Comparison of Diuron- and Phenol-Type Inhibitors: Additional Inhibitory Action at the Photosystem II Donor Site

Klaus Pfister * and Ulrich Schreiber

Botanisches Institut I, Universität Würzburg, D-87 Würzburg, Bundesrepublik Deutschland

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Inhibitors of photosystem II reactions from the "diuron-type" and "phenol-type" have been compared regarding their mechanism of action.

"Diuron-" as well as "phenol-type" inhibitors act at the acceptor site of photosystem II by displacing the secondary acceptor quinone Q_B from its binding site.

"Phenol-type" inhibitors additionally interfere with the donor site, which is demonstrated in studies of chlorophyll fluorescence and luminescence. This mechanism of action is shown to be similar but not identical to that reported for hydroxylamine.

Introduction

A variety of chemically different classes of herbicides and experimental inhibitors act by interrupting photosynthetic electron transport at the reducing site of PS II [1, 2]. One common feature of these chemicals – besides their inhibitory action upon PS II dependent photoreactions – is the competitive binding at a common receptor site in thylakoid membranes [3–5].

Several independent observations have led to the distinction between "diuron-type" inhibitors and "phenol-type" inhibitors; *e.g.* different inhibitory efficiency in trypsin treated thylakoids [6, 7], and in chloroplasts isolated from herbicide resistant weeds [4]; the involvement of different receptor proteins [8, 9]; a different residence time at their binding site [10]; an "essential" structural element, common only to the "diuron-type" inhibitors [1]; the requirement for different molecular parameters in QSAR studies [11] and a different modification of the thermoluminescence pattern in thylakoids [12].

The present study was undertaken to define the essential differences in the mechanism of action of these two types of inhibitors. We have compared representatives of both groups of herbicides with respect to their inhibitory action on the reducing and oxidizing site of PS II. Selected "diuron-type" compounds are: diuron (DCMU, a phenylurea), atrazine (a *s*-triazin), pyrazon (a pyridazinone). "Phenol-type": ioxynil (a benzonitrile), dinoseb (a nitrophenol), BNT (bromonitrothymol).

Material and Methods

All studies were performed with spinach chloroplasts, prepared intact and osmotically shocked immediately prior to use [13]. Chlorophyll fluorescence and luminescence were measured essentially as described before [14].

Results

1) Inhibitor induced f_0-rise

As first demonstrated by Velthuys and Amesz, the diuron induced increase of chlorophyll fluorescence oscillates with a period of 2, depending on the number of preilluminating flashes [15]. This inhibitor induced f_0-rise is caused by the displacement of the bound semiquinone Q_B^- from its binding site [16, 17], concomitant with a shift of the electron from Q_B^- towards Q_A. For a recent discussion of this mechanism, see ref. [18]. As shown in Fig. 1, inhibitor induced binary oscillations of chlorophyll fluorescence were found for all six inhibitors studied, which indicates a common mechanism of action at Q_B.

Abbreviations: Atrazine, 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine; BNT, 2-bromo-4-nitrothymol; DCMU (diuron), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; dinoseb, 2,4-dinitro-6-*sec*-butylphenol; f_0, fluorescence yield during weak monitoring light; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; PS, photosystem; pyrazon, 5-amino-4-chloro-2-phenylpyridazin-3-(2H)-one; Q_A, primary quinone electron acceptor of photosystem II; Q_B, secondary quinone electron acceptor; QSAR, quantitative structure activity relationship.

* *Present address:* CIBA-GEIGY Limited, Agrochemical Division, R1207, CH-4002 Basle, Switzerland.

Reprint requests to Dr. K. Pfister.

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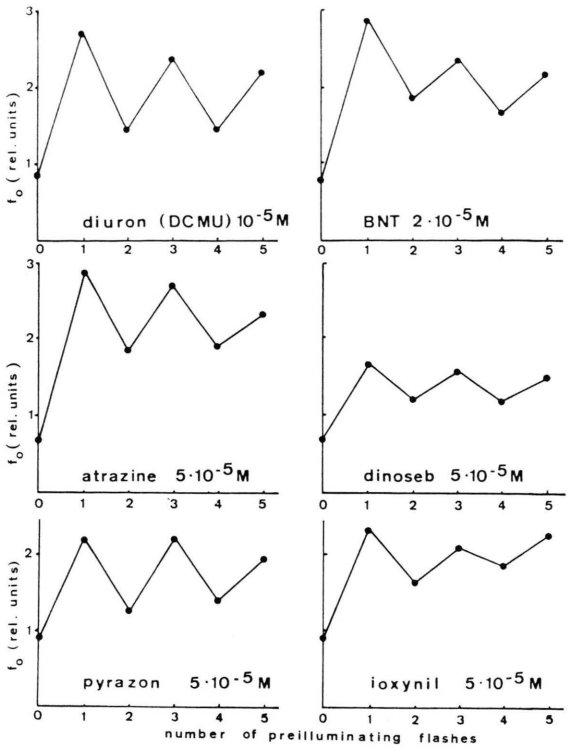

