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PIGMENT SYSTEMS AND ELECTRON TRANSPORT IN CHLOROPLASTS

I. QUANTUM REQUIREMENTS FOR THE TWO LIGHT REACTIONS IN SPINACH CHLOROPLASTS

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SUMMARY

From studies of electron-transport reactions of isolated spinach chloroplasts, we observe the following quantum requirements: (A) For the photoreduction of $NADP^+$, measured both aerobically and anaerobically, in a 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) poisoned system with ascorbate and reduced 2,6-dichlorophenolindophenol ($DCIPH_2$) present as electron donors, the quantum requirements are 1.0 $\pm$ 0.05 at wavelengths longer than 700 nm of actinic light, and 1.5–2.5 for wavelengths between 620 and 680 nm. (B) For the photoreduction of 2,6-dichlorophenolindophenol (DCIP) with water as the electron donor, the quantum requirements are 1.0 $\pm$ 0.05 in the range 630–660 nm. (C) For the photoreduction of $NADP^+$ with water as the electron donor, the quantum requirements are 2.0 $\pm$ 0.1 in the wavelength range 640–678 nm of actinic light, increasing to 6 or greater at wavelengths beyond 700 nm. These results are shown to be inconsistent with the "separate package" model for the two pigment systems in higher plant photosynthetic electron transport. The evidence is most easily interpreted using a "controlled spillover" model, in which the transfer of electronic excitation energy from one pigment system to the other is under the control of incompletely identified factors in the reaction mixture.

At moderate light intensities the steady state rate of the [ascorbate + $DCIPH_2$ $\rightarrow$ $NADP^+$] reaction (A) in the presence of DCMU and added ferredoxin can be increased more than 3 times when saturating amounts of plastocyanin and ferredoxin–NADP reductase are added to the chloroplasts. Similarly, the steady-state rate of the [$H_2O \rightarrow$ DCIP] Hill reaction (B) is increased about 3-fold by added $MgCl_2$ and plastocyanin, but added ferredoxin or ferredoxin–NADP reductase have no effect on this reaction. Plastocyanin appears to be the electron transport component which couples to DCIP, either in the oxidized or in the reduced form, in the reaction media. The steady-state rate of the [$H_2O \rightarrow NADP^+$] reaction (C) with saturating amounts of ferredoxin can be further increased more than 3-fold when $MgCl_2$, plastocyanin and ferredoxin–NADP reductase are added.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; $DCIPH_2$, reduced 2,6-dichlorophenolindophenol.

INTRODUCTION

In the evolution of models based on the original proposal by HILL AND BENDALL[1] of the two-light reaction or Z scheme for higher plant photosynthesis, two contrasting pictures of the organization and function of the chlorophyll pigments have emerged[2,3]. In the first of these, the "separate package" model, each light reaction is thought to be associated with its own array of light-absorbing pigment molecules, and no communication at the level of electronic excitation transfer is permitted between the two arrays. It is as if the two light reactions, each with associated pigment system, are physically separated from one another. In the second, or "spillover" model, the pigment arrays are still distinct, but electronic excitation transfer is permitted between them in order to make up for deficiencies in the activation of one or the other light reactions. The series two-light scheme of HILL AND BENDALL requires that both light reactions be driven at equal rates for optimum overall photosynthetic efficiency. An extreme modification of the "spillover" hypothesis, in which a major portion of the chlorophyll pigments is common to both photosystems, has been proposed by AVRON AND BEN-HAYYIM[4]. In this common pool one finds all, or nearly all, of the bulk pigments absorbing in the red region; *i.e.* at wavelengths shorter than about 680 nm. A common feature of all of the models is the assignment of far-red ($\lambda > 700$ nm) absorption almost exclusively to Photosystem I. This is thought to be responsible for the "red-drop" in the quantum yield of photosynthesis at long wavelengths first observed by EMERSON AND LEWIS[5,6].

There are several ways in which these models can be tested experimentally and in which they predict different results. One approach is to make careful measurements of quantum yields at different wavelengths for each light reaction operating in the absence of the other. According to the "separate package" hypothesis, photons absorbed by the pigment system of the functioning light reaction can be used photochemically and those photons absorbed by the pigments of the non-operative photosystem will necessarily be wasted. The latter will result in fluorescence or heat, but never in any photochemistry. By contrast, in the "spillover" hypothesis, photons absorbed in either pigment system have, by virtue of the possibility of electronic excitation transfer, the potential ability to do photochemistry. As pointed out in a previous publication[7], the "separate package" hypothesis requires that at a given wavelength of activation the sum of the quantum yields for the two light reactions measured separately ($\phi_I + \phi_{II}$) can never exceed 1.0 equiv·(einstein absorbed)$^{-1}$. According to the "spillover" hypothesis, the sum of quantum yields may exceed 1.0, and may approach a limiting value of 2.0 in the case of perfectly efficient electronic excitation transfer.

Until recently, such studies of quantum yield spectra have not produced convincing evidence of sums exceeding 1.0 (ref. 7–9). Unfortunately, this result is not conclusive, as it is always possible to argue that the chloroplasts under investigation were partially inactivated during preparation and this resulted in artificially low yields. Now AVRON AND BEN-HAYYIM[4] have presented experimental findings in which the quantum yield at 640 nm for the ferricyanide Hill reaction, probably associated solely with Photosystem II, is 1.0 and that for DCMU-blocked photoreduction of $NADP^+$ by ascorbate/$DCIPH_2$, a Photosystem I reaction, is about 0.5. With diquat or FMN as the electron acceptor even higher values were reported. Because

these results appeared to be in conflict with findings previously reported from this laboratory, we have undertaken a new study of this question. Using different assay systems and making extensive efforts to optimize chloroplast activities, we have now obtained results which are in essential agreement with those of AVRON AND BEN-HAYYIM[4]. A clear consequence of these findings is that the simple "separate package" hypothesis is no longer tenable. Which of several versions of the "spill-over" hypothesis is correct is a much more difficult question to answer.

An alternative approach to this question can be made by investigations of the Emerson enhancement effect on isolated chloroplasts. Results of such studies, which generally confirm the conclusions referred to above, are the subject of the following paper.

