The Inhibition of Photosynthetic Light Reactions by Halogenated Naphthoquinones

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Halogenated naphthoquinones act as inhibitors of photosynthetic electron flow. I_{50} concentration for inhibition of methylviologen reduction were found to range between 2×10^{-5} M to 2×10^{-6} M. Comparing their effects on several partial reactions of electron flow, the inhibition site of the naphthoquinones was found to be at the reducing site of PS II. Studies of fluorescence transients in presence of halogenated naphthoquinones give further evidence for a site action similar to that of diuron and different to that of DBMIB.

All naphthoquinones act as quenchers of chlorophyll fluorescence with pure chlorophyll a, and with much higher efficiency in green algae and chloroplasts.

It is concluded, that the halogenated naphthoquinones act similar to PS II-inhibitors like diuron, but do not share a common binding site at the PS II-complex. Implications of a possible involvement of phylloquinone K_1 in photosynthetic electron transport are discussed.

The synthesis of 2-chloro- as well as 2-bromo-3-isopropyl-1,4-naphthoquinone is described.

Introduction

Quinones are important functional constituents of plant biomembranes [1, 2]. Plant mitochondria contain ubiquinone-10 as a central electron carrier [3], whereas in chloroplast membranes plastoquinone-45 acts as a redox carrier [4, 5]. Both quinones are prenylsubstituted benzoquinones. Other regular quinoid constituents are the couple α-tocopherol/α-tocoquinone and the phylloquinone (vitamin K_1), a substituted naphthoquinone [1, 3, 6]. Though quinones are potential redoxcarriers and protontranslocators, no definite function is known as yet for the phylloquinones as well as for the α-tocopherol/α-tocoquinone couple [3].

A number of substituted benzoquinones are described as inhibitors of photosynthetic electron flow [7–14]. They are mostly halogen-alkyl-substituted benzoquinones. Depending on their substituents they act either before or after the plastoquinone pool [12, 13]. The most important and powerful experimental inhibitor is the well-known DBMIB, introduced and characterized by the group of Trebst [15]. Inhibitors from the benzoquinone group always act as inhibitors of the endogenous benzoquinone plastoquinone-9, either by preventing plastoquinone oxidation at the DBMIB site or by affecting the reduction of the PS II acceptor B [16–18], possibly a membrane-bound plastoquinone [19–21].

Compared to the number of benzoquinone-type inhibitors only a few naphthoquinones are described as inhibitors of photosynthetic reactions [7–9, 14]. Precise information on their mode of action is scarcely. In analogy to the benzoquinone-type inhibitors it seems possible that substituted naphthoquinones may act as inhibitors of the endogenous naphthoquinone phylloquinone (vitamin K_1).

The aim of the present paper is to study and to characterize the inhibitory effects of halogenated naphthoquinones on photosynthetic light reactions. In addition to their inhibitory properties the naphthoquinones are also strong quenchers of chlorophyll fluorescence [22, 23]. Therefore we studied also their quenching properties to get additional information about naphthoquinone function in photosynthesis.

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Abbreviations: B, secondary acceptor of PS II, site of action of PS II inhibitors; BIN, 2-bromo-3-isopropyl-1,4-naphthoquinone; BQ, *p*-benzoquinone; DBMIB, dibromomethyl-isopropylbenzoquinone; DCPIP, 2,6-dichlorophenol-indophenol; diuron, DCMU, 3,4-dichlorophenyldimethylurea; MV, methylviologen; PS I and II, photosystem I and II; Q, primary acceptor of PS II.

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Materials and Methods

1. Chloroplast isolation and measurements

Chloroplasts were isolated from 5 d old cotyledons of *Raphanus sativus* L. var. Saxa treib, grown under fluorescent light (fluora, 60 W) on humus soil. The leaves were grinded for 15 sec in a medium containing 0.3 M saccarose, 0.1 M phosphate buffer pH 7.4, 35 mM NaCl and 5 mM $MgCl_2$. After filtration through dense nylonfabric and 4 layers of cheese cloth the suspension was centrifuged at 1400 × *g* for 5 min. The medium for washing, resuspension and storage contained: 1 mM phosphate buffer pH 7.4, 35 mM NaCl and 5 mM $MgCl_2$.

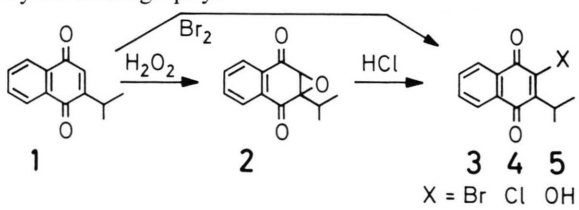
Cultures of the green algae *Scenedesmus obliquus* D_3 and *Chlorella pyrenoidosa* were grown at 22 °C and illuminated with fluorescent light (light intensity: 3000 lux). As nutrient medium a solution after Kessler [24] was used.

