Inhibition of Phytoene Desaturase – the Mode of Action of Certain Bleaching Herbicides

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Assay systems have been developed in order to differentiate between the modes of action of certain bleaching herbicides. These include inhibition of chlorophyll or carotenoid biosynthesis, and initiation of pigment degradation. Herbicidal compounds with phytoene desaturase as their primary target site were investigated in a cell-free carotenogenic system from *Aphanocapsa*. In a comparative study, the structural prerequisites for inhibition of phytoene desaturase were established for both benzophenone analogs and various *m*-phenoxybenzamides. This inhibitory action of the latter compounds is enhanced by lipophilic groups with certain steric properties.

Introduction

Herbicides are directed towards various processes in plants. A typical example of a plant-specific target is the (plastidic) photosynthetic apparatus. Here, the so-called bleaching herbicides interfere with the chlorophyll and carotenoid content of the organelle [1, 2].

The bleaching herbicides consist of a group of compounds which are heterogenous in their modes of action. The term "bleaching" refers to a decrease in the amount of pigment after a certain period of growth in the presence of a herbicide as compared to an untreated control. This pigment deficiency can be caused either by initiation of pigment degradation [3] or by inhibition of biosynthesis of either chlorophylls or carotenoids [4]. In this study, we report on several means to elucidate the primary mode of action of different types of bleaching herbicides. In addition, data are presented concerning the structural requirements of some groups of herbicidal compounds that inhibit phytoene desaturation.

Materials and Methods

The microalgae *Scenedesmus acutus* (276-3a, Algal Culture Collection of the University of Göttingen) and *Aphanocapsa* strain 6714 were grown in 1-l flasks under sterile conditions. Media and cultivation conditions for heterotrophic *Scenedesmus* and *Aphanocapsa* have been given elsewhere [5, 6]. Growth was determined as packed cell volume in graduated microcentrifuge tubes of 80 µl capacity.

Chlorophyll determination was carried out after hot methanol extraction (65 °C, 15 min) according to McKinney [7]. Carotenoids were extracted from cells with hot methanol containing 6% KOH (w/v). After transfer into petrol (b.p. 60 to 80 °C), the content of β-carotene was estimated from the 445-nm absorbance peak with an extinction coefficient $E^{1\%}_{1\,cm} = 2500$. The absorbance maximum at 287 nm was taken for calculation of the phytoene concentration.

Thylakoid membranes used for cell-free carotene biosynthesis were prepared by lysozyme treatment (3 mg/ml; 2-h incubation at 35 to 37 °C). The membranes were collected by centrifugation (3 min, $1000 \times g$), washed with 0.4 M Tris-HCl buffer, pH 7.8, and resuspended in the same buffer. Details of incubation with [2-^{14}C]geranylgeranyl pyrophosphate (GGPP) and herbicidal compounds, separation and purification of carotenes by TLC, as well as measurement of radioactivity incorporation have been previously described [8].

Lipid peroxidation was assayed by monitoring the degradation of plastidic sulfolipid of *Scenedesmus* cells in the presence of herbicides. The procedure of prelabeling the sulfolipid was described in a previous publication [9]. *Scenedesmus* was grown for

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2 days in a medium containing 10 μCi [^{35}S]sulfate per liter. Subsequently, the cells were transferred into media with unlabeled sulfate, then the herbicides were added and the cells kept under culture conditions for 48 h.

Herbicides (99% pure) were added from 10 mM stock solutions in methanol or methanol/dimethylformamide (1:1, v/v).

The herbicidal compounds used in this study were kindly provided by the following companies: BASF AG, Ludwigshafen/Limburgerhof, Germany, nos. 4, 5, 12, 14–24; Celamerck, Ingelheim, Germany, 4-chloro-m-phenoxy benzoic acids for the synthesis of no. 26; Ciba-Geigy, Basel, Switzerland, no. 9; Eli Lilly Co., Indianapolis, Indiana, USA, no. 6; ICI, Bracknell, UK, no. 12a; May and Baker Ltd., Dagenham, UK, no. 7; Mitsubishi Chemical Industries Ltd., Yokohama, Japan, no. 2; Rhône-Poulenc SA, Vitry-sur-Seine, France, nos. 1, 8; Rohm and Haas, Spring House, PA, USA, no. 11; Stauffer Chemical Company, Richmond, CA, USA, no. 10; Sandoz AG, Basel, Switzerland, no. 13; and Sumitomo Ltd., Takarazuka, Japan, nos. 25, 27.