Fig. 1. Inhibitor induced f_0-rise depending on the number of preilluminating flashes. Thylakoid membranes were illuminated with the indicated number of μs flashes, and 15 s after the last flash the inhibitor was added. The resulting increase in f_0 was measured in weak, monitoring light, which did not induce any fluorescence increase by itself.

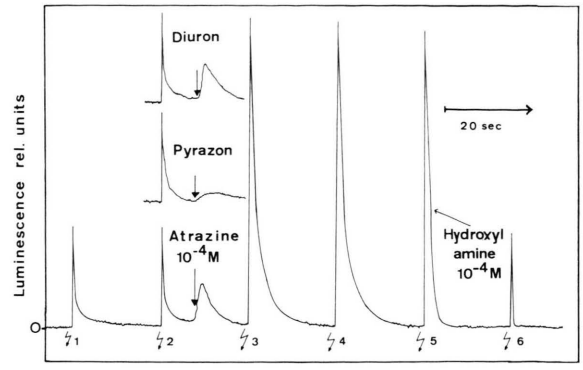

Fig. 2. Chlorophyll luminescence in presence of "diuron-type" inhibitors. Thylakoid menbranes were illuminated with μs flashes, spaced 20 s apart.

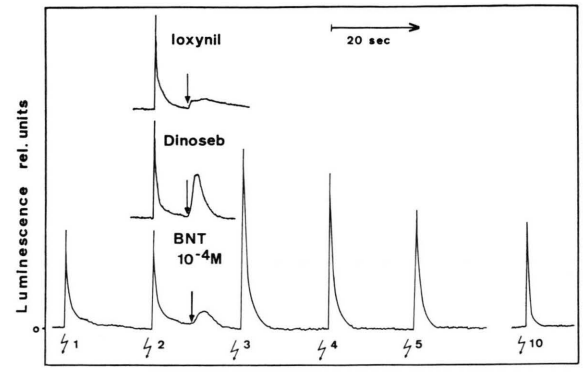

Fig. 3. Chlorophyll luminescence in presence of "phenol-type" inhibitors. Conditions as in Fig. 2.

2) Inhibitor and flash induced luminescence

Chlorophyll luminescence is thought to originate from charge recombination at PS II. Thus, luminescence depends on the concentration of reducing and oxidizing equivalents. We found for all six compounds tested an inhibitor induced luminescence burst, provided the inhibitor was added following a preilluminating μs flash (Fig. 2). Differences in the peak height of the luminescence burst as well as in peak integral can be partially explained by a different penetration speed towards the site of action, which has been reported for several "phenol-type" inhibitors [5, 7]. However, it is apparent from the data in Fig. 3 that "phenol-type" inhibitors (represented by BNT) have an additional effect on luminescence which is not observed with "diuron-type" inhibitors (represented by atrazine). When, following inhibitor addition, further flashes are given with "diuron-type" inhibitors, luminescence stays high and practically constant over a number of flashes. With "phenol-type" inhibitors, on the other hand, there is a gradual decline of the flash-induced luminescence, eventually resulting in a similar effect as that known for hydroxylamine (see effect in presence of atrazine, Fig. 2). Hence, similar to hydroxylamine, "phenol-type" inhibitors appear to affect charge recombination at PS II reaction centers. A special feature of that type of inhibition is its gradual development upon illumination.

3) Light-dependent inhibition of the PS II donor site

Upon an actinic flash in presence of diuron there is reduction of Q_A (high fluorescence) followed by Q_A^- reoxidation, which can be monitored *via* fluorescence decay to the "dark" level. In presence of hydroxylamine *and* diuron, fluorescence stays high

