Comparison of the Inhibition of Photosynthetic Reactions in Chloroplasts by Dibromothymoquinone, Bromonitrothymol and Ioxynil

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The inhibition of photosynthetic electron flow in broken chloroplasts by dibromothymoquinone and dibromothymohydroquinone ($DBMIBH_2$) is reversed by dithiothreitol (DTT) as well as by serum albumin. The reversal of $DBMIBH_2$ inhibition by DTT shows a time lag, that of DBMIB only, when chloroplasts and DBMIB had been preilluminated. This is to show that chloroplasts reduce DBMIB and that probably $DBMIBH_2$ is the actual inhibitor species.

Bromonitrothymol, ioxynil and related inhibitory phenolic compounds have a different relationship of inhibitory potency to chemical structure than DCMU and the analogous triazinone herbicide metribuzin but nevertheless inhibit photosynthetic electron flow at the same functional site. This is supported by the finding that labelled metribuzin is displaced from the thylakoid membrane by bromonitrothymol and ioxynil indicating identical binding sites. On the other hand inhibition by the phenolic inhibitors bromonitrothymol and ioxynil but not that of DCMU and metribuzin has a time lag of about 4 min.

Introduction

The inhibition of photosynthetic electron flow is of interest to photosynthesis and herbicide research. Two different inhibition sites at the acceptor side of photosystem II are of particular importance, one site beeing located before, the other after the main functional pool of plastoquinone [1, 2]. DCMU and many of its analogues are commercial herbicides which belong to the first group, inhibiting the reduction of plastoquinone after the primary quencher of photosystem II, whereas the other group, represented by DBMIB, inhibits the reoxydation of plastohydroquinone by plastocyanin (see reviews [1, 2]). DBMIB inhibition of electron flow in isolated chloroplasts is sensitive to thiol reagents [3]. We wish to show from the properties of the reversal of DBMIB inhibition by DTT as well as by serum albumin compared with that of reduced DBMIB ($DBMIBH_2$) that probably $DBMIBH_2$ is the actual inhibitor species.

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Abbreviations: BMNT, 2-bromo-4-nitro-3-methyl-6-*t*-butylphenol; BSA, bovine serum albumin; DBMIB, dibromothymoquinone; $DBMIBH_2$, dibromothymohydroquinone; DCMU, dichlorophenyldimethylurea; DCPIP, dichlorophenol indophenol; DTT, dithiothreitol; Fecy, K-ferricyanide; ioxynil, 3,5-diiodo-4-hydroxy-benzonitrile; metamitrone, 6-phenyl-4-amino-3-methyl-1,2,4-triazin-5-one; metribuzin, 4-amino-6-*t*-butyl-3-methylthio-1,2,4-triazin-5-one.

Recently we described new inhibitors of electron flow, found during studies on DBMIB analogues, which nevertheless inhibit electron flow at the DCMU inhibition site [4, 5]. This new group of herbicides consists of halogen and alkyl substituted nitrophenols, represented by bromonitrothymol. The long known herbicide and photosynthesis inhibitor ioxynil [6, 7 and loc. cit.] belongs to this group as well as do dinitrophenols [4, 8, 9] recognized recently to be photosynthesis electron flow inhibitors.

This new group of phenolic herbicidal photosynthesis inhibitors – represented by bromonitrothymol and ioxynil – appears to share the inhibition site with DCMU, in spite of a different basic chemical element responsible and essential for binding to the thylakoid membrane [4]. This paper wishes to document further similarities, but also differences of the inhibitory properties of the phenol herbicides as compared to DCMU. One difference is the reversal of inhibition of phenol herbicides *in vitro* by the addition of serum albumin, the other the time lag before maximal inhibition occurs.

Methods

Spinach chloroplasts were prepared according to Nelson *et al.* [10] 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 room temperature and 2.5×10^5 erg/cm^2·sec light through a Schott 12 G filter in a basic medium of 3 ml containing in μmol: Tricine-NaOH buffer pH 8.0 80; ADP 10, inorganic phosphate, 10, $MgCl_2$ 10 and as acceptor either ferricyanide 3 or NADP 3 + ferredoxin 0.01, and chloroplasts with 25 μg chlorophyll. Further additions and conditions are given in Tables and Figures.

