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THE ACTION OF INDOPHENOLS AND NITROPHENOLS ON THE DEACTIVATION REACTIONS IN THE WATER-SPLITTING SYSTEM OF PHOTOSYNTHESIS

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SUMMARY

The dependence of the relative average oxygen yield per flash φ for repetitive excitation with single flashes as a function of the dark time, t_d, between the flashes has been investigated.

The decrease of $\varphi = f(t_d)$ for long dark times (t_d) depends on the deactivation processes in the water-splitting system by which the number of precursors for photosynthetic oxygen evolution is diminished.

It is shown that the rate of the deactivation reactions can be either accelerated or retarded by indophenols and nitrophenols. These effects can be clearly correlated to the acidity of the hydroxyl group of these substances, but other factors have also to be considered in order to interpret completely the mode of action of these agents in the deactivation process. Possible mechanisms are discussed.

INTRODUCTION

In photosynthesis, an electron transport occurs from H_2O to $NADP^+$. This process is driven by two light reactions and is mediated by at least ten electron carriers. Fig. 1 shows a simplified scheme of the photosynthetic electron transport chain according to the results of Witt[1] and co-workers.

The molecular events taking place in the splitting of water in Photosystem II are still unknown. An important approach to the understanding of this process has been made by Joliot *et al.*[3] and by Kok *et al.*[4,5]. These authors have studied the activation–deactivation phenomena in the water-splitting system.

According to the hypothesis of Kok *et al.*[4], the formation of oxygen requires the accumulation of four positive charges in an activation period, involving four successive photoreactions;

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

Abbreviations: ADRY, Acceleration of the Deactivation Reactions of the water-splitting enzyme system Y; DCIP, 2,6-dichlorophenolindophenol; TCIP, 2,6,3′-trichlorophenolindophenol; TIP, 3-methyl-6-isopropylindophenol (thymolindophenol); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

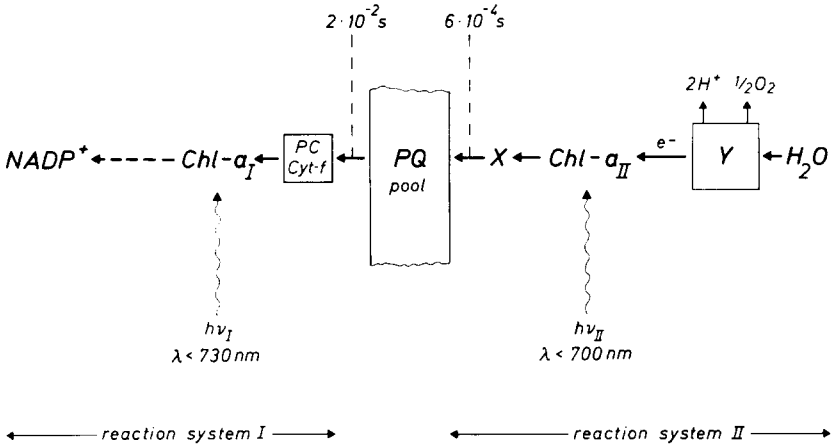

Fig. 1. Simplified electron transport chain in photosynthesis[1]. Chl-a_I, chlorophyll a_I; PC, plastocyanin; Cyt-*f*, cytochrome *f*; PQ, plastoquinone; X, primary electron acceptor of Photosystem II (Q in the notation of Duysens and Sweers[2]); Chl-a_{II}, chlorophyll a_{II}; Y, water-splitting enzyme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

Kok *et al.*[5] interpret the deactivation of the water-splitting system by single electron reduction processes;

$$S_3 \xrightarrow{R \to R^+} S_2 \xrightarrow{R \to R^+} S_1 \not\longrightarrow S_0$$

It has been shown[6,7] that the deactivation reactions give rise to luminescence emission. Joliot *et al.*[7] therefore argue that the characteristic pattern observed for the oxygen yield per flash as a function of the flash number can also be understood by the assumption of a two-step mechanism ($S_3 \to S_1$ and $S_2 \to S_0$), if an important fraction of the excitons formed in the deactivation reactions is reabsorbed by the same center. This would imply the participation of two-charge electron donors in the deactivation process. Renger[8-10] found that the deactivation reactions can be accelerated by substances like carbonyl cyanide phenylhydrazones or anilinothiophenes. This effect he calls ADRY effect (Acceleration of the Deactivation Reactions of the water-splitting enzyme system Y). He showed[11] that the acidic proton of such ADRY agents is an indispensable element for their action in the deactivation process.