MATERIALS AND METHODS

Spinach and preparation of chloroplasts

Chloroplasts were isolated from 6–8 week old spinach plants grown from seed in a growth chamber[10]; *Spinacia oleracea*, var. early hybrid No. 7. was used. In order to obtain the maximum photochemical capability, the spinach leaves were picked 4–8 h after the start of the illumination cycle in the growth chamber and just before the isolation procedure. The leaves were rinsed with cold distilled water, ribs were removed, and the leaves were then stored at —20° for 10 min. 10 g of leaves were homogenized between 10 and 15 sec in 50 ml of 0.5 M sucrose–0.1 M Tricine buffer (pH 7.6) in a Waring blendor. The resultant homogenate was strained through 8 layers of cheese-cloth and centrifuged at 200 × *g* for 1 min. The supernatant was then centrifuged at 1000 × *g* for 10 min and the precipitate resuspended in 1 ml of 0.5 M sucrose–0.05 M Tricine (pH 7.6) and stored at 0°. All the above procedures were carried out at 0°. Optimal activity of the chloroplasts could be obtained only within the first 6 h following isolation.

Reagents

$NADP^+$ was obtained from P-L Biochemical, Inc., Milwaukee, Wisc.; DCIP and Tricine from Sigma Chemical Co., St. Louis, Mo.; sodium ascorbate from Calbiochem, Los Angeles, Calif.; sucrose from J. T. Baker Chemical Co., N. J.; DCMU from E. I. DuPont de Nemours, Wilmington, Dela.; nitrogen gas directly from liquid nitrogen, obtained from Pacific Oxygen Co., Oakland, Calif.

Apparatus and light intensity measurements

Quantum yield measurements at various activation wavelengths were carried out using a modified Cary Model 14 spectrophotometer, as described by SAUER AND BIGGINS[9] for photoreduction of $NADP^+$, and by SAUER AND PARK[10] for photoreduction of DCIP. Measurements of light intensity were based on secondary standard lamps in the same way as SAUER AND BIGGINS[9]. The actinic light was obtained from a Bausch and Lomb monochrometer (red-blazed grating) with supplementary cut-off filters[10]. Monochromator slit widths were set at 3 mm, resulting in an actinic bandwidth of 10 nm. The quantity of light absorbed by chloroplasts was measured using the same method as SAUER AND BIGGINS[9].

Preparation of ferredoxin, NADP reductase and plastocyanin

Ferredoxin was prepared from commercial spinach according to the procedures of TAGAWA AND ARNON[11]. The ratio of absorbances at 420 nm and 274 nm of purified ferredoxin was 0.445. Ferredoxin–NADP reductase and plastocyanin were isolated in the same preparation as was ferredoxin. The NADP reductase was fractionated from the DEAE-cellulose column at $[Cl^-] = 0.12$ mole·l^{-1} and plastocyanin at $[Cl^-] = 0.26$ mole·l^{-1}. Both NADP reductase and plastocyanin were used without further purification with $(NH_4)_2SO_4$.

Reaction mixtures

The reaction mixture for the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction contained the following in μmoles·ml^{-1}: Tricine (pH 7.5), 45; $MgCl_2$, 7.5; DCIP, 0.053; DCMU, 0.01; sodium ascorbate, 5.0; $NADP^+$, 0.67; ferredoxin, 33.3 μg·ml^{-1}; NADP reductase, saturating amount or not added; plastocyanin, saturating amount or not added; chlorophyll, 13 μg·ml^{-1}.

The reaction mixture for the [$H_2O \rightarrow$ DCIP] reaction contained the following in μmoles·ml^{-1}: Tricine (pH 7.5), 45; $MgCl_2$, 4.5; DCIP, 0.027; plastocyanin, saturating amount; chlorophyll, 13 μg·ml^{-1}.

The reaction mixture for the [$H_2O \rightarrow NADP^+$] reaction contained the following in μmoles·ml^{-1}: Tricine (pH 7.5), 45; $MgCl_2$, 7.5; ferredoxin, 67 μg·ml^{-1}; $NADP^+$, 0.67; NADP reductase, saturating amount; plastocyanin, saturating amount; chlorophyll, 13 μg·ml^{-1}.

Procedures for obtaining anaerobic conditions

3 ml of reaction mixture was prepared in a small test tube (0.5 cm × 6 cm). The chloroplasts were added to the reaction mixture in the dark. The test tube was covered with a tight rubber cap and shaken well. The reaction mixture was flushed with nitrogen while shaking for 2 min. The nitrogen was passed through syringe needles penetrating the rubber cap on the test tube. The reaction mixture was transferred quickly with a nitrogen-flushed syringe into two cuvettes, on which rubber caps had been tied firmly with string, and then flushed with nitrogen through syringe needles for 2 min. These procedures were carried out in the dark.

Measurement of chlorophyll concentrations

The measurement of total chlorophyll and chlorophyll *a* and *b* concentrations[12,13] are according to the following equations, where the chlorophylls are in 80 % acetone solutions

$$\text{Chl } (a+b)\ (\text{mg}\cdot\text{ml}^{-1}) = 2.9\cdot 10^{-2}\,(A_{652\text{ nm}}) \quad (1)$$

$$\text{Chl } a\ (\mu\text{g}\cdot\text{ml}^{-1}) = 11.63\,(A_{665\text{ nm}}) - 2.39\,(A_{649\text{ nm}}) \quad (2)$$

$$\text{Chl } b\ (\mu\text{g}\cdot\text{ml}^{-1}) = 20.11\,(A_{649\text{ nm}}) - 5.18\,(A_{665\text{ nm}}) \quad (3)$$

where A_λ = the absorbance of the chlorophyll solution in 80 % acetone at wavelength λ.

RESULTS

Photosystem I: [Ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction with no added plastocyanin or NADP reductase

Typical time course for the photoreduction of $NADP^+$. The typical time course for the photoreduction of $NADP^+$ at high light intensity by isolated chloroplasts for

the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction is shown in Fig. 1. It shows that there is a rapid initial burst just after turning on the actinic light, as had been reported previously[9]. This suggests that there is a reservoir of electrons that can be rapidly transferred to $NADP^+$ *via* Photosystem I, and that the rate-limiting step is then the replenishment of this reservoir by the ascorbate/$DCIPH_2$ couple. The second portion of the slope is initially linear, corresponding to the steady-state photoreduction of $NADP^+$ in this system. There is a rapid small reversal at the time of turning off the exciting light, which may result from the reverse oxidation of NADPH in the dark by the depleted reservoir of reducing power.

