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EFFECT OF PHENOLIC HERBICIDES ON THE OXYGEN-EVOLVING SIDE OF PHOTOSYSTEM II

FORMATION OF THE CAROTENOID CATION

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Phenolic herbicides were added to suspensions of spinach chloroplasts or to oxygen-evolving Photosystem II membranes. Flash absorption spectroscopy at 21°C around 1000 nm reveals that these chemicals lead to a flash-induced absorption increase attributed to the radical-cation of a carotenoid. The herbicides studied can be arranged in the following order of decreasing efficiency for the reported effect: *i*-dinoseb, bromonitrothymol, trinitrophenol, ioxynil, dinitroorthocresol, 2,4-dinitrophenol. A similar effect was not observed with atrazine, DCMU or *o*-phenanthroline. For a given herbicide concentration, the amount of flash-induced carotenoid cation increases sharply when the pH is lowered below 5.5. A similar effect does not take place with other molecules which induce the formation of a carotenoid cation: tetraphenylboron, FCCP, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT-2p). The previous effects are observed in both oxygen-evolving Photosystem II and in preparations in which oxygen evolution is inhibited with alkaline Tris. In untreated material, the carotenoid cation is formed with a half-time of 10–35 μs. After Tris treatment, this half-time is a little longer at low than at high pH. These results indicate the existence of a specific site where phenolic inhibitors interact in the oxygen-evolving site of Photosystem II.

Introduction

Many of the herbicides of agricultural importance inhibit the functioning of Photosystem II (PS II). They can be grossly divided into two classes, according to their chemical structures and some aspects of their mode of interaction with PS II [1]: the DCMU-type herbicides and the phenolic herbicides. All of them efficiently inhibit electron transfer out of the primary plastoquinone Q_A, although binding experiments have indicated that these herbicides may bind to different proteins of the PS II reaction center [2–4]. In higher plants and green algae, it seems that DCMU-type herbicides have the single effect of blocking electron flow out of Q_A, whereas phenolic herbicides (or at least some of them) may also act on the oxygen-evolving side of PS II [5]. A recent study by EPR at low temperature showed that herbicides have different modes of interaction with reduced Q_A; it was also shown that the phenolic herbicide *i*-di-

Abbreviations: ANT-2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; *i*-dinoseb, 2,4-dinitro-6-isobutylphenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PS II, Photosystem II; Car^+, carotenoid radical cation; ΔA, absorption change; ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; Mes, 4-morpholine-ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

noseb additionally transforms cytochrome *b*-559 from the reduced high-potential form to the oxidized low-potential form and causes EPR Signal II_s, normally present in the dark, to disappear [6].

Similar effects on cytochrome *b*-559 and Signal II_s had already been reported in the presence of weak acids like FCCP and tetraphenylboron [7–9]. These chemicals, which are well-known for their property of accelerating the decay of the charge storage states of the O_2-evolving enzyme in PS II (the so-called ADRY effect; Ref. 10), had also been shown to induce the formation of a carotenoid radical cation in PS II upon flash excitation [11,12]. In an earlier report, we showed that addition of the phenolic herbicide *i*-dinoseb to PS II membranes resulted in a flash-induced absorption increase at 990 nm, which was initially attributed to carotenoid oxidation [6]. This incited us to conduct a more systematic study of phenolic herbicides. In this work, we report that all phenolic herbicides tested give rise to flash-induced carotenoid oxidation in normal and Tris-washed chloroplasts and PS II membranes.

