dissipated, presumably largely by a thermal process, and a high quantum requirement for the photo-reduction is observed. The kinetics are essentially independent of the wavelength of exciting light, with the possible exception of the normal reaction at long wavelengths. The limiting factor is apparently endogenous to the chloroplasts, since both PMPR and DCPIP (when present)
were demonstrated to be at concentrations near saturation levels.

A reasonable explanation of this effect can be made in terms of
the photosynthetic unit. Park and Biggins\(^2\) give evidence that the
quantasomes observed in spinach chloroplasts are the morphological
expression of the photosynthetic units. If we assume that there is
only one trapping site per photosynthetic unit and that its pigment
composition is that reported by Park and Biggins, we can estimate the
decay time of the excited traps from our quantum requirement versus
intensity data—specifically, from the values of \(k_D\).

An absorbed intensity of \(4 \times 10^{-7}\) einsteins/sec-liter is sufficient
to double the quantum requirement at 678 m\(\mu\) for NADP reduction by
reduced DCPIP in a suspension of spinach chloroplasts containing about
5 \(\times 10^{-6}\) moles/liter of chlorophyll \(a\). In accordance with the mechanism
proposed for the intensity dependence, we propose that under these con-
ditions at steady state only half the photosynthetic units are present
in their unexcited states. Park and Biggins\(^2\) estimate that each quanta-
some or photosynthetic unit contains 160 chlorophyll \(a\) molecules. The
above suspension of chloroplasts will contain 3.1 \(\times 10^{-8}\) moles of
quantasomes/liter, on this basis. Half the absorbed intensity, or
2 \(\times 10^{-7}\) einsteins/sec-liter, is actually effective in exciting the
traps and, under these conditions, each trap is excited on the average
6.5 times per second. A 50\% steady-state level of excited traps is
maintained under conditions where the rate of decay of excited traps
is roughly equal to their rate of formation. Hence, the decay time
of excited traps must be of the order of 0.15 sec for the DCMU-poisoned
system.
For the normal reaction, with water as electron donor, the light intensity required to double the quantum requirement of NADP reduction is \(12 \times 10^{-7}\) einsteins/sec-liter. The decay of excited units for this reaction is therefore about 0.05 sec. This compares favorably with values of 0.03 sec for the time between successive effective quantum absorption acts determined in intermittent illumination studies by Emerson and Arnold\(^{25}\). Because the mechanisms of the two reactions we have studied are certainly different, we cannot know whether the rate-limiting step occurs at the same point in each case. It does seem that the turnover time in isolated chloroplasts is three times faster when electrons are provided from water via pigment system II than when they derive from reduced DCPIP. Since DCPIP is present in saturating amounts, the slower rate-limiting step in the latter case implies the participation of an endogenous cofactor which is not common to the pathway where water acts as reductant. The dependence of quantum requirement on absorbed intensity for NADP reduction by reduced DCPIP is very similar for quanta-some and for chloroplast preparations. Hence, the lifetime of the excited intermediate must be nearly identical for the two systems. This is a further indication that the intermediate is endogenous to the chloroplast or quantasome structure.

**ACKNOWLEDGEMENTS**

The authors wish to express their appreciation to Dr. J. A. Bassham and Irwin D. Kuntz, Jr. for stimulating discussions concerning the interpretation of some of the observations. The investigations described in this report were sponsored, in part, by the United States Atomic Energy Commission.
APPENDIX

The relationship between quantum requirement and absorbed intensity for the mechanism presented in equations (1) - (4) can be derived using a modification of the conventional steady-state kinetics treatment. We assign a rate constant, $k_t$, to the sum of reactions (2) and (3) at low limiting light intensities, and an intrinsic probability, $p$, to characterize the fraction of C* disappearing by reaction (3). Under these circumstances the rate of reaction (3) is

$$\frac{dT^*}{dt} = k_t p C^*$$

whereas at higher light intensities it decreases to

$$\frac{dT^*}{dt} = k_t p \frac{T}{T_0} C^* = k_t p \frac{T - T^*}{T_0} C^*$$

owing to the reduction of the steady-state concentration of intermediates, $T$. By the steady-state approximation to the formation and disappearance of $T^*$ by reactions (3) and (4),

$$\frac{dT^*}{dt} = k_t p C^* \frac{T - T^*}{T_0} - k_1 T^* = 0$$

Similarly, for $C^*$

$$\frac{dC^*}{dt} = \phi I - k_t C^* = 0$$

Eliminating $C^*$ between these two equations and solving for $T^*$, we obtain

$$T^* = \frac{\phi I T_0}{k_1 T_0 + p \phi I}$$

By substituting this expression for $T^*$ into the equation for the rate of formation of products by reaction (4),

$$V = \frac{dP}{dt} = k_1 T^*$$

we obtain equation (5) directly. The final result does not depend on the value of $k_t$, but instead on the intrinsic probability of $C^*$ decaying.
via reaction (3) as opposed to reaction (2). It is this competition, plus the assumption that some reactive intermediate $T$ is appreciably depleted at higher reaction rates, that leads to the prediction of a linear increase in quantum requirement with increasing light intensity.

