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THE ACTION OF 2-ANILINOTHIOPHENES AS ACCELERATORS OF THE DEACTIVATION REACTIONS IN THE WATERSPLITTING ENZYME SYSTEM OF PHOTOSYNTHESIS

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SUMMARY

The effect of a number of 2-anilinothiophene derivates on the O_2 yield per flash obtained by excitation with repetitive flashes and repetitive double flash groups has been investigated. It was found that:

1. The decrease of the average O_2 yield per flash at long times (t_d) between the flashes caused by the natural deactivation reactions of the watersplitting enzyme system is strongly accelerated by various derivatives of 2-anilinothiophene.
2. The action of these agents as Acceleration of the Deactivation Reactions of the watersplitting Enzyme System Y (ADRY) substances is unequivocally proved.
3. The ADRY effect is dependent on the acidity of the imino group, present in all ADRY agents so far known.

These observations are discussed in connection with the mechanism of the activation–deactivation reactions of the watersplitting Enzyme System Y.

A new class of photosynthesis effectors is established: the class of the ADRY agents.

These substances provide a tool both to label and to modify the oxidizing equivalents which are responsible for the oxidation of water, in a similar way as the uncouplers can be used for the characterization of the high energy state in the phosphorylation process.

INTRODUCTION

The nature of the watersplitting Enzyme System Y mediating the oxidation of water induced by chlorophyll a_{II}[1,2] (see Fig. 1) is unknown.

For the oxidation of water to molecular O_2, either by electrolysis or by photolysis, the cooperation of four holes (oxidizing equivalents) is required. Therefore the question arises: In which way is this fundamental cooperativity realized in the O_2 evolving system of photosynthesis?

Abbreviations: ADRY, Acceleration of the Deactivation Reactions of the watersplitting Enzyme System Y; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; MES, 2-(*N*-morpholino)ethanesulphonic acid; ANT 2a, 2-(4-chloro)anilino-3,5-dinitrothiophene.

It is known that a single turnover in the photosynthetic watersplitting Enzyme System Y can only lead to an O_2 evolution if this System Y is in an activated state[7-10]. These active states are generated in a characteristic kinetic pattern[11,12] by a chlorophyll a_{II}-induced electron transport in System II[10,13]. From the above mentioned results it was concluded that the watersplitting enzyme systems act as independent units[12] and that they contain a storage device for the holes[11,12,14]. In the dark the active states of Y are exhausted by slow reactions[10,15,16] leading to the inactive state (or states) of the watersplitting enzyme system.

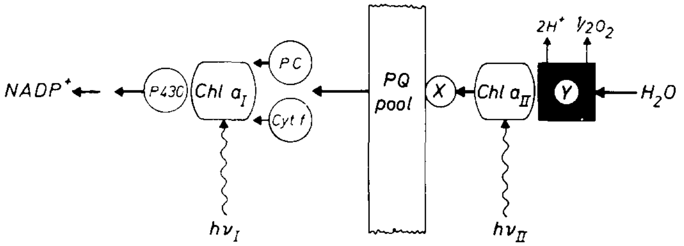

Fig. 1. Simplified electron transport chain in photosynthesis[3]. NADP⁺ = nicotinamide–adenine dinucleotide phosphate, P 430 = primary electron acceptor of System I (unidentified pigment, see ref. 4), Chl a_I = chlorophyll a_I, PC = plastocyanin, Cyt f = cytochrome f, PQ = plastoquinone pool interconnecting at least 10 electron transport chains[5], X = primary electron acceptor (or acceptors, see ref. 6) of System II, Chl a_{II} = chlorophyll a_{II}, Y = watersplitting enzyme system.

The molecular mechanism of the activation–deactivation reactions provides the clue for an understanding of the molecular events taking place in the photosynthetic oxidation of water. The nature of these reactions is unknown.

A first insight into this mechanism should be obtainable if one could influence the above-mentioned reactions in a well-defined way.

Recently it was found that agents like carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) accelerate the deactivation reactions of the watersplitting enzyme system[17,18]. The mechanism of the accelerative effect of CCCP and FCCP remains to be elucidated.

