The Inhibition of Photosynthetic Electron Flow in Chloroplasts by the Dinitrophenylether of Bromo- or Iodo-nitrothymol

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The O-dinitrophenyl derivatives of 2-bromo- and 2-iodo-4-nitrothymol are inhibitors of photosynthetic electron flow from water to NADP or methylviologen, yielding 50% inhibition at 0.5 μM. Photoreductions by either photosystem I or photosystem II alone are not inhibited. The inhibition site is bypassed by TMPD. The inhibition pattern of the new inhibitors is very similar but not identical to the one of dibromothymoquinone. It is suggested that the new inhibitors affect the reduction of plastoquinone.

Introduction

Studies of the sequence of carriers and the location of coupling sites in the photosynthetic electron transport system of chloroplasts have been greatly aided by inhibitors acting at the acceptor side of photosystem II in the area of plastoquinone function. Two principle classes of inhibitors have been differentiated, represented by DCMU and DBMIB [1, 2]. They inhibit electron flow either before (between Q and B) or after (between PQH_2 and PCy) the main plastoquinone pool (see also Fig. 4). Recently, however, some evidence for a third inhibition site in the area of plastoquinone function was obtained. A second inhibition site of DBMIB is seen, when DBMIB at higher concentrations shifts its point of inhibition toward the reduction site of plastoquinone [2, 3] (*i. e.* between B and Q) as do some other substituted quinones [4, 5] analogous to DBMIB. But also some stable radical compounds were reported to inhibit electron flow between the DCMU and primary DBMIB inhibition site [6].

We have recently reported on the effective inhibition of electron flow by alkyl substituted halogenated nitrophenols, like bromonitrothymol (BNT) [7]. Though the chemical substitution of the phenyl ring in BNT is somewhat analogous to dibromothymoquinone (DBMIB), the inhibition pattern of this compound is nevertheless alike DCMU [7, 11]. The O-dinitrophenyl derivative of bromonitrothymol (DNP-BNT) however, seemed to inhibit photosynthetic electron flow of chloroplasts analogous to and as effective as DBMIB [7]. Because DBMIB and analogous quinoid compounds are not entirely specific inhibitors because they shift the inhibitory site depending on concentration [3] as mentioned above and, as a quinone, have some disadvantageous side effects [2], the new compounds should be useful. We wish to present details of the inhibition of photosynthetic electron flow by the dinitrophenyl ethers of bromo- and iodonitrothymol (DNP-BNT and DNP-INT). The inhibition pattern does show great similarities to DBMIB inhibition, but also differences. We tentatively suggest that the site of action of DNP-BNT and DNP-INT is before the reduction of the main plastoquinone pool, *i. e.* between B and PQ.

Abbreviations: B, secondary acceptor of photosystem II; BNT, 2-bromo-4-nitrothymol; DAD, diaminodurene; DBMIB, 2,5-dibromothymoquinone; DCMU, dichlorophenyldimethylurea, DNP-BNT, 2,4-dinitrophenylether of bromonitrothymol; DNP-INT, 2,4-dinitrophenylether of iodonitrothymol; INT, 2-iodo-4-nitrothymol; MV, methylviologen; PCy, plastocyanin; PD, *p*-phenylenediamine; PMS, phenazine-metho-sulfate; PQ/PQH_2, oxidized and reduced plastoquinone; Q, primary acceptor of photosystem II; TMPD, N-tetramethyl-*p*-phenylenediamine.

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Methods

Spinach chloroplasts (salad in the experiments of Tab. VI) were prepared according to Nelson *et al.* [8] by homogenizing leaves in 0.4 M saccharose, 0.01 M tricine NaOH buffer pH 8.0, 0.01 M NaCl, 85 mg bovine serum albumin (BSA) and 500 mg

Na-ascorbate/100 ml. After washing, the centrifuged chloroplasts were osmotically shocked in 5 mM tricine NaOH buffer pH 8.5, centrifuged off and resuspended in the grinding medium except for BSA and ascorbate.

Photosynthetic activity of the broken chloroplasts was measured at 15 °C and 30 000 lux white light in a basic medium containing in 3 ml: tricine-NaOH buffer pH 8.0 80; ADP 10, inorganic phosphate, labelled with P^{32} 10, $MgCl_2$ 10 and as acceptor either ferricyanide 20 (in Table II the redox state poised with ferrocyanide 20), or NADP 6 + ferredoxin 0.01, or methylviologen 0.1 + sodium azide 1. Further additions and conditions are given in Tables and Figures.

Bromonitrothymol and iodonitrothymol and their phenylethers were synthesized as follows.

