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ON THE NATURE OF THE DEACTIVATOR OF THE WATER-SPLITTING SYSTEM IN PHOTOSYNTHESIS

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SUMMARY

In this paper the effect of 1,1-diphenyl-2-picrylhydrazine on the deactivation of the water-splitting system is investigated. The following results have been obtained:

1. In the presence of 10^{-5} M 1,1-diphenyl-2-picrylhydrazine (DPPH) the decrease of the relative average oxygen yield per flash φ as a function of the dark time between the flashes is strongly accelerated.

2. Under these conditions the amplitude of the absorption change of chlorophyll a_I at 705 nm decreases with increasing dark time between the flashes.

3. In the presence of 10^{-5} M DPPH, the amplitude of the absorption change of the primary electron acceptor X of the light reaction II, is independent of the frequency of excitation in the range of the decrease of $\varphi = f(t_d)$.

These results indicate that DPPH accelerates the deactivation of the water-splitting system and that in the presence of this agent the backflow of electrons to S_2 and S_3 occurs between X and chlorophyll a_I.

INTRODUCTION

According to the hypothesis of Kok *et al.*[3], the splitting of water in photosynthesis requires the accumulation of four positive charges in an activation period, involving four successive photoreactions

$$S_0 \xrightarrow{h\nu} S_1 \xrightarrow{h\nu} S_2 \xrightarrow{h\nu} S_3 \xrightarrow{h\nu} S_4 \rightarrow O_2$$

The stability of the positively charged precursors S_1–S_3 of the formation of oxygen depends on their accumulation state. S_1 is stable in the dark for a long time[4,5], whereas S_2 and S_3 are discharged within a few seconds[4–9]. Under natural

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting system Y; DPPH, 1,1-diphenyl-2-picrylhydrazine; MES, morpholino-ethanesulfonate; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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conditions these reactions therefore occur at least 100-fold more slowly than the rate-limiting step of photosynthesis, which is the oxidation of plastohydroquinone with a half-life time of $\tau_{1/2} \approx 20$ ms[10,11].

There is evidence that in the deactivation process S_2 and S_3 are reduced by intermediates of photosynthetic electron transport (refs 4, 12, 13 and Renger, G., unpublished). A simplified scheme of this process according to the results of Witt[1] and coworkers is shown in Fig. 1.

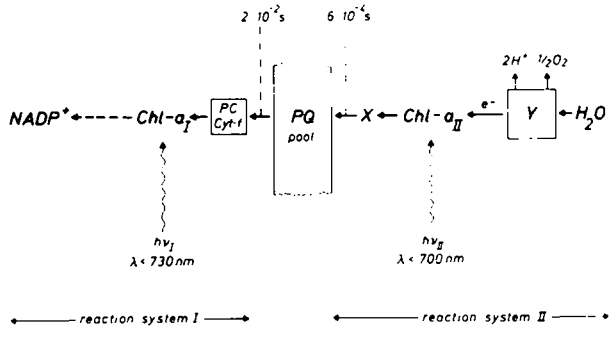

Fig. 1. Simplified electron transport chain in photosynthesis[1]. $NADP^-$, nicotinamide-adenine dinucleotide phosphate; Chl-a_I, chlorophyll a_I; PC, plastocyanin; Cyt *f*, cytochrome *f*; PQ, plastoquinone; X, primary electron acceptor of Photosystem II (Q in the notation of Duysens and Sweers[2]); Chl-a_{II}, chlorophyll a_{II}; Y, water-splitting enzyme system.

Assuming an endogenous substrate as the reductant of S_2 and S_3 the just mentioned kinetic relations can only be understood, if in the activation of the water-splitting system, electrons are trapped between the two light reactions or in Photosystem I. In this way the positive charges that accumulate in the activation period on the water-splitting complex can be neutralized by these electrons, though the forward reactions in the photosynthetic electron transport chain proceed much faster.

The chemical identification of the water-splitting complex as well as of its deactivators is still an open problem. The subject of this paper is, to obtain more information on the nature of the deactivator molecules.

Joliot *et al.*[4] have evidence that the reducing substrate of S_2 and S_3 is present in relatively high concentrations. Their results indicate that this substance is a doubly charged donor and that in the deactivation of the water-splitting system both one electron and two electron reduction processes are involved.