Electron transport was assayed in a medium described by Strotmann and Goesseln [11] and measured as oxygen evolution or consumption, respectively with a Clark-type electrode (YSI, Mod. 53). The actinic light intensity inside the measuring cell was 5×10^4 erg/cm² sec^{-1}. All measurements were done at 17 °C and a chlorophyll concentration of 20–30 µg chlorophyll/ml. As electron acceptors were used as indicated: MV, 0.2 mM in presence of 1 mM NaN_3; *p*-BQ, 0.5 mM. Electron donors: phenylenediamine, 33 µM; *p*-hydroquinone, 200 µM; DCPIP, 0.2 mM in presence of 5 mM Na-ascorbate.

Chlorophyll fluorescence was excited at 441 nm by a blue HeCd-laser (Fa. Linconix, Mod. 401) and detected by a RCA 7265 photomultiplier, guarded with a red RG 645 cutoff-filter (Fa. Schott) and a IF 680 interference (Fa. Shimadzu). The optical system consisted of a two-armed lightguide. The fluorescence signal was temporarily stored in the memory of a Nicolet Explorer III digital oscilloscope, photographed from the screen or recorded on a slow speed strip chart recorder. The overall response-time of the system incl. shutter opening time and the electrical time constants yielded fluorescence rise-times (f_0-level) well below 1 msec. Intensity of exciting light: 3×10^4 erg/cm² sec (= 6.6×10^{15} quanta/cm² sec).

2. Source and synthesis of naphthoquinones

1.4-Naphthoquinone and menadione were obtained from Merck, Darmstadt, phthiocol from Calbiochem (USA). Phylloquinone K_1 was a gift from Hoffmann-La Roche. 2-bromo-3-isopropyl-1,4-naphthoquinone (**3**) was prepared by direct bromination of 2-isopropyl 1,4-naphthoquinone (**1**). In the synthesis of the corresponding chloro derivative (**4**) the epoxide (**2**) was opened with hydrochloric acid. The resulting mixture with the hydroxy quinone (**5**) was separated by chromatography.



2-Isopropyl-1,4-naphthoquinone (**1**): 20.0 g isobutyric acid chloride was reacted with 14.6 g sodium peroxide in 100 ml cyclohexane at 0–5 °C according to Fieser's method [26–28]. After hydrolysis with ice-water the dried solution ($CaCl_2$) was concentrated in vacuo to 60 ml. 120 ml of such a solution of diisobutyryl peroxide was slowly added dropwise to a vigorously stirred solution of 7.9 g 1,4-naphthoquinone in 150 ml glacial acetic acid at 105 °C. After refluxing for 2 h the solvents were evaporated in vacuo. An ethereal solution of the resulting dark brown material was washed with aqueous sodium hydrogen carbonate, dried and evaporated. The residue was chromatographed on silica (0.05–0.2 mm) with eluent *n*-hexane/benzene (2:1) containing 3 vol. % ether. The first yellow fraction yielded 1.93 g (19%) golden yellow solid, m. p. 44 °C ([29]: 43–44 °C); IR (KBr) $\nu_{C=O}$ 1655 cm^{-1}; NMR (CCl_4) δ ppm: d 1.22 (2-CH_3, J = 7 Hz), sept. 3.19 (α H, J = 7 Hz), d 6.64 (1-H, J = 1 Hz), m 7.80 (4 arom. H).

2-Bromo-3-isopropyl-1,4-naphthoquinone (**3**): 2.31 g bromine was added to a solution of 2.6 g 2-isopropyl-1,4-naphthoquinone (**1**) and 6.0 g sodium acetate in 20 ml glacial acetic acid at 50–60 °C. The solution was allowed to stand for 2 d at room temperature and then poured onto ice. The yellow solid precipitate was chromatographed with benzene/ethyl acetate and finally recrystallised from cyclohexane; 1.50 g (42%) yellow needles, m. p. 84–85 °C; IR (KBr) $\nu_{C=O}$ 1670, 1650 cm^{-1}; NMR (CCl_4) δ ppm: d 1.40 (2-CH_3, J = 7 Hz), sept. 3.57 (α H. J = 7 Hz). m 7.55–8.15 (4 arom. H), sublimed at high vacuum for analysis

$C_{13}H_{11}BrO_2$ (279.1)
calc. C 55.93 H 3.97 Br 28.63
found C 56.05 H 4.06 Br 27.12.