NADP reduction was measured at 340 nm, ferricyanide reduction at 420 nm.

In the DCPIP experiments the reaction mixture contained in mM: Tricine puffer pH 6.5 40; $MgCl_2$ 6; KCl 10; gramicidin D 0.001; DCPIP 0.05 and chloroplasts with 10 μg chlorophyll. Illumination with 4×10^5 erg/cm^2·sec white light and recording at 623 nm.

In the binding studies chloroplasts with 50 μg chlorophyll were preincubated with 0.035 μM ^{14}C labelled metribuzin with 45 μC/mg *i.e.* 1.5×10^3 cpm/probe under the measuring condition with a liquid scintillation counter. After adding the compounds given in Figs 7 and 8 and Table IV, the chloroplasts were filtered through a membrane filter (Sartorius SM 1/305) and the radioactivity of the supernatant (chloroplast with bound inhibitor) and the solution (free inhibitor) was measured.

^{14}C labelled metribuzin as well as metribuzin, metamitrone, bromonitrothymol and BMNT are gifts of Bayer AG via Dr. W. Draber, Bayer AG, Forschungszentrum, Wuppertal.

Results

DBMIB inhibits photosynthetic electron flow from water to an acceptor of photosystem I like NADP or methylviologen. The inhibition of NADP reduction by 2 μM DBMIB is easily reversed by addition of 50 μM thiol like DTT, mercaptoethanol or glutathion, as already reported[3]. The reason is probably a 1,4-addition of the nucleophilic thiol to the quionone moiety, which then rearranges and splits off the halogen essential for an inhibitory property. If correct, then the inhibition of electron flow by the reduced hydroquinone form of DBMIB ($=DBMIBH_2$) should be less susceptible to a thiol.

As Fig. 1 indicates, DTT reverses the inhibition by both DBMIB oxidized and $DBMIBH_2$ reduced of NADP reduction in broken chloroplasts. The time needed for complete restoration of photosynthetic activity is, however, much longer in the case of the inhibition by the hydroquinone. In the experiment of Fig. 1, DTT had been added to the reaction mixture after the light was turned on. If this is turned around, and DTT and DBMIB are mixed before the light is turned on then oxidized DBMIB does not inhibit the chloroplast reaction any more, indicating that DBMIB in the quinone form was already inactivated when the photosynthetic experiment was started. The inhibition of NADP reduction by reduced $DBMIBH_2$ hydroquinone, however, is still there and disappears only after a few minutes in the light (Fig. 2).

The experiments actually suggest, that DBMIB is reduced by the choroplast system in the light and that the reduced form is the actual inhibitor species. The remaining small pool of oxidized form of

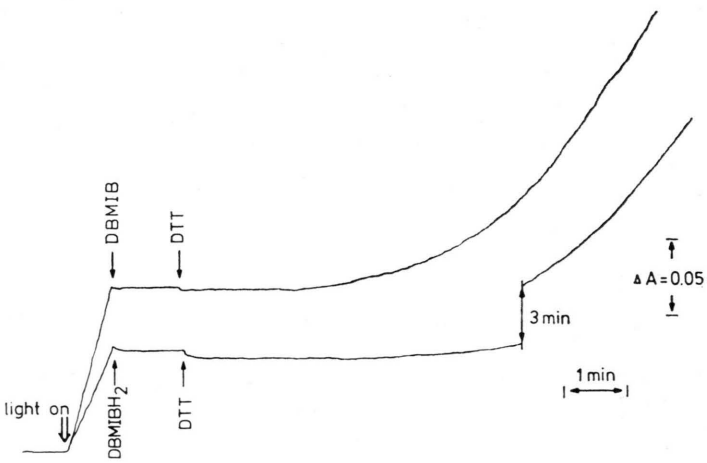

Fig. 1. Reversal of DBMIB inhibition (0.5 μM oxidized or reduced=$DBMIBH_2$) of photosynthetic NADP reduction by 50 μM DTT. NADPH formation measured at 340 nm. Note that inhibitor and chloroplasts are preilluminated before DTT is added. Rate: 150 μmol NADPH formed/mg chlorophyll and h.

