Inhibition of Photosynthetic Electron Flow by Phenol and Diphenylether Herbicides in Control and Trypsin-Treated Chloroplasts

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Alkyl substituted 2-halogen-4-nitro-phenols (like bromonitrothymol) are potent inhibitors of photosynthetic electron flow in chloroplasts. There inhibition site is identical to that of DCMU, though the phenols do not contain their essential chemical element responsible for inhibition nor do they follow their correlation of chemical structure and biochemical function. Binding of labelled metribuzin on the chloroplast membrane and its replacement by the phenol inhibitors support the notion of identical binding sites, however.

In trypsin treated chloroplasts, the primary acceptor of photosystem II becomes accessible to ferricyanide, whose photoreduction is therefore DCMU insensitive. After trypsin treatment also the sensitivity of ferricyanide reduction to phenol inhibitors is lost as is the binding of labelled metribuzin.

The dinitrophenylethers of bromonitrothymol and of ioxynil and other diphenylethers are potent inhibitors of photosynthetic electron flow. Their site of inhibition is after the function of plastoquinone, *i. e.* they are DBMIB analogues.

Introduction

The comparison of structure activity relationships in urea and phenol herbicides

Photosynthetic electron flow from water to NADP in thylakoid membranes from chloroplasts may be inhibited at various places. From these possibilities several have been realized by commercial herbicides. Most prominent – scientifically as well as commercially – is the inhibition of electron flow between the primary (Q) and secondary (B) quencher of photosystem II by substituted urea-, triazine-, triazinone-, anilide-, etc. herbicide families. These compounds have in common an essential chemical element responsible for inhibition: a $-N-C=$ group bound to a lipophilic alkyl- or arylrest. The biological potency depends on chemical parameters, in particular on lipophilicity (a π contribution) and electronic substituents (a σ contribution) (see review [1, 2]).

Recently certain phenols have been recognized to be also potent inhibitors of photosynthetic electron flow at photosystem II [2–5]. Among these alkyl substituted 2-halogen-4-nitro- or 2,4-dinitrophenols have particular inhibitor potency, some of them herbicides since a very long time, like DNOC or dinoseb. Also the well known photosynthesis inhibitor ioxynil belongs to this group of phenolic herbicides. In addition to an uncoupling these compounds inhibit photosynthetic electron flow exactly at the same place as the diuron family, *i. e.* between the primary (Q) and secondary (B) acceptor of photosystem II, as indicated by biochemical and biophysical studies of their effect on photosynthetic functions of chloroplasts [2–5]. In spite of identical mode of action these phenolic compounds do not contain the basic chemical element essential in the urea type herbicides. We have reported [2], furthermore, that the dependence of biological (inhibitory) potency on chemical parameters shows no correlation to π and σ values but a dependence, instead, on steric parameters only. To accomodate two different herbicide families with identical inhibition patterns, we proposed [2] that the binding area for both groups of herbicides in the thylakoid membrane at the function of photosystem II (on the protein which has the enzymic activity of catalysing electron transfer from the primary quencher Q to plastoquinone via its prosthetic group, the secondary quencher B, see Fig. 1) can interact with both urea and phenol type herbicides in such a way, that overlapping

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Abbreviations: BNT, 2-bromo-4-nitrothymol; DAD, diaminodurol; DBMIB, dibromothymoquinone; DCMU, dichlorophenyldimethylurea; DNP-BNT, 2,4-dinitrophenylether of 2-bromo-4-nitrothymol; DNP-INT, 2,4-dinitrophenylether of 2-iodo-4-nitrothymol; ioxynil, 2,6-diiodo-4-cyano-phenol; metribuzin, 2-amino-6-*t*-butyl-3-methylthio-triazin-5-one; MV, methylviologen; PD, *p*-phenylenediamine.

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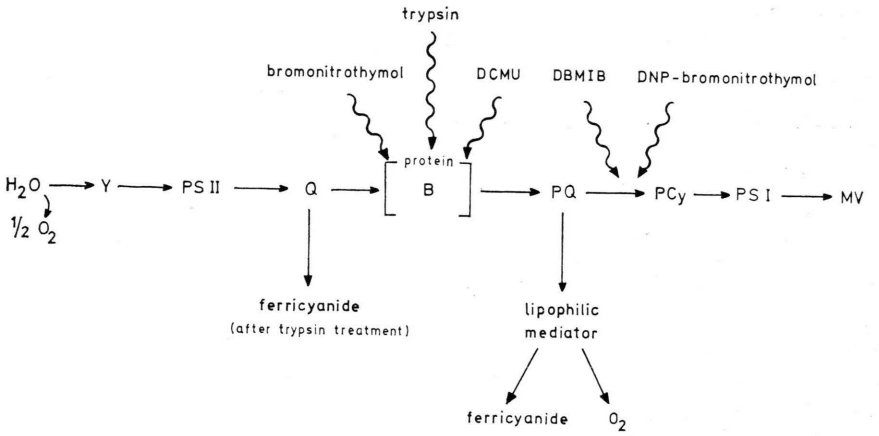