One way to attack this problem is to search for a number of substances acting in the same way as CCCP and FCCP and then to find out the common chemical and physical properties of these agents which could be responsible for the accelerative effect on the deactivation reactions.

In the present paper the action of substituted 2-anilinothiophenes, recently discovered as uncouplers of the oxidative phosphorylation by BÜCHEL AND SCHÄFER[19], on the deactivation reactions of the watersplitting Enzyme System Y is described. The results show that a few derivatives of the 2-anilinothiophenes act as accelerators of the deactivation reactions in the same way as CCCP and FCCP.

The accelerative effect is dependent on the acidity of the NH group. Since CCCP and FCCP also contain a NH-acid group it is concluded that this part of the Acceleration of the Deactivation Reactions of the watersplitting Enzyme System Y (ADRY) agents is important for the observed effect.

MATERIALS AND METHODS

Spinach chloroplasts were isolated from market spinach according to the method of WINGET *et al.*[20]. 5 % dimethylsulphoxide was added as a protective agent during storage in liquid N_2. O_2 was measured with a Clark-type electrode[21] with the flash polarography equipment described elsewhere[22]. The sensitivity of the O_2 measuring device was not sufficient to allow resolution of the O_2 yield produced by excitation with a short single flash. Therefore a repetitive technique was used.

In this method chloroplasts were excited with m short flashes (or flash groups) and the total amount of O_2 produced by the m flashes (or flash groups) was measured (see Fig. 2). From the signal obtained the average O_2 yield per flash was determined.

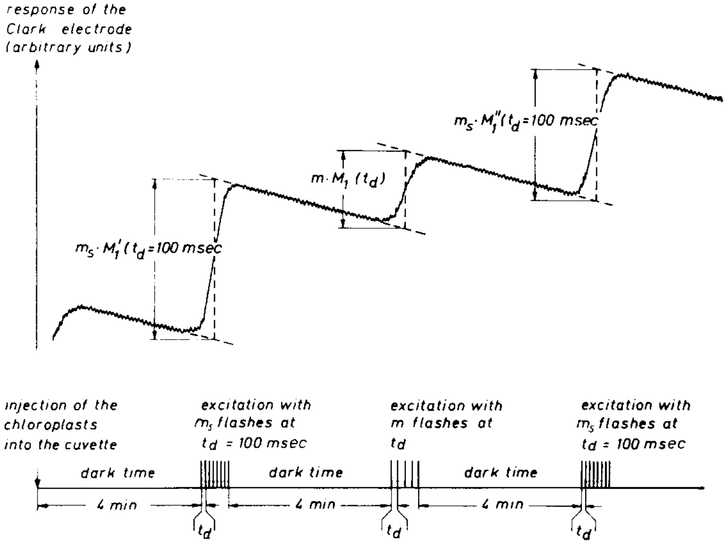

Fig. 2. Response of the Clark electrode as a function of the excitation conditions and of the measuring time. The electrode response caused by the flash light is calibrated by injection of air saturated water into the cuvette. The calibration measurement response is not shown. The dark decline of the curves is created by the O_2 consumption of the electrode. It is also dependent on the chloroplast preparation.

In order to avoid misinterpretations in the comparison between results obtained by the repetitive flash polarography technique and the results obtained using the highly sensitive O_2 measuring device developed by JOLIOT AND JOLIOT[23], it seems worthwhile to give a short characterization of the quantity "average O_2 yield per flash".

The average O_2 yield per flash, designated as M_n (t_d, t_v, τ_h), is given by:

$$M_n(t_d,t_v,\tau_h) = \frac{1}{4} [N_{turnover}(t_d,t_v)]_n \cdot [P_h(t_d,t_v,\tau_h)]_n \cdot [Y_o] \qquad (1)$$

n = number of the flash in each flash group (in this paper n = 1 or 2), t_d = time between the repetitive flashes or flash groups, t_v = time between the flashes of each flash group, τ_h = life time of the holes (oxidizing equivalents) stored in the water-splitting Enzyme System Y, $[N_{turnover}(t_d,t_v)]_n$ = average number of turnovers per flash induced in the electron transport of System II, $[P_h(t_d,t_v,\tau_h)]_n$ = probability for the transformation of a hole (oxidizing equivalent) produced in System II by the nth flash of the flash group, into molecular O_2 and $[Y_0]$ = number of intact watersplitting enzyme systems.