following a flash, as the backreaction is blocked (see Fig. 4 in ref. [4]). Thus, an elevated f_0-level found after a flash in presence of an inhibitor provides an indication of a donor site inhibition. We subjected chloroplasts in presence of inhibitors to a series of strong actinic flashes of 25 ms duration spaced 20 s apart and measured the f_0-level with a weak monitoring beam. With "diuron-type" inhibitors a series of flashes did not lead to any appreciable increase in the f_0-level (data not shown). With "phenol-type" inhibitors (see Fig. 4 for ioxynil), the f_0-level was found to be increased at the end of each 20 s dark interval. This behaviour demonstrates the gradual development of inhibition at the PSII donor site as indicated already by the luminescence data from Figs. 2 and 3. An additional feature of this type of inhibition is shown by the experiment of Fig. 4b. In this experiment the illumination time was increased by a factor of ten with respect to the conditions in Fig. 4a. Surprisingly, it was found that a 250 ms flash produces about the same f_0-rise as a 25 ms flash. Actually, ten 25 ms flashes are about ten times as effective as one single 250 ms illumination, although the same amount of light was given. Similar results as shown for ioxynil (Fig. 4) were obtained also with BNT and dinoseb (data not shown). The dependence of the donor site effect upon the spacing of the actinic flashes was further studied by varying the dark interval and keeping

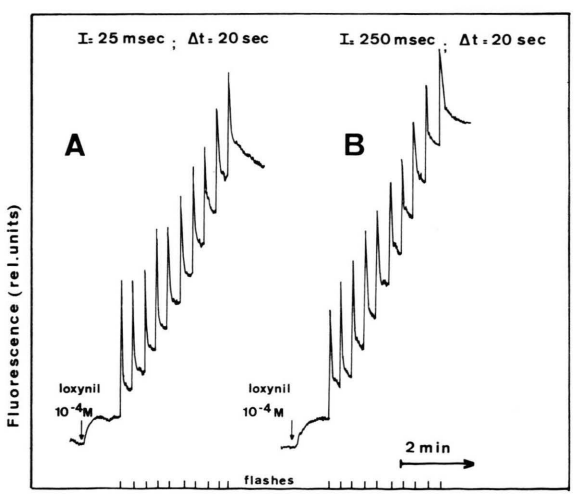

Fig. 4. Increase of f_0-level upon repeated flash illumination in presence of ioxynil. Illumination time a: 25 ms; b: 250 ms. Chlorophyll fluorescence was recorded in weak measuring light.

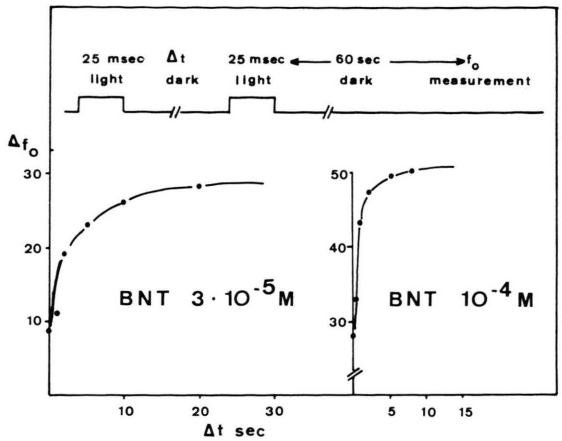

Fig. 5. Flash induced increase of f_0 in dependence on the darktime between flashes. f_0 was measured in weak monitoring light. Outline of the experiment as indicated in the upper part.

the flash duration constant. As a representative result, it is demonstrated in Fig. 5 for BNT that a dark time of >20 s (at 3×10^{-5} M) or >7 s (at 10^{-4} M) is required for the maximal increase in f_0 following a single flash.

From these results we conclude that light is required for the full development of the donor site effect and that light must be given in properly spaced light/dark cycles for maximum efficiency. There was no enhancement of the donor site effect by preilluminating flashes given before the addition of the phenols. From this the involvement of long living *S*-states seems unlikely. One may assume that the observed effect depends on a product which is formed in a photoact and becomes subsequently modified in a dark reaction.

Discussion

A common property of "diuron"- and "phenol-type" inhibitors is their mechanism of action at the acceptor site of PSII, as can be deduced from a large number of experiments with different kind of approaches, *e.g.* studies of chlorophyll fluorescence induction, inhibition of PSII dependent photoreactions and competitive binding assays [1, 2]. The inhibitor-induced binary oscillations of fluorescence (Fig. 1) provide further evidence for a common mechanism, namely the displacement of the bound quinone Q_B from its binding site. Also stimulation

of chlorophyll luminescence (Figs. 2, 3) is in accordance with this view. Contrasting results about a lack of an ioxynil or BNT induced luminescence burst have been reported [19, 20]. These authors utilized dark adapted *Chlorella* cells without preilluminating flashes for studying "dark luminescence". Presumably with this type of luminescence, no electrons supplied by Q_B^- are involved.