Because the activation–deactivation reactions are the clue to an understanding of the molecular events in the water-splitting enzyme complex, it is of great importance to get more information on the mode of action of ADRY agents. Therefore in this paper two new classes of such compounds are introduced, the indophenols and the nitrophenols. It is shown that indophenols, depending on their substituents, can not only accelerate but also retard the deactivation reactions. It is demonstrated that the acidic OH-group of these molecules is important for their action in the deactivation process.

MATERIALS AND METHODS

Whole spinach chloroplasts were prepared according to the method of Winget *et al.*[12]. Photosynthetic oxygen production was measured with a Clark electrode, type

17026 from Instrumentation Laboratory Inc., Boston. The flash polarography apparatus used for these measurements has been described previously[13].

Joliot *et al.*[3] have shown that in dark-adapted chloroplasts a damped oscillation of the oxygen yield per flash Y_n as a function of the flash number n occurs. After about 20–25 flashes a nearly constant value Y_{ss} is observed, which indicates the steady state of the activation–deactivation processes in the water-splitting system.

In all experiments reported in this paper the steady state oxygen yield Y_{ss} has been measured as described by Renger[10].

The limit of sensitivity of the Clark electrode is reached if the dark times between the flashes are longer than 10 s. Therefore the oxygen yield per flash for $t_d > 10$ s must be recorded with a more sensitive oxygen measuring device.

Xenon flashlamps 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 used.

All experiments were performed at 22 °C.

RESULTS

The relative oxygen yield per flash φ as a function of the dark time t_d between the flashes

The relative oxygen yield per flash φ for repetitive excitation with single flashes was examined as a function of the dark time between the flashes in the presence of $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ as the electron acceptor. According to Emerson and Arnold[14], the rising part of $\varphi = f(t_d)$ to the maximum of φ is mainly determined by the time course of the rate-limiting step of photosynthesis. Stiehl and Witt[15,16] have shown that this process is the oxidation of reduced plastoquinone by Photosystem I.

For higher dark times $\varphi = f(t_d)$ decreases, becauses the number of precursors for photosynthetic oxygen evolution is diminished by the deactivation reactions in the water-splitting system. In the presence of $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ as the electron acceptor, the decrease of $\varphi = f(t_d)$ starts at t_d = approx. 500 ms. This phase of $\varphi(t_d)$ is shown in Fig. 2 (Curve a).

As is apparent from a plot of the reciprocal steady state oxygen yield per flash $1/Y_{ss}$ as a function of t_d in Fig. 3 (Curve a) second order kinetics with equal initial concentrations of the reaction partners gives a good fit for the decreasing part of $\varphi = f(t_d)$

$$\frac{1}{Y_{ss}} - \frac{1}{Y_{ss\,max}} = k_{app} t_d \tag{1}$$

$$\varphi = \frac{Y_{ss}}{Y_{ss\,max}} = \frac{1}{1 + k_{app} Y_{ss\,max}\, t_d} \tag{1a}$$

From the intersection of the extrapolation of the linear relationship $1/Y_{ss} = f(t_d)$ with the ordinate at $t_d = O$, $1/Y_{ss\ max}$ can be determined. $Y_{ss\ max}$ is a measure of the photosynthetic unit. According to Joliot[17] this functional unit corresponds to the number of photochemically active reaction centers II per total number of chlorophylls. From the value for $Y_{ss\ max} = 34.8 \cdot 10^{-4}$ equiv O_2/mole chlorophyll per flash extrapolated in Fig. 3 (Curve a) it follows that one reaction center II is present per approx. 290 mole-