In all experiments reported here short flashes (approx. 20 μsec) of saturating intensity were used. From experiments performed by WEISS AND SAUER[24] and by KOK *et al.*[12] it can be concluded that these flashes can be considered in a first order approximation as single turnover flashes, that means: The flash duration is short enough to ensure that one (because of the saturating intensity) and only one (because of the short duration t_f) turnover per flash in the System II can occur if t_d and t_v are long enough. Therefore, the effect of t_f and of the flash intensity is not considered in Eqn. 1. The influence of misses and double hits (see ref. 12) will not be taken into account.

Furthermore, in the experiments presented here, the times t_d and t_v are chosen to be of a length that, to a good approximation, the influence of rate limiting effects of the electron transport chain is negligible (see refs. 25 and 26). Hence, in the experiments reported in this paper it is assumed that each flash induces one turnover, *i.e.* $[N_{\text{turnover}}(t_d,t_v)]_n = 1$ for n = 1 or 2.

Because of the dark reactions leading to the deactivation of the watersplitting Enzyme System Y only a fraction $[P_h(t_d,t_v,\tau_h)]_n$ of the holes produced by System II leads to the oxidation of water to molecular O_2. The value of $[P_h(t_d,t_v,\tau_h)]_n$ depends on the life time τ_h of the System II holes and on the times t_d and t_v. This function will be discussed in detail in this paper (see RESULTS and DISCUSSION).

The watersplitting Enzyme System Y is very sensitive to irreversible destruction[27–30]. Therefore, during measurements a time-dependent decrease of Y_0 takes place[31]. Since the watersplitting enzyme systems were found to be functionally independent of each other[12] the inhibitory effects can be eliminated by a suitable standardization.

For standard conditions a time of t_d = 100 msec was chosen for the excitation of the chloroplasts with repetitive single flashes and t_d = 250 msec, t_v = 5 msec for the excitation with repetitive flash groups. The activity loss of the chloroplasts (decrease of $[Y_0]$) during measurement was nearly eliminated by a linear approximation. Generally the experiments were performed in the following way (see Fig. 2):

4 min after the injection of the chloroplasts into the cuvette m_s flashes were given, with the standard time t_d = 100 msec, leading to an average O_2 yield per flash designated as M'_1 (t_d = 100 msec). After the next dark time of 4 min m flashes, with a time t_d between the flashes, were given, inducing an average O_2 yield per flash of $M_1(t_d)$. Finally, after a dark time of 4 min, m_s flashes, with the standard time t_d = 100 msec, were given, resulting in an average O_2 yield per flash of M''_1 (t_d = 100 msec). In most of the experiments reported here $m_s = m = 120$.

Flash-group experiments were performed in an analogous way. For subsequent investigations of the ADRY effect the relative average O_2 yield per flash $\varphi(t_d)$, defined by Eqn. 2, is introduced:

$$\varphi(t_d) = \frac{M_1(t_d)}{1/2\,[M'_1(t_d = 100\ \text{msec}) + M''_1(t_d = 100\ \text{msec})]} \qquad (2)$$

If absolute values for the chloroplast activity are given (Fig. 5) they are always related to the results obtained in the first measurement of each experimental series (see Fig. 2). In order to increase the signal/noise ratio of the O_2 measuring device the O_2 content of the suspension was decreased by flushing with N_2 before the addition of the chloroplasts.

The complete reaction mixture contained (cuvette volume, 3.24 ml): $5\cdot10^{-5}$ M chlorophyll; 10^{-4} M $K_3[Fe(CN)_6]$ + 10^{-4} M $K_4[Fe(CN)_6]$ as electron acceptor; 10^{-2} M KCl; $2\cdot10^{-3}$ M $MgCl_2$; 10^{-2} M 2-(*N*-morpholino)ethanesulphonic acid (MES)–NaOH; pH 6.5; temperature, 21°.

Simple inorganic or organic reagents were of the highest purity commercially obtainable. The 2-anilinothiophenes were kindly supplied by Dr. Büchel.