Materials and Methods

Biological materials. Chloroplasts were isolated from spinach leaves in an isotonic buffer (0.35 M sucrose/0.01 M NaCl/0.02 M Tricine (pH 7.8)) and centrifuged. Oxygen-evolving PS II membranes were prepared as described by Berthold et al. [13] with the modifications of Ford and Evans [14]. Inhibition of oxygen evolution by alkaline Tris was effected by 10 min of incubation in 0.4 M Tris at pH 9.0. For studies at various pH, pellets of chloroplasts or PS II membranes were washed once in 0.01 M NaCl. All pellets were finally resuspended and homogenized in 0.01 M NaCl and stored on ice. The following buffers were used for the spectroscopic measurements: 0.35 M sucrose/0.01 M NaCl/0.02 M Mes (pH 6.0) (buffer 1); 0.01 M NaCl/0.05 M Hepes (pH 8.0 and 7.0); 0.02 M NaCl/0.05 M Mes (pH 6.0 and 5.5); 0.02 M NaCl/0.05 M succinate (pH 5.0 and 4.6). Except when mentioned, 0.005 M $MgCl_2$ and 80 μM *p*-phenylbenzoquinone (with PS II membranes) or 500 μM potassium ferricyanide (with chloroplasts) were added.

Chemicals. Atrazine and *i*-dinoseb were provided by Dr. Van Assche (Procida, Marseille); bromonitrothymol and ioxynil were gifts from Dr. Oettmeier (Ruhr University, Bochum). *o*-Phenanthroline (Merck), trinitrophenol (Rhône-Poulenc), dinitro-*o*-cresol (Fluka AG) and 2,4-dinitrophenol (Merck) were purchased. Recrystallized DCMU was provided by Dr. J. Farineau and ANT-2p by Dr. J. Garnier.

Stock solutions in ethanol were kept at −15°C.

Absorption change measurements. The biological material was suspended in a 10 × 10 mm cuvette, at 21°C, and excited by the pulse from a dye laser (15 ns; broad band around 600 nm) the energy of which was attenuated to just saturate Photosystem II. The absorption change in the region 820–1080 nm was measured as previously described, with a time resolution of 1 μs [12]. The observations that we report here are strongly affected by preillumination, since the studied chemicals are often inhibitors of Q_A reoxidation. Except when mentioned, we thus measured the effect of a first laser flash given after the following protocol: the stock of biological material was suspended in the buffer, ferricyanide or *p*-phenylbenzoquinone was added in darkness, the inhibitor was added and incubated in darkness for 120 s before firing the laser flash.

Results

Some original data of flash-induced ΔA are shown in Fig. 1, for chloroplasts without addition

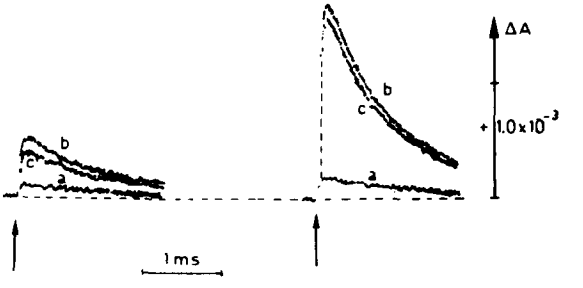

Fig. 1. Absorption changes at 990 nm induced by one flash in chloroplast suspensions (chlorophyll concentration, $3 \cdot 10^{-5}$ M) with 0.5 mM $K_3Fe(CN)_6$. Left traces, buffer at pH 7. Right traces, buffer at pH 5. a, no further addition; b and c, addition of 1 mM 2,4-dinitrophenol (DNP) at the first and second flash, respectively (separated by 15 s).

(traces a) or with 1 mM dinitrophenol. A small absorption increase takes place without addition, but a much bigger ΔA is seen in presence of dinitrophenol. It is also shown that the ΔA is smaller at the second flash. The diminution of ΔA upon the second flash is much more pronounced when $MgCl_2$ and/or electron acceptor are omitted; it is also more pronounced at higher pH, whereas the first and subsequent flashes have nearly the same effect at pH 5.0 or 4.6. These properties, which we found for all of the phenolic inhibitors studied, are in agreement with known reoxidation properties of Q_A in the presence of DCMU [15]. Similar properties were observed with PS II membranes. The spectrum of flash-induced ΔA for chloroplasts with dinitrophenol, at pH 4.6, is shown in Fig. 2. This spectrum presents a well-defined peak centered at 990 nm; the shape is identical to that obtained with tetraphenylboron (Fig. 2) and attributed to the radical cation of a carotenoid [12]. Very similar spectra were obtained with other phenolic inhibitors and also with PS II membranes. We thus conclude that Car^+ is formed after a flash in PS II in the presence of phenolic herbicides.