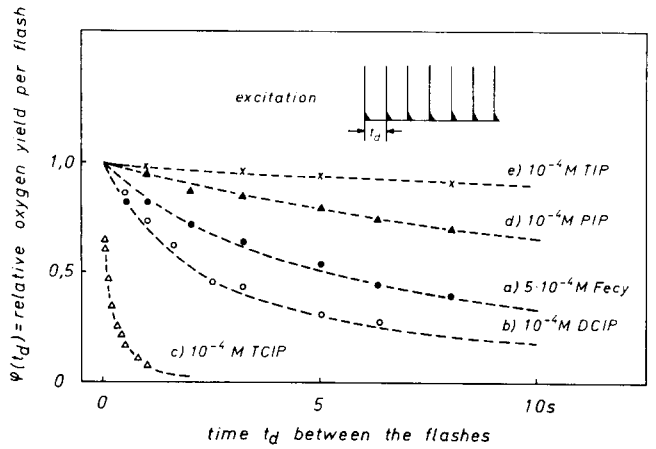

Fig. 2. Relative average O_2 yield per flash $\varphi(t_d)$ as a function of the time t_d between the flashes. Only the decreasing part of $\varphi = f(t_d)$ is shown. Chlorophyll concentration of the chloroplast suspensions: $3 \cdot 10^{-5}$ M. Activity of O_2 production: $53/161 \cdot 10^{-3}$ equiv O_2/mole chlorophyll per s (—/+uncoupler NH_4Cl; $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ as the electron acceptor). Suspension medium for the chloroplasts: $2 \cdot 10^{-2}$ M KCl, $2 \cdot 10^{-2}$ M tricine buffer, pH 7.2; electron acceptor as indicated in the figure. The dotted curves a–e show the second order kinetics for the decrease of $\varphi = f(t_d)$ calculated according to Eqn 1a with the data $Y_{ss\,max}$ and k_{app} determined from the straight lines $1/Y_{ss} = f(t_d)$ in Fig. 3. PIP, phenolindophenol; Fecy, $K_3Fe(CN)_6$.

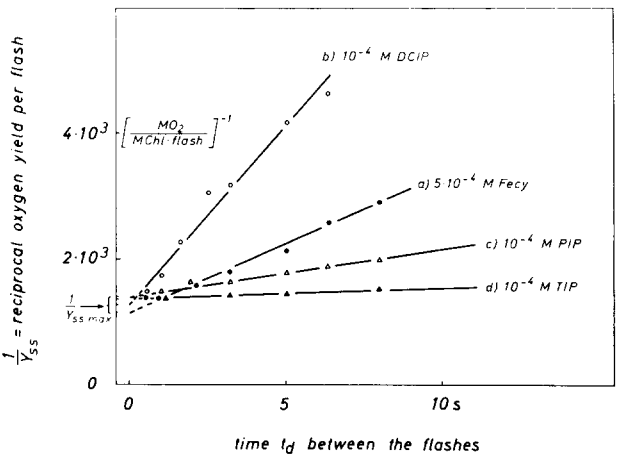

Fig. 3. Reciprocal average oxygen yield per flash $1/Y_{ss}$ as a function of the time t_d between the flashes. Experimental conditions as described in Fig. 2. PIP, phenolindophenol; Fecy, $K_3Fe(CN)_6$.

cules of chlorophyll. This is in good agreement with the functional unit estimated for whole cells of *Chlorella*[14,17,18]. From the slope of the straight line in Fig. 3, Curve a, an apparent second order rate constant $k_{app} = 54.5$ equiv $^{-1} \cdot s^{-1} \cdot$ mole chlorophyll can be determined for the decrase of $Y_{ss} = f(t_d)$.

With the values of k_{app} and $Y_{ss\ max}$, $\varphi = f(t_d)$ has been calculated according to

Eqn 1a. The resulting second order kinetics with a half life time $(\tau_{1/2})_{app} = 5.3$ s is represented by the dotted curve in Fig. 2a.