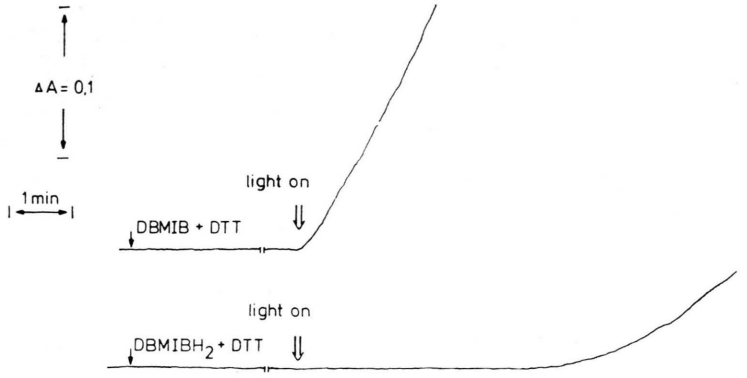

Fig. 2. Insensitivity of photosynthetic NADP reduction to DBMIB (0.5 μM oxidized or reduced = $DBMIBH_2$) when 50 μM dithiothreitol is also present. NADPH formation measured at 340 nm. Note that inhibitor and DTT were mixed for 30 sec, before the light was turned on. Condition as in Fig. 1.

DBMIB would not be able to saturate all inhibition sites. This conclusion depends on the assumption that the ratio of active inhibitor to electron flow change is about one, as indeed reported by Haehnel [11].

Also inhibitory analogues of DBMIB, like dibromo-dimethyl-benzoquinone [5] or diazidothymoquinone [12] (DATQ), described recently, are inactivated by DTT. The half time of inactivation is very fast in the later case, indicating perhaps a light dependent chemical degradation [12] (Fig. 3).

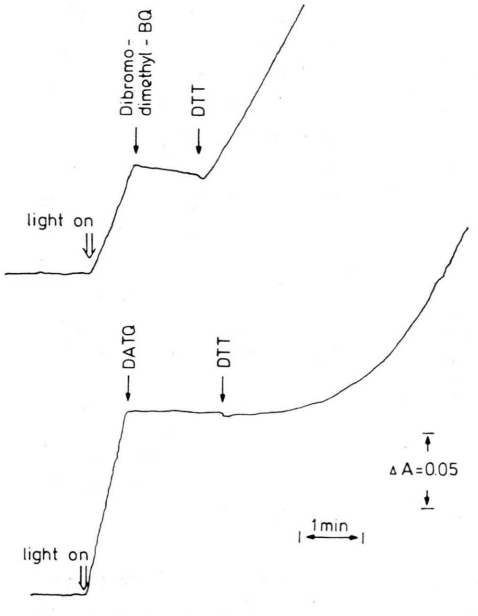

Fig. 3. Reversal of inhibition of photosynthetic NADP reduction by 10 μM dibromo-dimethyl-benzoquinone or by 0.5 μM diazido thymoquinone (DATQ) after the addition of DTT (500 μM in the first, 100 μM in the latter case). NADPH formation measured at 340 nm. Conditions as in Fig. 1.

The DBMIB inhibition of photosynthetic electron flow may also be reversed by addition of bovine serum albumin (BSA). The inhibition of DBMIB as well as of $DBMIBH_2$ is reversed by BSA instantaneously and equally well (Fig. 4). 1 mg/ml BSA, but also serum albumin of other organisms, reverse the inhibition of photosynthetic NADP reduction, though egg albumin is not very effective at the concentration used in Table I. As known, addition of BSA has a stimulatory effect on the control rate, as also seen in Table I. The reversal effect is probably due to an adsorption of DBMIB on serum albumin and therefore pretreatment to remove lipids from BSA greatly increases its effectivness (Table I). Other proteins, chosen at random, like globulin or a chloroplast protein like carboxydismutase, do not reverse DBMIB inhibition (Table I), nor does lecithin.