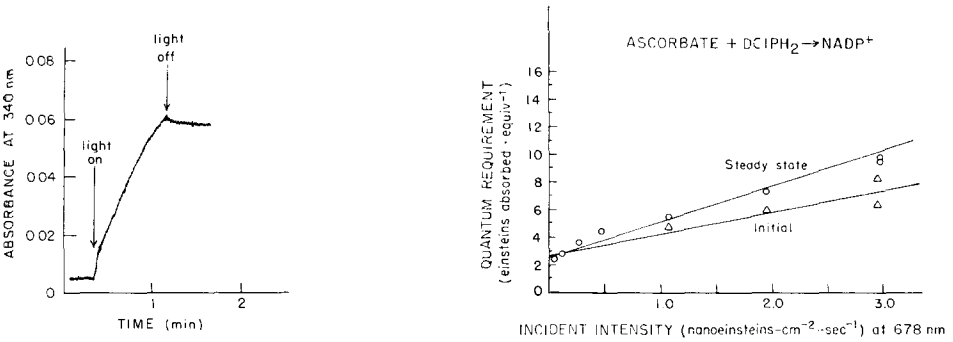

Fig. 1. Typical time course for the photoreduction of $NADP^+$ by isolated chloroplasts in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction. The reaction mixtures are given in the text, but plastocyanin and NADP reductase were not added. Actinic light at 678 nm; incident intensity, 3.0 nanoeinsteins·cm^{-2}·sec^{-1}; chlorophyll concentration, 13 $\mu g \cdot ml^{-1}$. The molar absorptivity for NADPH at 340 nm is $6.2 \cdot 10^3$ $l \cdot mole^{-1} \cdot cm^{-1}$.

Fig. 2. The relationship between the quantum requirement for $NADP^+$ reduction and incident intensity in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction; no plastocyanin or NADP reductase added. ○, obtained from the steady-state rate; △, obtained from the rapid initial phase. Illumination and chlorophyll concentration as in Fig. 1.

Primary quantum requirement. The intensity dependence of the quantum requirement (the reciprocal of the quantum yield) for the photoreduction of $NADP^+$ is shown in Fig. 2. The intrinsic quantum requirements are obtained by extrapolation to zero incident intensity. In most cases the quantum requirements obtained from the steady-state rate of the photoreduction of $NADP^+$ at different intensities lie fairly well on a straight line. The quantum requirements obtained from the rapid initial rates are plotted separately in Fig. 2. They also lie in a straight line, but with a smaller slope. The primary quantum requirements at zero intensity, obtained from either initial rates or steady-state rates, are identical. The rapid initial phase disappears at the lowest intensities, where light absorption becomes the rate-limiting step.

Action spectrum. The spectrum of the zero-intensity quantum requirements for the photoreduction of $NADP^+$ in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction is given as the solid curve in Fig. 3. The quantum requirements at wavelengths above 700 nm are near 1.0. Removal of oxygen does not produce any change in the quantum requirements or in the rate of $NADP^+$ photoreduction in this system.

Effect of ferredoxin. Ferredoxin is a protein containing non-heme iron and has one of the most negative redox potentials in chloroplasts[14]. It is known to be readily water soluble and essential to the electron transport chain. The rate of photoreduction

of $NADP^+$ was less than $\Delta A_{340\,nm} \cdot min^{-1} = 0.002$ when no ferredoxin was added to the reaction mixture together with the isolated chloroplasts. If other factors, such as light intensity, DCIP and $NADP^+$ concentrations, *etc.*, were not rate-limiting, the rate of photoreduction of $NADP^+$ increased to $\Delta A_{340\,nm} \cdot min^{-1} = 0.062$ as a linear function of the concentration of added ferredoxin, which increased from zero to 13 $\mu g \cdot ml^{-1}$ in the reaction mixture. The rate of $NADP^+$ photoreduction reached saturation at about $\Delta A_{340\,nm} \cdot min^{-1} = 0.084$ when the ferredoxin concentration was 27 $\mu g \cdot ml^{-1}$ of reaction mixture. In our standard reaction mixture 100 μg of ferredoxin in 3 ml of reaction mixture was used.

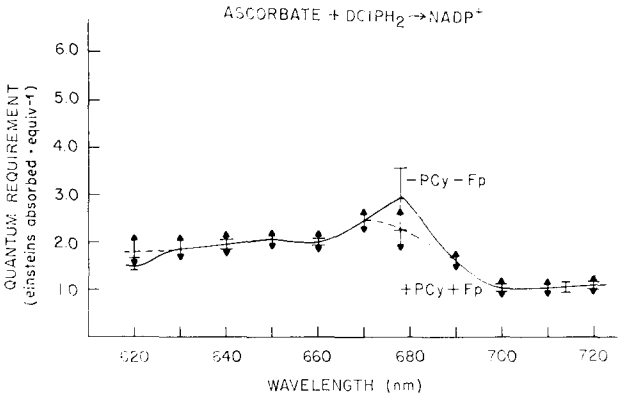

Fig. 3. Action spectrum of the zero-intensity quantum requirement of photoreduction of $NADP^+$ in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction, with and without added plastocyanin (PCy) and NADP reductase (Fp). Upper curve, sample without added plastocyanin or NADP reductase, shown as "bars". Lower curve, sample with added saturating plastocyanin and NADP reductase, shown as "arrow bars". Saturating ferredoxin was added to both reaction mixtures; other components are given in the text.

Effect of oxygen. It was thought that a portion of the electrons transported by Photosystem I in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction would leak to oxygen *via* ferredoxin[14]. For this reason, the reaction was studied under anaerobic conditions. Experiments showed, on the other hand, that the presence of air had no effect on the rates or quantum requirements of this reaction. Apparently the leak of electrons to oxygen in this system is negligible under our experimental conditions.