The decrease of $\varphi = f(t_d)$ for chloroplasts in the presence of $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ in Fig. 2 (Curve a)has been taken as a reference for the experiments with indophenols and nitrophenols.

The decrease of $\varphi = f(t_d)$ in the presence of indophenols

Indophenols are widely used as electron donor–acceptor substances and as uncouplers of phosphorylation for the study of photosynthesis and respiration. In a series of experiments the indophenol dyes listed in Table I have been used at concentrations of 10^{-4} M. For all experiments the decrease in the concentrations of the tested indophenols in the light was negligible (maximally 5%).

TABLE I

COMPARISON OF THE EFFECTS OF INDOPHENOLS ON THE KINETICS OF THE DEACTIVATION PROCESS IN THE WATER-SPLITTING SYSTEM (EXPRESSED BY THE RATE CONSTANT k_{app} OR THE HALF LIFE TIME $(\tau_{1/2})_{app}$ OF THE APPARENT SECOND ORDER DECREASE OF $\varphi(t_d)$) AND THE pK VALUE OF THE OH-GROUP OF THESE AGENTS

Agent (10^{-4} M)	*$Y_{ss\,max}$ (equiv O_2/mole chlorophyll per flash)*	*k_{app} (equiv$^{-1} \cdot s^{-1} \cdot$ mole chlorophyll)*	*$(\tau_{1/2})_{app}$ (s)*	pK
Phenolindophenol	$28.6 \cdot 10^{-4}$	18.75	18.6	8.1*
TIP	$29.4 \cdot 10^{-4}$	4	85	8.8*
DCIP	$31.4 \cdot 10^{-4}$	142	2.3	5.7**
TCIP	$27.0 \cdot 10^{-4}$	3750	0.1	5.8**

* Ref. 19. ** Ref. 20.

In this paper the effect of these compounds on the decrease of $\varphi = f(t_d)$ has been examined. The results of these measurements are summarized in Table 1. From Fig. 2, Curves b–e it is apparent that 2,6-dichlorophenolindophenol (DCIP) and 2,6,3′-trichlorophenolindophenol (TCIP) accelerate, and phenolindophenol and 3-methyl-6-isopropylindophenol (thymolindophenol) (TIP) retard, the decay of $\varphi = f(t_d)$ compared to the kinetics observed in the presence of $K_3Fe(CN)_6$ as the electron acceptor (Fig. 2, Curve a). Because the acceleration of the decrease of $\varphi = f(t_d)$ is especially high in the presence of 10^{-4} M TCIP, $\varphi = f(t_d)$ and $1/Y_{ss} = f(t_d)$ observed under these conditions are shown in Fig. 4 separately.

From Fig. 3 (Curve b) and the insert of Fig. 4 it is apparent that in the presence of 10^{-4} M DCIP and TCIP $1/Y_{ss}$ can well be approximated by a straight line in the range of the decrease of $\varphi = f(t_d)$. Therefore this part of $\varphi = f(t_d)$ can also be described by second order kinetics under these conditions, as shown for the decrease of $\varphi = f(t_d)$ with $K_3Fe(CN)_6$ as the electron acceptor. On the other hand, Renger[9,10] found that, in the presence of carbonyl cyanide phenylhydrazones or anilinothiophenes, the decrease of $\varphi = f(t_d)$ can be approximated to by first order kinetics. From the plot of $1/Y_{ss}$ as a