Fig. 3 shows the effect of inhibitor concentration of the size of ΔA at 990 nm induced by a single flash in PS II membranes at pH 6.0. In a first domain of concentration, the ΔA increases linearly with inhibitor concentration. If we accept that the slope gives an index of activity, and giving the index 100 for the most active species, we obtain the following relative activities: *i*-dinoseb, 100; bromonitrothymol, 71; trinitrophenol, 41; ioxynil, 35; dinitro-*o*-cresol, 13; dinitrophenol, 7. On the same scale and under similar conditions, ANT-2p, tetraphenylboron and FCCP have relative activities of about 1300, 1400 and 80 (see also Ref. 12). We found no effect of DCMU, atrazine or *o*-phenanthroline at concentrations up to 165 μM, 130 μM or 9 mM, respectively. Ethanol, up to 2%, has no effect either. At a higher concentration of phenolic inhibitor, the curves tend to saturate and even to decrease, an effect that we have observed with *i*-dinoseb and bromonitrothymol (Fig. 3) and which had been observed earlier with ANT-2p [12].

The amount of Car^+ formed by one flash is also affected by the pH of the suspending medium. As shown in Fig. 4 (left panel) for untreated chloroplasts, the flash-induced ΔA increases slightly between pH 8 and 6, and then more dramatically until pH 4.5. Lower pH values have not been used

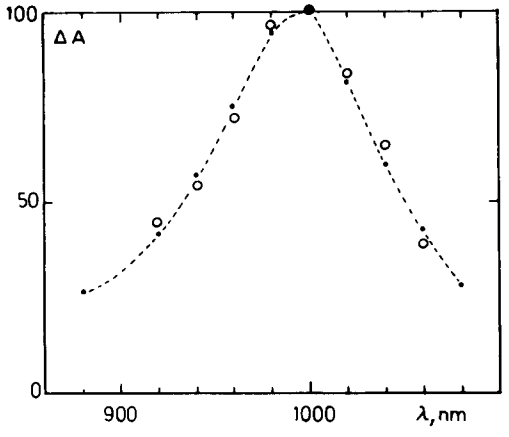

Fig. 2. Spectrum of ΔA induced by one flash in chloroplast suspensions (chlorophyll concentration, $3 \cdot 10^{-5}$ M). (●) Buffer at pH 4.6; addition of 1 mM 2,4-dinitrophenol; 0.5 mM $K_3Fe(CN)_6$; average of two flashes; $\Delta A = 100$ corresponds to $4.9 \cdot 10^{-3}$ absorbance unit. (○) Buffer at pH 6; addition of 0.5 μM tetraphenylboron; one flash; $\Delta A = 100$ correspond to $2.0 \cdot 10^{-3}$ absorbance unit.

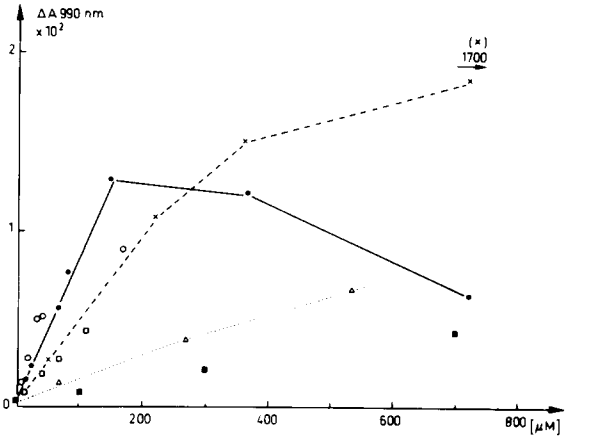

Fig. 3. Absorption change at 990 nm induced by one laser flash in Photosystem II particles (chlorophyll concentration, $4.5 \cdot 10^{-5}$ M) in buffer at pH 6 (with 80 μM *p*-phenylbenzoquinone) in the presence of various amounts of inhibitor: *i*-dinoseb (○), bromonitrothymol (●), trinitrophenol (×), ioxynil (□), dinitro-*o*-cresol (△), dinitrophenol (■). The drawn lines are for bromonitrothymol, trinitrophenol and dinitro-*o*-cresol.