The luminescence studies (Figs. 2, 3) suggest for the "phenol-type" inhibitors a mechanism of action at the PS II donor site which resembles that of hydroxylamine. From electron transport studies (inhibition of silicomolybdate reduction) a second site of action has been already proposed for ioxynil [19]. Further experiments demonstrating a donor site inhibition using fluorescence induction studies are described in ref. [21]. Interestingly, in addition to the light requirement for the donor site inhibition by the phenols, there is also the need for relatively long dark periods between the actinic illumination. Under our experimental conditions no indications for a PS II donor site inhibition were found for the "diuron-type" inhibitors.

It should be noted that in most of our experiments the inhibitors were used in concentrations far above the I_{50}-concentration for inhibition of electron transport. As proposed in [17], and more extensively discussed in [16] and [18], binding of PS II inhibitors is much weaker when PS II centers are in the Q_A/Q_B^- state. This was the case in our fluorescence (Fig. 1) as well as in luminescence experiments (Figs. 2, 3), which explains the requirement for rather high inhibitor concentrations in contrast to those used in continuous light studies, *e.g.* I_{50}-determinations. The significance of the observed donor site inhibition by the phenols as a contribution to their inhibitory efficiency or herbicidal phytotoxicity is difficult to evaluate. It is possible that the light-dependent increase of the donor site inhibition (Figs. 4, 5), similar to the previously described light- and time requirement for maximal inhibition [7] and binding [22], becomes more pronounced in continuous light and increases herbicidal efficiency of the phenols *in vivo*.

In view of the current understanding of PS II herbicide binding, we propose the following model: the PS II herbicide binding site consists of a 32 kD protein ("Q_B-protein") and a 41–44 kD reaction center protein. Binding of a "diuron-type" herbicide results in an almost exclusive inhibition at the reducing site of PS II *via* the 32 kD protein. Phenols affect additionally – although weaker – the donor site possibly *via* an interaction with the 41 kD protein.

[1] A. Trebst, in: Photosynthesis, **Vol. VI,** pp. 507–520 (G. Akoyunoglou, ed.), Balaban Int. Sci. Services, Philadelphia 1981.
[2] K. Pfister and W. Urbach, in: Encyclopedia of Plant Physiology, **Vol. 12 D,** pp. 329–391 (L. Lange *et al.*, eds.), Springer, Berlin 1983.
[3] W. Tischer and H. Strotmann, Biochim. Biophys. Acta **460,** 113–125 (1977).
[4] K. Pfister and C. J. Arntzen, Z. Naturforsch. **34 c,** 996–1009 (1979).
[5] H. Laasch, K. Pfister, and W. Urbach, Z. Naturforsch. **37 c,** 620–631 (1982).
[6] K. Steinback, K. Pfister, and C. J. Arntzen, Z. Naturforsch. **36 c,** 98–108 (1981).
[7] P. Böger and K. J. Kunert, Z. Naturforsch. **34 c,** 1015–1020 (1979).
[8] K. Pfister, K. Steinback, G. Gardner, and C. J. Arntzen, Proc. Natl. Acad. Sc. USA **78,** 981–985 (1981).
[9] W. Oettmeier, K. Masson, and U. Johannigmeier, Biochim. Biophys. Acta **679,** 376–383 (1982).
[10] W. F. J. Vermaas, G. Renger, and C. J. Arntzen, Z. Naturforsch. **39 c,** 368–373 (1984).
[11] A. Trebst and W. Draber, in: Advances in Pesticide Science, **Vol. 2** (H. Geissbühler, ed.), 223–234, Pergamon Press, Oxford, New York 1979.
[12] I. Vass and S. Demeter, Biochim. Biophys. Acta **682,** 496–499 (1982).
[13] U. Schreiber and K. Pfister, Biochim. Biophys. Acta **680,** 60–68 (1982).
[14] U. Schreiber, FEBS Letters **122,** 121–124 (1980).
[15] B. Velthuys and J. Amesz, Biochim. Biophys. Acta **333,** 85–94 (1974).
[16] J. Lavergne, Biochim. Biophys. Acta **682,** 345–353 (1982).
[17] B. Velthuys, FEBS Letters **126,** 277–281 (1981).
[18] W. Urbach, H. Laasch, and U. Schreiber, Z. Naturforsch. **39 c,** 397–401 (1984).
[19] C. J. van Assche, in: Photosynthesis, **Vol. II,** pp. 227–236 (G. Akoyunoglou, ed.), Balaban Int. Sci. Services, Philadelphia 1981.
[20] J. Lavorel, Biochim. Biophys. Acta **596,** 385–399 (1980).
[21] K. Pfister and U. Schreiber, VI. Intern. Congr. Photosynthesis, in press (1983).
[22] H. Laasch, U. Schreiber, and W. Urbach, FEBS Letters **159,** 275–279 (1983).