RESULTS

The influence of the rate of the deactivation reactions of the watersplitting Enzyme System Y on the relative average O_2 yield per flash

If rate-limiting effects of the electron transport chain can be excluded (*i.e.* at longer times t_d, see MATERIALS AND METHODS), under repetitive single flash excitation conditions the relative average O_2 yield per flash $\varphi(t_d)$ is determined only by the probability function $[P_h(t_d,\tau_h)]_1$:

$$\varphi(t_d) = \text{const}\cdot[P_h(t_d,\tau_h)]_1 \tag{3}$$

with const $= [P_h(t_d = 100 \text{ msec}, \tau_h)]_1^{-1}$.

Hence the function $[P_h(t_d,\tau_h)]_1$ can be directly experimentally determined. Furthermore t_d is known, so that it should be possible to determine τ_h. But because different active states of the watersplitting Enzyme System Y are included in the trapping of holes (see refs. 11, 12 and 14) there is not only one value for τ_h, but several, since different life times (for each active state at least one) have to be taken into account.

Hence, the function $[P_h(t_d,\tau_h)]_1$ is very complex and cannot be described by an explicit algebraic form. It can be evaluated only stepwise by numerical computation depending on the mechanism postulated for the activation–deactivation reactions of the watersplitting Enzyme System Y[32]. Therefore, in the present paper only a qualitative discussion of the function $[P_h(t_d,\tau_h)]_1$ will be given.

In Fig. 3 the relative average O_2 yield per flash, $\varphi(t_d)$, as a function of the time t_d between the flashes is depicted in the absence and in the presence of $2\cdot10^{-6}$ M 2-(4-chloro)anilino-3,5-dinitrothiophene (ANT 2a). It is seen that, in comparison to the decrease of $[P_h(t_d,\tau_h)]_1$ caused by the natural deactivation reactions, a strong acceleration of this decrease is observed in the presence of ANT 2a.

In Fig. 4 a semilogarithmic plot of the ANT 2a-induced decrease of $\varphi(t_d)$ is given. As can be seen, the function $[P_h(t_d,\tau_h)]_1$ is approximately described by first order kinetics for $t_d > 100$ msec. Therefore, in this time range the function

$$\varphi(t_d) = \text{const}\cdot[P_h(t_d,\tau_h)]_1 = e^{-\frac{(t_d - 100\text{ msec})\cdot\ln 2}{(\tau_{h\frac{1}{2}})_{app}}} \tag{4}$$

can be used as an approximate description. The reciprocal value of the half time $(\tau_{h\frac{1}{2}})_{app}$ of this apparent first order decay will be used here as a qualitative measure of the deactivation reactions of the holes stored in the watersplitting Enzyme System Y.

Up to now it was assumed that ANT 2a acts as an ADRY agent. Now an unequivocal proof needs to be given for the assumed ADRY effect of ANT 2a. This can be given in the following way. It was inferred from fluorescence measurements that only the activation-deactivation mechanism of the watersplitting Enzyme System Y is responsible for the O_2 evolution kinetic pattern[11,33]. Therefore one must

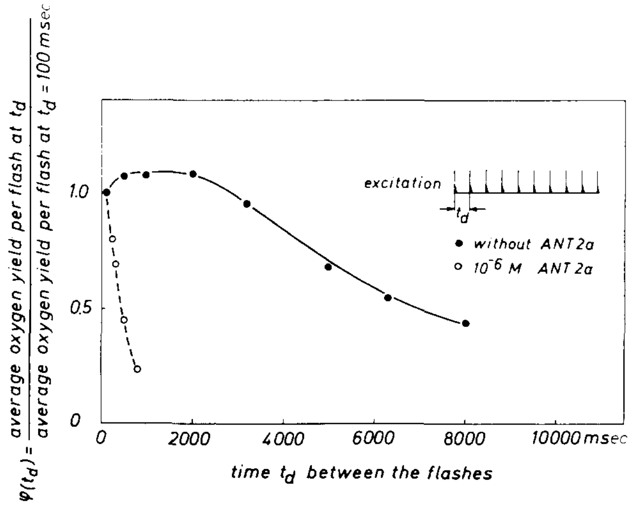

Fig. 3. Relative average O_2 yield per flash, $\varphi(t_d)$, as a function of the time t_d between the flashes in chloroplasts. Chlorophyll concentration: $5 \cdot 10^{-5}$ M. Activity of O_2 production: 35/168 moles O_2/moles Chl·h (—/+ uncoupler NH_4Cl). ANT 2a (2-(4-chloro)anilino-3,5-dinitrothiophene) addition as indicated in the figure.