2-Chloro-3-isopropyl-1,4-naphthoquinone (**4**): 4.6 ml hydrogen peroxide (35%) was added dropwise to a stirred solution of 2.0 g 2-isopropyl-1,4-naphthoquinone (**1**) in 7.0 ml ethanol at 70 °C. Then 1.15 g sodium carbonate in 4.0 ml water was added slowly during which a brown oil deposited under effervescence. Stirring was continued for 15 min, then 80 ml water was added and the product was extracted with ether. After drying and evaporation the crude oxide (**2**) (2.03 g) was disolved in 4.5 ml glacial acetic acid and 2.1 ml 35% hydrochloric acid added dropwise to the solution at 75 °C. A brown oil separated but stirring was continued for 30 min. The solution was diluted with 100 ml water and then extracted with ether, yielding 2.1 g of a crystalline mixture. This was separated by chromatography on silica (0.05–0.2 mm) with *n*-hexane/benzene (2:1) containing 5% ether. The first yellow band yielded 1.01 g (43%) pale-yellow platelets, m.p. 63 °C, sublimed for analysis at 60 °C in vacuo, IR (KBr) $\nu_{C=O}$ 1665 cm^{-1}; NMR (CCl_4) δ ppm: d 1.38 (2-CH_3, $J = 7$ Hz), sept. 3.61 (α H, $J = 7$ Hz), m 7.91 (4 arom. H); u.v. (CH_3OH) λ_{max} ($\varepsilon \times 10^{-3}$): 242 (17.3), 245 (18.7), 250 (16.2), 268 (11.5), 275 (12.6), 334 (2.8).

$C_{13}H_{11}ClO_2$ (243.7)
calc. C 66.52 H 4.79 Cl 15.10
found C 66.39 H 5.01 Cl 15.18.

The second red band yielded 518 mg (24%) 2-hydroxy-3-isopropyl-1,4-naphthoquinone (**5**), m.p. 94 °C ([27]: 94–95 °C).

The corresponding reaction sequence with 2-methyl-1,4-naphthoquinone furmished the epoxide (64%, m.p. 94–95 °C from ethanol ([28]: 94.5–95.5 °C) and after treatment with hydrochloric acid and chromatographic separation as above 2.8 g (44%) 2-chloro-3-methyl-1,4-naphthoquinone, m.p. 144 °C ([30]: 143–144 °C) as well as ca. 10% 2-hydroxy-3-methyl-1,4-naphthoquinone, m.p. 173 °C ([31]: 173 °C). Both compounds recrystallised from ethanol.

Results

a) Inhibition of photosynthetic electron transport reactions

The activity of the whole chain electron transport was studied in the $H_2O \rightarrow MV$ system which involves the activity of both pigment systems. The efficiency of an inhibitor was characterized as I_{50}-concentration (pI_{50}-value).

The results in Table I show, that the inhibitory efficiency can be largely increased by introduction of an halogen in 3-position. A bromo-substitution yields a better electron transport inhibitor as the comparable chloro-derivate. A similar dependence was observed by Oettmeier for halogene-substitution of halogenated benzoquinones [12]. Also an isopropyl-chain instead of a methyl substitution improves the inhibitory properties of the naphthoquinones.

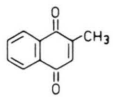
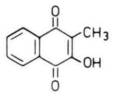
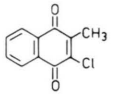
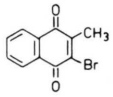
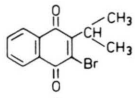
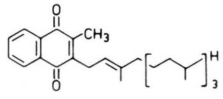
The most effective inhibitor was found to be the 2-bromo-3-isopropyl-1,4-naphthoquinone, which gave a I_{50}-concentration of 2 μM ($pI_{50} = 5.7$). For this compound also the chlorophyll concentration independent inhibition constant K_i was determined by measuring I_{50}-concentrations at different chlorophyll concentrations, as described by Tischer and Strotmann [32]. This determination gave a K_i of 5.8 for Raphanus chloroplasts and a K_i of 5.5 for spinach chloroplasts.

1,4-naphthoquinone and menadione cannot be considered as true inhibitors because their unphysiological high I_{50}-concentrations are in the millimolar range. It was surprising to see that also with phylloquinone a certain degree of inhibition was obtained although a 50% inhibition required a millimolar concentration. This concentration, however, was calculated from the amount of phylloquinone added to the chloroplast suspension. Because lipophilic phylloquinone is a very poor watersoluble compound, the actually available concentration responsible for the observed inhibition will certainly be lower. From this we can assume that the true inhibitory efficiency of phylloquinone is much better.

In a quantitative structure activity relationship (QSAR) the biological activity (measured as pI_{50}, data from Table I) was correlated with the lipophilicity (P) as only molecular parameter (Fig. 1). The good correlation ($r = 0.9714$) demonstrates the importance of lipophilicity as major molecular property influencing the efficiency of an inhibitor. Similar correlations were found by Oettmeier for a series of substituted benzoquinones [12].

The results obtained, using the $H_2O \rightarrow MV$ test-system, clearly show an inhibitory effect of the naphthoquinones studied on the photosynthetic electron transport. Further localization of the site of action can be obtained by comparing the effects of

Table I. Inhibition of methylviologen reduction by naphthoquinones in isolated Raphanus chloroplasts.