Bromonitrothymol (2-Bromo-3-methyl-4-nitro-6-isopropyl-phenol). To a solution of 35.7 g (0.183 mol) 4-nitro-2,5-isopropyl-methylphenol in 250 ml CH_3OH/125 ml H_2O 10.2 ml (0.2 mol) Br_2 were added dropwise at 15 °C. After 3 hours of stirring at roomtemperature the precipitated product was filtered, washed with CH_3OH/H_2O (7:3) and dried at 50 °C to yield 45.1 g (90%) of colorless crystals, m. p. 107 °C (ref. [9]) 107 – 108 °C).

Iodonitrothymol (2-Iodo-3-methyl-4-nitro-6-isopropylphenol). To a solution of 9.8 g (0.05 mol) 4-nitro-2,5-isopropyl-methylphenol in 50 ml CH_3OH/25 ml H_2O 8.1 g (0.05 mol) ICl were added dropwise under stirring. The product precipitates as an oil which slowly crystallizes. It was filtered, washed with petrolether and dried at 50 °C. Yield 10.8 g (67%) m. p. 70 – 72 °C (ref. [10] not given. The compound is mentioned there as the product of the reaction of 2,4-diiodothymol with $NaNO_2$ in AcOH without further details and physical properties).

Dinitrodiphenylether 0.05 mol of 2-halo-4-nitro-3-methyl-6-isopropylphenol and 0.05 mol (16.6 g) 4-chloro-1,3-dinitro-benzene were dissolved in 100 ml DMF, 6.3 g $NaHCO_3$ in 50 ml H_2O were added and the mixture was refluxed for 16 hours. After cooling the mixture was diluted with 1 l H_2O, extracted three times with 150 ml portions of AcOEt, the organic phase washed three times with 100 ml portions of a $NaHCO_3$-solution and three times with H_2O, dried and evaporated in vacuo. The residual oil is recrystalized from ether/petrolether (10.4 g, (47%), m. p. 141 °C (Br); 3.9 g (16%), m. p. 169 – 71 °C (I).

Elemental analyses, ^{1}H-NMR-, IR- and mass-spectra of all new compounds were in accordance with the structures.

DNP – BNT DNP – INT

2',4'- Dinitrophenylether of

2-Bromo-4-nitro-thymol 2-Iodo-4-nitro-thymol

Results

Halogenated alkyl-substituted-nitrophenols are effective inhibitors of photosynthetic electron flow, 2-bromo-4-nitro-thymol (BNT) and 2-iodo-4-nitro-thymol (INT) having a PI_{50} value of 6.7, *i. e.* inhibiting electron flow to an extent of 50% at a concentration of 0.5 μM [7]. Their inhibition site is identical to that of DCMU. O-alkylation of such nitrophenols in general diminishes the inhibitor potency [7, 11]. This is, except for the dinitrophenyl-ether derivatives which do remain active [7]. The following results are to indicate that the dinitrophenylether of BNT and of INT are effective electron flow inhibitors but with an inhibition pattern unlike that of DCMU or the parent BNT or INT, but, instead, comparable, though not entirely identical, to DBMIB.

Fig. 1 is to indicate the inhibition of photosynthetic electron flow from water through both photosystems to either NADP or MV as terminal acceptor. At the chloroplast concentration used (0.2 mg chlorophyll) 50% inhibition (the I_{50} value) is obtained at about 0.1 μM for the iodine derivative and 0.3 μM for the bromine derivative, the I_{50} value being slightly different in a NADP or MV acceptor system. The usual dependence of the I_{50} of an inhibitor on chlorophyll concentration is shown for DNP-INT in Fig. 2. At low chlorophyll concentration the chloroplast electron flow is inhibited to 50% already at 0.04 μM.

The inhibition site of the dinitrophenylethers is not located in the area of photosystem I activity, as artificial donor system for photosystem I like DAD/