In Tables II and III it is indicated that BSA not only reverses the inhibition by DBMIB of the photoreduction of NADP but also of the reduction of ferricyanide. Furthermore also the inhibiton by DBMIB as well as by its analogues like diiodobutyl-benzoquinone is reversed by BSA. Note that DBMIB never inhibits ferricyanide reduction more than about 70 to 80% because of a photosystem II dependent portion. The two Tables (II and III) also indicate the effect of BSA on other inhibitors of photosynthetic electron flow which have a different inhibition site. Bromonitrothymol, an effective inhibitor alike DCMU is also rendered ineffective by BSA as inhibitor of NADP (Table III) or of ferricyanide reduction (Table II). The same reversal of inhibitory efficiency by addition of BSA is observed for diiodothymol and to some extent for ioxynil and bromonitro-butylphenol (Tables II and III). These compounds also belong to a group of herbicidal phenols

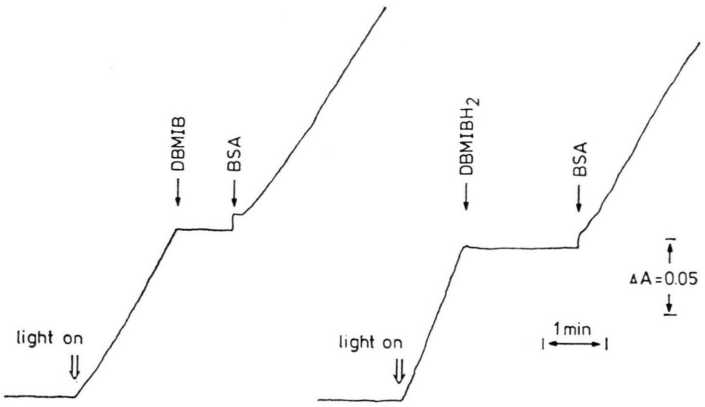

Fig. 4. Reversal of the inhibition of photosynthetic NADP reduction by 0.5 μM DBMIB or $DBMIBH_2$ upon addition of 1 mg/ml bovine serum albumin (BSA). NADPH formation measured at 340 nm. Conditions as in Fig. 1.

Table I. Effect of different proteins on the inhibition of photosynthetic NADP reduction by DBMIB, rate of electron flow = 140 μmol NADPH formed/mg chlorophyll and h.

Protein (1 mg/ml)	No inhibitor	+0.5 μM DBMIB
control	=100	0
bovine serum albumin		
BSA (fatty acid free)	117	88
BSA	118	16
human serum albumin	124	77
egg albumin	103	13
lactalbumin	86	63
α-globulin	84	0
hemoglobin	72	0
apoferritin	96	0
carboxydismutase (Ri-bis-P-carboxylase)	118	0
aldolase		0
lecithin	116	0

Table II. Effect of bovine serumalbumin (BSA) on the inhibition of photosynthetic FeCy reduction by DBMIB and analogues, rate = 300 μmol ferricyanide reduced/mg chlorophyll and h, control = 100%.

Inhibitor	Inhibitor concentration	Inhibition electron flow in percent		Concentration of BSA
	[μM]	−BSA	+BSA	[mg]
DBMIB	0.5	70	80	2
	0.5	78	46	10
diiodo-*t*-butyl-benzoquinone	0.5	88	47	6
bromonitro-thymol	0.5	91	32	2
ioxynil	0.5	93	41	2

acting on electron flow at the DCMU inhibition site [4, 5]. On the other hand, the inhibition of electron flow by DCMU, a triazine (simazin) and two triazinone herbicides (metribuzin and metamitrone) is not changed upon addition of BSA (Table III). The reversal of certain inhibitors of electron flow by BSA might also occur by endogenous proteins of chloroplasts in vivo or even *in situ*, this way perhaps responsible for a selectivity of herbicidal action. Inhibition of NADP reduction by bathophenanthroline, an inhibitor between the two light reactions [13], is also less effective in the presence of (larger) amounts) of BSA (Tab. III).