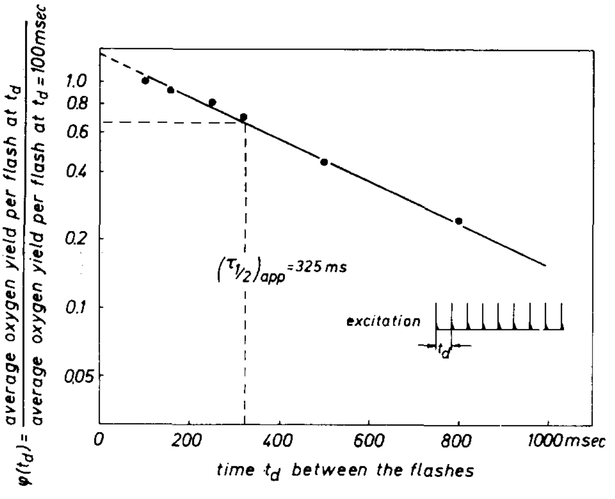

Fig. 4. Relative average O_2 yield per flash, $\varphi(t_d)$, as a function of the time t_d between the flashes in chloroplasts. ANT 2a concentration: $2 \cdot 10^{-6}$ M. Other experimental conditions as described in Fig. 3. Ordinate: logarithmic.

show that ANT 2a influences this characteristic behaviour of System Y. By the use of a repetitive method the characteristics of the above mentioned kinetics can be investigated in the simplest way by excitation of photosynthesis with repetitive double flash groups (see ref. 18).

The decrease of the average O_2 yield per flash under repetitive double flash-group excitation conditions

The average O_2 yield per double flash group is given by the sum:

$$M_{II}(t_d,t_v) = M_1(t_d,t_v) + M_2(t_d,t_v) \tag{5}$$

where $M_{II}(t_d,t_v)$ = total average O_2 yield per double flash group at the times t_d and t_v, and $M_1(t_d,t_v)$ and $M_2(t_d,t_v)$ are the average O_2 yields of the first and of the second flash of each double flash group (see MATERIALS AND METHODS, Eqn. 1).

Since rate-limiting effects of the electron transport chain are negligible (see MATERIALS AND METHODS) from Eqn. 1 it follows that:

$$M_1(t_d,t_v = 5\text{ msec}) = \frac{1}{4}\,[Y_o]\,[P_h(t_d,t_v = 5\text{ msec},\tau_h)]_1 \tag{6a}$$

$$M_2(t_d,t_v = 5\text{ msec}) = \frac{1}{4}\,[Y_o]\,[P_h(t_d,t_v = 5\text{ msec},\tau_h)]_2 \tag{6b}$$

Because $[Y_0]$ is a constant the transformation probabilities $[P_h(t_d,t_v = 5\text{ msec}, \tau_h)]_1$ and $[P_h(t,_d t_v = 5\text{ msec}, \tau_h)]_2$ of the System II holes can be directly experimentally determined.

At $t_v = 5$ msec there is practically no influence of the deactivation reactions of the holes on the transformation probabilities, so that in the subsequent investigation only the effect of t_d is discussed. Since a quantitative treatment of the transformation probabilities as a function of t_d is impossible, only two extreme conditions for t_d will be considered:

(A) t_d is small in comparison to $(\tau_{h\frac{1}{2}})_{app}$. In that case practically all of the holes produced by System II contribute to the water oxidation, *i.e.* $[P_h(t_d,\tau_h)]_1 = [P_h(t_d,\tau_h)]_2 = 1$.