Photosystem I: [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction using saturating amounts of plastocyanin and NADP reductase

Plastocyanin. Plastocyanin is a copper-containing protein in chloroplasts and is readily water soluble. The addition of plastocyanin to the spinach chloroplast preparation restores part of what was lost during the isolation process. If other factors such as light intensity, ferredoxin concentration *etc.*, are not rate-limiting, the steady-state rate of photoreduction of $NADP^+$ in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] system can be increased more than 2-fold when optimal amounts of plastocyanin are added; and this incident intensity is 3.0 nanoeinsteins $\cdot cm^{-2} \cdot sec^{-1}$ at 678 nm (absorbed intensity is 0.23 nanoeinstein $\cdot (\mu g\ chl)^{-1} \cdot sec^{-1}$). The relationship between the rate of photoreduction of $NADP^+$ and plastocyanin concentration for this system is shown in Fig. 4.

Ferredoxin–NADP reductase. Ferredoxin–NADP reductase is another readily water-soluble protein in chloroplasts and is essential to the Photosystem I electron transport chain leading to $NADP^+$ (ref. 14). With optimal concentrations of ferredoxin and plastocyanin, the rate of photoreduction of $NADP^+$ can be increased further by added ferredoxin–NADP reductase. The relationship between the rate and the NADP reductase concentration is shown in Fig. 5. When the absorbed intensity was

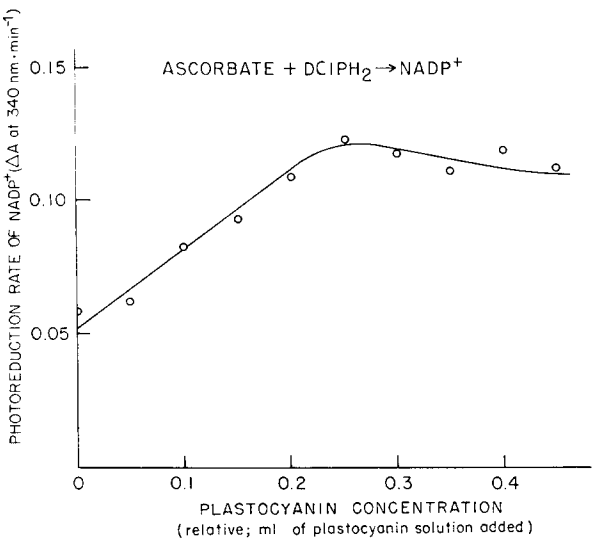

Fig. 4. The influence of plastocyanin concentration on the rate of photoreduction of $NADP^+$ in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction. The reaction mixture is given in the text; plastocyanin concentration unknown. Actinic light at 678 nm; incident intensity, 2.8 nanoeinsteins·cm^{-2}·sec^{-2}; chlorophyll concentration, 13 $\mu g \cdot ml^{-1}$.

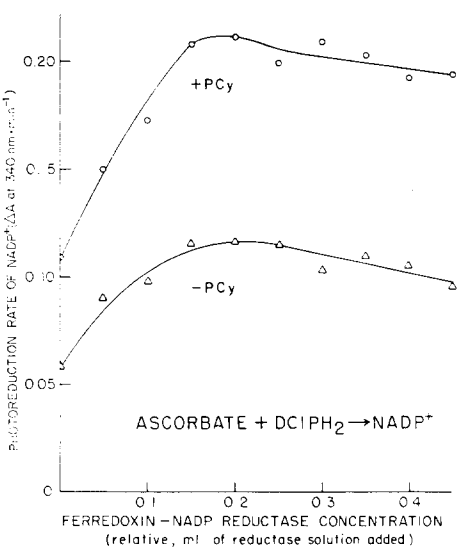

Fig. 5. The influence of plastocyanin and NADP reductase concentrations on the rate of photoreduction of $NADP^+$ in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction. ○—○, with added plastocyanin in saturating amounts. △—△, without added plastocyanin. Absolute concentrations of plastocyanin and reductase are unknown. Illumination conditions and chloroplast concentration as in Fig. 4.

0.23 nanoeinstein·(μg chl)$^{-1}$·sec^{-1}, or incident intensity was 3.0 nanoeinsteins·cm^{-2}·sec^{-1} at 678 nm, the steady-state rate of $NADP^+$ photoreduction, $\Delta A_{340\,nm}$·min^{-1}, was 0.058 when no plastocyanin or NADP reductase was added, and increased to 0.115 when optimal plastocyanin was added, and to 0.21 when both optimal plastocyanin and NADP reductase were present. The final rate with optimal plastocyanin and NADP reductase is more than 3 times greater than the rate without these additions.

Primary quantum requirements and action spectrum. Fig. 6 shows the intensity dependence of the [ascorbate + $DCIPH_2$ → $NADP^+$] reaction of chloroplasts in the presence of saturating amounts of both plastocyanin and ferredoxin–NADP reductase.

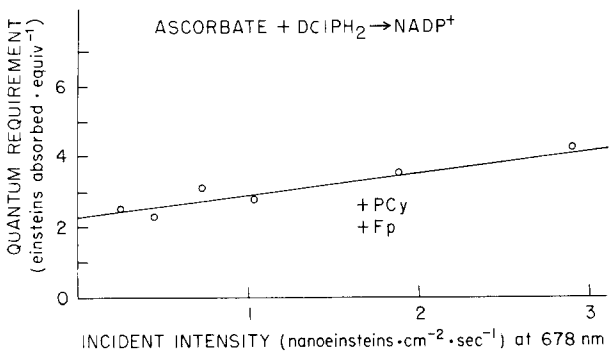

Fig. 6. The relationship between the quantum requirement of $NADP^+$ photoreduction and incident intensities in the [ascorbate + $DCIPH_2$ → $NADP^+$] reaction with saturating amounts of plastocyanin (PCy) and NADP reductase (Fp). Illumination conditions and chloroplast concentration as in Fig. 4.

Comparison with Fig. 2, where reaction conditions were similar except that no plastocyanin or reductase were added, shows that the reaction proceeds about 2.5 times faster in the former case at high intensities, but that both sets of data extrapolate to nearly the same quantum requirements at zero intensity, from 2.3 to 2.5 (einsteins absorbed)·equiv^{-1}.