Fig. 1. Inhibition sites in the photosynthetic electron transport chain of chloroplasts. The DCMU or BNT binding protein is visualized as the membrane bound enzymic system catalyzing electron flow from the primary quencher of photosystem II (Q) to plastoquinone (PQ) via the prosthetic group B. Trypsin treatment effects this protein, such that electron flow to PQ is blocked, but Q becomes accessible to the hydrophilic ferricyanide. In intact thylakoids photosystem II driven ferricyanide or oxygen reduction is possible only via a lipophilic mediator, like PD or methylene-dioxy-methyl-*p*-benzoquinone.

binding sites, some identical others not, are responsible for the conformational change of this protein, which in turn effects its enzymic function in photosynthesis.

Methods

Washed thylakoid membranes from spinach chloroplasts were obtained in the usual way. Photosynthetic activity was measured at pH 7.0 in 3 ml, containing 80 μmol hepes buffer, 20 μmol $MgCl_2$, 15 μg gramicidin, chloroplasts with 30 μg chlorophyll and 1.5 μmol of ferricyanide or 0.1 μM methylviologen or methylene-dioxy-dimethylbenzoquinone [14]. The photoreduction of the first was measured at 420 nm or by oxygen evolution, the later two by oxygen uptake in a Clark type oxygen electrode and 20 °C and 2.5×10^5 ergs/cm^2/sec red light.

For trypsin treatment chloroplasts (washed thylakoids) with 300 μg chlorophyll were incubated at either pH 8.0 (tricin buffer) or pH 7.0 (hepes buffer) with 10 μmol $MgCl_2$ and 200 μg trypsin (bovine pancreas, Boehringer) in 3 ml at 20 °C. After the times indicated in the figures 300 μg trypsin inhibitor (hen egg white, Boehringer) was added. Then an aliquot was tested as above.

In the binding studies chloroplasts (control or trypsin treated) with 50 μg chlorophyll in 2 ml with 300 μmol hepes buffer pH 8.0 were incubated with a concentration range (see figures) of ^{14}C-labelled metribuzin (34 μCi/mg = 2.900 cpm/2 ml for 0.1 μM) for 5 min. After filtration through a Satorius membrane filter (SM 11305, pore size 0.65, size 25 mm) the chloroplast precipitate as well as an aliquot of the filtrate were counted in aquasol in a liquid scintillation counter. Those compounds not commercially available have been synthetized by Bayer AG and obtained through Dr. W. Draber.

Results and Discussion

A. Binding of herbicides on the thylakoid membrane

A direct approach to indicate identical or non identical binding sites is the method of Tischer and Strotmann [6], in which a radioactive labelled inhibitor is replaced from its binding site by an anlogous inhibitor. Such replacement is possible only when they share binding sites. The urea, triazine, biscarbamate, triazinone herbicides do indeed replace each other from the chloroplast membrane [6, 7]. As reported, also phenolic herbicides, like bromonitrothymol or ioxynil, do replace radioactive metribuzin, a triazinone herbicide [8]. This is also shown in Fig. 2, in which the amount of radioactivity (in ^{14}C-labelled metribuzin) bound to the thylakoid membrane is lowered, when either diuron (DCMU), ioxynil or bromonitrothymol is added (the other compounds are discussed below). This then is in line with the conclusion, that urea