(B) $t_d \rightarrow \infty$. If t_d increases *ad infinitum* the repetitive excitation with m double flash groups degenerates to a single excitation with only one double flash group. For the single excitation condition of dark-adapted chloroplasts the transformation probability of the System II holes is known from the experiments of JOLIOT *et al.*[11] and of KOK *et al.*[12]. Hence it follows that:

$$\lim_{t_d \rightarrow \infty} [P_h(t_d,\tau_h)]_1 = 0 \tag{7a}$$

$$\lim_{t_d \rightarrow \infty} [P_h(t_d,\tau_h)]_2 \approx 0.3 \tag{7b}$$

Because of comparable flash durations (see refs. 11 and 12) Eqn. 7b is applicable for the results reported in this paper. The function $[P_h(t_d,\tau_h)]_1$ has been already discussed (see Figs. 3 and 4), but for an unequivocal proof of the assumed ADRY action of ANT 2a the probability $[P_h(t_d,\tau_h)]_2$ has to be analyzed. This function can be experimentally determined according to Eqn. 6b by measurement of the average O_2

yield caused by the second flash of each double flash group M_2 ($t_d,t_v = 5$ msec) as a function of t_d. From the comparison of the transformation probability of the System II holes produced by the second flash of each double flash group under Conditions A and B, a decrease of $[P_h(t_d,\tau_h)]_2$ by a factor of approx. 3 is expected if t_d increases. M_2 ($t_d,t_v = 5$ msec) was determined in an indirect way as the difference between M_{II} ($t_d,t_v = 5$ msec) and $M_1(t_d)$ obtained by excitation with repetitive single flashes (see Eqn. 5).

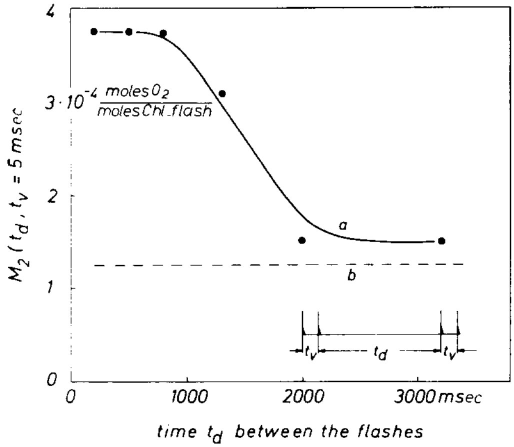

Fig. 5. Average O_2 yield of the second flash of each double flash group M_2 ($t_d, t_v = 5$ msec) as a function of the time t_d between the double flash groups in chloroplasts. ANT 2a concentration: $2 \cdot 10^{-6}$ M. Other experimental conditions as described in Fig. 3. Dotted line: value of M_2 ($t_d, t_v = 5$ msec) expected for $t_d \rightarrow \infty$ (see ref. 18).

In Fig. 5 M_2 ($t_d,t_v = 5$ msec) is depicted as a function of t_d. It is clearly seen that the expected decrease of M_2 ($t_d,t_v = 5$ msec) in the presence of $2 \cdot 10^{-6}$ M ANT 2a occurs in a time range of t_d, when the effect of the natural deactivation reactions of the watersplitting Enzyme System Y is small (see Fig. 3). By comparison of $M_1(t_d)$, depicted in Fig. 3 as the normalized form $\varphi(t_d)$, and M_2 ($t_d,t_v = 5$ msec) (Fig. 5) a remarkable slower decrease of M_2 ($t_d,t_v = 5$ msec) with increasing t_d is observed. This result is qualitatively understandable by the fact that in each double flash group two holes per System II 'are produced, so that a longer time t_d is required for the discharge of these two holes to the same average level as it is reached in repetitive single flashes (producing only one hole per System II) at a shorter time t_d.

Earlier investigations[32] have shown a good agreement between experimental results and numerical computations. Therefore, the decrease of M_2 ($t_d,t_v = 5$ msec) with increasing t_d is only explicable by the assumption of an ANT 2a-induced ADRY effect. Now an investigation of the effect of a few derivatives of the 2-anilinothiophenes is required in order to obtain information about the mechanism of the ADRY action.

The influence of substituted 2-anilinothiophenes on the transformation probability of System II holes into molecular O_2

The substituted 2-anilinothiophenes analyzed as possible ADRY agents in this paper are presented in Fig. 6. The effect of these substances, at a concentration of

$4 \cdot 10^{-7}$ M, on the decrease of the average O_2 yield per flash, $\varphi(t_d)$, as a function of the time t_d is shown in Fig. 7.

