

Induction of Reversible Tolerance of Algal Cells to Various Herbicides. I. Inhibition of Photosynthesis by Phenol Herbicides and Dibromothymoquinone, Its Reversal and Development of Insensitivity to Different Herbicides

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Dedicated to Prof. Dr. W. Simonis on the Occasion of His 70th Birthday

Photosynthesis, Algal Cells, Reversal of Inhibition, Development of Tolerance, Phenol Herbicides, Dibromothymoquinone

Inhibition of photosynthetic O_2 evolution of algal cells by phenol herbicides (e. g. ioxynil, di-iodonitrophenol, dinitrothymol) and dibromothymoquinone is reversed during treatment within one or two hours. Addition of the same or related inhibitory phenolic compounds as well as of dibromothymoquinone in higher concentrations to these pretreated algae causes no inhibitory effect. The cells become temporarily resistant to these inhibitors. Algal cells pretreated with phenol herbicides are still sensitive to diuron and representatives of some other groups of herbicides. After treatment with dibromothymoquinone, however, the sensitivity of cells to diuron is lowered.

Introduction

The phytotoxicity of many commercial herbicides is caused by inhibition of the photosynthetic electron transport chain at photosystem II. Well known herbicides with this mode of action are substituted phenyl ureas like diuron (DCMU), triazines, triazinones and pyridazinones [1–3]. Recently a new group of photosynthetic inhibitors represented by alkyl substituted 2,4-dinitrophenols like dinitrothymol (DNT) or halogen substituted 4-nitrophenols like bromonitrothymol (BNT) (see Fig. 1), which also effect electron transport chain at the inhibition site of DCMU has been described by Trebst and Draber [4]. However, also the long known herbicides ioxynil [5–7] and 4,6-dinitro-o-cresol (DNOC) [6, 8, 9] belong to this group of phenol herbicides. Concerning the inhibitory effect of ioxynil it was already shown that it can lose its herbicidal activity in plants or even in soil, where it is metabolized by microorganisms [6, 10–12].

In our experiments *in vivo* we observed: a) a reversal of phenol herbicide inhibition of the photosynthetic O_2 evolution in algae and b) that the treated algal cells became insensitive to these herbicides.

A comparable effect was observed if algal cells were treated with the well known plastoquinone antagonist dibromothymoquinone (DBMIB), which inhibits photosynthetic electron transport at a different site than the phenol herbicides or DCMU, *i. e.* after the main functional pool of plastoquinone [13, 14]. In isolated chloroplasts DBMIB inhibition of the electron transport is reversed by decomposition of this compound as well as by addition of thiol reagents (DTT) or serum albumine [15–17].

This paper describes the effects of phenol herbicides and DBMIB on algal cells in regard to a reversal of photosynthetic O_2 evolution and a development of tolerance to these compounds.

Material and Methods

Ankistrodesmus braunii (Naegeli) was grown synchronously [18] in an inorganic medium [19] with a light-dark rhythm of 14 to 10 hours (8000 lx white light, 30 °C, pH 6.2, air + 1.5% CO_2).

All tested compounds were dissolved in methanol before added to the suspension of algal cells or to the culture medium in long term experiments. Final concentration of methanol in the nutrient solution was 1%.

Abbreviations: Aglypt, 3-methylthio-6-phenyl-4-amino-1,2,4-triazin-5-one; BNT, 2-bromo-4-nitrothymol; Chl, chlorophyll; DBMIB, 2,5-dibromo-3-ethyl-6-isopropyl-p-benzoquinone; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DINP, 2,6-diido-4-nitrophenol; DNOC, 4,6-dinitro-o-cresol; DNT, 2,4-dinitrothymol; DTT, dithiothreitol; fenuron, 3-phenyl-1,1-dimethylurea; Mes, 2-(N-morpholino)-ethanesulfonic acid; metribuzin, 3-methylthio-6-t-butyl-4-amino-1,2,4-triazin-5-one; pyramin, 5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone; sencor, metribuzin.