Substance		pI_{50}	I_{50}
1.4-naphthoquinone		2.7	$2000 \cdot 10^{-6}$
menadione (K_3)		3.3	$500 \cdot 10^{-6}$
phthiocol		3.8	$160 \cdot 10^{-6}$
2-chloro-3-methyl-1.4-naphthoquinone		4.8	$16 \cdot 10^{-6}$
2-bromo-3-methyl-1.4-naphthoquinone		5.1	$7.9 \cdot 10^{-6}$
2-bromo-3-isopropyl-1.4-naphthoquinone (BIN)		5.7	$2 \cdot 10^{-6}$
phylloquinone (K_1)		3.0	$1000 \cdot 10^{-6}$

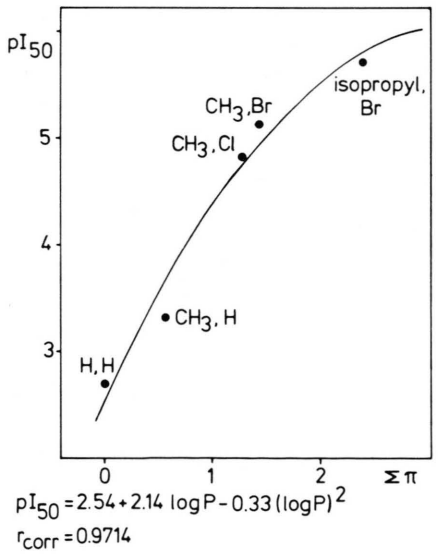


Fig. 1. Correlation between measured pI_{50} values (Table I) and hydrophobic molecular parameters for 5 substituted naphthoquinones. Substitution ($-CH_3$, Cl, Br, isopropyl, $-H$) in position 2 and 3 of the naphthoquinone nucleus as indicated. The calculation of the quantitative structure-activity relation from our data was kindly performed by Dr. W. Oettmeier, which is gratefully acknowledged.

these inhibitors on several partial reactions of electron flow (Table II).

The activity of PS I in the $DCPIPH_2 \rightarrow MV$ system is not affected by the naphthoquinones studied, whereas PS II dependent reactions ($H_2O \rightarrow BQ$) are inhibitor sensitive. At high naphthoquinone concentrations a complete inhibition of these reactions can be obtained. The observations that the benzoquinone reduction and the methylviologene reduction are affected by the naphthoquinones to about the same degree is an indication that the naphthoquinones do not act similar to DBMIB but instead show a behaviour similar to diuron. Because of the fact that the benzoquinone reduction is only to about 40% DBMIB sensitive (DBMIB conc.: 4×10^{-6} M), a complete inhibition of the benzoquinone reduction can be expected only by inhibitors of the diuron-type. Our data demonstrate (Table III), that a complete inhibition in the $H_2O \rightarrow BQ$ system by addition of the halogenated naphthoquinones can be achieved. This clearly shows, that the halogenated naphthoquinones have an inhibition site different from that of DBMIB and before the plastoquinone pool.

Table II. Inhibition of photosynthetic electron transport reactions by naphthoquinones in isolated Raphanus chloroplasts. (Values in μM O_2/mg Chl · h.)

Substance	Conc. [M]	$H_2O \rightarrow BQ$	$H_2O \rightarrow MV$	DCPIP/ Asc → MV
control	–	92	80	96
2-chloro-3-methyl-1,4-naphthoquinone	10^{-5}	52	47	92
2-bromo-3-methyl-1,4-naphthoquinone	10^{-5}	36	32	82
2-bromo-3-isopropyl-1,4-naphtoquinone (BIN)	10^{-5}	14	12	86
vitamin K_1	10^{-3}	49	43	91
menadione (K_3)	10^{-3}	33	22	90
phthiocol	10^{-3}	16	27	82

To rule out a possible inactivation of the water-splitting system by the naphthoquinones applied which could also explain the previous results, we studied their action in heat-inactivated chloroplasts. In this heat-treated system the O_2-evolution is abolished and artificial donors supplied electrons to the oxidizing site of PS II. These testsystems (phenylenediamine/ascorbate → MV and *p*-hydroquinone/ascorbate → MV) turned out to be also naphthoquinone sensitive (Table IV). Together with the previous results (Table III) this indicates that the halogenated naphthoquinones actually act at the reducing site of PS II, and not at the water splitting system or at the donor site of PS II. The remaining reaction rates are insensitive to higher naphthoquinone concentrations and even to diuron. These rather low rates are explained by the fact that the reduced electron donors *p*-phenylenediamine and *p*-hydroquinone can act to some extent also as electron donors for PS I [33, 34]. Therefore the reaction rates in presence of high naphthoquinone concentrations must be considered as a pure PS I dependent photoreaction.

Table III. Inhibition of *p*-benzoquinone reduction in isolated Raphanus chloroplasts by halogenated naphthoquinones in presence of DBMIB.

Addition	μM O_2/ mg Chl · h	Reaction rate (% of control)
control	75	100
+ DBMIB (4×10^{-6} M) (no naphthoquinone added)	46	59
+ DBMIB (4×10^{-6} M) + 2-bromo-3-methyl-1,4-naphthoquinone (10^{-4} M)	< 2	< 3
+ DBMIB (4×10^{-6} M) + 2-bromo-3-isopropyl-1,4-naphthoquinone (10^{-4} M)	< 2	< 3

The halogenated naphthoquinones inhibit the formation of the Δ pH with the same efficiency as they

Table IV. Inhibition of photosynthetic electron transport reactions in heat-inactivated Raphanus chloroplasts by halogenated naphthoquinones. Values in % of control rate: 80 μM O_2/mg Chl · h. Inactivation of chloroplasts: 5 min at 45 °C.