Renger *et al.*[13,14] demonstrated that in the presence of accelerators of the deactivation reactions (ADRY agents) like carbonylcyanidephenylhydrazones[15] or anilinothiophenes[16], for example, the deactivators of S_2 and S_3 are localized on the acceptor side of Photosystem I.

In this paper the effect of 1,1-diphenyl-2-picrylhydrazine (DPPH) on the deactivation process is investigated. The reported results indicate that this substance also accelerates the deactivation reactions and, in contrast to the agents tested by Renger *et al.*[13,14], in the presence of DPPH, the electrons for the discharge of S_2 and S_3 are trapped between the two light reactions.

MATERIALS AND METHODS

Whole chloroplasts of spinach were prepared, according to the method of Winget *et al.*[17]. The photosynthetic oxygen production was measured with a Clark electrode (type 17026 from Instrumentation Laboratory Inc., Boston) by a repetitive technique[18]. The steady-state oxygen yield per flash Y_{ss} was always determined, as has been described by Renger[16]. For the spectroscopic measurements an improved repetitive double-beam flash photometer was employed similar to that described in ref. 19. Details of this apparatus will be discussed in Hähnel, W., unpublished. Xenon flash lamps of the type OSRAM XIE 200 were used. In all experiments flashes of saturating intensity with a half-width of approx. $2 \cdot 10^{-5}$ s were applied. All experiments were performed at 21 °C. DPPH, purchased from Fluka AG, was used without further purification.

RESULTS

(1) The acceleration of the decrease of the relative oxygen yield per flash φ as a function of the dark time between the flashes by DPPH

The kinetics of both the rate-limiting step of photosynthesis and the deactivation of the water-splitting system can be analyzed by the measurement of the relative average oxygen yield per flash φ as a function of the dark time between the flashes. φ is defined as $Y_{ss}(t_d)/Y_{ss\ max}$ $Y_{ss}(t_d)$ is the steady-state oxygen yield per flash for a dark time t_d between the flashes. $Y_{ss\ max}$ is the maximal attainable steady-state oxygen yield per flash.

In Fig. 2, $\varphi = f(t_d)$ is shown in the presence of 10^{-5} M DPPH and without this agent. The rising part of $\varphi = f(t_d)$ in Fig. 2 to the maximum of φ is mainly determined by the time course of the rate-limiting forward reaction, as has first been shown by Emerson and Arnold[20]. At longer dark times $\varphi = f(t_d)$ decreases, because

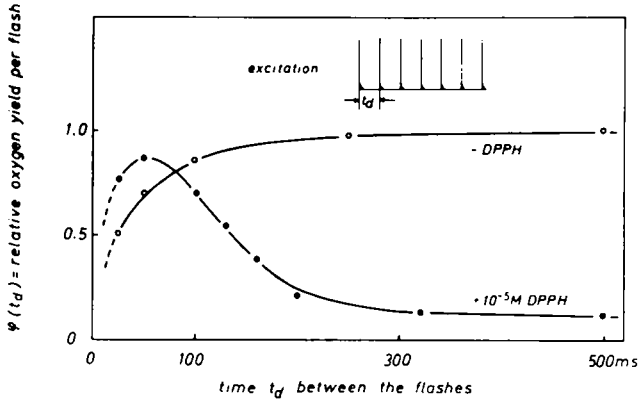

Fig. 2. Relative average oxygen yield per flash φ as a function of the time between the flashes. Chlorophyll concentration of the chloroplast suspensions: $3 \cdot 10^{-5}$ M. Activity of the oxygen production: 141/25.6 M O_2/M chlorophyll·h (+/− uncoupler NH_4Cl; $5 \cdot 10^{-4}$ M $K_3[Fe(CN)_6]$ as the electron acceptor). Suspension medium for the chloroplasts: (a) $2 \cdot 10^{-2}$ M Tricine buffer, pH = 7.2, $2 \cdot 10^{-2}$ M KCl; $5 \cdot 10^{-4}$ M $K_3[Fe(CN)_6]$ as the electron acceptor. (b) The same as for (a) + 10^{-5} M DPPH.

the number of precursors for photosynthetic oxygen production is diminished by the deactivation reactions. The decrease of $\varphi = f(t_d)$ is characteristic for the overall deactivation process. With the repetitive technique for the measurement of photosynthetic oxygen production described under Materials and Methods, the deactivation of the oxygen precursors S_2 and S_3 cannot be measured separately. At a first approximation, however, the steady-state oxygen yield per flash $Y_{ss}(t_d)$ for the decreasing part of $Y_{ss} = f(t_d)$ indicates the steady-state concentration of S_3, if "double hits"[3,5] and "misses"[3,5] are neglected.