Saturating amounts of plastocyanin and ferredoxin–NADP reductase had no significant effect on the zero intensity quantum requirements for the [ascorbate + $DCIPH_2$ → $NADP^+$] reaction for activation wavelengths between 620 and 720 nm. The general agreement is shown in Fig. 3.

Photosystem II: [H_2O → DCIP] reaction

pH dependence. In order to obtain the optimum rate, the photoreduction of DCIP was studied from pH 6.0 to 9.0, using 45 mM Tricine as the buffer to adjust the pH. The dependence of the photoreduction rate of DCIP on pH is shown in Fig. 7. 5–7 independent experiments were carried out at each pH. Although the extinction coefficient of DCIP decreases below pH 7 (ref. 15), we compare only the measured rate of change of absorbance at 580 nm. As shown in Fig. 7, the optimum rate of photoreduction of DCIP is at pH 7.5.

DCIP concentration. Since DCIP has an absorption maximum at 600 nm with a broad spectrum in the visible region, its presence diminishes the effective absorption of chloroplasts. Therefore we tried to find the optimum DCIP concentration, which

will not screen the absorption of chloroplasts too much but will approach saturation as a substrate. The concentration dependence of the rate of photoreduction of DCIP is shown in Fig. 8. A rather sharp maximum in the photoreduction rate occurs at a DCIP concentration of $2.7 \cdot 10^{-5}$ mole$\cdot$l^{-1}, and the activity falls off rather quickly at higher concentrations. In a previous study, where brief flashes of illumination were used, the activity remained high to $6 \cdot 10^{-5}$ mole$\cdot$l^{-1} of DCIP[16]. This indicates that the pattern and intensity of illumination are important factors in determining the optimal concentrations of substrates.

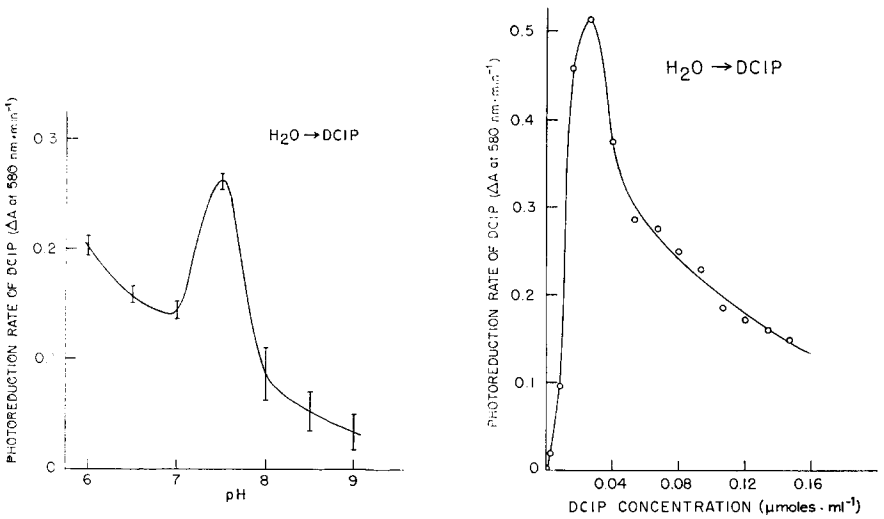

Fig. 7. The pH dependence of the rate of photoreduction of DCIP in the reaction [$H_2O \rightarrow$ DCIP]. Results obtained from 5–7 independent experiments at each pH. The reaction mixture is given in the text, except that no plastocyanin was added in these experiments. Illumination conditions and chloroplast concentration as in Fig. 4.

Fig. 8. The rate of photoreduction of DCIP as a function of DCIP concentration. The reaction mixture is given in the text, except that no plastocyanin was added. Illumination conditions and chloroplast concentration as in Fig. 4.

$MgCl_2$. Both Mg^{2+} and Cl^- are essential for photosynthesis. As in other reactions involving the transfer of a phosphate group, Mg^{2+} is required for phosphorylation by chloroplasts[17]. We found that by adding $MgCl_2$ the rate of the [$H_2O \rightarrow$ DCIP] reaction could be increased about 60 % over the rate in the absence of added $MgCl_2$ (Fig. 9).

Plastocyanin. Since plastocyanin has been implicated in non-cyclic electron transport from water to NADP[18–20] and also is readily water soluble, we investigated its effect on the rate of photoreduction of DCIP in the [$H_2O \rightarrow$ DCIP] reaction at incident intensity about 3.5 nanoeinsteins$\cdot$cm$^{-2}\cdot$sec^{-1} at 678 nm. Fig. 10 shows that the rate of photoreduction of DCIP is increased more than 40 % when saturated plastocyanin is added. Similar increases were observed in 6 separate experiments. After optimizing all the above conditions, the steady-state rate of photoreduction of DCIP was increased about 3-fold from $\Delta A_{580\ nm} \cdot min^{-1} = 0.28$–$0.70$. The incident intensity was near 3.0 nanoeinsteins$\cdot$cm$^{-2}\cdot$sec^{-1} throughout this part of the investigation.

Ferredoxin and NADP reductase. Both ferredoxin and NADP reductase were investigated as to their effects on the photoreduction of DCIP by the chloroplasts. Each of these components was added, separately or together, at different concentrations to the reaction mixture. No significant improvement on the photoreduction rate of DCIP was found when low concentrations of either (or both) ferredoxin or NADP reductase were added. A small decrease in the rate occurred when higher concentrations of either or both of them were added. Apparently, they are not involved in this part of the electron transport chain.

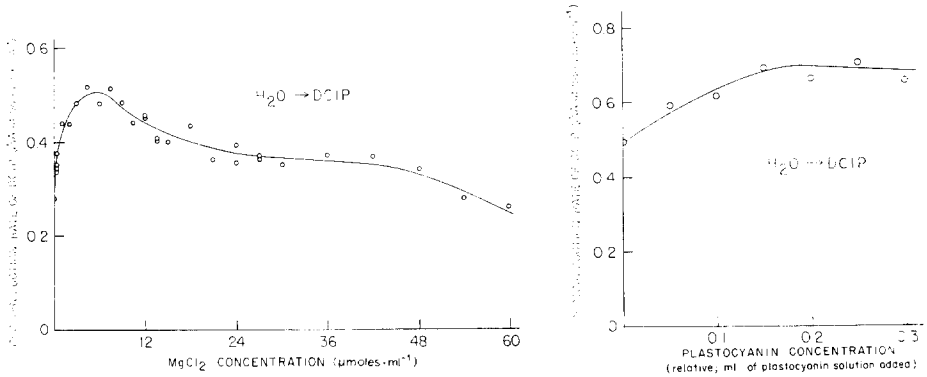

Fig. 9. The rate of photoreduction of DCIP as a function of $MgCl_2$ concentration. The reaction mixture is given in the text, except that no plastocyanin was added. Illumination conditions and chloroplast concentration as in Fig. 4.