Results and Discussion

The green alga *Scenedesmus* can be grown under heterotrophic conditions in the dark with a fully developed photosynthetic apparatus, thus preventing photooxidation of pigments. The effects of certain compounds on the contents of chlorophylls, colored carotenoids, and phytoene under these growth conditions are shown in Table I. Compounds of group (A) affect the chlorophyll concentration, but do not interfere with the carotene content. As can be seen from the applied concentrations, MK-616 (no. 2) is more effective than oxadiazon (no. 1) and DTP (no. 3). We tentatively assume that these three compounds are inhibitors of chlorophyll formation. Specific assay systems have to be developed to demonstrate the inhibitory site in the chlorophyll-biosynthetic pathway.

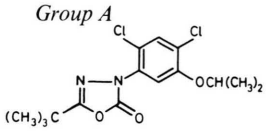
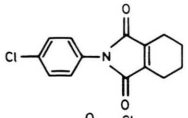
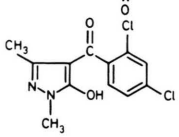
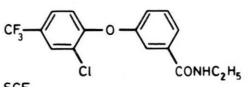
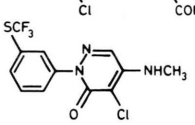
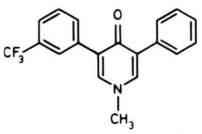
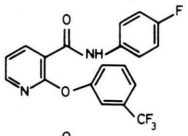
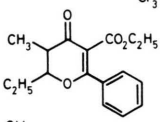
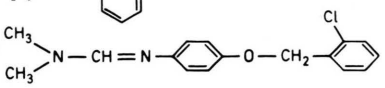
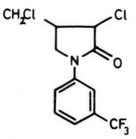
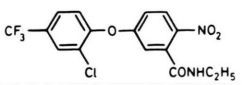
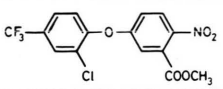
Compounds in group (B) and (C) decrease both chlorophylls and carotenoids. In the presence of the compounds (B), inhibition of carotenoid formation is about twice as strong as inhibition of chlorophyll formation. In contrast with (C), application of these compounds results in a similar decrease of *both* the chlorophyll and carotenoid content. Accumulation of phytoene was evident with compounds of group (B) only. This strongly suggests that these chemicals interfere with carotene biosynthesis as proposed previously for compounds nos. 4 [10], 5 [11], 6 [12], 8 [13], and 10 [14]. A concurrent decrease in chlorophyll content with chemicals of group (B) is regarded as a secondary response to carotenoid deficiency of the cells which is much smaller or nil using shorter herbicide treatments, *e.g.* 1 day instead of 2 [10, 15].

Acifluorfen methyl (no. 11) and the other nitrodiphenyl ether (no. 12) of group (C) are regarded as peroxidative compounds [16]. Specific assays for peroxidative action and for inhibitors of carotene biosynthesis are available [4, 17]. This allows a better discrimination of both types of bleaching herbicides. Therefore, the influence of oxadiazon, a substituted phenoxybenzamide, and its corresponding nitro derivative (no. 12), each taken from one of the three herbicide groups of Table I, on a cell-free carotenogenic enzyme system and on the content of a plastidic lipid was investigated.

As shown in Table II, [2-^{14}C]GGPP is efficiently converted into β-carotene, with comparatively low incorporations into phytoene and phytofluene. In the presence of the phenoxybenzamide (no. 4), however, the flow of radioactivity into β-carotene is inhibited and an accumulation of radioactivity into phytoene and phytofluene can be observed. A useful parameter for inhibition of cell-free carotene biosynthesis, the ratio of phytoene/β-carotene, increased over 30-fold. In contrast, oxadiazon, the nitrophenoxybenzamide (no. 12), and fomesafen gave no pronounced effect. The latter finding corroborates the results with intact *Scenedesmus* [10]: a p-NO_2 group apparently alleviates inhibition of carotenogenesis by a compound like no. 4.