From the comparison of the results in Fig. 2 it is apparent that 10^{-5} M DPPH strongly accelerates the decrease of $\varphi = f(t_d)$ without inhibition of photosynthetic electron transport.

Under these conditions this phase of $\varphi = f(t_d)$ can neither be fitted exactly by first-order nor by second-order kinetics. The first-order approach is, however, a good fit for an approximate determination of the half-life time of the decrease of $\varphi = f(t_d)$. In this way an apparent first-order half-life time $(\tau_{1/2})_{app} = 100$ ms was found at pH = 7.2. Without addition of DPPH a half-life time of approx. 5 s was estimated[8,9].

Table I shows the dependence of $(\tau_{1/2})_{app}$ in the presence of 10^{-5} M DPPH as a function of the pH value. From these results it follows that with increasing pH value a decrease of $(\tau_{1/2})_{app}$ is observed.

TABLE I

THE HALF-LIFE TIME $(\tau_{1/2})_{app}$ OF $\varphi = f(t_d)$ AS A FUNCTION OF THE pH VALUE IN THE PRESENCE OF 10^{-5} M DPPH

The decrease of $\varphi(t_d)$ was in first approximation fitted by first-order kinetics. Buffers used: $2 \cdot 10^{-2}$ M *N*-tris(hydroxymethyl)methylglycine (Tricine)–NaOH at pH = 8.0 and pH = 7.2; $2 \cdot 10^{-2}$ M morpholino-ethanesulfonate (MES)–NaOH at pH = 5.5. Other experimental details see Fig. 2.

pH	$(\tau_{1/2})_{app}$ (*ms*)
8.0	55
7.2	100
5.5	1400

(2) The effect of DPPH on the absorption change at 705 nm

In fig. 3 measurements of the relative average oxygen yield per flash φ have been correlated with the measurement of the absorption changes at 705 nm and at 265 nm to identify the deactivator of S_2 and S_3. The absorption changes at 705 nm are characteristic for the redox reaction of chlorophyll a_I[21–24], the active pigment of Photosystem I. The fast decrease at 705 nm immediately after the flash indicates the photooxidation of chlorophyll a_I. If in the presence of DPPH the backflow of electrons to S_2 and S_3 in the deactivation process occurs between the two light reactions, the amplitude of the absorption changes of chlorophyll a_I at 705 nm should decrease for $t_d > 50$ ms in a similar way as $\varphi = f(t_d)$. If on the other hand the deactivator molecules are localized on the acceptor side of Photosystem I, these transients should be

independent of the frequency of excitation for $t_d > 50$ ms under these reaction conditions.

From Fig. 3 it is apparent that the amplitude at 705 nm is the same for $t_d = 50$ ms with addition of 10^{-5} M DPPH and without this agent. For $t_d = 500$ ms in the presence of 10^{-5} M DPPH the amplitude of the absorption changes of chlorophyll a_1 is, however, reduced to about 25% of the amplitude without this agent.

