Mode of Inhibition of Photosynthetic Electron Transport by Substituted Diphenylethers

W. Draber, H. J. Knops, and A. Trebst

Forschungszentrum Wuppertal, Bayer AG und Ruhr-Universität Bochum

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Dedicated to Prof. Grünewald *on the Occasion of His 60th Birthday*

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Several substituted diphenylethers were found to be effective inhibitors of photosynthetic electron flow in isolated thylakoid membranes from spinach chloroplasts. Their site of inhibition was localized with artificial acceptor and donor systems. The phenylether of an alkyl substituted nitrophenol is primarely inhibiting electron flow after plastoquinone function whereas a dinitrophenylether of a phenyl substituted nitrophenol is inhibiting before plastoquinone function. Therefore certain diphenylethers interfere with plastoquinone function at the oxidation or reduction site, depending on the substitution.

Introduction

Diphenylether derivatives are important herbicides [1–3]. Their mode of action, though, has not been clearly established. Some with a certain substitution (in *ortho* position) are assumed to be photosynthesis inhibitors [2, 3]. We have recently described diphenylethers that are highly inhibitory to photosynthetic electron transport [4, 5]. In particular, the dinitrophenylether of iodonitrothymol (DNP-INT) stops completely photosynthetic oxygen evolution in isolated chloroplast thylakoid membrane preparations at 1 µm. The inhibition site at the electron transport system was localized in plastoquinone function [4–6], similar to the inhibition pattern of DBMIB [7].

We wish to report further details on the structure activity relationship of substituted diphenylethers effective as inhibitor of photosynthesis. The results indicate that changes in the substitution shift the site of inhibition in the electron transport system from a point after plastoquinone function (DBMIB site) to one before (DCMU site).

Abbreviations: DBMIB, dibromothymoquinone; DCMU, dichlorophenyldimethylurea; DQH_2, durohydroquinone; DNP-INT, dinitrophenylether of iodonitrothymol; MDBQ, methylenedioxydimethylbenzoquinone; MV, methylviologen; TMPD, N-tetramethyl-*p*-phenylenediamine.

Reprint requests to Prof. Dr. A. Trebst, Ruhr-Universität Bochum, Abteilung Biologie, Lehrstuhl für Biochemie der Pflanzen, D-4630 Bochum 1.

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Methods

Spinach chloroplasts were prepared by homogenizing leaves in 0.4 M saccharose, 0.01 M tricin NaOH buffer pH 8.0, 0.01 M NaCl and 85 mg bovine serum albumine. After centrifugation the chloroplasts were osmotically shocked in 5 µM tricine buffer pH 8.5.

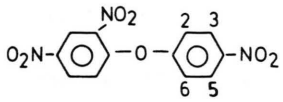
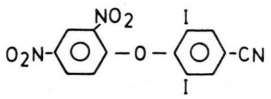
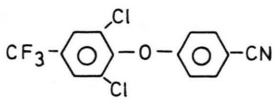
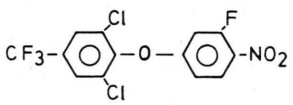
Photosynthetic activity was measured in a volume of 3 ml with 0.2 M tricin buffer pH 8.0, 0.1 mM $MgCl_2$, 10 mM Na-azide, 15 µg gramicidine and chloroplasts thylakoids with 20 µg chlorophyll. Oxygen uptake was measured with a teflon covered oxygen electrode with an illumination of red light of 2.5×10^5 erg./cm² × sec. The inhibitors were either preincubated with the chloroplasts for 2 min or the rate is given after 2 min illumination.

For the binding studies chloroplasts with 100 µg chlorophyll were incubated with 0.1 µM [^{14}C]metribuzin at pH 8.0. Further details are given in reference 15. The synthesis of the inhibitors is described in reference 9 or was performed accordingly.