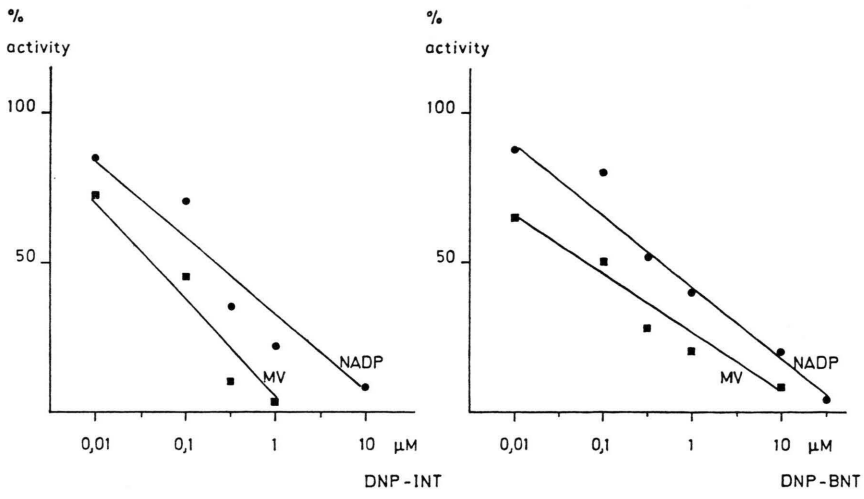

Fig. 1. Inhibition of the photoreduction of NADP or MV in chloroplasts by the dinitrophenylether of bromo- or iodo-nitrothymol.
Conditions: Coupled chloroplasts with 0.2 mg chlorophyll illuminated for 12 min. Rate of NADP reduction 110 µmol NADPH/mg chlorophyll and hour; rate of MV reduction 125 µmol oxygen/mg chlorophyll and hour.

Table I. Effect of DNP-bromo- or -iodo-nitrothymol on donor systems for NADP reduction by photosystem I. Conditions: coupled chloroplasts with 0.2 mg chlorophyll, 0.1 mM DAD or TMPD, 10 mM Na-ascorbate, 2 µM DCMU and 12′ light under nitrogen.

Donor	Inhibitor [µM]	µmol NADPH formed	µmol ATP formed	P/e_2
DAD/asc.	—	3.3	3.2	1
	7.5 DNP-BNT	2.7	2.7	1
	3.5 DNP-INT	2.6	2.1	0.8
TMPD/asc.	—	1.5	none	
	7.5 DNP-BNT	1.75		
	3.5 DNP-INT	1.45		

ascorbate or TMPD/ascorbate are not inhibited at all by a concentration 10 times the I_{50} value, indicated in Table I for a NADP system. The ATP formation coupled to the DAD → NADP system is also not influenced (Table I). Also cyclic electron flow of photosystem I and ATP formation coupled to it, catalysed by PMS, is not much affected by even very high concentration of either DNP-BNT or -INT (Table II). Because the measuring parameter is ATP formation, the slight inhibition observed (in comparison to the DCMU control) may be due to an effect on either electron flow (poising of the system) or ATP formation. The menadione catalysed cyclic electron flow and phosphorylation system on the other hand is inhibited by both inhibitors. The inhibition is reversed by the addition of a catalytic amount of TMPD (Table II). This is identical to the inhibition pattern of DBMIB and will be taken up later in connection with the TMPD bypass (Tables VI and VII).

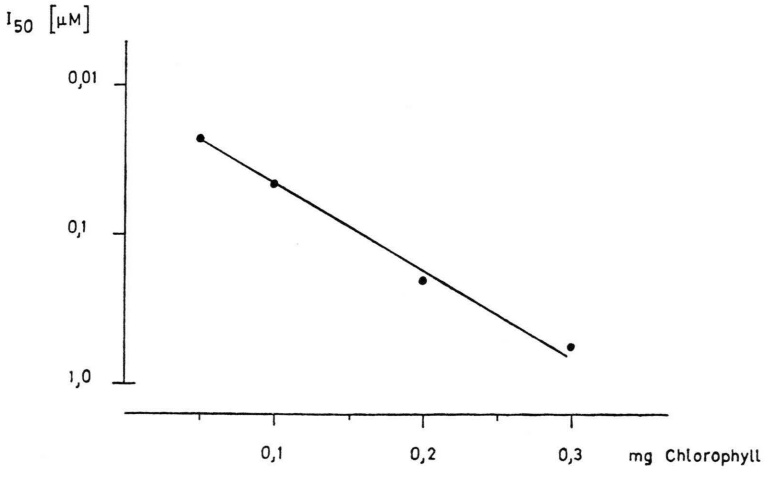

Fig. 2. Dependance of the inhibition of photosynthetic electron flow (given as I_{50} = concentration to inhibit 50%) by DNP-INT on the chlorophyll concentration.
Conditions: NADP reduction by coupled chloroplasts illumination 12 min, rate 100 µmol NADPH/mg chlorophyll and hour.

The addition of an uncoupler, like gramicidin, to an electron flow system from water to MV or NADP in the presence of DNP-INT partially reverses inhibition (Table III). Though the reversal of an electron flow inhibition by an uncoupler is typical for an energy transfer inhibitor, the ATP formation unaffected by the inhibitor in donor systems and cyclic eletron flow systems for photosystem I argues against this interpretation. For comparison it is shown in Table III that the DBMIB inhibition of electron flow is independent of the presence of an uncoupler.