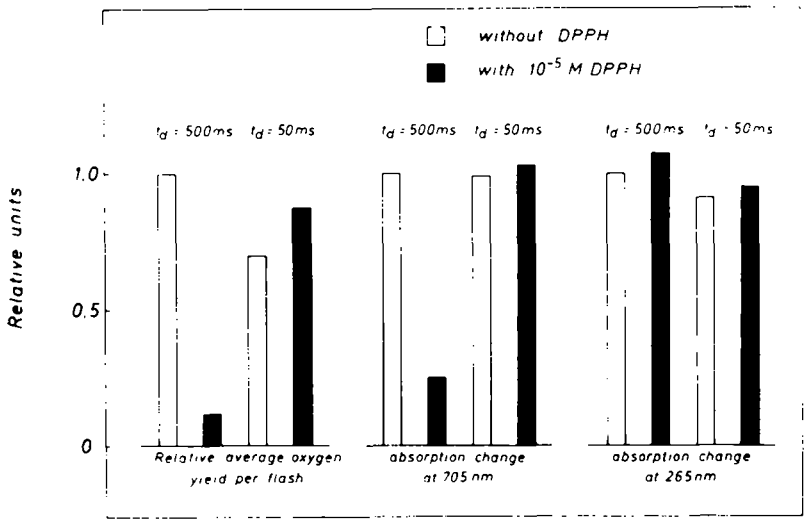

Fig. 3. A comparison of the relative average oxygen yield per flash, the absorption change of chlorophyll a_1 (Chl-a_1) at 705 nm and the absorption change of X at 265 nm for $t_d = 50$ ms and $t_d = 500$ ms in the presence of 10^{-5} M DPPH and without this agent. All data were normalized on the corresponding result for $t_d = 500$ ms without addition of DPPH (full steady-state activation of the water-splitting system, no limitation by the rate-limiting forward reaction). Oxygen measurements: for experimental details see Fig. 2. Absorption changes at 265 nm: electron acceptor in the chloroplast suspension, 10^{-4} M benzylviologen. Excitation: BG 23/6 mm. Optical path length: 0.12 cm; monitoring light: < 50 erg·cm^{-2}·s^{-1}; optical band width: $\Delta\lambda = 3$ nm; electrical band width: 10–500 cps. For other experimental details see Fig. 2. Absorption change at 705 nm: monitoring light, < 200 erg·cm^{-2}·s^{-1}; optical band width, $\Delta\lambda = 2$ nm. For other experimental details see above and Fig. 2.

(3) The effect of DPPH on the absorption changes at 265 nm

Stiehl and Witt[10,11] have demonstrated that in the ultraviolet part of the spectrum, absorption changes of the primary electron acceptor X of the light reaction II and of plastoquinone PQ interfere. At 265 nm, therefore, the kinetics of the absorption changes of X and plastoquinone are superimposed. According to the results of Stiehl and Witt[11], X is photoreduced within $< 10^{-5}$ s to X^-. X^- is reoxidized by the pool of plastoquinone with a half-life time $\tau_{1/2} = 6 \cdot 10^{-4}$ s[25,26]. Plastohydroquinone PQ^{2-} is formed in this reaction[10,11]. PQ^{2-} is oxidized by plastocyanin and/or cytochrome *f* with a half-life time of approx. 20 ms[11].

The absorption change of X is biphasic with a predominant fast phase ($\approx 80\%$; ($\tau_{1/2} = 6.10^{-4}$ s) and a minor slow phase ($\approx 20\%$; $\tau_{1/2} \approx 2 \cdot 10^{-2}$ s)[25,26]. Because X is reduced within $< 10^{-5}$ s, the reduction of plastoquinone by X^-, that takes place

much more slowly ($\tau_{1/2} = 6 \cdot 10^{-4}$ s), cannot be resolved in the ultraviolet. The amplitude of the absorption changes at 265 nm, which is reached immediately after the flash, indicates, therefore, the amount of X, that is reduced by one turnover of the light reaction II.

If in the presence of 10^{-5} M DPPH the electrons, that are transferred in Photosystem II in the activation of the water-splitting system, are trapped by X, and S_2 and S_3 are reduced by X^- in the deactivation process, this amplitude of the absorption changes at 265 nm should decrease for $t_d > 50$ ms similar to $\varphi = f(t_d)$. Otherwise this amplitude should be independent of the frequency of excitation in this range of t_d.

From Fig. 3 it follows that at 265 nm almost equal amplitudes are measured for $t_d = 50$ ms and $t_d = 500$ ms with and without DPPH.

DISCUSSION

From the reported results it follows that DPPH has the typical properties of the accelerators of the water-splitting system, introduced by Renger[15,16]. DPPH is characterized by an acidic NH group and the presence of delocalizable π orbitals favoring the solubility of its anion in media of low dielectric constant. Renger[27] found that a high concentration of the anion form of these accelerators generally causes a high acceleration effect. This was confirmed also for the acidic NH group of DPPH, as is apparent from the dependence of the half-life time of the decrease of $\varphi = f(t_d)$ on the pH value in Table I. With increasing pH value, *i.e.* with increasing concentration of the anion of DPPH, $(\tau_{1/2})_{app}$ decreases. Therefore, one can conclude that the effect of this agent on the decrease of $\varphi = f(t_d)$ indicates an acceleration of the deactivation process.