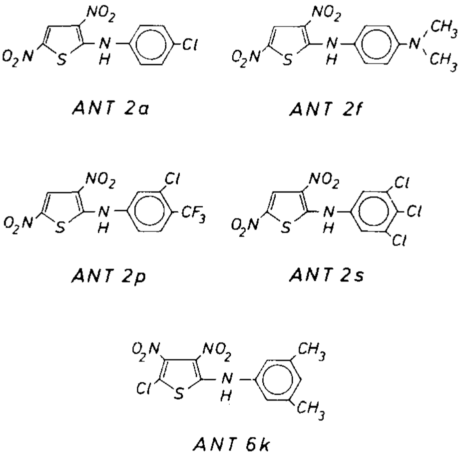

Fig. 6. The chemical formulae for the derivatives of 2-anilinothiophene investigated in this paper. The symbols for these agents are related to those of ref. 19.

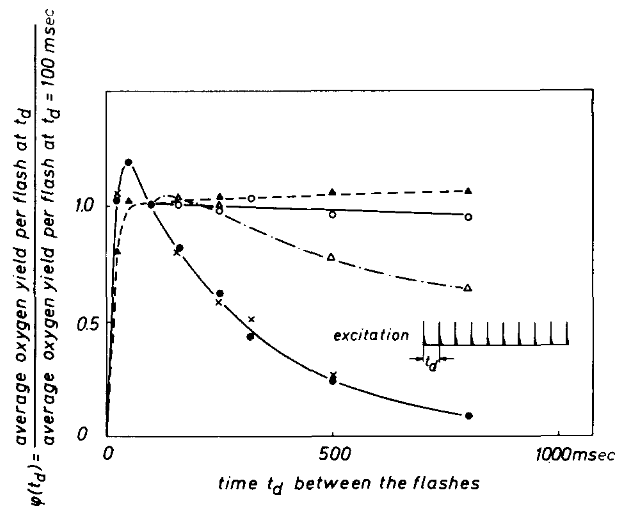

Fig. 7. Relative average O_2 yield per flash, $\varphi(t_d)$, as a function of the time t_d between the flashes in the presence of various derivatives of 2-anilinothiophene in chloroplasts. Anilinothiophene concentration: $4 \cdot 10^{-7}$ M. Other experimental conditions as described in Fig. 3. △, ANT 2a; ▲, ANT 2f; ×, ANT 2p; ●, ANT 2s; ○, ANT 6k.

Two agents, ANT 2p and ANT 2s, were found to be very potent ADRY substances, ANT 2a provides a moderate ADRY effect, whereas ANT 2f and ANT 6k give only a poor, or no, acceleration of the deactivation reactions of the watersplitting Enzyme System Y.

DISCUSSION

The results reported in this paper clearly show the discovery of a new class of ADRY agents: the 2-anilinothiophenes. The 2-anilinothiophenes were found to act in the same way as the carbonylcyanidephenylhydrazones, CCCP[17] and FCCP[13]. With these results a new class of biological effectors is established: the class of the ADRY agents.

The ADRY effect is important for two reasons: (a) It must be taken into consideration in all experiments where the rate of the photoreaction is of the same order of magnitude as the ADRY agent-induced rate of deactivation reactions in the watersplitting Enzyme System Y, in order to avoid misinterpretations of the results obtained. This is the case in experiments carried out at low intensity continuous light and at low frequency repetitive flash light. Under these excitation conditions a fixed fraction of the oxidizing equivalents produced by chlorophyll a_{II} is not transformed to O_2, but is wasted by deactivation reactions, also in the steady state in respect to the activation of the watersplitting Enzyme System Y. The magnitude of this fraction, given by $1 - P_{\mathrm{h}}(\tau_{\mathrm{h}})$ (the variables characterizing the excitation conditions are omitted), depends on the intensity in continuous light or on the times t_{d} and t_{v} (see MATERIALS AND METHODS) in flash light experiments.