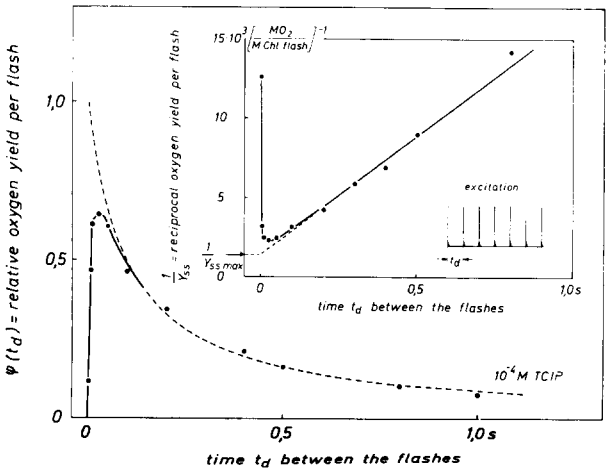

Fig. 4. Relative average O_2 yield per flash $\varphi(t_d)$ as a function of the time t_d between the flashes in the presence of 10^{-4} M TCIP as the electron acceptor. Experimental conditions as described in Fig. 2 (Curve c). The dotted curve represents the second order kinetics for the decrease of $\varphi = f(t_d)$ calculated according to Eqn 1a with the values of $Y_{ss\,max}$ and k_{app} determined from the straight line $1/Y_{ss} = f(t_d)$ in the insert of this figure.

function of t_d in Fig. 3(Curve b) and the insert of Fig. 4, $Y_{ss\ max}$ and the apparent second order rate constant k_{app} have been determined (see Table I). With these values the decrease of $\varphi = f(t_d)$ has been calculated according to Eqn 1a. The resulting second order kinetics are represented by the dotted curves in Figs 2b and 4.

In the presence of 10^{-4} M TCIP, the deactivation reactions are so strongly accelerated that the rate of these processes is comparable to the rate of the slowest forward reaction in the photosynthetic electron transport chain, which is the oxidation of plastohydroquinone[15,16]. Therefore the oxygen yield per flash in the maximum of the curve $\varphi = f(t_d)$ in Fig. 4 is only 65% of $Y_{ss\ max}$. For $t_d > 50$ ms the course of $\varphi = f(t_d)$ is exclusively determined by the deactivation reactions in the water-splitting system.

In the presence of 10^{-4} M phenolindophenol and TIP, only a small initial range of the decrease of $\varphi = f(t_d)$ can be measured, because the limit of the sensitivity of the Clark electrode is reached for dark times $t_d > 10$ s. The measurable data therefore are not sufficient for a precise kinetic analysis of this part of the $\varphi = f(t_d)$ curve. If it is assumed, however, that in the presence of phenolindophenol and TIP the decrease of $\varphi = f(t_d)$ for $t_d > 1$ s can also be approximated to by second order kinetics, $Y_{ss\ max}$ and k_{app} can be determined from a plot of the available data of $1/Y_{ss}$ in the range of 1–8 s (Fig. 3, Curves c and d).

The decrease of $\varphi = f(t_d)$ in the presence of nitrophenols

In another series of experiments, the effects of 2,4-dinitrophenol and picric acid (2,4,6-trinitrophenol) on the kinetics of $\varphi = f(t_d)$ have been examined. In this case $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ was added as the electron acceptor to the suspension medium of the chloroplasts. 2,4-Dinitrophenol as well as picric acid induce an acceleration of the deactivation reactions in the water-splitting system, as is apparent from Fig. 5.

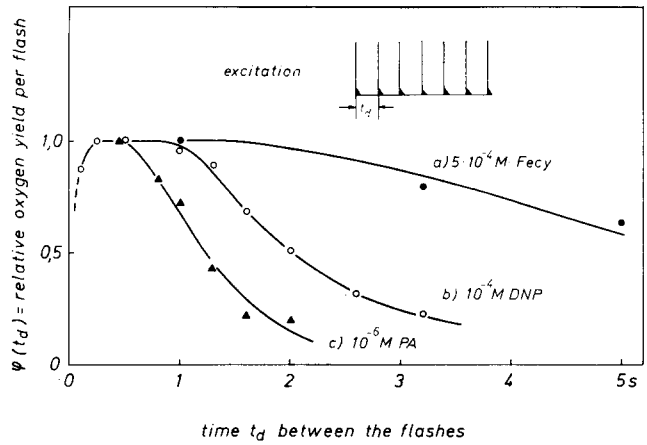

Fig. 5. Relative average O_2 yield per flash $\varphi(t_d)$ as a function of the time t_d between the flashes. Chlorophyll concentration of the chloroplast suspensions: $3 \cdot 10^{-5}$ M. Activity of O_2 production: $30/172 \cdot 10^{-3}$ equiv O_2/mole chlorophyll per s (−/+uncoupler NH_4Cl; $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ 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; $5 \cdot 10^{-4}$ $K_3Fe(CN)_6$ as the electron acceptor. (b) The same as for (a), $+10^{-4}$ M 2,4-dinitrophenol (DPN). (c) The same as for (a), $+10^{-6}$ M picric acid (PA).