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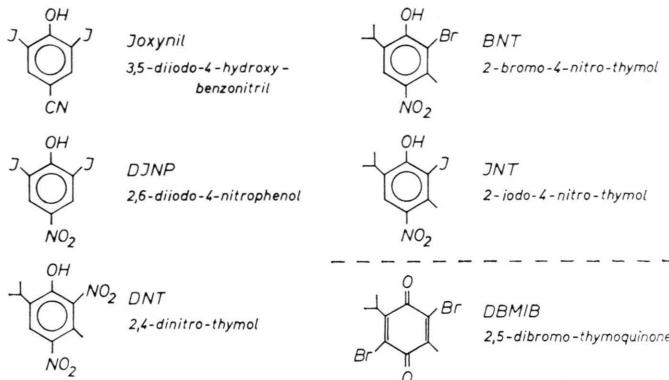


Fig. 1. Chemical structure of the compounds tested in the experiments.

O_2 evolution was measured polarographically with a Beckman electrode in a temperature controlled cuvette which was illuminated with red light (664 nm) by a modified slide projector. The final volume of the reaction mixture was 2.0 ml containing the algal suspension (7–8 μg chlorophyll/ml), MES-buffer (pH 6.5) or phthalat-buffer (pH 5.8) and KHCO_3 (1 mM).

Chlorophyll was determined according to Arnon [20]. Further experimental details are given in the legends to the figures.

Results and Discussion

Ioxynil (Fig. 1) is known for long time as a strong inhibitor of photosynthetic processes [6, 21–23]. In view of the experiments of Trebst and Dräger [4] with chloroplasts using related halogen or alkyl substituted phenol herbicides it was of interest to investigate the mode of action of some of these compounds which are shown in Fig. 1 also *in vivo*. In comparison to these phenolic inhibitors

which block photosynthetic electron transport at the inhibition site of DCMU [4, 16] also the effect of the well known plastochinon antagonist DBMIB with a different site of inhibition [13, 14] should be tested in intact cells.

Photosynthetic O_2 evolution of the green alga *Ankistrodesmus braunii* is inhibited almost completely by a concentration of 10^{-5} M ioxynil, DNT or DINP at pH 5.8 in the medium. The pI_{50} value (the negative logarithm of the concentration for 50% inhibition) for ioxynil and DNT is about 5.7. DINP ($\text{pI}_{50} \sim 5.1$) as well as DBMIB ($\text{pI}_{50} \sim 5.3$) are somewhat less effective. A distinctly higher inhibitory effectiveness in algal cells has been observed with BNT ($\text{pI}_{50} \sim 7$). Measuring the effect of these compounds on photosynthetic O_2 evolution in algae for a period of time (about 60 min or longer), a more or less fast reversal of inhibition during treatment could be realized (Fig. 2). An inhibition of photosynthetic O_2 evolution in *Ankistrodesmus braunii* by ioxynil or DINP (10^{-5} M) up to 20% of the control rate is completely recovered after

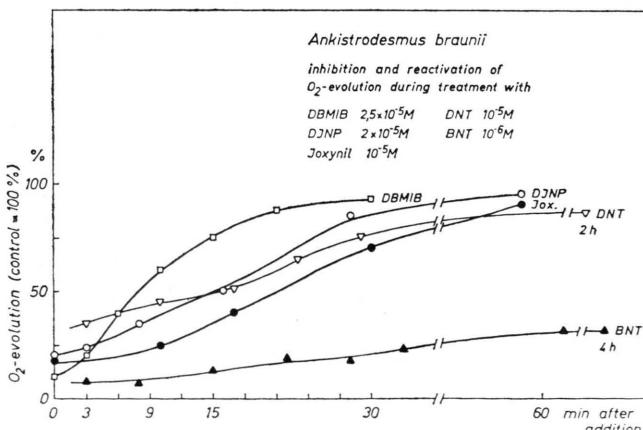


Fig. 2. Time course of reversal of the inhibition of photosynthetic O_2 evolution in *Ankistrodesmus b.* at pH 6.5 in the medium (see Material and Methods). Inhibitors were added at zero to give the final concentrations indicated. Untreated algae evolve about $250 \mu\text{M} \text{ O}_2 \times \text{mg Chl}^{-1} \times \text{h}^{-1}$ (control = 100%).