Inhibitor	Intact chloroplasts $H_2O \rightarrow MV$	Heat-inactivated chloroplasts		
		$H_2O \rightarrow MV$	PhD/Asc → MV	HQ/Asc → MV
control	100	5	83.7	76.2
2-chloro-3-methyl-1,4-naphthoquinone	7.5	–	16.2[d]	11.2[d]
2-bromo-3-methyl-1,4-naphthoquinone[b]	5	–	10.0[d]	75.0[d]
2-bromo-3-isopropyl-1,4-naphthoquinone (BIN)[c]	~ 0	–	13.7[d]	6.2[d]

[a] Inhibitor concentration 8×10^{-4} M.
[b,c] Inhibitor concentration 8×10^{-5} M.
[d] The remaining reaction rates are diuron-insensitive.

inhibit the electron transport [35]. In contrast to this menadion (2×10^{-5} M) and K_5 (1-hydroxy-2-methyl-naphthaline-4-ammoniumchloride, 10^{-4} M) stimulate the Δ pH by 20% and 85%, respectively. This demonstrates that halogenated naphthoquinones do not mediate a PS I dependent cyclic proton translocation.

b) Influence of sulfhydryl-compounds on the inhibitory efficiency of halogenated naphthoquinones

Reactions between quinones and SH-dependent enzymes or SH-reagents are often discussed as a possible mode of action of quinones in enzymatic reactions, in antibacterial and antifungal applications [36, 37] or in herbicidal studies [7]. Our results show a strong influence of the amino acid cystein on the inhibitory efficiency of the halogenated naphthoquinones (Table V). The inhibition of the photochemical activity ($H_2O \rightarrow MV$) by the naphthoquinones can be abolished only to a small extent when cystein is added to the inhibited chloroplasts. If cystein was added first to the reaction mixture and the naphthoquinone inhibitor was added 3 min later, only a very small degree of inhibition was detectable. Similar results were obtained using as SH-reagent glutathione in the same concentration as cystein.

Reimer and Trebst reported that the action of DBMIB can be prevented by previous addition of glutathione, but not reversed, if the inhibition has already occurred [38]. This observation is similar to ours, except that DBMIB acts at a different site on the photosynthetic electron flow.

The fact that inhibition of photosynthetic electron flow by naphthoquinones can be prevented by cystein and glutathione, is probably due to unspecific chemical reactions between SH-reagents and the applied quinones without special involvement of chloroplast membrane constituents. Such reactions between quinones and SH-agents are well-known in chemistry. The possibility, that the naphthoquinones bind to endogenous SH-groups at the inhibitor binding protein can not be ruled out.

c) Fluorescence measurements in presence of naphthoquinones

Studies of the chlorophyll fluorescence induction curve (Kautsky effect) are an important tool for detailed investigations of the photochemical properties of PS II [39]. For recent reviews see ref. [40, 41]. Fluorescence studies were performed to provide further evidence for the localization of the site of action of the naphthoquinones at the reducing site of PS II.

The modification of the fluorescence induction curve by diuron is characterized by a fast fluorescence rise during the first milliseconds after onset of illumination (Fig. 2) starting at the f_0 level (also called 0-level). The maximal fluorescence level (P or f-max) is reached, using the green algae *Scenedesmus* after about 40–50 msec in presence of diuron. This fast fluorescence rise is due to the fast and complete reduction of the primary acceptor pool Q of PS II because the flow of electrons into the plastoquinone pool via the acceptor B is prevented in presence of diuron. Recent studies, initiated by Velthyus and by Boughes-Boquet [16, 17], indicate the acceptor B as target for diuron and other PS II inhibitors. In presence of DBMIB the combined

Table V. Inhibition of methylviologen reduction by 2-bromo-3-isopropyl-1,4-naphthoquinone in presence or in absence of cystein.

Inhibitor concentration	Control	Reduction of methylviologen ($H_2O \rightarrow MV$) in % of control		
	(no inhibitor)	+ Inhibitor	Cystein + inhibitor	Inhibitor + cystein
7×10^{-5} M	100[a]	2[b]	95[c]	10[d]
7×10^{-6} M	100[a]	41[b]	96[c]	48[d]

[a] No inhibitor present, control rate: 47 μM O_2/mg Chl · h = 100%.
[b] After addition of inhibitor.
[c] 3 min cystein incubation (8×10^{-4} M), than addition of inhibitor. Rates measured after 1 min.
[d] 1 min inhibitor incubation, than addition of cystein (8×10^{-4} M). Rates measured after 3 min cystein incubation.