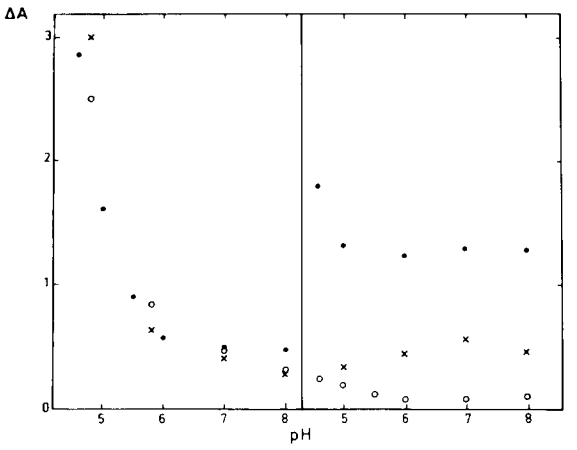

Fig. 4. Effect of pH on the size of ΔA at 1000 nm induced by one flash in chloroplast suspensions (chlorophyll concentration, $3 \cdot 10^{-5}$ M) with 0.5 mM $K_3Fe(CN)_6$. Left panel: addition of 45 μM picric acid (○), 1 mM 2,4-dinitrophenol (●) or 40 μM *i*-dinoseb (×). Right panel: no addition (○), addition of 0.5 μM tetraphenylboron (●) or 0.8 μM FCCP (×). Ferricyanide was omitted when tetraphenylboron was added.

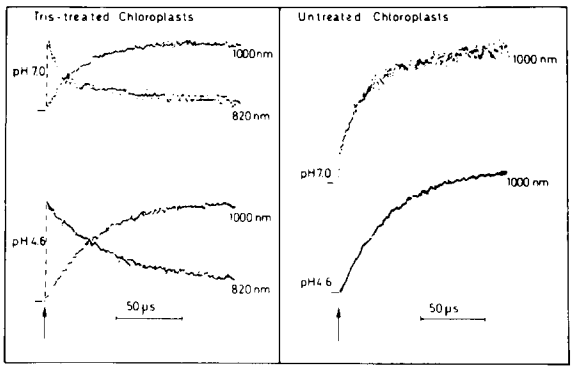

Fig. 5. Kinetics of absorption change at 820 or 1000 nm, induced by a laser flash in Tris-treated (left panel: chlorophyll concentration, $5.5 \cdot 10^{-5}$ μM) or untreated chloroplasts (right panel: chlorophyll concentration, $6.7 \cdot 10^{-5}$ μM) in buffers at pH 4.6 or 7.0. Addition of 0.5 mM $K_3Fe(CN)_6$ and 1 mM dinitrophenol. Average of 4–6 experiments. Maximum absorption changes: Tris-treated chloroplasts (pH 4.6: $1.3 \cdot 10^{-3}$ at 820 nm, $2.3 \cdot 10^{-3}$ at 1000 nm; pH 7.0: $0.7 \cdot 10^{-3}$ at 820 nm, $1.6 \cdot 10^{-3}$ at 1000 nm); untreated chloroplasts (pH 4.3: $4.3 \cdot 10^{-3}$; pH 7.0: $0.4 \cdot 10^{-3}$).

because of chloroplast floculation. It appears that the pH effect is nearly identical with trinitrophenol, dinitrophenol or *i*-dinoseb. Without any addition, the formation of Car^+ also increases slightly at low pH (Fig. 4, right panel), but little effect of pH was obtained with FCCP or tetraphenylboron. A similar effect of pH on Car^+ formation in the presence of phenolic herbicides was obtained with PS II membranes. With both types of material, inhibition of oxygen evolution by alkaline Tris does not change the formation of Car^+ and also does not modify the increase of Car^+ formation at low pH in experiments with dinitrophenol. Maximum signals were obtained at pH 4.6 with trinitrophenol, corresponding to one Car^+ per 1250 chlorophylls in chloroplasts and one per 810 chlorophylls in PS II membranes (we assumed an extinction coefficient of 125 000 $M^{-1} \cdot cm^{-1}$ for Car^+ at 990 nm).