60 minutes. A full restoration of O_2 evolution in presence of DNT takes about 2 hours whereas inhibition by BNT, which is the most effective of all tested phenolic compounds, shows only a very slow reversal. It is not yet known why there exists a different inactivation of these phenol herbicides. The results, however, may indicate that the ability of algal cells to metabolize these compounds is variable [24, 25]. Also *in vitro* a reversal of inhibition by phenol herbicides caused by addition of serum albumine to isolated chloroplasts has been found recently probably due to the known binding capacity of this protein to phenolic compounds [16]. Possibly in intact cells endogenous proteins act in a similar way.

In comparison to phenol herbicides the inhibition of photosynthesis by DBMIB shows a faster reversal (Fig. 2). About 30 minutes after addition of this inhibitor photosynthetic O_2 evolution in algal cells is near fully restored. A similar reversal of DBMIB inhibition was already found also in isolated chloroplasts by addition of thiol reagents (DTT) or serum albumine [15, 16] but in contrast to phenol herbicides even without any addition of inactivation agents [17]. The latter finding indicates that a decomposition of this or related compounds in chloroplasts takes place. In algal cells the observed fast inactivation of DBMIB may also be caused by endogenous thiols and proteins.

The restoration of O_2 evolution after inhibition by phenol herbicides or DBMIB is faster in the light than in the dark (Fig. 3) and is slowed down at lower temperatures (not shown). At zero degree no reversal of inhibition of photosynthesis can be observed. The reactivation of photosynthetic O_2 evolution is independent from the pH level of the medium. Besides *Ankistrodesmus* other green algae (*Chlorella*, *Dunaliella*) have been tested which show a comparable recovery of photosynthesis after inhibition. As yet no reactivation of photosynthetic O_2 evolution has been found in the blue green alga *Anacystis nidulans* as well as in intact spinach chloroplasts.

Restoration of photosynthesis in algal cells takes place although still a sufficient concentration of herbicides is present in the medium to cause a partial inhibition. By repeated addition of the herbicides (e.g. ioxynil) to the recovered algae it was not possible to inhibit photosynthetic O_2 evolution again (Fig. 4, upper part). The pretreated algal cells have

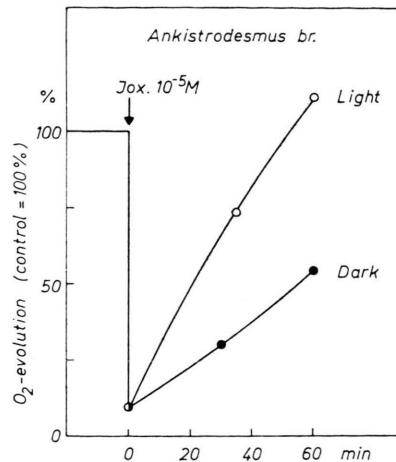


Fig. 3. Restoration of photosynthetic O_2 evolution of *Ankistrodesmus b.* after addition of ioxynil at pH 6.5, if kept the algae in the light or in the dark. Ioxynil (final concentration 10^{-5} M) was added at the time indicated (↓).

become resistant to these herbicides. Even by adding a five to ten fold higher concentration of the herbicides to the tolerant algae, no effect on photosynthesis could be observed. Also treatment of algal cells with lower concentrations of herbicides for a longer period of time induces resistance which can not be abolished by addition of higher concentrations (e.g. up to 30 fold). Culturing these resistant algae for about 24 hours in presence of these herbicides they still remain tolerant. Resuspending in a medium without herbicides, however, the algal cells slowly lose their resistance.