Bromonitrothymol and ioxynil, as representative of herbicidal phenols, which do not contain the basic chemical element of the urea herbicides responsible for inhibition [4] show a further difference in their mode of action on chloroplasts reactions. The inhibition of electron flow by ioxynil and bromonitrothymol is time dependent. Maximal inhibition is obtained only after a lag of a few minutes (Fig. 5). The longer the preincubation in the dark, the shorter the time lag (Fig. 5). The time lag of about 5 minutes for ioxynil inhibition is seen more clearly from the (same) data of Fig. 5 b, in which the total incubation time dark + light is indicated. Another inhibitor of photosystem II of quite different chemical structure, metribuzin, does not show this time lag (Fig. 5 a and 5 b). Bromonitrothymol, another phenolic inhibitor with chemical and functional similarities to ioxynil, also inhibits photosynthesis only after a certain time lag (Fig. 6). From Fig. 6 it is seen that the time lag also depends on the concentration. The lag is shorter at higher concentrations.

From these results it follows that ioxynil and bromonitrothymol differ from DCMU and metribuzin in their inhibitory properties in these respects: reversal of inhibition by serum albumin and a time lag in addition to a different essential basic chemical element. Though the inhibition site was shown to

Table III. Effect of bovine serumalbumin (BSA) on the inhibition of photosynthetic NADP reduction by DBMIB, DCMU and their respective analogues conditions as in Table I.

Inhibition		Inhibitor concentration	Inhibition of electron flow in percent		Concentration of BSA
Site	Inhibitor				
		[μM]	−BSA	+BSA	[mg]
DBMIB analogues					
	DBMIB	0.5	100	12	1
		0.5	100	7	2
	diiodo-*t*-	0.2	77	31	1
	butyl-quinone	0.5	97	43	2
	dibromo-di-methyl-quinone	4	71	21	2
	dibromo-naphto-quinone	0.3	72	18	1
	bathophenanthroline	10	52	12	5
DCMU analogues					
	bromonitrothymol	1	90	34	2
	bromonitro-*t*-butyl-phenol	0.05	86	52	2
	ioxynil	0.5	87	51	1
	2.4-diiodothymol	4	77	12	5
	DCMU	0.5	91	89	1
		0.5	89	83	10
	metribuzin	0.5	75	77	1
	metamitrone	2	41	51	5
	simazin	2	81	80	5