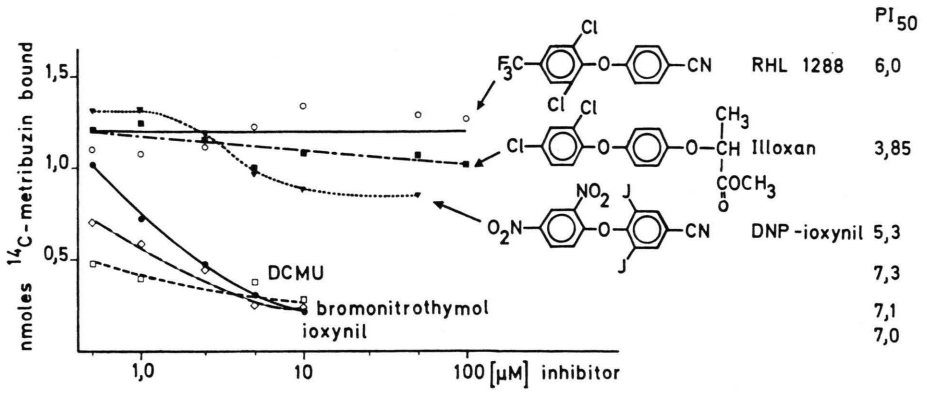

Fig. 2. Competetive displacement of [^{14}C]metribuzin by DCMU and phenols but not by diphenylethers from the thylakoid membrane. The pI_{50} value is the concentration of the compounds for 50% inhibition of electron flow.

and phenol herbicides are bound to the thylakoid membrane at the same binding protein and therefore effect photosynthetic function in an identical fashion.

B. The effect of trypsin treatment on ferricyanide photoreduction and on metribuzin binding

Further support comes from trypsin treated chloroplasts. Experiments by Regitz and Ohad [9], later extended by Renger [10, 11], indicated that the binding protein for DCMU is changed upon trypsin treatment of the membrane in such a way, that a DCMU insensitive photoreduction becomes possible in a photosystem II driven reaction. In trypsin treated chloroplasts the reduction of plastoquinone is not longer possible because the secondary acceptor B becomes disconnected from Q [10]. As Fig. 3 is to show, thylakoid preparations from spinach chloroplasts treated with an equal amount of trypsin at either pH 7.0 or 8.0 do show an impairment of photosynthetic ferricyanide reduction (and oxygen evolution) with time of trypsin incubation. But the remaining photosynthetic activity is no longer fully inhibited by even large concentrations of DCMU. It had been shown very early [12, 13], that after trypsin treatment, photoreduction of photosystem I acceptors, like methyl viologen, is no longer possible. As Table I indicates, the photosystem I driven photoreduction of methylviologen at the expense of an artificial electron donor DAD/ascorbate is, however, not impaired by trypsin treatment. The reduction of an artificial acceptor system for photosystem II, methylene-dioxy-dimethyl-p-benzoquinone [14], is also inhibited by trypsin

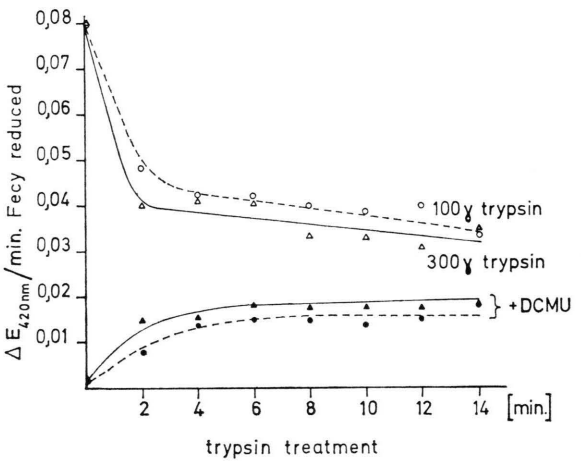

Fig. 3. Effect of trypsin treatment (at pH 8.0) of the thylakoid membrane on the rate and DCMU (5 µM) sensitivity of photosynthetic ferricyanide reduction (uncoupled at pH 7.0). Rate=500 µmol/mg chlorophyll/h in the control.

treatment, in line with conclusions by Renger [10] on phenylene-diamine reduction. The photoreduction of ferricyanide of photosystem II, however, is not very much inhibited by trypsin treatment (Table I). The loss of DCMU sensitivity of ferricyanide reduction (measured also as oxygen evolution) is shown in more detail in Fig. 4. Because complete inhibition by DCMU is not obtained any more in the trypsin treated chloroplasts, no true pI_{50} value can be computed. A somewhat incorrect pI_{50} taken graphically from Fig. 4 is a 100 fold lower than that of the control. This loss of DCMU sensitivity is also obtained for other inhibitors like triazines or triazinones (not shown). Important for the discussion on the mode of action of phenolic

Table I. Effect of trypsin-treatment on photosynthetic reactions in chloroplasts.