TABLE II

COMPARISON OF THE EFFECTS OF 2,4-DINITROPHENOL AND PICRIC ACID ON THE KINETICS OF THE DEACTIVATION PROCESS IN THE WATER-SPLITTING SYSTEM (EXPRESSED BY THE EXPERIMENTALLY DETERMINED HALF LIFE TIME $(\tau_{1/2})_{exp}$ OF THE DECREASE OF $\varphi = f(t_d)$) and THE pK VALUE OF THE OH-GROUP OF THESE AGENTS

Agent	$(\tau_{1/2})_{exp}(s)$	*pK*
2,4-Dinitrophenol (10^{-4} M)	2	4.0*
Picric acid (10^{-6} M)	1.25	0.38**

* Ref. 21. ** Ref. 22.

(See Table II for kinetic data.) Under these conditions the decrease of $\varphi = f(t_d)$ can neither be described by first order kinetics nor by second order kinetics. Therefore, the experimentally determined half life time $(\tau_{1/2})_{exp}$ of $\varphi = f(t_d)$ for $t_d > 500$ ms in Fig. 5 is taken as the measure for the effect of these agents on the kinetics of the deactivation reactions. In the presence of 10^{-4} M 2,4-dinitrophenol, a half life time $(\tau_{1/2})_{exp}$ of approx. 2 s is found from Fig. 5 (Curve b). For comparison, a half life time $(\tau_{1/2})_{exp} = 6.75$ s was observed for the same preparation of chloroplasts with $K_3Fe(CN)_6$ alone (Fig. 5, Curve a).

At a concentration of 10^{-4} M 2,4-dinitrophenol acts as a weak inhibitor of photosynthetic electron transport. Y_{ss} in the maximum range of the function $\varphi = f(t_d)$ is reduced to about 75% of the value found without 2,4-dinitrophenol. Picric acid is a more effective inhibitor. Even at a concentration of 10^{-6} M, Y_{ss} in the maximum

range of $\varphi = f(t_d)$ decreases to about the same extent as with 10^{-4} M 2,4-dinitrophenol. But even at this two orders of magnitude lower concentration, picric acid shows a higher acceleration effect on $\varphi = f(t_d)$ than 10^{-4} M 2,4-dinitrophenol. A half life time $(\tau_{1/2})_{exp} = 1.25$ s has been determined under these conditions from Fig. 5 (Curve c).

DISCUSSION

It is generally assumed that the deactivation of the water-splitting system is caused by the recombination of charges separated by the light reaction II in the activation of this system[5-7,23].

The decrease of $\varphi = f(t_d)$ obtained with the repetitive technique described under Materials and Methods is characteristic for the overall deactivation process. With this technique the deactivation of the oxygen precursors S_2 and S_3 cannot be measured separately. It is therefore difficult to discuss the reaction orders that have been found for the decrease of $\varphi = f(t_d)$ relating to the deactivation mechanism.

Renger[9,10] has demonstrated that the deactivation process can be accelerated by agents like carbonyl cyanide phenylhydrazones or anilinothiophenes (ADRY effect). Analogous effects have been described for the indophenols and the nitrophenols in this paper. The experiments with indophenols show that if the decrease of $\varphi = f(t_d)$ observed for chloroplasts in the presence of $5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$ as the electron acceptor is taken as a reference, these molecules can not only accelerate but also retard the deactivation reactions, depending on their substituents. These effects can be interpreted as a consequence of the direct action of indophenols on the deactivation process in the water-splitting system.