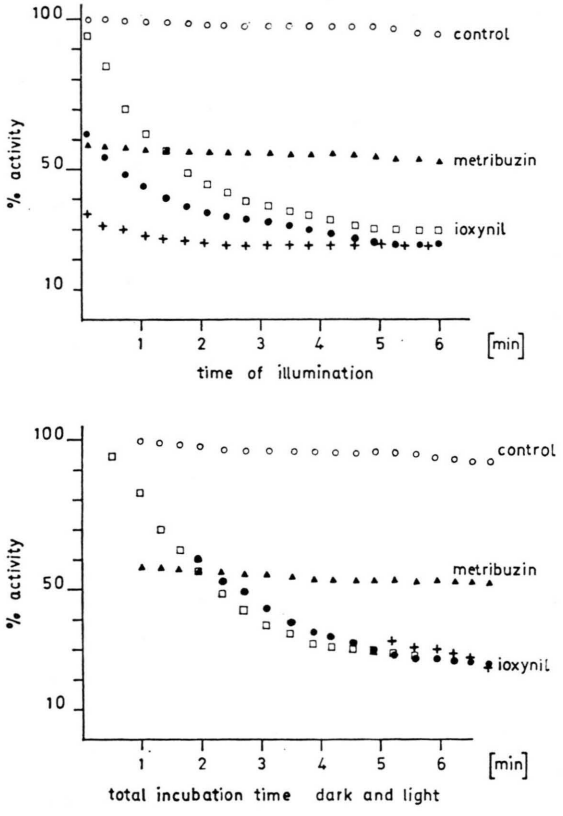

Fig. 5. Time lag in the inhibition of photosynthetic DCPIP reduction by ioxynil but not by metribuzin. In Fig. 5 a the time of illumination after a dark preincubation of 30 sec (□−□), 2 min (●−●) or 5 min (+−+) is indicated. In Fig. 5 b the same data on percent inhibition are drawn against the total incubation time dark+light. Activity: 500 μmol DCPIP reduced/mg chlorophyll and h. Chloroplasts with 5 μg chlorophyll/2 ml, 0.05 μM ioxynil or metribuzin. DCPIP reduction measured at 623 nm.

be identical for bromonitrothymol and DCMU [4, 5], it was important to show further that they nevertheless inhibit at the DCMU site. The procedure of Tischer and Strotmann [13] to show identical binding sites of two chemically unrelated inhibitors consists in the replacement of a radioactive labelled inhibitor from the thylakoid membrane by another analogue.

Fig. 7 and Table IV indicate the results of such a displacement experiment, in which chloroplasts and radioactive labelled metribuzin were incubated with a number of phenolic inhibitors. The data show that labelled [^{14}C]metribuzin, bound to the thylakoid membrane, is removed from its binding site not only by DCMU, as known from studies by Tischer and Strotmann [14], and by another triazinone herbicide metamitrone, but also by bromoni-

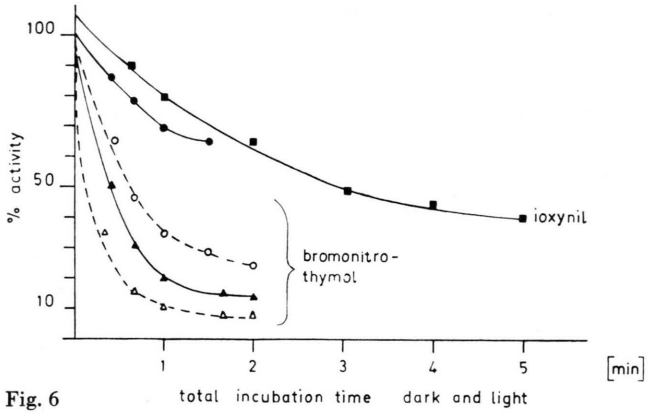

Fig. 6

Fig. 6. Time lag in the inhibition of photosynthetic DCPIP reduction by bromonitrothymol depending on the concentration of the inhibitor. Photosynthetic activity obtained is drawn against total incubation time of the chloroplasts with the inhibitor in the dark plus light. Conditions as in Fig. 5. 0.05 μM ioxynil (■—■) or bromonitrothymol 0.05 μM (●—●), 0.1 μM (○—○), 0.2 μM (▲—▲) or 0.5 μM (△—△).