Results

The inhibition of photosynthetic electron flow by a number of herbicidal diphenylethers has first been observed by Moreland [8]. We latter reported on the much more effective inhibition of electron flow by DNP-BNT and DNP-INT [4] and extended this to some further diphenylether derivatives [5, 6]. Others have been investigated by Bugg *et al.* [9]. For comparison and for further details our diphenyl-

Table I. The inhibition of photosynthetic electron flow (coupled MV reduction) in spinach thylakoids by the dinitrophenylethers of iodonitrothymol (DNP-INT) and related diphenylethers.

No.	Abbreviation	Synthesis code	Formula	Substituent at			pI_{50} (coupled Hill-reaction)	inhibition pattern like	
				R_2	R_3	R_6		DBMIB	DCMU
1	DNP-INT	KNJ 724		I	methyl	isopropyl	7.0	+	
2	DNP-BNT	KNJ 723		Br	methyl	isopropyl	6.5	+	
3		KNJ 721		Br	H	cyclohexyl	5.0	+	+
4		KNJ 726		Br	H	phenyl	5.2		+
5	DNP-ioxynil	KNJ 942					5.3	+	
6		RHL 1288					6.0	+	
7		RHL 2473					5.4	+	

ethers reported on already [4–6] are included in this report. Table I indicates the chemical structure of the diphenylethers testes. Their inhibitory potency is indicated as a pI_{50} value *i. e.* the logarithm of that concentration, leading to 50% inhibition of coupled photosynthetic electron flow from water to methylviologen by isolated thylakoid membranes from spinach chloroplasts. As reported the I_{50} depends on the coupling conditions [4]. Under uncoupling conditions the inhibitory potency is smaller (for explanation see [4]). This explains that the inhibition potency of the compound seems to vary in the Tables of this paper. Furthermore, there is a lag in inhibition after adding the compounds to the chloroplasts. Therefore, the inhibition value is taken after 1 to 2 min preincubation. Table I also indicates the principal inhibition site in the electron flow system alike DBMIB or DCMU as is to be shown by the following data.

All diphenylethers tested inhibit Hill reaction depending on both photosystems *i. e.* a photosynthetic NADP or MV reduction with water as the electron donor. Unlike DCMU the plastoquinone antagonists like DBMIB and DNP-INT at moderate concentrations much less effect Hill reactions driven by photosystem II as reported for some of the compounds already [4]. A convenient way to measure a photosystem II driven Hill reaction is to follow oxygen uptake catalyzed by methylenedioxydimethylbenzoquinone (MDBQ) as acceptor instead of methylviologen [10]. Table II compares the substituted diphenylethers in their effect on these two types of Hill reactions.

Table II. Effect of diphenylether on Hillreaction driven by both light reaction (MV acceptor) or by photosystem II only (MDBQ) measured as oxygen uptake under uncoupled conditions.

Acceptor		MV 100 µM	Methylenedioxydimethylbenzoquinone (MDBQ)	
			50 µM	500 µM
control (in µmol oxygen taken up)		170	110	110
addition of inhibitor (µM)		percentage of inhibition		
0.1	DNP-INT	40	20	0
1	DNP-INT	85	60	30
1	DNP-BNT		50	0
2	DNP-BNT	82	56	21
1	DNP-ioxynil	60	25	
10	DNP-ioxynil	80	50	21
10	KNJ 721	50		45
0.5	KNJ 726	30	26	31
5	KNJ 726	60	60	60
5	RHL 1288		36	26
10	RHL 1288	40	50	21
1	RHL 2473	73	30	6
10	RHL 2473		52	6
0.05	DCMU	65	64	64

DNP-INT, DNP-BNT, DNP-ioxynil and the two RHL derivatives inhibit the PS II dependent oxygen uptake much less than that driven by both photosystems via methylviologen. Complete inhibition of electron flow is obtained only in the methylviologen system but not in that with MDBQ. This indicates that the main block of these compounds is beyond the point, where the mediator MDBQ becomes reduced *i.e.* after plastoquinone reduction with inhibition pattern like DBMIB. The inhibition of the photosystem II driven reaction is dependent on the concentration of the mediator MDBQ. An inhibition at lower but already saturating concentrations (see control rate) of the mediator is overcome by adding more of the mediator. This indicates that a second inhibition site of these compounds at the reduction of plastoquinone can be overcome and may indicate a competition of inhibitor and mediator for the same binding area (on the B-protein). On the other hand KNJ 721 and 726 inhibit MV and MDBQ reduction to the same extent like DCMU does and there is no concentration dependence of the mediator. These two compounds then have their major inhibition site before plastoquinone.