The results in Fig. 4 (upper part) indicate that after restoration of photosynthesis in presence of ioxynil for instance not only other phenol herbicides like DNT or BNT, which block the electron transport chain at the same site, but also DBMIB, which inhibits at another site, provoke no effect on photosynthesis of the ioxynil pretreated algal cells. The same characteristics show also algae pretreated with the other phenolic compounds (e.g. DNT or DINP). In untreated cells all these compounds cause a decrease of photosynthetic O_2 evolution to 20–40% of the control rate (Fig. 4, columns). On the other hand herbicides like DCMU, sencor (metribuzin) or pyramin (pyrazon), interrupting photosynthetic electron transport at the same site as the phenol herbicides, inhibit the ioxynil pretreated algae to about the same degree as the untreated cells (Fig. 4,

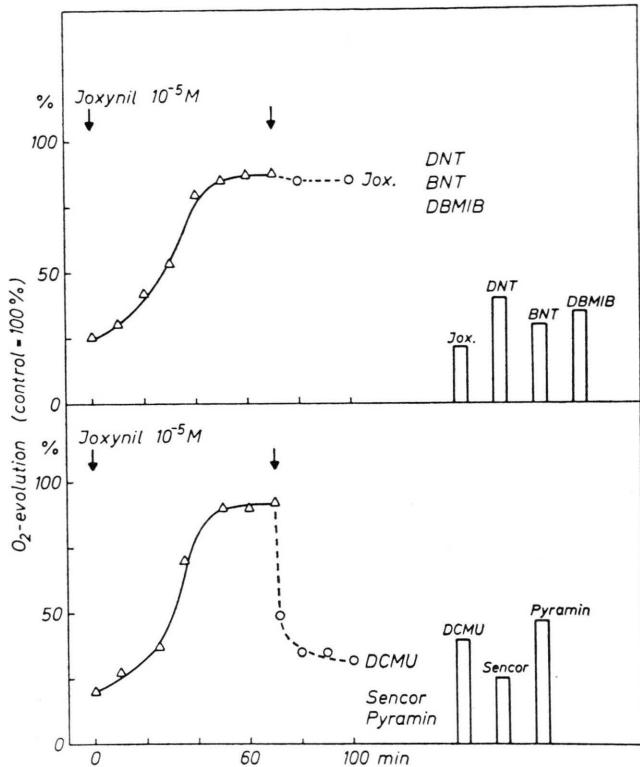


Fig. 4. Restoration of photosynthetic O_2 evolution of *Ankistrodesmus b.* after addition of ioxynil ($10^{-5} M$), see Fig. 2. At the time indicated by an arrow following substances were added (final concentrations): (above) ioxynil ($10^{-5} M$), DNT ($2 \times 10^{-5} M$), BNT ($7.5 \times 10^{-7} M$), DBMIB ($5 \times 10^{-6} M$); (below) DCMU ($2 \times 10^{-7} M$), sencor ($5 \times 10^{-7} M$), pyramin ($10^{-5} M$). The columns show the inhibitory effects of these compounds in algae without pretreatment by ioxynil.

below). The same is true with algae after treatment with DNT or DINP. That means that algal cells pretreated with ioxynil or other phenol herbicides become resistant to this herbicidal group but not to other herbicides like phenyl ureas (DCMU), triazinones (sencor) or pyridazinones (pyramin), although all these herbicides affect the photosynthetic electron transport chain at the same inhibition site [4, 16]. A comparable difference in regard to the mode of action between ioxynil and BNT on one hand and DCMU and metribuzin on the other hand has been shown recently by Reimer *et al.* [16].