The acceleration of the decrease of $\varphi = f(t_d)$ in the presence of DCIP or TCIP could also be caused by competition of $DCIPH_2$ or $TCIPH_2$ formed in the light with water as the natural electron donor, since it is known[24,25] that DCIP/$DCIPH_2$, for example, can mediate a cyclic electron flow in Photosystem I. This interpretation can be excluded however, because DCIP can also accept electrons between the two light reactions[26,27]. On the other hand, an electron donor effect of $DCIPH_2$ at the oxidizing end of Photosystem II has not been observed[25].

Renger[11] suggested that the presence of an acidic group is an indispensable functional element for the action of agents like carbonyl cyanide phenylhydrazones *etc.* in the deactivation of the water-splitting system. He found that a high anion concentration generally causes a great acceleration effect. Therefore it is to be expected that the action of indophenols and nitrophenols as catalysts in this process depends on the acidity of their OH-group. This is consistent with the data in Tables I and II. Phenolindophenol and TIP, with relatively high pK values between 8 and 9, retard the deactivation reactions, while DCIP and TCIP, with pK values around[6], show an acceleration effect.

Similar results have been obtained for the nitrophenols. Picric acid with a very low pK value of 0.38, shows a distinctly higher acceleration of the deactivation kinetics than 2,4-dinitrophenol even at a 100-fold lower concentration The catalytic activity of these agents in the deactivation process is, however, not only determined by the acidity of their OH-group. For instance, the different acceleration effects of DCIP and TCIP which have similar pK values imply that there are other factors still to be considered.

Compounds like indophenols and nitrophenols are lipophilic substances which are potent uncouplers of phosphorylation. It can be expected therefore that these molecules establish partition equilibria between the suspension medium and the thylakoid. The partition coefficients for these equilibria, and the mobility of these compounds in the thylakoid, may be of great importance to their catalytic activity in the deactivation process. On the other hand, it seems likely that, besides these thermodynamic properties, electronic, hydrophobic and steric substituent parameters are significant in this respect, as has for instance been shown by Draber *et al.*[28] for the uncoupling activity of a great number of α-acyl-α-cyanocarbonyl phenylhydrazones. Also, for the uncoupling of phosphorylation by such substances, there is a requirement for an acidic OH- or NH-group, as has been pointed out by Mitchell[29] in his chemiosmotic hypothesis of phosphorylation. Quantitative structure–activity studies are needed to analyze the influence of all these factors on the kinetics of the deactivation of the water-splitting system.

The mechanism of the action of ADRY agents still needs to be elucidated. Several suggestions regarding this problem have been made by Renger and coworkers[9,10,30,31]. From the results reported in this paper it seems likely that substances like indophenols and nitrophenols are concerned in proton transfer reactions in the deactivation process. This conclusion is however not decisive. It is also possible that these agents participate in electron transfer reactions in the water-splitting system. Also, in this case the dependence of the kinetics of the deactivation reactions on the acidity of the OH-group of these compounds can be explained. The following mechanisms seem to be relevant in this respect;

(1) ADRY agents may function as electron carriers between the oxygen precursors and their deactivators. If these carriers move along a potential gradient in the thylakoid membrane, ionized species will migrate faster and therefore reduce the oxygen precursors faster than neutral protonized forms.

(2) ADRY agents may modify the activation energy for the recombination of the opposite charges that accumulate in the activation of the water-splitting system. This can, for instance, be accomplished by the formation of charge transfer complexes between ADRY agents and components of the photosynthetic electron transport chain.

Because, generally, the rate of the reaction of charged species is higher than that of neutral molecules, it is to be expected that the ionized intermediates react faster with the oxygen precursors than the neutral protonized forms. In this case the effect of agents like indophenols or nitrophenols in the deactivation of the water-splitting system depends on the pK values of the complexes formed in their interaction with compounds of photosynthetic electron transport.

The mode of action of ADRY agents can only be understood completely if the reactants involved in the deactivation process are identified. New results on the nature of the deactivator for the oxygen precursors in the water-splitting system will be published elsewhere (Vater, J., unpublished).

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