Like DCMU, DBMIB and DNP-INT do not inhibit donor systems for photosystem I feeding into plastocyanin (like DAD/ascorbate) [4]. But unlike DCMU, DBMIB does inhibit donor systems feeding into the Rieske FeS center [11, 12] (which is part of the cytochrome b_6/f complex). The effect of the other diphenylethers on the durohydroquinone (DQH_2) donor system is indicated in Table III in comparison with DBMIB and metribuzin, the later an inhibitor like DCMU which does not inhibit the DQH_2 system. The data indicate that DNP-INT, DNP-BNT, DNP-ioxynil and the two RHL derivatives inhibit the durohydroquinone system as DBMIB does. KNJ 721 and in particular KNJ 726 even at high concentrations, have definitely a lower inhibitory effect of the donor system when compared with the control with water as electron donor system. This indication for two different major inhibitory effects of different diphenylethers is also observed in the TMPD bypass. As reported [13] a DBMIB like inhibition can be reversed by adding a catalytic amount of TMPD (but not ascorbate, which would make it a PS I donor system for plastocyanin). A DCMU (or metribuzin) inhibition cannot be reversed by a TMPD bypass. The data in Table III indicate no such bypass for the inhibition by KNJ 721 and 726, an appreciable reversal for DNP-INT and DNP-BNT and a complete bypass for DNP-ioxynil and RHL 1288. An incomplete bypass indicates that the inhibitor has two inhibition sites, one like DCMU with low and one like DBMIB with high affinity.

A radioactive inhibitor of photosynthetic electron flow will bind to the thylakoid membrane. An analogue of such a labelled inhibitor will displace it from the membrane and lower the membrane bound radioactivity, when the two compounds share the binding sites [14]. An inhibitor at a different inhibition site will not displace it. This technique has been introduced by Tischer and Strotmann [14] and has been used extensively now in recent (photo-

Table III. Effect of diphenylether derivatives on methylviologen reduction with water or with durohydroquinone as electron donor and their effect on a TMPD bypass (uncoupled system).

	H_2O as electron donor		DQH_2 as electron donor (5×10^{-4} M)
control rate (μmol oxygen taken up)	–	146 + 0.1 μM TMPD	662 (including the dark rate)
addition of inhibitor (in μM)		percentage of inhibition	
1 DNP-INT	85	40	80
1 DNP-BNT	70	25	60
20 KNJ 721	75	70	47
1 KNJ 726	43	43	8
5 KNJ 726	68	75	18
5 DNP-ioxynil	60	0	42
10 RHL 1288	40	0	54
5 RHL 2473	73	41	63
1 metribuzin	87	87	0
0.1 DBMIB	77	0	77

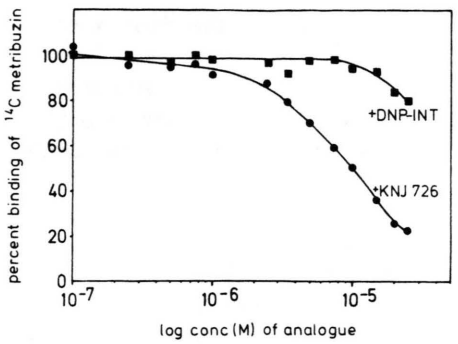


Fig. 1. Displacement of the radioactive labelled inhibitor metribuzin from the thylakoid membrane by either DNP-INT or the dinitrophenylether of phenylbromonitrophenol (KNJ 726).

synthesis) herbicide research, for example to indicate that phenol type inhibitors also replace the DCMU type inhibitor family [15]. Oettmeier had already shown that DNP-INT does not replace [^{14}C]metribuzin [6]. This is shown in Fig. 1 again. Only at a concentration 100 times above its I_{50} value does DNP-INT have an effect in displacing [^{14}C]-metribuzin. KNJ 726 however, easily removes [^{14}C]-metribuzin from the membrane already at the concentration of its I_{50} value.