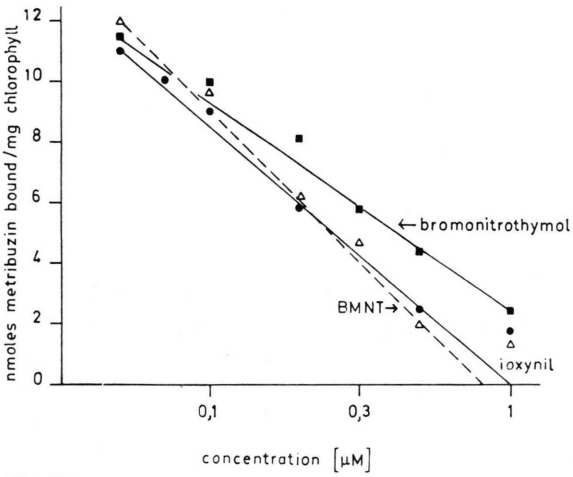

Fig. 7 a

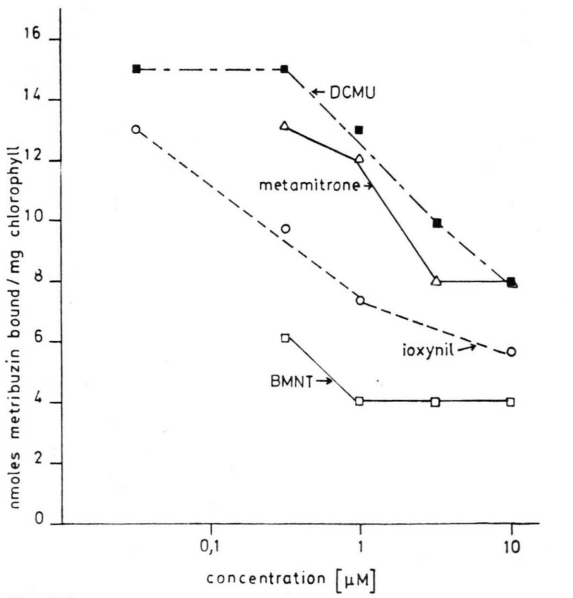

Fig. 7 b

trothymol, bromonitromethyl-*t*-butyl-phenol (BMNT) and ioxynil. The extent of displacement, of course, depends on the concentration of the analogue (Fig. 7 and Table IV). This then would indicate identical binding sites of the classical photosystem II inhibitors, like DCMU and the triaziones as well as

Table IV. Displacement of [^{14}C]metribuzin from its binding site by analogues (pH 6.5, 100 μg chlorophyll, 0.14 μM [C^{14}]metribuzin).

	% binding of metribuzin (control+100%)				
addition of	at a concentration of [μM]				
	0.05	0.1	0.2	0.5	1
ioxynil	96.5	78.2	50.3	21.8	14.3
bromonitrothymol	100	87.8	71.0	37.6	20.5
bromonitromethyl-*t*-butyl phenyl	100	84.4	52.4	17.3	11.7

of the new phenolic herbicides. Tischer and Strotmann had shown earlier [14], that DBMIB would not compete with the DCMU inhibition site. Interestingly, also the replacement of labelled metribuzin by ioxynil has a time lag (Fig. 8) similar to the one in the onset of inhibition (Fig. 5). The time lag depends on the chlorophyll concentration used.

Fig. 7. Displacement of bound [^{14}C]metribuzin from the thylakoid membrane by other inhibitors. Bound [^{14}C]metribuzin is given as nmoles per mg chlorophyll. a) Addition of bromonitrothymol (■—■), ioxynil (●—●) and 2-bromo-4-nitro-3-methyl-6-*t*-butyl phenol (BMNT) (○—○). b) Addition of DCMU (■—■), metamitrone (△—△), ioxynil (○—○) and BMNT (□—□).

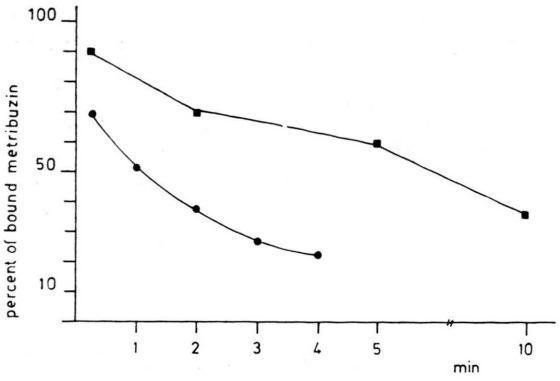

Fig. 8. Time lag and dependence on chlorophyll concentration in the displacement of bound [^{14}C]metribuzin from the thylakoid membrane by ioxynil (in percent of the control). (15 μmol [^{14}C]metribuzin/mg chlorophyll), ■—■ 0.075 μM ioxynil and chloroplasts with 185 μg chlorophyll, ●—● 0.01 μM ioxynil and chloroplasts with 42 μg chlorophyll.