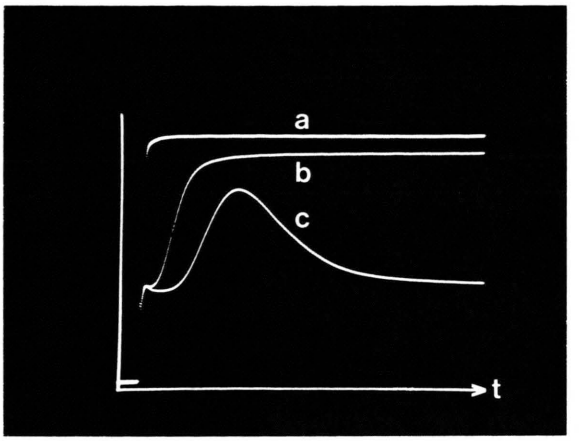


Fig. 2. Chlorophyll fluorescence transients of *Chlorella pyrenoidosa* in presence of 10^{-6} M diuron (a); 7×10^{-6} M DBMIB (b); no addition (c). Total recording time: 2 sec. Ordinate: chlorophyll fluorescence relative units.

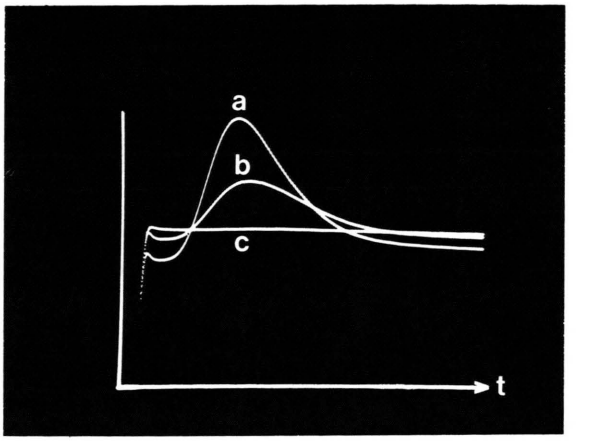


Fig. 3. Chlorophyll fluorescence transients of *Chlorella pyrenoidosa* in presence of 2-bromo-3-isopropyl-1,4-naphthoquinone (BIN). a: no addition; b: 3×10^{-6} M BIN; c: 6×10^{-5} M BIN. Total recording time: 2 sec. Ordinate: chlorophyll fluorescence in relative units.

reducible pool sizes (Q, B and plastoquinone) are much larger than in the presence of diuron. Therefore the time to drive the Q-pool into the fully reduced state is much longer than it is in presence of diuron. As a result DBMIB induces only a slight fluorescence rise during the first 100 msec (Fig. 2). The *P*- or f_{max}-level is reached in more than 400 msec. At higher DBMIB concentrations, however, ($> 8 \times 10^{-5}$ M) we observed a modification of the fluorescence induction curve which was similar to that obtained with diuron. This property of DBMIB to shift its inhibition site before the plastoquinone pool was already observed in electron transport studies in isolated chloroplasts [42, for a review see 43].

Fluorescence measurements with isolated chloroplasts in presence of naphthoquinones are very difficult to perform due to strong quenching of the variable parts of the fluorescence induction [44, 45] seen already at low inhibitor concentrations (see Table VI). To overcome this difficulty we have studied the modification of the fluorescence induction curve by halogenated naphthoquinones using green algae, which showed less sensitivity to quenching as compared to thylakoid membranes.

In experiments using 2-bromo-3-isopropyl-1,4-naphthoquinone (BIN), (Fig. 3) and 2-bromo-3-methyl-1,4-naphthoquinone (Fig. 4) inhibition of photochemical activity is clearly indicated by the modification of the fluorescence transient. BIN induces a fast rise to the f_{max}-level accompanied by a strong quenching of the variable part of the fluorescence curve ($f_{max} - f_0$). At concentrations below 10^{-5} M only

Table VI. Quenching of chlorophyll fluorescence by naphthoquinones. k_{685}, k_{730}: quenching constants determined after Fig. 6 for the quenching of the chlorophyll fluorescence emitted at 685 ± 10 nm or 730 ± 10 nm, respectively.

Substance studied	k_{685} chlorophyll a in methanol [a]	k_{685} diuron-inhibited *Scenedesmus* [b]	k_{685} *Raphanus* chloroplasts [b]	k_{730} *Raphanus* chloroplasts [b]
menadione (vitamin K_3)	59	12000	16000	13600
phylloquinone (vitamin K_1)	54	400	1430	1300
2-methyl-3-chloro-1,4-naphthoquinone	51	11000	–	–
2-methyl-3-bromo-1,4-naphthoquinone	53	6600	681000	470500
2-isopropyl-3-bromo-1,4-naphthoquinone	55	8500	115000	99600

[a] Conc. 5×10^{-6} M.
[b] Adjusted to an extinction of 0.1 at the maximum of the red absorption band.