A ferricyanide Hill reaction is as sensitive to DNP-INT as the NADP system (Fig. 3). As in the NADP system (Table III) the reversal of electron flow inhibition by an uncoupler is also apparent in the inhibition of ferricyanide photoreduction at medium concentrations of DNP-INT (Fig. 3). Furthermore, if a ferricyanide Hill reaction is run in the presence of phenylenediamine (PD), a mediator for a photosystem II reduction, only a very slight inhibition of electron flow by DNP-INT is observed (Fig. 3). The insensitivity of a photosystem II dependent ferricyanide reduction (but not the gramicidin

Table II. Effect of DNP-bromo- or -iodo-nitrothymol on cyclic photophosphorylation. Conditions: Chloroplasts with 0.2 mg chlorophyll and 12′ light at 15 °C under nitrogen.

Cofactor [0.1 mM]	Inhibitor [μM]	μmol ATP formed	
PMS	control	6.7	
	control + 2 μM DCMU	7.5	
	0.4 DNP-BNT	6.8	
	7.5 DNP-BNT	6.8	
	0.35 DNP-INT	6.5	
	3.5 DNP-INT	5.6	
menadione		no TMPD	+ 0.1 mM TMPD
	control	1.8	7.2
	0.75 DNP-BNT	0.1	6.8
	7.5 DNP-BNT	3.8	4.8
	0.35 DNP-INT	2.0	6.3
	3.5 DNP-INT	0.06	5.2

Table III. Effect of an uncoupler on the inhibition of a photosystem I Hill-reaction by DNP-iodonitrothymol. Conditions: coupled chloroplasts with 0.2 mg chlorophyll, 12′ light at 15 °C under air (MV) or nitrogen (NADP).

Acceptor	Inhibitor [μM]	No gramicidin		Plus 15 γ-gramicidin	
		Oxygen uptake [μmol]	% Inhibition	Oxygen uptake [μmol]	% Inhibition
MV	control	−4.7		−7.0	
	0.1 DNP-INT	−2.6	44	−5.7	18
	0.5 DNP-INT	−1.1	76	−3.2	54
	1 DNP-INT	−0.5	89	−2.2	68
NADP		μmol NADPH formed	% Inhibition	μmol NADPH formed	% Inhibition
	control	2.3		3.1	
	0.2 DNP-INT	1.0	60	3.2	0
	2 DBMIB	0.3	87	0.25	92

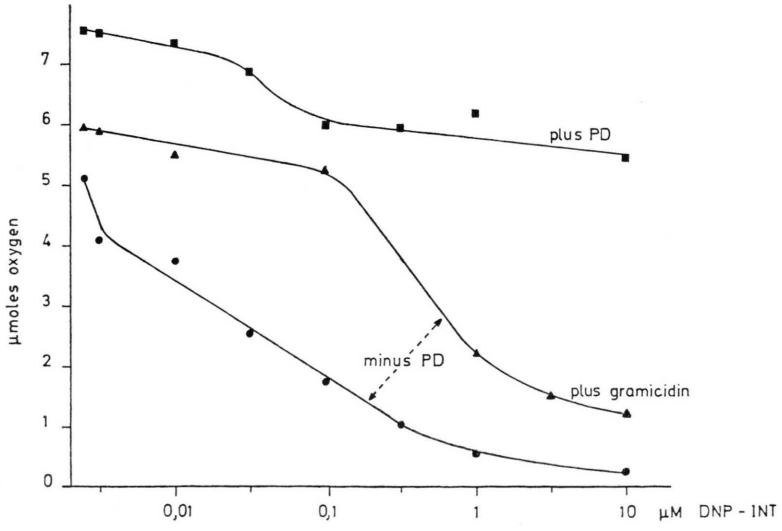

Fig. 3. Effect of DNP-INT on the photoreduction of ferricyanide in the presence or absence of an uncoupler (15 γ gramicidin) or an acceptor of photosystem II (0.1 mM phenylenediamine PD).
Conditions: Coupled chloroplasts with 0.2 mg chlorophyll 12 min light at 15 °C under nitrogen.

reversal) is reminiscent of the effect of DBMIB on the ferricyanide system with that difference that DBMIB, as against DNP-INT, inhibits ferricyanide reduction in the absence of PD not completely even at large concentrations, because DBMIB itself – a quinone unlike DNP-INT – is a mediator between photosystem II and ferricyanide [19].