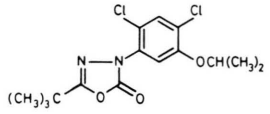
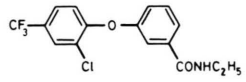
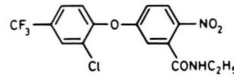
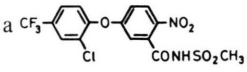
Table III shows the initiation of peroxidative degradation of prelabeled sulfolipid in *Scenedesmus* cells. Over a growth period of 2 days, the amount of sulfolipid was unchanged and no physiological degradation was observed [9]. Oxadiazon and the phenoxybenzamide exhibited no peroxidative properties at concentrations of 1 μM, whereas the nitro derivative (no. 12) is responsible for a 50% degradation of prelabeled sulfolipid. The peroxidative properties of compound no. 12, a nitrodiphenyl ether, were already demonstrated through light-induced formation of short-chain hydrocarbons by *Scenedesmus* cells [10].

Table I. Pigments in heterotrophic *Scenedesmus* grown for 2 days in the presence of inhibitors of chlorophyll (A) and carotenoid (B) biosynthesis or in the presence of peroxidative herbicides (C).

No.	Herbicides added	Concentration applied [μM]	Chlorophyll [mg/ml pcv], %	Total colored carotenoids [mg/ml pcv], %	Phytoene accumulation [mg/ml pcv]	Abbreviation or number of compound
	Control	–	4.8 (100)	0.58 (100)	n.d.	
	Group A					
1		1	3.5 (73)	0.55 (95)	n.d.	Oxadiazon
2		0.1	3.8 (79)	0.53 (91)	n.d.	MK-616
3		5	3.7 (77)	0.57 (98)	n.d.	DTP
	Group B					
4		1	3.8 (79)	0.273 (47)	0.041	BAS 174639
5		0.1	2.0 (41)	0.104 (18)	0.383	–
6		0.1	1.9 (39)	0.099 (17)	0.418	Fluridone
7		0.1	2.2 (47)	0.098 (17)	0.438	MB 38183
8		0.1	3.2 (66)	0.173 (30)	0.115	LS 80.707
9		0.1	2.1 (44)	0.158 (27)	0.123	CGA 22867
10		1	4.1 (86)	0.279 (48)	0.052	R-40244
	Group C					
11		1	1.1 (23)	0.15 (26)	n.d.	Acifluorfen-methyl
12		1	3.1 (64)	0.40 (69)	n.d.	BAS 177985

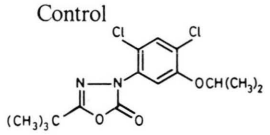
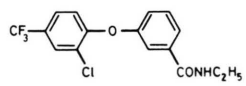
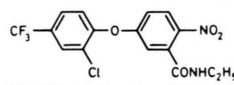
n.d. = not detectable.

Table II. Cell-free carotene synthesis in the presence of bleaching herbicides (10 μM).

No.	Addition	Incorporation of radioactivity [dpm] into			Phytoene/ β-carotene ratio
		Phytoene	Phytofluene	β-Carotene	
	Control	68	50	1004	0.07
1		89	59	986	0.090
	Oxadiazon				
4		1562	174	494	3.16
12		137	53	1112	0.12
12a		61	53	902	0.07
	Fomesafen				

Isolated *Aphanocapsa* thylakoids incubated with 0.7 μCi [2-^{14}C]GGPP, 2 h, at 20 °C (see Methods and [8]).

Table III. Degradation of ^{35}S-prelabeled sulfolipid in *Scenedesmus* cells by bleaching herbicides (1 μM).

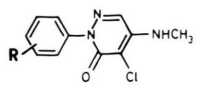
No.	Addition	Radioactivity in sulfolipid (10^3 dpm/l suspension)
	Control	223
1		239
	Oxadiazon	
4		226
12		104

Incubation time was 2 days, with herbicides added, under culture conditions.

From the results of Tables II and III it can be seen that the mode of action of bleaching herbicides is strongly dependent on certain substituents of the molecule. The insertion of a nitro group into the phenoxybenzamide (no. 4) alters an inhibitor of carotene biosynthesis into a compound with peroxidative properties. A substantial additional contribution of carotene-biosynthesis inhibition to bleaching activity can be excluded with our cell-free carotenogenic system.