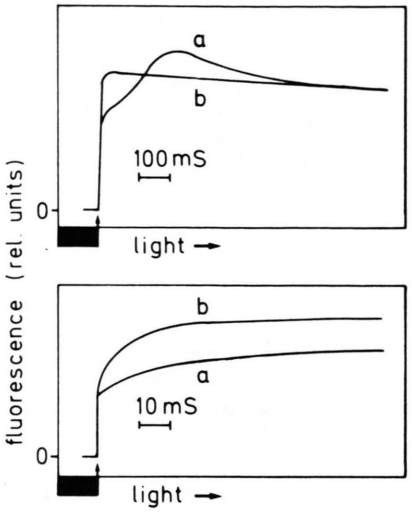


Fig. 4. Chlorophyll fluorescence transients of *Scenedesmus* in presence of 2-bromo-3-methyl-1,4-naphthoquinone. a: no addition; b: 10^{-5} M inhibitor.

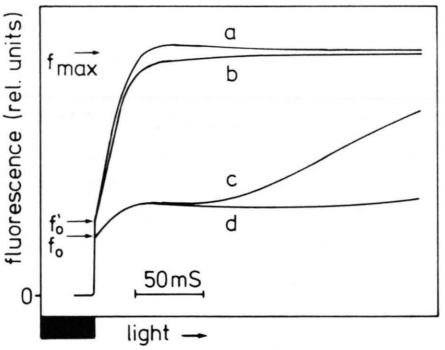


Fig. 5. Comparison of chlorophyll fluorescence rise curves of *Chlorella pyrenoidosa* treated with a: 2-bromo-3-isopropyl-1,4-naphthoquinone (BIN); b: diuron (10^{-5} M); c: DBMIB (3×10^{-6} M); d: no addition. Note: The transient of the BIN-containing sample (taken from Fig. 3c) was normalized to the same variable fluorescence ($f_{max}-f_0$) as was observed in a diuron treated sample (Fig. 2a), in order to compensate quenching of chlorophyll fluorescence. Ordinate: chlorophyll fluorescence in relative units.

the variable part and not the constant part (f_0-level) are affected by quenching. After normalizing the quenched variable part of the transient of the BIN-treated sample to the same value of the variable fluorescence as is observed in a diuron-treated sample (Fig. 2a), the two transients become very similar (Fig. 5). By comparing the fast fluorescence rise of the normalized, BIN-treated sample and of a diuron-treated sample, it is very obvious that BIN modifies

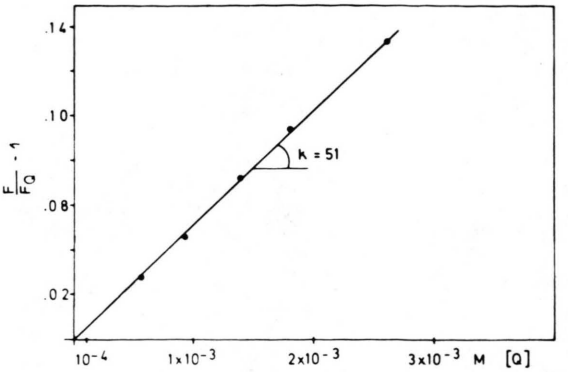


Fig. 6. Fluorescence quenching by 2-chloro-3-methyl-1,4 naphthoquinone with pure chlorophyll a in methanol. Chlorophyll concentration 10^{-5} M.

the fluorescence transient in a way nearly identical to diuron, but clearly different to the action of DBMIB. This gives further evidence for an action of the halogenated naphthoquinones at the reducing site of PS II.

Quenching of chlorophyll fluorescence by quinones has been described before [22, 23, 46, 47]. This quenching by quinones is quantitatively studied by analysis of the fluorescence decrease observed after addition of certain quencher concentrations in a Stern-Volmer plot (Fig. 6) according to the Stern-Volmer equation:

$$F/F_Q = 1 + k\,[Q]$$

where F and F_Q denotes the fluorescence without or with quencher added (Q), the concentration of quencher present and k the quenching constant. k actually is the product of the rate constant and of the chlorophyll fluorescence lifetime. The reciprocal value of k gives the molar concentration of the quenching agent required for a 50% decrease of the fluorescence intensity, as can be easily seen from the Stern-Volmer equation.

The constants determined from Stern-Volmer plots with pure chlorophyll a in methanol are very similar for all compounds studied (Table VI). With algae and especially with isolated, broken chloroplasts a drastic increase of the quenching by the halogenated naphthoquinones is observed, indicating for isolated chloroplasts a very strong quenching of the chlorophyll fluorescence already at μM concentrations (conc. for 50% quench and for the bromomethyl substituted compound: 1.5×10^{-6} M). This illustrates the difficulties in obtaining meaningful