Fig. 10. The rate of photoreduction of DCIP as a function of plastocyanin concentration. The reaction mixture is given in the text. Plastocyanin was isolated from spinach; its absolute concentration was unknown. Illumination conditions and chloroplast concentration as in Fig. 4.

Action spectrum of the quantum requirement. When the rate of photoreduction of DCIP was optimized, the quantum requirements of this reaction were measured over a range of light intensities at several wavelengths. The action spectrum of the zero-intensity quantum requirements is shown in Fig. 11. The values at each wavelength are averaged from the 5–7 independent experiments where chloroplasts were most active, from a total of more than 15 experiments performed. Quantum requirements of 1.0 $\pm$ 0.05 were obtained when the actinic light was between 630 and 660 nm.

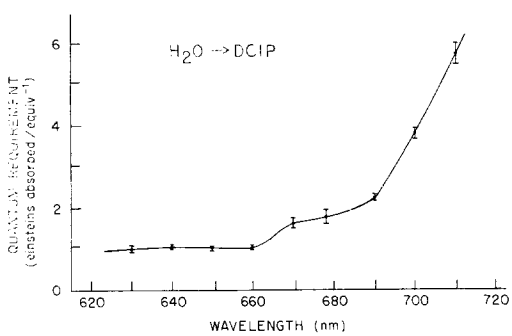

Fig. 11. Spectrum of the quantum requirements of photoreduction of DCIP in the [$H_2O \rightarrow$ DCIP] reaction, extrapolated to zero light intensity.

Higher values of the quantum requirement were obtained when the actinic light was at 670 nm or longer wavelengths.

Photosystem (I and II): [$H_2O \rightarrow NADP^+$] reaction

Optimum rate of photoreduction of $NADP^+$. The rate of photoreduction of $NADP^+$ by chloroplasts, as for the other reactions, is dependent on pH, the concentration of $MgCl_2$, plastocyanin, ferredoxin, and NADP reductase, method of preparation of chloroplasts and the age of spinach. Experiments have been carried out similar to those for the [$H_2O \rightarrow$ DCIP] reaction in order to optimize the reaction rate of the system. The optimum conditions are given as the standard reaction mixture in MATERIALS AND METHODS. $MgCl_2$, ferredoxin, plastocyanin and NADP reductase are all essential for this reaction. High rates of photoreduction of $NADP^+$ were obtained from pH 7.0 to 8.0, with a maximum at pH 7.5, which was used in our experiment. The rate of the reaction when saturating ferredoxin was added was 20-fold more than the rate when no ferredoxin was added. With the optimum concentration of $MgCl_2$, 7.5 μmoles$\cdot$ml^{-1}, the rate could be increased about 30% from $\Delta A_{340\,nm} \cdot min^{-1} = 0.065$–$0.090$, over the rate when no $MgCl_2$ was added.

With the optimum concentration of $MgCl_2$, ferredoxin and pH the steady-state photoreduction rate of $NADP^+$ of the reaction was more than doubled by adding saturating NADP reductase, and more than tripled by adding both saturating NADP reductase and plastocyanin. These experiments were carried out using an incident light intensity about 2.9 nanoeinsteins$\cdot$cm$^{-2}\cdot$sec^{-1} at 678 nm.

Action spectrum of quantum requirements. The action spectrum of zero-intensity quantum requirements for the [$H_2O \rightarrow NADP^+$] reaction is shown in Fig. 12. Only the optimized conditions for the reaction have been used for obtaining the quantum requirements. The results shown in Fig. 12 are the values obtained from the best 5–7 independent experiments where chloroplasts were most active from a total of more than 15 experiments performed. When chloroplasts were not active we were not able to obtain these low quantum requirements. The quantum requirements from 620 to 660 nm and at 678 nm of actinic light are very close to 2.0 $\pm$ 0.1, and they are slightly higher at 670 and 690 nm. The photoreduction of $NADP^+$ becomes very inefficient when the actinic light is at 700 nm or longer wavelengths.

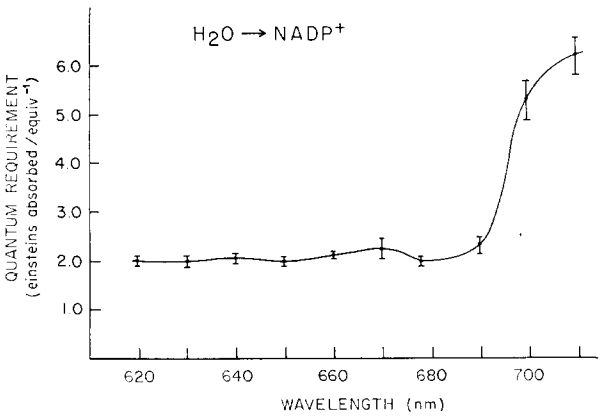

Fig. 12. Spectrum of the quantum requirements of photoreduction of $NADP^+$ in the [$H_2O \rightarrow NADP^+$] reaction, extrapolated to zero light intensity.

DISCUSSION

The normal course of photosynthetic electron transport in higher plant chloroplasts leads to the reduction of $NADP^+$ to NADPH and the oxidation of water to molecular oxygen. A current version of the mechanism of this process, based on the series two-light reaction scheme originally proposed by HILL AND BENDALL[1] and elaborated for the [$H_2O \rightarrow NADP^+$] reaction by LOSADA *et al.*[21], is shown in Fig. 13.