The kinetics of rise and of decay of Car^+ may provide information on the mode of action of the phenolic inhibitors. After a flash, the absorption rise at 990 nm always includes a small submicrosecond phase (Fig. 5), as already reported [12]; this phase is relatively more important at high pH. The major rise has a $t_{1/2}$ between 10 and 35 μs. This $t_{1/2}$ appears to be little affected by experimental conditions. With untreated chloroplasts, the $t_{1/2}$ varies less than by a factor of 2 when changing the concentration of dinitrophenol (0.1–1.3 mM), of trinitrophenol (0.1–1.5 mM) or *i*-dinoseb (0.03–0.2 mM); with 1 mM dinitrophenol, it increases somewhat when the pH is lowered, as shown in Fig. 5 for pH 4.6 and 7.0. In the pH domain studied, from 4.6 to 8.0, the $t_{1/2}$ changes by a factor of 2, from 28 to 15 μs in the average. The same trend was observable with Tris-treated chloroplasts (Fig. 5). At pH 4.6–6.0, the absorption rise at 1000 nm in the presence of dinitrophenol matches closely the decay of absorption at 820 nm, without dinitrophenol, which is attributed to $P\text{-}680^+$. At higher pH, however, the decay of $P\text{-}680^+$ is greatly accelerated, whereas the absorption rise at 1000 nm remains rather slow ($t_{1/2} = 12$ μs at pH 8.0, whereas $P\text{-}680^+$ decays with $t_{1/2} = 3$ μs). These different kinetics are illustrated for pH 7.0 in Fig. 5.

The kinetics of decay of Car^+ have not been studied in detail. In the whole series of measurement, the $t_{1/2}$ varied bteween 0.25 and 2 ms. The concentration of inhibitor does not affect greatly the decay, but two factors are of importance. Firstly the pH of the medium: the $t_{1/2}$ decreased by nearly a factor of 2 between pH 8 and 4.6 in

measurements with *i*-dinoseb, dinitrophenol and trinitrophenol. The $t_{1/2}$ of decay also varies greatly with the nature of the inhibitor. A rather fast decay was observed with bromonitrothymol, ioxynil and dinitro-*o*-cresol ($t_{1/2}$ = 0.25–0.6 ms); it was slower with dinitrophenol and *i*-dinoseb (0.5–1.2 ms) and still slower with trinitrophenol (1–2 ms).

Discussion

The present results demonstrate that a group of chemicals with phenolic function(s) induce the formation of a carotenoid radical cation in PS II following flash excitation. These chemicals belong to the class of so-called phenolic herbicides [1]. We also show that some other well-known PS II inhibitors, DCMU, atrazine, *o*-phenanthroline, do not exert the same effect. All of these inhibitors are known to block electron transfer from the primary plastoquinone, Q_A, to the secondary plastoquinone Q_B. This has been proved, in parallel with these measurements, by flash absorption spectroscopy in the ultraviolet (unpublished work in collaboration with Dr. J. Farineau). There are indications in the literature that the phenolic herbicides ioxynil and *i*-dinoseb also act on the oxygen-evolving side of PS II, since they inhibit chemically triggered luminescence in chloroplasts [5]. An index of inhibitory activity has been derived for PS II inhibitors: the pI_{50}, which is the logarithm of the reciprocal of concentration for which the Hill reaction is decreased by 50%. The phenolic compounds that we have used have the following pI_{50} values: bromonitrothymol, 6.9; ioxynil, 6.2; *i*-dinoseb, 5.9; trinitrophenol, 5.3; dinitro-*o*-cresol, 3.7; and dinitrophenol, 3.0 [1,16]. It appears that the order of decreasing efficiency is about the same as measured for the formation of Car^+, although we had to use higher inhibitor concentrations. This similar order of efficiency raises the question of a common property between inhibition of Hill reaction and Car^+ formation, perhaps a similar binding site. This could equally be explained as being simply due to the relative lipophilicity of the herbicides, i.e., if a more lipophilic species could have easier access to a site on the acceptor side of the PS II center, it would also have easier access to a site on the donor side. However, Trebst and Draber [1] showed that lipophilicity is not a major factor in the activity of nitrophenols.