	Rate of electron flow in equivalents 0 (2e) taken up or Fecy (1e) reduced/mg chlorophyll·h	
	control chloroplasts	4 min trypsin treated chloroplasts
Methylviologen reduction		
uncoupled control	90	2,5
+ DAD/ascorbate	280	330
Methylene-dioxy-dimethyl-*p*-benzoquinone	315	30
+ 1 μM DBMIB	235	25
Ferricyanide	504	398
+ 0,1 mM phenylenediamine	550	326
+ 1 μM DCMU	48	226

Trypsin incubation at pH 8.0, uncoupled electron flow measured at pH 7.0, pseudocyclic electron flow in methylviologen (photosystem I driven) and methylene-dioxy-dimethyl benzoquinone (photosystem II driven) reduction, measured as oxygen uptake.

herbicides is, that also the sensitivity to bromonitrothymol and ioxynil is lost, as has been reported at the meeting, but will be printed in more detail separately.

The high activity of ferricyanide photoreduction and oxygen evolution after trypsin treatment is most easily explained by an accessibility change of the primary quencher Q towards ferricyanide, when trypsin has effected the protein part of the secondary quencher B. This way the inhibition sites of DCMU and bromonitrothymol are bypassed (see Fig. 1). But trypsin treatment of the membrane also changes its binding capacity for metribuzin as has been discussed by Tischer and Strotmann [7]. Fig. 5 compares the binding of ^{14}C metribuzin in control chloroplasts with those treated with trypsin at either pH 7 or 8. At pH 7.0 no specific binding of metribuzin is observed any more. At pH 8.0 the number of binding sites is reduced, compare nmoles metribuzin bound on the X-axis after correcting for unspecific binding. Also the binding affinity is changed, a Lineweaver-Burk plot shows a decrease of pK_I from 7.4 in the control to 6.44 in the trypsin treated chloroplasts.

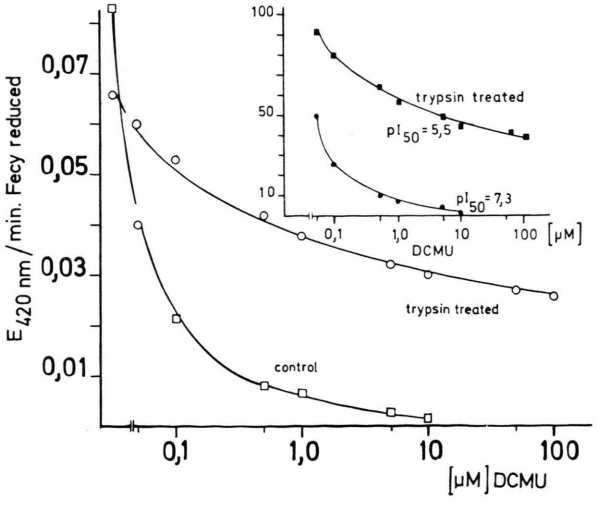

Fig. 4. Loss of DCMU sensitivity of photosynthetic ferricyanide reduction after trypsin treatment of the thylakoid membrane. Trypsin treatment at pH 8.0, uncoupled electron flow measured at pH 7.0. The insert shows the same values plotted in percent activity to indicate the change in pI_{50} values.

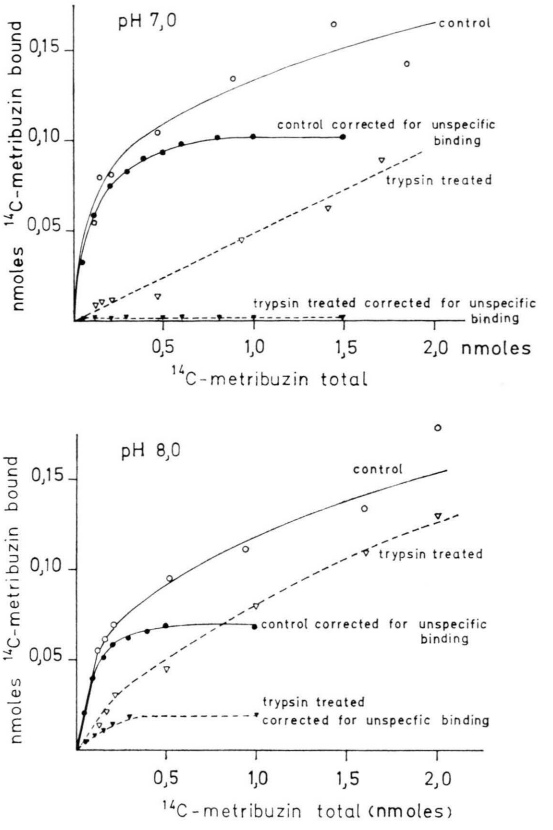

Fig. 5. Binding of [^{14}C]metribuzin in control and trypsin treated thylakoid membranes. Binding at pH 7.0, trypsin at either pH 7.0 or 8.0. The saturation curve for specific binding is obtained by substracting the unspecific binding of high concentrations of metribuzin.