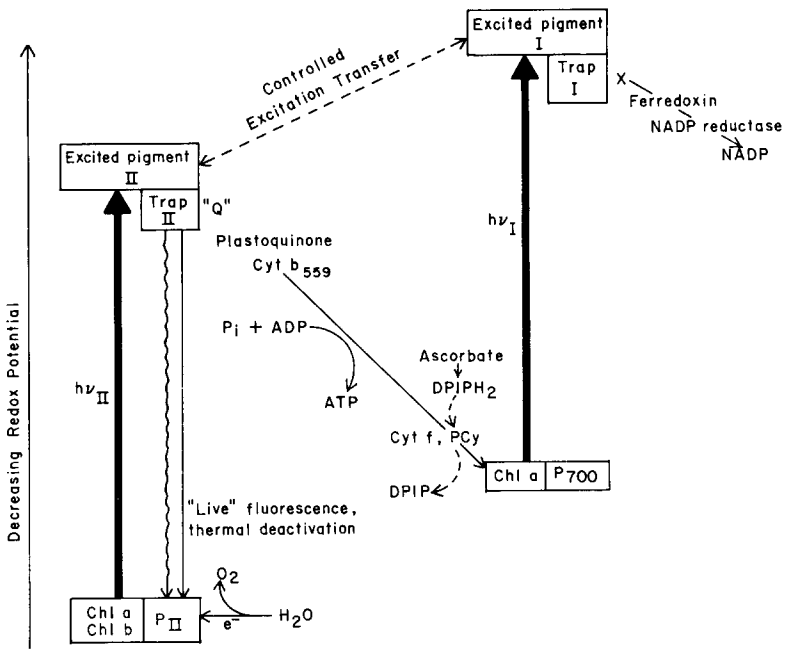

Fig. 13. Electron transport scheme for the two-light reaction mechanism in chloroplasts. Pcy, plastocyanin.

DCMU is a potent inhibitor of the reduction of $NADP^+$ (ref. 22), and this inhibition can be relieved, in part, by the addition of the couple $DCIPH_2$ *plus* ascorbate[23]. Based on the action spectrum of quantum requirements, HOCH AND MARTIN[8] and SAUER AND BIGGINS[9] suggested that the photoreduction of $NADP^+$ in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction is associated solely with Light reaction I. In the absence of DCMU, the oxidized form of DCIP serves as an electron acceptor for the chloroplast Hill reaction, leading to the photooxidation of water to molecular oxygen. This reaction has been assigned to Light reaction II alone, on the basis of action spectra and the absence of enhancement in the presence of long wavelengths of light, but it has not been possible to rule out the possibility that Light reaction I participates as well. A detailed discussion of this question was given by SAUER AND PARK[10]. On the basis of the results of the present study, where quantum yields of 1.0 equiv·(einstein absorbed)$^{-1}$ are observed for the [$H_2O \rightarrow$ DCIP] reaction using actinic wavelengths from 630 to 660 nm, we can now unambiguously assign this reaction to Light reaction II alone. If both light reactions were involved, then at least two absorbed photons would be required for each electron transferred and the quantum

yield would never exceed 0.5 at any actinic wavelength. From the analogous results of AVRON AND BEN-HAYYIM[4], the ferricyanide Hill reaction can also be assigned to Light reaction II alone. In our studies of the DCIP Hill reaction, the absence of any effect resulting from the addition of ferredoxin and/or ferredoxin–NADP reductase supports the assignment to Light reaction II.

The effect of added plastocyanin in increasing the rate of electron transport in the [$H_2O \rightarrow$ DCIP] reaction at moderate light intensities suggests that the DCIP accepts electrons close to Photosystem I in broken chloroplasts. Presumably added plastocyanin is relieving a rate-limiting step in this reaction under conditions where light is not limiting. This observation is consistent with the finding that methylamine, an uncoupler of non-cyclic photophosphorylation, also serves to accelerate electron flow in the [$H_2O \rightarrow$ DCIP] reaction[10,16]. The site of non-cyclic photophosphorylation is thought to occur between Photosystem II and plastocyanin. We believe that the influence of added plastocyanin on this System II reaction indicates that DCIP accepts electrons either directly from plastocyanin or else from an adjacent electron transport component whose oxidation state is controlled by plastocyanin.

Associated with each of the two photosynthetic reaction centers is an array of light absorbing or antenna pigment molecules (chlorophylls *a* and *b* and carotenoids in the case of higher plants). Many models have been proposed to describe the arrangement of these molecules[2–4]. In one model, the "separate package" hypothesis, each pigment molecule is associated with one or the other of the light reactions and no communication, by way of electronic excitation transfer, is permitted between them. As a consequence of this model, photons absorbed by a particular pigment molecule can be transferred only to the reaction center of its own pigment system. If that reaction center happens not to be functioning, then the excitation will be wasted and will appear only as fluorescence or heat. If we measure the quantum yields at low light intensities, where the arrival of photons is rate limiting, for each of the light reactions operating separately, then according to the "separate package" hypothesis the sum of the quantum yields at each wavelength should not exceed 1.0 (ref. 7). In Table I we present the zero-intensity quantum yields at several wavelengths between

TABLE I

QUANTUM YIELDS EXTRAPOLATED TO ZERO INTENSITY FOR $NADP^+$ REDUCTION BY [ASCORBATE + $DCIPH_2$] AND BY WATER AND FOR THE DCIP HILL REACTION OF SPINACH CHLOROPLASTS AT VARIOUS WAVELENGTHS

λ (nm)	*Quantum yield (equiv · (einstein absorbed)$^{-1}$)*			
	[*Ascorbate* + $DCIPH_2 \rightarrow NADP^+$] ϕ_I	[$H_2O \rightarrow DCIP$] ϕ_{II}	$\phi_I + \phi_{II}$	[$H_2O \rightarrow NADP^+$] $\phi_{(I+II)}$
620	0.67 ± 0.04			0.50 ± 0.02
630	0.61 ± 0.04	1.00 ± 0.04	1.61 ± 0.08	0.50 ± 0.02
640	0.53 ± 0.04	0.95 ± 0.05	1.48 ± 0.09	0.49 ± 0.02
650	0.51 ± 0.03	1.00 ± 0.05	1.51 ± 0.08	0.50 ± 0.02
660	0.50 ± 0.03	0.96 ± 0.05	1.46 ± 0.08	0.48 ± 0.03
670	0.43 ± 0.03	0.63 ± 0.05	1.06 ± 0.08	0.45 ± 0.05
678	0.38 ± 0.11	0.56 ± 0.06	0.94 ± 0.17	0.50 ± 0.03
690	0.61 ± 0.04	0.44 ± 0.03	1.05 ± 0.07	0.43 ± 0.04
700	1.00 ± 0.05	0.25 ± 0.03	1.25 ± 0.08	0.19 ± 0.03
710	0.96 ± 0.05	0.18 ± 0.03	1.14 ± 0.08	0.16 ± 0.01
720	0.96 ± 0.05			