The reversal of the inhibition by DNP-INT of ferricyanide photoreduction by an artificial electron acceptor (mediator) for photosystem II is shown in Table IV for a number of phenylenediamines and quinones. Phenylenediamine (PD), above 0.1 mM, diamine-toluene (DAT) and DAD are effective mediators, but TMPD and DCPIP are not (Table IV). Also 2,6-dimethyl-benzoquinone is not much effective, but methylen-dioxy-dimethyl-benzoquinone [12] is. For comparison, an experiment with DBMIB is also indicated in Table IV, to show similarities and differences. The similarity in the inhibition pattern of DBMIB and DNP-INT lies a) in the insensitivity of photosystem II driven electron flow and b) that this electron flow sequence from water to PS II is coupled to ATP formation. The difference between 2 μM DBMIB and DNP-INT is in the stoichiometry of electron flow to ATP formation of a photosystem II Hill reaction. Whereas in the case of DBMIB (at the concentration of 2 μM!) the P/e_2 ratio is just slightly below half (*i. e.* 0.4) of the value of non cyclic electron flow from water to photosystem I (*i. e.* 1.0), the P/e_2 ratio in the case of DNP-INT is definitely lower, on average about on fourth (*i. e.* 0.25) (Table IV). For more data on P/e_2 ratios see also Tables V and VIII.

Table V is to indicate that pseudocyclic electron flow from water to oxygen driven by photosystem II only via the autoxidizable methylen-dioxy-dimethyl-benzoquinone [12] is not very sensitive to even high concentrations of DNP-BNT and DNP-INT, again similar to DBMIB. Again, as in the ferricyanide system (Table IV) the ATP formation coupled to the system is less in the case of the diphenylether inhibitors as compared to DBMIB (Table V).

Table IV. Reversal by various mediators of the inhibition by DNP-iodonitrothymol in the photoreduction of ferricyanide by photosystem II. Conditions: coupled chloroplasts with 0.2 mg chlorophyll, 7 mM ferri- +7 mM ferrocyanide as acceptor, 12′ light at 15 °C under nitrogen.

Mediator [mM]	controls			+3.5 μM DNP-INT		
	μmol oxygen evolved	μmol ATP formed	P/e_2	μmol oxygen evolved	μmol ATP formed	P/e_2
–	6.6	6.0	0.91	1.1	0.14	
0.05 PD	10.3	6.5	0.63	4.4	1.43	0.32
0.1 PD	10.6	5.4	0.51	7.6	2.0	0.26
0.1 DAT	10.4	4.6	0.44	6.7	1.6	0.24
0.1 DAD				4.2	1.0	0.24
0.1 TMPD	7.4	4.0	0.4	1.7	0.4	0.23
0.1 DCPIP	5.0	–	–	1.9	–	–
0.1 2.6-dimethyl-BQ	4.4	2.9	0.66	1.9	0.31	0.16
0.1 methylendioxy dimethyl-BQ	5.8	2.7	0.45	3.7	0.77	0.21
0.1 PD	instead of DNP-INT 2 μM DBMIB			9.5	3.8	0.4

Table V. Pseudocyclic electron flow, driven by photosystem II via methylene-dioxy-dimethyl-benzoquinone (0.2 mM). Conditions: coupled chloroplasts with 0.2 mg chlorophyll 0.5 mM $MnCl_2$ and 0.1 mM Na-azide, 12′ light at 15 °C in air.

Inhibitor [μM]	μmol oxygen taken up	μmol ATP formed	P/e_2
control	−5.6	2.3	0.41
2 DBMIB	−3.9	1.4	0.36
0.75 DNP-BNT	−2.8	0.6	0.21
0.35 DNP-INT	−3.2	0.82	0.26

A different stoichiometry of the coupling eletron flow system to ATP formation, insensitive to DNP-BNT and DNP-INT, as compared with DBMIB, is also observed in the TMPD bypass. As described for the DBMIB inhibition [3, 13], a catalytic amount of TMPD (no ascorbate!) reconnects photosystem II and oxygen evolution back onto photosystem I in NADP or MV reduction. The P/e_2 value in a TMPD bypass of DBMIB inhibition is approaching the value of the control which was taken as indication for an internal bypass [3, 13]. Also DNP-BNT and DNP-INT inhibition of NADP or MV reduction is reversed by a TMPD bypass

Table VI. TMPD bypass of the inhibition by DNP-bromo- or -iodonitrothymol in MV reduction. Conditions: Coupled chloroplasts with 0.1 mg chlorophyll, 0.1 mM Na-azide, 12′ light at 15 °C in air.

Inhibitor [μmol]	0.1 mM TMPD	μmol oxygen taken up	μmol ATP formed	P/e_2
control	−	−3.9	3.5	0.90
	+	−5.5	4.2	0.77
7.5 DNP-BNT	−	0.1	—	—
	+	−3.3	1.45	0.44
3.5 DNP-INT	−	0.1	—	—
	+	−3.5	1.5	0.43
2 DBMIB	−	0.1	—	
	+	−6.2	−4.7	0.76

Table VII. TMPD bypass of the inhibition by DNP-bromo- or iodonitrothymol in NADP reduction. Conditions: salad chloroplasts (0.1 mg) 12′ light at 15 °C in nitrogen.