This effect poses serious problems if one compares experiments carried out under different excitation conditions, since in the presence of ADRY substances $P_{\mathrm{h}}(\tau_{\mathrm{h}})$ is strongly dependent on excitation conditions. Theoretically the same problem also arises in the absence of ADRY agents, but the effect of the natural deactivation reactions of the watersplitting Enzyme System Y is significant only at very low light intensities (in the order of a few $\mathrm{ergs \cdot cm^{-2} \cdot sec^{-1}}$, see ref. 10), so that under the excitation conditions frequently used the above mentioned effect can be neglected.

The details of these effects will be discussed elsewhere[34].

(b) The ADRY agents provide a powerful tool for the investigation of the molecular events occurring along the watersplitting Enzyme System Y, especially for the elucidation of the nature of the oxidizing equivalents stored in this system. These ADRY substances can be used both to label and to modify the active states, *i.e.* the stored positive charges, of system Y in a similar way as the uncouplers were used for the modification and for the identification of the high energy state in phosphorylation.

The derivatives of the 2-anilinothiophenes differ strongly in their ADRY efficiency (see Fig. 7) depending on the substitution of the anilino moiety (see Fig. 6). These substituents influence the acidity of the NH group in the 2-anilinothiophenes. Therefore, it could be possible that there exists a correlation between the acidity of the imino group and the observed ADRY effect. A similar behaviour was earlier found for the inhibitory effect of phenylurea derivatives[35]. It was shown that the inhibitory power of substituted phenylurea is correlated to the relative acidity of the imino group.

In Table I the p*K* values of the ADRY agents and the apparent ADRY-induced rate of the deactivation reactions of the watersplitting Enzyme System Y are presented.

It can be seen that the p*K* values of the strong ADRY substances FCCP, ANT 2s and ANT 2p are below or close to 6.0. For the weak acids ANT 2f and ANT 6k no ADRY effect was observed. From these results it is concluded that the acidity of the imino group in the ADRY agents is important for the ability to induce an ADRY effect. It remains to clarify the molecular basis for the role of the NH-acid group, also whether this NH-acid group is an indispensible functional element of all ADRY substances. Another chemical property of the ADRY agents is the presence of π-orbitals being able to delocalize the negative charge of the anion form. This charge delocalization favours the solubility of the anion in a low dielectric medium[37], *i.e.* these anions are soluble in lipids.

TABLE I

COMPARISON BETWEEN THE ADRY EFFECT (EXPRESSED BY THE RECIPROCAL HALF TIME $(\tau_{h\frac{1}{2}})_{app}^{-1}$ OF THE APPARENT FIRST ORDER DECAY OF $\varphi(t_d)$) AND THE pK VALUE OF THE NH-ACID GROUP IN THE ADRY AGENTS

ADRY agent	$(\tau_{h\frac{1}{2}})_{app}^{-1}$ (*sec*$^{-1}$)	*pK*
ANT 2a	1.3	7.0*
ANT 2f	<0.2	8.0*
ANT 2p	4.8	5.9*
ANT 2s	4.9	5.6*
ANT 6k	<0.2	7.4*
FCCP	4.6	5.8**
CCCP	2.0	6.0**

* Ref. 19. ** Ref. 36.

Because of the above-mentioned behaviour all ADRY agents, so far known, are potent proton translocators across biological and artificial membranes[38–40] and act as strong uncouplers of phosphorylation[19,36,41,42]. Therefore, it could be possible that these properties are responsible for the observed ADRY effect. Further investigations are required in order to clarify whether the oxidizing equivalents stored in the watersplitting Enzyme System Y are stabilized by a proton gradient or are correlated with a high energy intermediate state in the thylakoid.

Another possible mode of action of the ADRY substances is the induction of conformational changes[43,44] in the protein (or lipoprotein) of the watersplitting Enzyme System Y (see ref. 14) which destabilize the stored oxidizing equivalents. Furthermore, one has to take into account the possibility of an electron donor effect of the ADRY agents thereby directly discharging the stored positive charges.

Up to now the nature of the mechanism of the ADRY action still remains an open question, though recently some new results were obtained[45] leading to the conclusion that the ADRY effect is not influenced by the electrical field across the thylakoid membrane and is not caused by ADRY agent-induced irreversible conformational changes in the chloroplasts or by the reduction of the System II holes with ADRY agents as irreversible electron donors.

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