620 and 720 nm for the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction attributed to Photosystem I and for the [$H_2O \rightarrow$ DCIP] reaction attributed to Photosystem II. The sums of the two quantum yields at each wavelength are given in the 4th column. Although the sums are reasonably close to unity at long wavelengths (670–710 nm) they exceed unity by 45–60 % in the shorter wavelength range (630–660 nm) of activating light. Light reaction II itself has a quantum yield near unity in this wavelength range, and the quantum yield of Light reaction I is around 0.5. These results, together with those reported previously by AVRON AND BEN-HAYYIM[4], are clearly inconsistent with the "separate package" hypothesis and suggest that it is no longer tenable.

An alternative model, the "spillover" hypothesis, includes the possibility that excitation present in one of the pigment systems may, if it is not needed there, be transferred to the other reaction center and used there. The efficiency of transfer may depend on the wavelength of activation, on the conditions of the reaction and on the rates of competing excitation-loss processes. In an extreme version of the "spillover" hypothesis, proposed by AVRON AND BEN-HAYYIM[4], the distinction between the two pigment systems disappears (at least at all but the longest wavelengths) and photons absorbed anywhere in the antenna may ultimately reach either type of reaction center. It should be recognized that proximity of a given antenna pigment molecule to a reaction center may be important in determining the probability of transfer of its excitation, particularly where the time required for all of the necessary transfer steps to occur is comparable with the chlorophyll fluorescence lifetime. These spillover models, in common with most others, assume that the longest wavelengths of activating light involve photons with insufficient energy to activate the reaction center of Light reaction II.

If there is a single pigment system absorbing in the red region (630–660 nm) and if both types of reaction center can receive excitation from this pigment system, then we might expect the quantum yields for both Light reaction I and for Light reaction II to be the same for these actinic wavelengths. Our measurements show, however, that the quantum yield of Light reaction II is about twice as large as that for Light reaction I in the red region. This suggests that the two types of reaction centers are not symmetrically disposed with respect to the antenna pigments. When Reaction center I is not operating because of the absence of suitable electron acceptors ($NADP^+$ and/or ferredoxin) all excitation in the red region is able to be trapped and utilized for Light reaction II photochemistry. On the other hand, when Light reaction II is blocked by DCMU, only half the red photons absorbed are capable of producing electron transfer in Light reaction I. We know that this is not due to inactivation of System I reaction centers, because they are fully effective for long wavelength photons (700–720 nm). Also, in order to account for a quantum yield of 0.5 at short wavelengths for the [$H_2O \rightarrow NADP^+$] reaction, where both light reactions operate in series, it is necessary to assume that the photons absorbed are partitioned equally between the two light reactions and that each light reaction is able to use essentially all of the excitation that it receives.

One measure of the fate of excitation not utilized by Light reaction I is the fluorescence associated with Photosystem II. This fluorescence, which has an emission spectrum that is consistent with its origin in Photosystem II pigments, has been interpreted as containing a "live" portion and a "dead" portion[24]. The "live" portion is seen to compete with photochemistry carried out by Light reaction II and attains

a maximum efficiency when photochemistry is saturated at high light intensities. The "dead" portion, on the other hand, contributes a constant yield of fluorescence that is independent of the state of Photosystem II. If this indeed is the case, then it is unlikely that the "dead" fluorescence comes from the same site as is responsible for the "live" fluorescence and for photochemistry. The "dead" fluorescence may come from a non-functional trap or from the antenna pigment molecules themselves. In either case, it represents a constant leak of excitation energy from the chloroplast membranes. The addition of DCMU, which blocks Photosystem II light reactions, causes the "live" fluorescence to rise to its maximum value. This suggests that DCMU does not close the traps of Light reaction II as energy sinks. In fact they appear to operate just as efficiently in this respect in the presence or in the absence of DCMU. This observation is difficult to reconcile with our quantum yield measurements, for virtually all of the short wavelength photons are trapped by Light reaction II in the [$H_2O \rightarrow$ DCIP] reaction in the absence of DCMU, whereas in the presence of DCMU in the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction at least half of these short wavelength photons must reach Light reaction I. Yet the maximum yield of "live" fluorescence from Photosystem II is the same in both cases.

Perhaps the simplest way around this apparent inconsistency is in the alternative model of AVRON AND BEN-HAYYIM[4] in which a control mechanism determines the fraction of excitation that reaches each reaction center. This control mechanism is under the influence of factors which differ from one set of reaction conditions to another. The most likely candidates for this factor are the redox potential of the system and the concentrations of added cofactors. Experiments by MURATA[25] suggest that Mg^{2+} concentration plays a role in excitation transfer among the antenna pigments; however; the effects observed here could result from a complex interaction among several such components. Additional results reported by AVRON AND BEN-HAYYIM[4], where FMN or diquat are the electron acceptors for the Photosystem I reaction, show that in these cases 640-nm photons are fully effective in producing electron transfer. This contrasts sharply with the [ascorbate + $DCIPH_2 \rightarrow NADP^+$] reaction and is further evidence that some control mechanism is determining the fraction of red light excitation that is available to Photosystem I.

In summary, the results of quantum yield measurements for the separate Photosystem I and Photosystem II reactions show that a simple separate package model for the antenna pigment molecules is inconsistent with the experimental findings. The variability of the fraction of photons in the red region of the spectrum that is available to each of the reaction centers suggests the presence of a control mechanism that regulates electronic excitation transfer among major portions of the antenna pigment molecules. The investigation and identification of the mechanism of this "controlled spillover" hypothesis will be an important field for future research.

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