The acceleration of the decrease of $\varphi = f(t_d)$ in the presence of 10^{-5} M DPPH could also be explained by competition of this agent with water as the natural electron donor, because Haveman *et al.*[28] recently found that hydrazine compounds, like hydrazobenzene, for example, can function as efficient donors for Photosystem II. Such an effect of DPPH seems, however, improbable. In the presence of benzylviologen as the electron acceptor, both light reactions are operating. Therefore, the amplitude of the absorption change of chlorophyll a_1 at 705 nm should be independent of the frequency of the flashes for $t_d > 50$ ms in contrast to the results in Fig. 3.

From the correlation of the oxygen measurements with the optical experiments in Fig. 3 it follows that the amplitude of X at 265 nm is nearly independent of the activation state of the water-splitting system. On the other hand the amplitude of the absorption change at 705 nm is decreased to about 25%, if in the presence of 10^{-5} M DPPH the steady-state concentration of S_3 is reduced to about 10–20% of its maximal value. From these results it follows that in the presence of this agent the backflow of electrons to S_2 and S_3 in the deactivation process occurs between the primary electron acceptor X of the light reaction II and chlorophyll a_1.

The negative charges, that accumulate on the reducing side of the light reaction II in the activation of the water-splitting system, must, therefore, be trapped either in the pool of plastoquinone or by plastocyanin and/or cytochrome *f*. These electrons cannot be trapped by X. In this case the amplitude of the absorption changes

at 265 nm, that is reached immediately after the flash, should strongly depend on the frequency of excitation for $t_d > 50$ ms in the presence of 10^{-5} M DPPH. This interpretation can be excluded from Fig. 3.

It is, however, possible that the negative charges that may be trapped in the pool of plastoquinone redistribute between PQ and X, because the reaction of X and PQ is reversible[18]. In this way the opposite charges separated in the activation of the water-splitting system could recombine at least partly *via* X^-.

From the results known so far it seems likely that a similar mechanism is also relevant for the deactivation process under natural conditions. It has been shown[12,29] that the reduced primary electron acceptor participates in the deactivation reactions in the absence of accelerating agents.

On the other hand Joliot *et al.*[4] suggest the existence of another reducing substrate for S_2 and S_3, which is a doubly charged donor and which is present in relatively high concentrations in the photosynthetic electron transport chain. Such properties are characteristic for plastoquinone.

Also Lemasson and Barbieri[6] observed that the deactivation reactions proceed faster, if the pool A between the two light reactions, which mainly consists of plastoquinone[11], is in the reduced state. By the participation of plastoquinone in the deactivation reaction the positive charges on the water-splitting complex could be balanced by negative charges on the reduction side of Photosystem II, without affecting appreciably the forward reactions in the electron transport chain.

The results in Fig. 3 are different from the observation of Renger *et al.*[13,14] that in the presence of carbonylcyanide phenylhydrazones and anilinothiophenes, which are also effective accelerators of the deactivation process, the amplitude of the absorption change at 705 nm is independent of the activation state of the water-splitting system. In the presence of these agents reductants on the acceptor side of Photosystem I function as the deactivators of S_2 and S_3. Renger, G., unpublished, recently demonstrated however, that, if the electron flow in Photosystem I is selectively inhibited, S_2 and S_3 can also be deactivated by reductants localized between the two light reactions. From these results it follows that the site for the backflow of electrons S_2 and S_3 in the deactivation process depends on the reaction conditions.

The results of this paper may also be explained by other cyclic reactions around the light reaction II involving DPPH and oxidation or reduction products of this substance possibly formed in the light. A quantitative correlation of the kinetics of the deactivation of S_2 and S_3 and of the oxidation of the intermediates between the two light reactions is needed to exclude such interpretations definitely.

Further work is also necessary to investigate the mechanism of the action of ADRY agents. From the results of this paper it seems likely that some of these lipophilic substances such as DPPH, for example, interact with reduced plastoquinone in the lipid layer of the thylakoid. Such agents may function as carriers for electrons and/or protons between reduced plastoquinone and S_2 and S_3 or may modify the activation energy for the recombination of the opposite charges in the deactivation process.

This can be for instance accomplished by the formation of electron and/or proton donor–acceptor complexes with reduced plastoquinone. The detection of absorption changes of the intensively coloured DPPH, that arise from its action as

a catalyst in the deactivation of the water-splitting system, may point out an approach to clarify these phenomena.

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