Inhibitor [μM]	TMPD [0.1 mM]	μmol oxygen evolved	μmol NADPH formed	μmol ATP formed	P/e_2
control	−	3.2	2.8	2.6	0.93
	+	3.1	2.9	2.4	0.85
2 DBMIB	−		0.1		
	+	2.6	2.7	2.1	0.8
6 DNP-INT	−		0.3		
	+	3.2	2.2	1.3	0.58
8.5 DNP-BNT	−		0.4		
	+	2.8	2.5	1.1	0.4

(Tables VI, VII). The P/e_2 value of coupling ATP formation, however, approaches only about half the P/e_2 ratio of the control (Table VI and VII). This TMPD bypass is responsible also for the insensitivity to DNP-INT of cyclic electron flow catalysed by menadione + TMPD (Table II).

A photoreduction of ferricyanide by photosystem II *i. e.* in the presence of DBMIB via certain (amino group containing) artificial mediators was found to be − surprisingly − not stimulated by an uncoupler but even inhibited [12, 14, 15] (at pH 8 and if the illumination time is long enough to actually permit turnover of the mediator). This was taken as indication for a reduction of the mediator in the internal pH space, whose low pH is equilibrated with the external pH by an uncoupler [12, 16]. The same inhibition of a photosystem II Hill reaction by an uncoupler is observed in the presence of DNP-BNT and DNP-INT. If a phenylenediamine (positively charged) is used as a mediator, gramicidin severely inhibits electron flow. Gramicidin does not inhibit − actually slightly stimulates − if a hydroquinone (negatively charged) is used as mediator (Table VIII). This behaviour of an uncoupler in a photosystem II dependent Hill reaction is the same whether DBMIB or the new inhibitors are used.

Discussion

DBMIB as inhibitor of photosynthetic electron flow at the level of plastoquinone function (between PQH_2 and PCy) has proven very useful in photosynthesis research. In the presence of DBMIB, electron flow from photosystem II to photosystem I is blocked but a photoreduction of a suitable artificial electron acceptor by photosystem II only is still possible (for review see refs [1, 2]). DBMIB inhibition was instrumental in particular to prove the existence of a second ATP coupling site in such photosystem II dependent Hill reactions [17, 18]. DBMIB has disadvantages, though. Being a quinone, it is a redox compound and as such acts as electron

Mediator [mM]	Inhibitor [μM]	μmol oxygen evolved	μmol ATP formed	P/e_2	+15 Gramicidin μmol oxygen evolved
PD	control	9.7	4.0	0.4	9.9
	2 DBMIB (low)	8.1	3.2	0.4	3.4
	5 DBMIB (high)	6.4	1.7	0.27	
	7.5 DNP-BNT	6.9	1.2	0.17	2.4
	3.5 DNP-INT	6.8	1.75	0.26	2.1
"BQ"	control	6.6	3.0	0.45	7.7
	2 DBMIB	4.3	1.4	0.32	3.5
	7.5 DNP-BNT	3.7	1.0	0.27	4.3
	3.5 DNP-INT	4.3	0.8	0.18	4.6

Table VIII. Effect of uncouplers in the reduction of ferricyanide by photosystem II via phenylenediamine or a benzoquinone in the presence of DNP-bromo- or iodonitrothymol. Conditions: 0.2 mg chlorophyll, 0.1 mM PD or methylendioxidimethyl-benzoquinone = "BQ", 12′ light in nitrogen.

acceptor for photosystem II and as mediator for electron flow from photosystem II to ferricyanide [19]. Also due to its quinoid character nucleophiles, particularly SH compounds, quickly inactivate DBMIB [20]. At very high concentrations (100 μM) it is a very effective quencher of chlorophyll fluorescence [21]. At 1 μM concentration DBMIB acts specifically on the inhibition of PQH_2 oxidation, but at medium high concentrations (5 – 10 μM) it is less specific and it shifts from its primary site of inhibition toward a secondary one at the reducing site of plastoquinone [2, 3, 22, 23]. A number of DBMIB analogues – some as effective – are now available [4, 5] but they are also halogenated or hydroxylated alkylsubstituted quinones and therefore with the same side effects. It was desirable therefore to design a DBMIB like inhibitor without redox properties. Recently Robinson *et al.* [24] reported that trifluraline (a N-alkyl dinitroaniline derivative) is an inhibitor of electron flow in much the same way as DBMIB. Other N-alkylated dinitroaniline derivatives (herbicides) act the same way [7]. Bathophenanthroline has been described as an inhibitor between PQ and PCy function, but at a different site than DBMIB [25]. The inhibitory potency of these latter compounds is less than DBMIB but they have the advantage of not being redox compounds. Inhibitors of both high potency and no redox properties are reported on here.