C. Dinitrophenylethers of phenols as analogues of DBMIB

DBMIB is an inhibitor of photosynthetic electron flow in the area of plastoquinone function [15], but at quite a different site than that of DCMU (see Fig. 1). As is now well recognized, in the presence of DBMIB, photosystem II driven photoreduction of ferricyanide is possible provided a lipophilic mediator like phenylene diamine is added to the chloroplast system. We have recently described [16] new effective DBMIB analogues and the efficient inhibition of electron flow by the dinitrophenylethers of halogenated phenols: DNP-bromonitrothymol and DNP-iodonitrothymol. These compounds at 0.5 μM inhibit electron flow alike DBMIB, *i. e.* they block electron flow from photosystem II to photosystem I, but neither photosystem II or photosystem I driven photoreductions [16]. There are some differences to DBMIB inhibition, like the lower sensitivity of uncoupled chloroplasts to DNP-iodonitrothymol [16]. This does not indicate a different binding site. We explain it by a better binding of the inhibitor to the membrane on the inner surface in the energized state, *i. e.* at the lower pH inside the thylakoid vesicle in the coupled state. We have now found that also the dinitrophenylether of ioxynil is an inhibitor alike DBMIB and DNP-INP (Trebst, Draber and Knops, to be published).

It is interesting to realize that the parent compounds of the effective dinitrophenylethers are those phenol herbicides, which effectively inhibite alike DCMU, *i. e.* bromonitrophymol and ioxynil. The different binding site of the diphenylether inhibitors from that of the phenolic inhibitors is clearly established by the replacement technique of Tischer and Strotmann [6]. As Figs. 2 and 6 show, the dinitrophenylether of bromonitrothymol and of ioxynil as well as RHL 1288 do not replace (or very little) radioactive metribuzin as against the parent phenols. They behave like DBMIB, which also does not replace metribuzin (Fig. 6). In Fig. 2 also a diphenylether derivative is indicated, illoxan, which has lower inhibitory potency than DNP-INT or DNP-ioxynil (it is a phenoxyacetic derivative with a different mode of action *in vivo* than photosynthesis inhibitors). More interesting is that this diphenylether is not a specific DBMIB analogue, because it does inhibit photoreductions by photosystem II (results not shown here). It does not replace metribuzin either (Fig. 2).

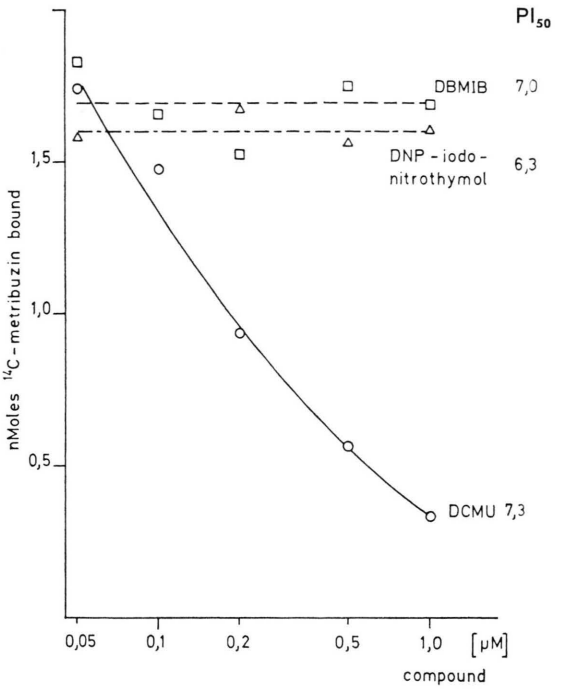

Fig. 6. Displacement of [^{14}C]metribuzin by DCMU but not by DBMIB and DNP-INT. The pI_{50} value is given for inhibition of electron flow.

The complexity of the photosynthetic system and inhibition sites in the plastoquinone area becomes obvious this way. Electron flow may be inhibited at the reduction site of plastoquinone by compounds like DCMU and phenols at the same binding site but also by compounds which nevertheless have a different binding site. Examples of the later are also diphenylamine-derivatives, described by Oettmeier [17]. On the other hand chemically related compounds like the phenols and the dinitrophenyl derivatives of these phenols behave quite differently and inhibit before or after the plastoquinone function respectively. One might almost speculate that the oxidation site of plastoquinone is structurally in the membrane not very far from its reduction site, a concept quite at variance with vectorial electron flow.

Acknowledgements

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