Discussion

The most useful inhibitors of plastoquinone function in photosynthetic electron flow are DCMU and DBMIB. DCMU, metribuzin and their analogues interfere with plastoquinone function at a site between the primary (Q) and secondary (B) acceptor of photosystem II (for review see [7]). DBMIB, on the other hand, interferes with plastohydroquinone oxidation by interacting with the Rieske FeS center [11]. The inhibition pattern used in this paper easily distinguish between the two type of inhibitors: 1) Inhibitors like DCMU inhibit all Hill reactions, whereas inhibitors like DBMIB do not effect Hill reaction driven by photosystem II only [10]. 2) The inhibition of methylviologen reduction by DBMIB, but not by DCMU, can be reversed by a TMPD bypass [13]. 3) The durohydroquinone donor system is inhibited by DBMIB, but not by DCMU or metribuzin [11, 12]. 4) A radioactive inhibitor is displaced from the membrane by an analogue with a shared binding site only [14, 15].

The our first report on the effect of the dinitrophenylether of iodonitrothymol (DNP-INT) we reported that this derivative has an inhibitor pattern on photosynthetic electron transport of chloroplasts very similar to DBMIB [4]. Some properties on the other hand let us to suggest that DNP-INT reacts somewhat differently than DBMIB and possibly closer to B [4]. However, DNP-INT would not share a binding site with inhibitors of the DCMU family because it did not displace radioactive metribuzin from the membrane [6]. A further significant information on the inhibitory properties of DNP-INT comes from experiments by Binder and Selman [16], who report that cyclic photophosphorylation catalyzed by ferredoxin is much less inhibited by DNP-INT than is non cyclic electron flow.

We reported already on the inhibitor potency of RHL 1288 [5] and of DNP-ioxynil [6]. Some other inhibitory diphenylethers acting similarly like DNP-INT (or DBMIB) have been reported by Bugg *et al.* [9] among them a commercial diphenylether like nitrofluorfen.

It should be mentioned that diphenylether derivatives have also other effects on the photosynthetic system like the energy transfer inhibitory capacity of nitrofen [17] or their bleaching effect [18].

In this paper we present further details of some alkyl substituted diphenylether derivatives which are strong inhibitors of photosynthetic electron flow. Their inhibition pattern suggest a principal site of inhibition after plastoquinone function, *i.e.* similar to DBMIB. However, there is also some effect on the B-protein at high concentrations, which interestingly can be overcome by adding a high concentration of an artificial quinone acceptor for this area. Still DNP-INT can not displace [^{14}C]metribuzin from the membrane. A phenyl substituted diphenylether (KNJ 726) however interferes with the electron flow system in the opposite way. At low concentrations it interferes with plastoquinone reduction and it does compete with metribuzin for its binding site. Only at higher concentration does it also interfere at the DBMIB site. The cyclohexyl derivative (KNJ 721) is between the alkyl and aryl substituted diphenylethers in that it interferes with both sites at the same concentration.

Diphenylethers therefore have two sites of inhibition in the photosynthetic electron flow before and after plastoquinone function. The site of inhibiton can be shifted by changing the concentration of the

compound or by changing the chemical substituent of the inhibitor.

The exact mode of inhibition of the diphenylethers nervertheless remains unclear. Though they either behave like DBMIB or DCMU, it seems unlikely that they neither inhibit in a similar fashion as either of those nor are they direct plastoquinone analogues. But surely they interfere with the binding protein(s) for plastoquinone on either the reduction or oxidation site. The shift in inhibition pattern is not that surprising, because surely the binding protein(s) for either plastoquinone or plastohydroquinone (*i.e.* the B protein or the cytochrome b_6/f complex, respectively) share major properties. Therefore they may both bind the same inhibitor though with low or high affinity respectively and depending on nuances in the chemical substitution.

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