Table IV compares inhibition of carotene biosynthesis either in intact cells or in cell-free preparations of *Aphanocapsa* by *meta*-substituted 2-phenylpyridazinones [11]. Column (2) shows a decrease in β-carotene formation by the cells. Concurrently, an accumulation of phytoene and phytofluene was observed with all active analogs. For compounds nos. 5 and 13 these precursors were determined quantitatively. With *Aphanocapsa* cells phytofluene is always formed with these inhibitors in contrast to *Scenedesmus*, where phytoene alone is seen. Column (1) demonstrates the degree of inhibition in the cell-free *Aphanocapsa* system by the "inhibition index" which is defined as the ratio of ^{14}C incorporated into phytoene to ^{14}C-labeled β-carotene as referred to the same ratio of the untreated control. Thus a figure higher than 1 indicates inhibition. The 2-phenylpyridazinones are arranged in the order of decreasing inhibition of cell-free carotenogenesis. This arrangement of compounds also matches inhibition of β-carotene formation in *Aphanocapsa* cells except for the m-NO_2 (no. 21) and p-CF_3 (no. 23) analogs which show higher relative activity in intact *Aphanocapsa* cells than in its cell-free system.

Table IV. Inhibition of carotene synthesis by norflurazone derivatives (phenyl-ring substituents) in both the cell-free and cellular system of *Aphanocapsa*.

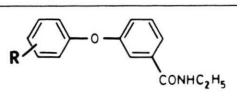
No.		Concentration	(1) Inhibition index[d] of the cell-free system	(2) Inhibition of β-carotene formation in intact *Aphanocapsa* cells % control
		Control	1	100[a]
5	*m*-SCF_3		85	11[b]
13	*m*-CF_3		14	33[c]
14	*m*-OSO_2CH_3		1.9	90
15	*m*-CN	1 μM	1.4	72
16	*m*-NO_2		1.2	61
17	*m*-OCH_3		1	96
18	*m*-H		1	92
19	*m*-OSO_2CH_3		27.3	48
20	*m*-CN		8.6	67
21	*m*-NO_2	60 μM	3.0	21
22	*m*-OCH_3		2.8	77
23	*p*-CF_3		1.5	47
24	*m*-H		1.4	75

[a] Equivalent to 620 μg β-carotene/ml pcv; [b] additional accumulation of phytoene (1330 μg/ml pcv) and phytofluene (247 μg/ml pcv); [c] phytoene 1026 μg/ml pcv and phytofluene 297 μg/ml pcv; [d] for definition see Fig. 1; here, the index is referred to 1 μM or 60 μM of herbicide, respectively.

Apparently, most inhibitors of carotene biosynthesis affect the same target – the phytoene-desaturase complex. Three potential sites of herbicide interference, all resulting in phytoene accumulation, can be postulated. As desaturation of phytoene to phytofluene is an oxidative reaction in *Aphanocapsa* [8] which may involve an electron-transfer system [18], inhibition of either the dehydrogenation reaction or of electron transfer to oxygen is possible. In addition, isomerization has to occur somewhere between 15-*cis* phytoene, the product of phytoene synthase, and the colored carotenoids which have the all-*trans* configuration. Further experiments will have to show whether isomerization of *cis*- to *trans*-carotenes occurs at phytoene or phytofluene. If this takes place at the level of phytoene, the isomerase may be another target. However, herbicide-induced accumulation of phytoene together with phytofluene as reported for difunones [19] and shown here for *m*-phenoxybenzamides (Table II) or 2-phenylpyridazinones (Table IV) excludes inhibition of phytoene isomerization for these compounds, since phytofluene is also accumulated.

The structures of the inhibitors of phytoene desaturation are very different (see Table I, group B). For diphenylamine- and benzophenone-type compounds Rilling [20] suggested competition with phytoene for the catalytic site of the phytoene-desaturase complex. In Figure 1, we have tried to establish a structural relationship between inhibitors and phytoene. A phytoene segment is shown, and the arrow indicates the position where the double bond is inserted by desaturation. The benzophenone compounds chosen in Fig. 1 exhibit maximum inhibition activity in our cell-free system, when parts of their structure resemble the phytoene segment. For example, it can be assumed that the

Table V. Inhibition of carotenogenesis by *m*-phenoxybenzamides (10 μM): influence of phenoxy substituents on inhibition of phytoene desaturase (cell-free *Aphanocapsa* system).

No.	 Substituent R	Lipophilicity π*	Inhibition index
25	H	0	14.6
26	4-chloro	0.71	19.0
27	2,5-dimethyl	1.12	29.4
12	2-chloro, 4-trifluoro-methyl	1.59	45.1

* Values for π were taken from [22]; for definition of the inhibition index see legend of Fig. 1.