The O-dinitrophenyl derivatives of halogenated nitrothymols are inhibitors of electron flow in chloroplasts very much like DBMIB. Both DNP-BNT and DNP-INT are very potent inhibitors of electron flow from water to an acceptor of photosystem I (like NADP or MV), yielding 50% inhibition at about 0.5 μM and 100% inhibition at 5 μM (depending on chlorophyll concentration). The inhibition pattern of the two compounds is identical to the one of DBMIB inhibition (more precisely to the primary one by low concentrations of DBMIB) in these respects:

1. The photoreduction of ferricyanide via *p*-phenylenediamine or pseudocyclic electron flow mediated by methylene-dioxy-dimethyl-benzoquinone [12], both dependent on photosystem II only, are not inhibited.

2. Photosystem II dependent reactions (in the presence of either DBMIB or DNP-INT) are coupled to ATP formation.

1. Coupled electron flow is more sensitive to DNP-BNT or DNP-INT than is uncoupled or basal electron flow, *i. e.* gramicidin at a certain concentration range of the inhibitor reverses inhibition. Actually such a behaviour is typical for an energy transfer inhibitor. However, because PMS cyclic photophosphorylation and phosphorylation in other insensitive electron flow systems are not blocked by DNP-BNT, there has to be another explanation for this lower sensitivity of uncoupled systems, not obvious to us yet. A similar energy transfer inhibition effect has been reported for $HgCl_2$ inhibition, which was explained by higher sensitivity of coupling site I, as against coupling site II [26]. We do not think this explanation appropriate, because it implies an unlikely heterogenity of coupling factors for the two coupling sites. Also Robinson *et al.* [24] observed an uncoupler effect in their studies on trifluraline inhibition.

2. A ferricyanide Hill reaction (driven by photosystem I) is inhibited by even very high concentrations of DBMIB only to about 60 to 80%, but is completely sensitive to the new inhibitors. This is easily explained, though, by the redox properties of DBMIB, which itself acts as mediator between photosystem II and ferricyanide [19] and this way can bypass its own inhibition site.

3. Uncouplers inhibit photosystem II driven reactions (*i. e.* in the presence of DNP-INT) when an amine, but not when a quinone is the mediator, as observed earlier in the respective systems in the presence of DBMIB [12, 14 – 16].

4. Photoreductions by photosystem I only, like NADP reduction at the expense of DAD or TMPD/ascorbate or cyclic photophosphorylation catalyzed by PMS, are not or very little sensitive to the new compounds.

5. The menadione catalyzed cyclic photophosphorylation system is sensitive, but not if TMPD is also present.

6. A TMPD bypass [3, 13] of the DNP-INT inhibition site in NADP reduction is also possible; this bypass is coupled to ATP formation as it is in a similar DBMIB experiment.

In all these respects the new inhibitors show identical inhibition pattern to that of DBMIB (at 1 μM DBMIB, a concentration suffice to block electron flow after PQH_2).

However, there are differences between DNP-BNT and DBMIB inhibition also.

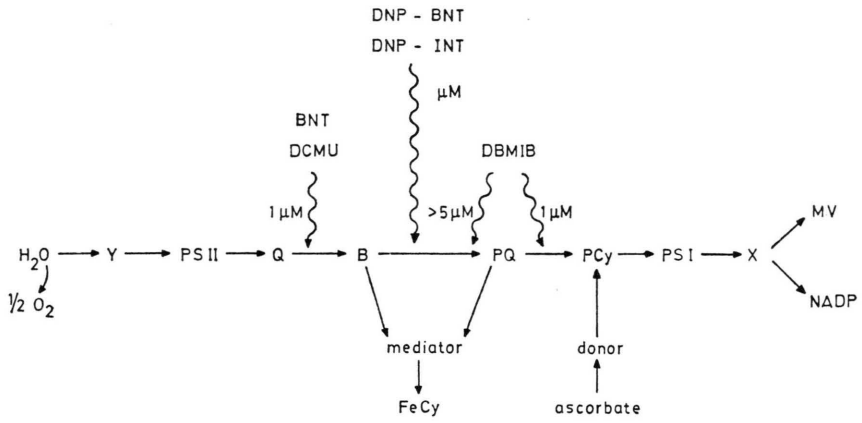

Fig. 4. Scheme of photosynthetic electron flow with inhibition sites of various compounds in the area of plastoquinone function.