ABBREVIATIONS USED

- $\text{NADP}$ nicotinamide adenine dinucleotide phosphate
- $\text{NADPH}_2$ " " " " reduced form
- $\text{ADP}$ adenosine 5'-diphosphate
- $\text{DCMU}$ 3-(3,4-dichlorophenyl)-1,1-dimethylurea
- $\text{PPNR}$ photosynthetic pyridine nucleotide reductase
- $\text{Tris}$ tris(hydroxymethyl) aminomethane
- $\text{DCPIP}$ 2,6-dichlorophenolindophenol
REFERENCES

15. R. Emerson and C. M. Lewis, Amer. J. Bot. 30 (1943) 165.


24 R. S. Park and J. Biggins, Science 144 (1964) 1009.

TABLE I

Quantum requirement for NADP reduction by spinach chloroplasts at wavelengths from 550 to 740 μm—comparison with literature values. Q₀ is the zero intensity quantum requirement in einsteins/equivalent at wavelength λ (μm).

<table>
<thead>
<tr>
<th>Normal System (H₂O Donor)</th>
<th>Hoch &amp; Martin⁵</th>
<th>Govindje, Govindje, &amp; Hoch⁶</th>
<th>This study</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>λ</td>
<td>Q₀</td>
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<tr>
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<td>λ</td>
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LEGENDS FOR FIGURES

Fig. 1. Apparatus; sample compartment of the Cary Model 1462 Scattered-Transmission Accessory, showing the modifications for illumination of the sample cuvette. Filters IF 1 (or monochromator) and CF 1 select the actinic wavelengths from a tungsten lamp. Filters IF 2 and CF 2 prevent actinic light and chloroplast fluorescence from interfering with the photomultiplier.

Fig. 2. Absorption spectra of spinach chloroplasts from 550 to 800 nm. Upper curve (solid): normal transmission spectrum using Cary Model 14 Spectrophotometer with normal cell compartment and HITV R-136 photomultiplier (see Ref. 13). Lower curve (solid): scattered-transmission spectrum using Cary Model 1462 Scattered-Transmission Accessory with Dumont 6911 photomultiplier. Dashed curve: opal glass spectrum, using Cary 14 with normal cell compartment and R-136 photomultiplier; matched opal glass diffusing plates in sample and reference beams; superimposed on scattered-transmission spectrum out to 700 nm. All three measurements were made on the same chloroplast suspension.

Fig. 3. Absorption spectra of spinach chloroplasts and quantasomes at long wavelengths using Cary 14 Spectrophotometer with Model 1462 Scattered-Transmission Accessory and Dumont 6911 photomultiplier. Curves are normalized to an absorbance of 10.0 at 678 nm. Lower curve (dashed): computed quantasome absorption corrected for light scattering.
LEGENDS FOR FIGURES (Continued)

Fig. 4. Rayleigh scattering of spinach quantasomes from 750 to 900 μm. Turbidity of quantasomes (middle curve of Fig. 3) versus inverse fourth power of wavelength.

Fig. 5. A trace of the time course of the photoreduction of NADP by isolated chloroplasts excited at 720 μm. (DCMU/DCPIP/ascorbate system). A molar extinction coefficient of $6.2 \times 10^3 \text{ l-mole}^{-1}\text{cm}^{-1}$ for NADPH$_2$ at 339 μm was used.

Figs. 6 and 7. The relationship between the quantum requirement for NADP reduction and incident intensity. ———, the normal reaction where H$_2$O is electron donor and ———, ascorbate/DCPIP as electron donor in the DCMU-poisoned reaction.

Fig. 8. The action spectrum for NADP reduction by isolated chloroplasts for the normal (——o——) and DCMU-poisoned (——o——) systems. The quantum requirements are values obtained from extrapolations to zero intensity.

Fig. 9. The effect of the concentration of DCPIP on the quantum yield of NADP reduction by chloroplasts excited at 678 μm. The standard reaction mixture was used with sufficient chloroplasts (1 cm path) to give an absorbance of 0.80 at 678 μm. Incident intensities used were 0.20 (filled circles) and 0.57 (open circles) nano-einstains/cm$^2$-sec.
CARY 14 SPECTROPHOTOMETER

REFERENCE

SAMPLE

LENS

IF 1

IF 2

CF 1

CF 2

PHOTOMULTIPLIER

WATER FILTER

INFRARED-ABSORBING FILTER

ACTINIC LIGHT

Fig. 1

MUB-2802
Fig. 2
ABSORPTION AND LIGHT SCATTERING

Fig. 3
SPINACH QUANTASOMES
Rayleigh Scattering
A_{678} = 16.2
PHOTOREDUCTION OF NADP BY SPINACH
CHLOROPLASTS

TIME (Min)

ABSORBANCE 339 m

FIG. 5

MUB-2803

LIGHT OFF

LIGHT ON
NADP REDUCTION BY CHLOROPLASTS AT 720 mp

-  * = \( \text{H}_2\text{O} \)
-  * * = Na-ascorbate, DCPIP, DCMU

\[ \text{QUANTUM REQUIREMENT: einsteins} \times 10^9/\text{cm}^2 \times \text{sec.} \]

\[ \text{INCIDENT INTENSITY: einsteins} \times 10^9/\text{cm}^2 \times \text{sec.} \]

**Fig. 7**
PHOTOREDUCTION OF NADP BY SPINACH CHLOROPLASTS

- $\text{H}_2\text{O}$
- ascorbate / DCPIP / DCMU

QUANTUM REQUIREMENT einsteins/equivalent

WAVELENGTH, m$\mu$

Fig. 8
NADP REDUCTION BY CHLOROPLASTS
Dependence on DCPIP Concentration

$\text{Quanum Yield (equivalents einstein)}$

$\text{DCPIP Concentration (\(\mu\) moles/ml)}$

$I_0 = 0.20 \text{ nanoeinstins} \frac{\text{cm}^2}{\text{sec}}$

$I_0 = 0.57 \text{ nanoeinstins} \frac{\text{cm}^2}{\text{sec}}$

MUB-3257

Fig. 9
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