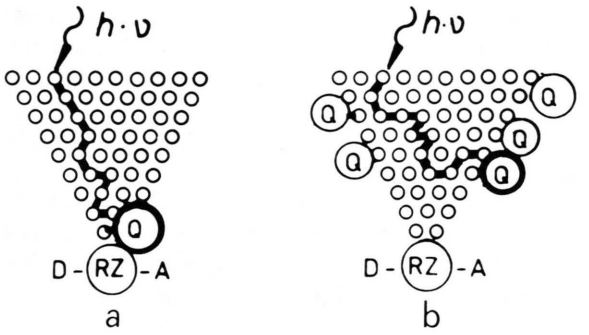


Fig. 7. Model for high efficient quenching of chlorophyll fluorescence by naphthoquinones a) at the reaction center and b) at the antenna system. a) quenching of variable part of the fluorescence by forming artificial traps close to the reaction center chlorophyll. b) quenching of constant part of fluorescence (f_0-level) occurring at high quencher concentrations. D = donor for PS II-reaction center. A = acceptor for PS II-reaction center. RC = reaction center. Q = artificial quencher.

data by recording transients of the fluorescence induction with isolated chloroplasts. The much higher efficiency to quench chlorophyll fluorescence in algae and in chloroplasts, as compared to the results obtained with pure chlorophyll a, can be explained by an action of the naphthoquinones at or near the reaction center chlorophyll of PS II (Fig. 7). This explains their much higher quenching efficiency, already in low concentrations, as compared to the quenching of excited chlorophyll in solution. This interpretation is also consistent with the observation, that in low concentrations preferentially the variable part of the chlorophyll fluorescence is quenched. This fluorescence is believed to be originating from the antennae chlorophyll when a reaction center trap is in the closed state. If the naphthoquinones form artificial traps [46] near the reaction center of PS II, it is possible that a reasonable amount of excitation energy is dissipated and therefore lost for the useful conversion into chemical energy. This mechanism should be considered as a possible second mode of action of the halogenated naphthoquinones.

Discussion

The results obtained by studying photosynthetic electron transport reactions in presence of halogenated naphthoquinones showed that the naphthoquinones act as inhibitors of the electron transport chain at the reducing side of PS II. Their mode of action is clearly different to that of the plastoquinone antagonist DBMIB. No effect of the halogenated naphthoquinones on the oxidizing site of PS II (water-splitting system) was found. These results gave an indication that the inhibition site of the naphthoquinones is very similar to that diuron. This conclusion is supported by fluorescence measurements on green algae, even when the observed fluorescence kinetics are superimposed by a strong quenching of chlorophyll fluorescence.

The halogenated naphthoquinones induce a typical modification of the fluorescence induction transient, which is similarly seen in presence of diuron. These modified transients (Figs. 3 and 4) are clearly different from those measured in presence of low DBMIB concentrations (Fig. 5). High DBMIB concentrations, however show an intermediate pattern of the fluorescence induction curve [35] between the shape of a typical diuron-type curve and a curve obtained at low DBMIB concentrations (10^{-5} M).

The inhibition site at the reducing site of PS II is not only sensitive to diuron (an urea derivate), but also to a wide variety of chemically different inhibitors, *i. e.* triazines, triazinones, amides, pyridazinones, carbamates, uraciles, nitrophenoles and others [43, 48–50]. All PS II inhibitors seem to bind at the same site at the thylakoid membranes as shown by Tischer and Strotmann [32], but they also involve different subunits at this binding site [18, 51, 52]. For all of the inhibitors mentioned above, except the phenols, it is believed, that they contain as an essential structural element the configuration $-\overset{\overset{\|}{}}{\underset{}{C}}-\overline{N}-$, bound to lipophilic substituents or side chains. It must be pointed out, that this element is not present in the naphthoquinones though our data indicate a very similar site of action as the other PS II inhibitors.

There are several reports now on binding- and competition experiments which indicate, that all PS II inhibitors have the same binding area as a common target [32, 52–54]. Due to the lack of radioactive naphthoquinones, binding-experiments involving these inhibitors have not yet been performed. The observation, that 2-bromo-3-methyl-1,4-naphthoquinone poorly competes with radioactive, bound atrazine for a common binding site in contrast to all other PS II inhibitors studied so far, gives the naphthoquinones a special rank in the group of PS II inhibitors (Pfister, unpublished results). This observation establishes a slightly dif-

ferent binding site for naphthoquinones at the PS II complex, which may still be located at the recently identified PS II inhibitor binding protein (B-protein) [55].

In addition to the inhibition of the photosynthetic electron flow, an additional mode of action of the halogenated naphthoquinones, their strong quenching properties should be considered. The mechanism of energy dissipation via quenching of the excited state of chlorophyll may possibly increase the efficiency of the naphthoquinones as inhibitors of photosystem II reactions.

In contrast to other naphthoquinones [56–59] no indication was found that the halogenated naphthoquinones induce a proton translocating cyclic electron flow around PS I. If the halogenated naphthoquinones should act as antagonists against the endogenous phylloquinone, then this function should be associated with the function of PS II because the site of action of the halogenated naphthoquinones was shown to be there. At first view this seems to be different to older proposals, which assumed that phylloquinone is involved in cyclic electron transport reactions around PS I [56–60]. The assumption that cycling electrons enter the photosynthetic electron transport before the plastoquinone pool [61, 62] would require an entry site at the reducing site of PS II. At such a position phylloquinone could act as a possible mediator because, as a naphthoquinone, it is reducible and a potential proton translocator [6].

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