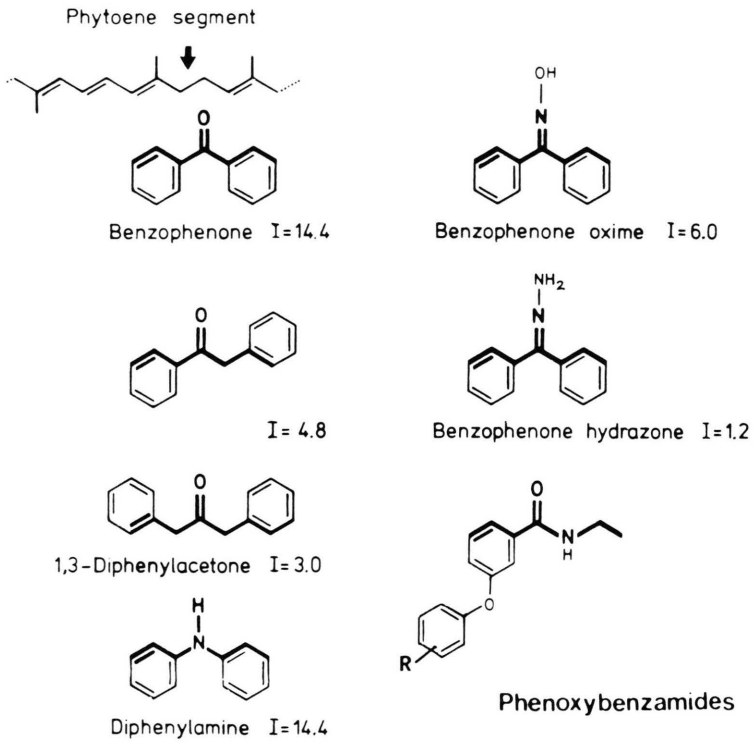


Fig. 1. Structural relationship of inhibitors of phytoene desaturase. Inhibition index *I* is defined as the ratio of ^{14}C incorporated into phytoene to β-carotene in the presence of 10 μM inhibitor as referred to the same ratio of the untreated control. See Table V for inhibition indices of *m*-phenoxybenzamides.

left-hand benzene ring of benzophenone mimics the chromophore of phytoene, while the central portion fits into the active site which is then blocked by the oxo group. When the right-hand benzene ring is brought out of phase by insertion of an additional CH_2-group, thus hindering the attachment of the inhibitor, inhibition decreases as indicated by lower values of the inhibition index *I*. When both benzene rings are out of phase, inhibition is found to be even lower. Substitution of the carbonyl group by an amine results in a similarly effective inhibition, whereas replacement by an oxime or hydrazone decreases the activity since, apparently, these substituents are too bulky to allow a close contact between the inhibitor and the desaturase complex. These differential inhibitory properties can also be observed with a fungal carotenogenic enzyme system [21]. By analogy to the benzophenone derivatives it can be assumed for m-phenoxybenzamides that part of the molecule resembling the phytoene structure (bold-drawn part) is essential for inhibition of phytoene desaturation. In addition, the phenoxy moiety apparently enhances the inhibitory activity by increasing the lipophilicity of the molecule (Table V). The lipophilicity of substituents and inhibition index of the corresponding compounds increase concurrently.

Phytoene desaturation in cell extracts from *Phycomyces* is under negative control of neurosporene, lycopene, β-zeacarotene, and γ-carotene which are all intermediates of β-carotene biosynthesis [23]. Therefore, we cannot exclude the possibility that some compounds of Fig. 1 might act in a similar way on phytoene desaturase at an allosteric site. If this were the case, non-competitive inhibition by these compounds would be expected. Experiments are under way to show whether inhibition is of a competitive or non-competitive type. However, these data are difficult to obtain, since (^{14}C-labeled) phytoene has to be solubilized with detergents and these – in turn-decrease the activity of the carotenogenic system.

Detailed knowledge about targets of phytotoxic chemicals and their effective structural elements should be helpful in the development of new herbicidal compounds. These data represent a first biochemical approach towards elucidating the mode of action of bleaching herbicides which inhibit "phytoene desaturase". The recent development of a cell-free system including photosynthetically active

membranes provides a means of characterizing the enzyme complex responsible for formation of β-carotene and of predicting structures of potential inhibitors directed towards the enzymes of carotene biosynthesis.

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