3. The stoichiometry of ATP formation to electron flow in photoreductions by photosystem II only in the presence of DNP-BNT at DNP-INT is definitely lower than in the respective DBMIB experiment with a DBMIB concentration just sufficient to fully block electron flow from PQ to PCy (*i. e.* 1 μM). The same is true for the P/e_2 ratio in the TMPD bypass of the inhibition site in NADP reduction. This third effect of the DNP-ethers is identical to the effect of a five fold excess of DBMIB, Then also the rate of electron flow as well as the P/e_2 ratio in photosystem II driven Hill reactions as well as in the TMPD bypass is lowered [3, 14]. We had described the changed inhibition pattern of DBMIB above 5 μM (as against 1 μM) to a shift in the inhibition site from the primary inhibition site after PQH_2 to a second inhibition site before PQ and as an intermingling of DBMIB with the PQ pool, assumed to shuttle electrons and protons across the membrane [3, 16].

If this is accepted, we would then suggest that DNP-BNT and DNP-INT inhibit photosynthetic electron flow between B (or R) and PQ as do high concentrations of DBMIB (Fig. 4). Does this explain a low stoichiometry of ATP formation to electron flow?

According to the present model of vectorial electron flow the reduction site of plastoquinone via B is assumed to be on the external (matrix) site of the thylakoid. A reduction of mediators of photosystem II driven Hill reactions by B (plastoquinol-anion) on the external side would not necessarily involve the protonisation to the plastoquinol, as in the case when PQH_2 on the inner side of the membrane is the reduction site, as in an experiment with a low DBMIB concentration. In an external reduction only the protons of the water splitting reaction are available for coupling, whereas in an inside reduction both proton liberating sites are operative, though half the protons are chanelled back to the outside via the reduced mediator (stoichiometric uncoupling [16]). In both cases the proton electron ratio would theoretically be the same (1 H^+/e) although Gould and Izawa [27] observed a H^+/e ratio of only 0.5 in photosystem II reductions (as against Ausländer and Junge [28]). A shift in inhibition site from the inside to the outside should not necessarily change the P/e_2 ratio and therefore does not yet satisfactorily explain the lowered P/e_2 ratio in photoreductions by photosystem II in the presence of DNP-INT. A similar case is the controversal coupling of DCMU insensitive silicomolybdate photoreduction via Q on the external side of the membrane. Ben Hayyim and Neumann [29] suggested that in this system the Δ pH formed is below the threshold Δ pH required for ATP synthesis. For the TMPD bypass an external rather than internal bypass could indeed be the explanation for the lowered P/e_2 ratio in the DNP-INT experiment, because then only one proton liberating site (at H_2O oxidation) is involved, whereas there are two such sites in an internal bypass in a DBMIB experiment. Surely the lowered P/e_2 ratio is – in our opinion – not due to an energy transfer inhibition, uncoupling or any other interactio of the e-flow inhibitors with the coupling system in the usual strict sense. We think that instead these characteristics in the case with the inhibitors here are a reflection – not understood in mechanism – of the effect of the inhibitors on the electron flow system at the site proposed.

If the inhibition site for the new inhibitors is tentatively accepted to be between B and PQ an interesting problem arises. The properties of photoreductions by photosystem II (uncoupler inhibition, when an amine is the mediator) had been explained for the DBMIB system as being a reflection of the influence of internal pH on the protonisation of the amine and in this way on the imine/amine ratio inside, affecting the rate of electron flow [16]. There was no conflict to assume a reduction site in the internal pH-space because DBMIB does inhibit at PQH_2 oxidation, surely a site in the electron flow path at the inside of the membrane. The effect of internal pH is, however, also present with the new inhibitors, but for which we had just assumed to inhibit electron flow before PQ, *i. e.* at the matrix side of the thylakoid. Does this indicate that the reduction and protonisation from the outside of the main PQ pool occurs already in the internal pH space with the barrier inside/outside located very much towards the external surface? PQ reduction would then be the driving force for proton translocation across the membrane rather than PQH_2 oxidation.

These problems, relevant for the understanding of the sidedness of the thylakoid membrane, vectorial electron flow and the possibility of microspaces in the formation of a pH gradient, require, however, a more convincing localisation of the inhibition site.

It is worth remembering, that the parent compounds of the DNP-derivatives, *i. e.* bromonitrothymol and iodonitrothymol – are also very potent inhibitors of electron flow but at the DCMU site [7, 11]. The diphenylether of BNT and INT have a completely changed inhibition pattern as reported here. But one could perhaps argue that because of chemical similarities the binding area of both parent and derivative compound is close and overlapping and located in the protein shield [30] covering PS II, Q, B and PQ reduction. This may be taken as another, though weak, argument that DNP-BNT inhibits the reduction of PQ rather than oxidation. It should be noted, that some other diphenylethers (herbicides) do behave also like DNP-BNT, but others do not [7]. As yet we do not understand the chemical structural features of phenols and phenylethers responsible for the binding at different inhibition sites.

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