In their capacity to induce the formation of Car^+, phenolic herbicides appear to behave like ANT-2p, tetraphenylboron or FCCP. They are less active than the first two, but as active as FCCP [12]. These three molecules have been shown to belong to a class of so-called ADRY compounds, which deactivate the precursors of oxygen evolution [10]. However, Car^+ formation takes place in Tris-treated as well as in untreated, oxygen-evolving chloroplasts or PS II particles; thus, there is no obvious relation between the classical ADRY effect and Car^+ formation. The effect we observe may be more akin to the transformation of cytochrome *b*-559 from the high-potential to the low-potential form, which can be induced by *i*-dinoseb [6] as well as by FCCP or CCCP [7], and which concerns the immediate donor side of the PS II reaction center.

In previous works, it has been proposed that Car^+ formation was related to the ability of some molecules to form lipophilic anions [11,12]. Many properties of FCCP and other uncouplers have also been interpreted in that manner [17]. If this were true, and if it were also true for phenolic herbicides, there should be a relation between the effect of pH on Car^+ formation and the pK of the inhibitors. It appears, however, that the effect of FCCP is independent of pH (Fig. 4) on either side of its pK (5.9), and that the stimulation of Car^+ at lower pH with phenolic compounds is not related to their pK (trinitrophenol: pK = 0.2; dinitrophenol: pK = 4.0). Oettmeier and Masson [18] found that the binding of *i*-dinoseb increases at low pH, but that pH does not change the binding of picric acid (trinitrophenol) [19]. It is thus more likely that the pH effect observed here is due to an integral component of PS II which, when protonated, somehow gives rise to a stimulation of the herbicide effect on carotenoid oxidation. It may be noted that the $t_{1/2}$ of carotenoid oxidation (approx. 10–35 μs) corresponds approximately to a minor phase of $P\text{-}680^+$ reduction, the relative contribution of which increases at low pH [20]. It is thus possible that the carotenoid oxidation that we report here corresponds to a direct electron transfer from the carotenoid to $P\text{-}680^+$ in reaction

centers where P-680$^+$ decays according to the so-called '35-μs' phase.

Several effects of inhibitors on the donor side of PS II seem to be closely related: deactivation of the S states (ADRY effect) [10], conversion of cytochrome *b*-559 to the low-potential form [7,9], disappearance of the EPR Signal II_s [8], acceleration of the decay of Signal II_f, and induction of carotenoid oxidation. The origin of these effects is not clear. The acceleration of the decay of Signal II_f has been interpreted in terms of direct electron donation from the inhibitor to Z^+, the species which gives rise to Signal II [21]. This proposal, however, does not permit a simple interpretation of all phenomena. It may be noted that phenolic herbicides bind to a 41 kDa polypeptide which belongs to the PS II reaction center [4], as also do cytochrome *b*-559 and the protein bearing the species Z [22,23]. In order to interpret most of the data, we propose that those inhibitors of PS II electron transfer which are also uncouplers in a classical sense bind to a site in the PS II reaction center, perhaps the 41 kDa polypeptide, and induce structural changes which modify the normal electron-transfer path. All the reported modifications of electron transfer in the donor side of PS II could originate in a lowered redox potential of a carotenoid, due to the interaction with bound inhibitor, as suggested previously [12]. This could permit a direct oxidation of the carotenoid by P-680$^+$ and by Z^+, which would explain the observed carotenoid oxidation as well as the effects on Signal II_s, Signal II_f and the S states (if we assume some equilibrium between the redox state of Z and of the S states). The kinetic effects reported for Signal II and for the S states can be easily explained in this model if the inhibitor is not permanently bound to a reaction center but shuttles from one center to another.

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Note added in proof (September 26th, 1984)

The effect of phenol-type inhibitors on the chlorophyll fluorescence and luminescence has also been attributed recently to an inhibitory action at the donor side of Photosystem II [24].

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