Discussion

DBMIB proved to be a very useful inhibitor in studies on the mechanism of photosynthetic electron flow in isolated chloroplasts, because it effectively blocks the connection between photosystem I and II between plastohydroquinone and plastocyanin, but nevertheless permitting at the same time both photosystem I and II photoreductions to proceed (for review see [1, 2]). DBMIB is, however, much less effective in studies with intact organisms probably due to a rapid inactivation of the compound. Because of the quinone moiety of DBMIB the easy chemical inactivation of DBMIB upon nucleophilic attack by thiols – as shortly reported [3] – is not surprising. Further results here with chloroplasts show, as expected, that the inhibition of electron flow by the hydroquinone form of DBMIB is much less sensitive in time to DTT reversal.

The pipetting order is of importance. DTT has inactivated DBMIB already, if added before the light experiment starts. If DTT is added to the chloroplast system + DBMIB in the light there is a time lag of reversal. This lag is even larger if the reduced form of DBMIB is used. This indicates that the DBMIB is present in steady state photosynthesis mostly in the reduced form, protecting it for a certain time from a SH attack. From this it might be concluded in turn that $DBMIBH_2$ is the actual inhibitor of the oxidation site of plastohydroquinone by plastocyanin/cytochrome f. The pool of oxidized DBMIB is to small to saturate all inhibition sites, with the assumption that about one inhibitor molecule is needed to block one chain, as indeed measured by Haehnel [11].

Serum albumines from various sources also reverse DBMIB as well as $DBMIBH_2$ inhibition of electron flow immediately. This is very probably due to the known binding capacity by serum albumin of lipophilic compounds and of quinones and phenolic compounds in particular [15, 16]. A nucleophilic inactivation by serum albumin might be also part of the reversal of inhibition [16, 17]. Other proteins do not seem to inactivate DBMIB. An effect of BSA on DBMIB inhibition has been noted already by Berg and Izawa [18]. Chloroplasts are usually prepared in the presence of BSA; they may show a lowered sensitivity to DBMIB, if BSA is not washed out before the experiment. The chemical sensitivity of DBMIB to thiol inactivation and albumin binding makes this inhibitor much less useful for photosynthesis studies *in vivo*.

Because of this binding of lipophilic penols it is not surprising that serum albumin also reverses the inhibition of electron flow by bromonitrothymol, analogous nitrophenols as well as ioxynil. This inactivation of the herbicidal phenol inhibitors distinguishes these from other herbicidal inhibitors of photosynthesis like DCMU or metribuzin. Both groups of compounds do inhibit electron flow at the same site in photosynthetic electron flow after the primary quencher Q of photosystem II [4, 5]. An identical inhibition pattern of DCMU and a dinitrophenol-herbicide (dinitrocresol = DNOC) has been studied in great detail by van Rensen *et al.* [9, 19]. Nevertheless urea and phenol herbicides do not contain the same chemical structural element essential for inhibition. It has been shown that a $N-\overset{\|}{C}\text{-}sp^2$ hybrid is essential for inhibition by DCMU and its many herbicidal analogues (see [4]), a group not present in bromonitrothymol or ioxynil. This has been reported recently [4]. Another property distinguishes bromonitrothymol and ioxynil inhibition from that of DCMU and metribuzin. This is the definite time lag between addition of the inhibitor to the chloroplast and maximal inhibition. The time lag is dependent on concentration and seems to be independent of dark or light pretreatment.

In spite of such differences in chemical structure and inhibitory properties, the general binding area of alkyl substituted dinitrophenols and halogen

nitrophenols is identical to the one of DCMU. This is concluded from the repalcement of radioactive labelled metribuzin, which does bind to the DCMU site, by bromonitrothymol and ioxynil. According to Tischer and Strotmann [14] this replacement procedure is a very direct way of establishing identical binding sites of inhibitors of different chemical structure but identical inhibitory mechanisms. Tischer and Strotmann had actually also mentioned – but not yet published – that ioxynil may replace a labelled DCMU analogue. The replacement of [^{14}C]metribuzin by ioxynil also shows a time lag as does the onset of inhibition.

The replacement technique and the inhibition pattern of DCMU and analogues as well as of the herbicidal phenol compounds indicate a similar binding area. But special, though overlapping, binding sites in this area have to be postulated to accomodate that compounds with different essential chemical elements affect the same functional site. This is discussed in more detail elsewhere [4].

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