From our results it follows so far that the different phenol herbicides tested in our experiments show very similar effects like DBMIB in respect to a reversal of photosynthesis inhibition and development of resistance to this group of herbicides, although both, DBMIB and phenol herbicides, inhibit electron transport at different sites. It can be seen again from Fig. 5 (upper part) that DBMIB treated algal cells neither will be affected by DBMIB itself in concentrations which inhibit photosynthesis in untreated cells to about 20% of the control rate (column) nor by adding phenol herbicides like

ioxynil, DNT, DINP or BNT in similar concentrations as used in Fig. 4.

In contrast to the effect of DCMU shown in Fig. 4, DBMIB treated algal cells had become temporary resistant to DCMU (Fig. 5, middle). Addition of $2.5 \times 10^{-7} M$ DCMU which reduces the rate of photosynthesis in untreated cells to about 50% (column) causes no inhibition in the first 15 minutes after addition to the DBMIB treated algae. But with time a slow inhibition of O_2 evolution by DCMU can be observed. Like DCMU act representative compounds of the triazinone herbicides, *e.g.* metribuzin and aglypt, which normally cause a strong inhibition of photosynthesis at relatively low concentrations (pI_{50} 6.8–6.3) and without a time lag.

If herbicidal compounds, which are less potent inhibitors ($pI_{50} \sim 4.6$) *e.g.* pyramin or even fenuron, a representative of the phenyl ureas like DCMU, were added to DBMIB pretreated algal cells, no resistance was observed (Fig. 5, below). From this finding it may be concluded that there is a difference between the tolerance of algae pretreated with iox-

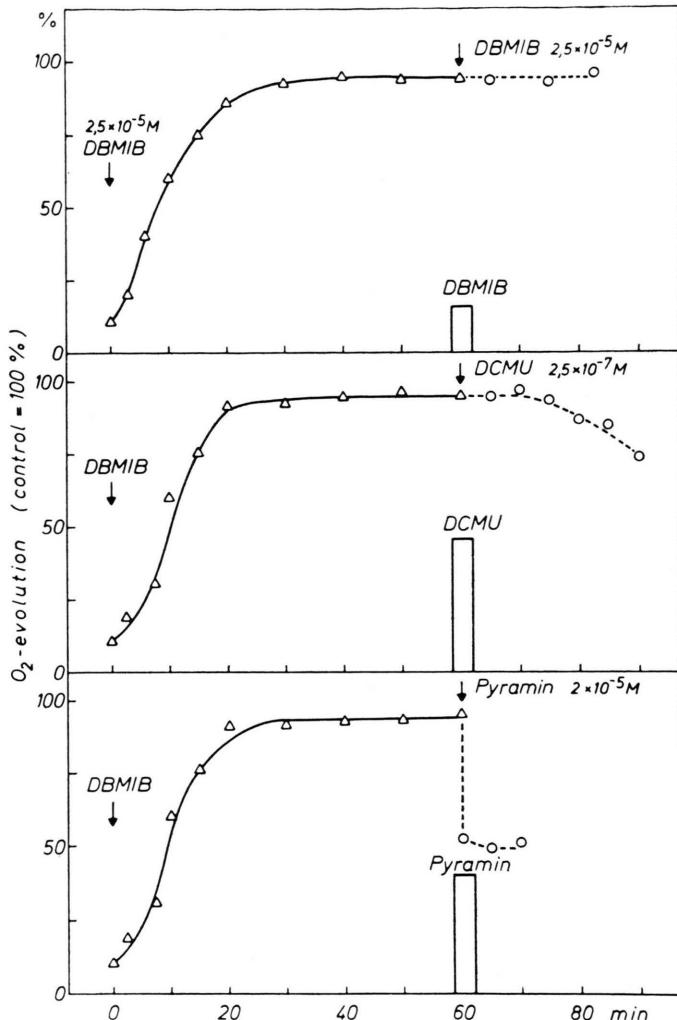


Fig. 5. Restoration of photosynthetic O_2 -evolution of *Ankistrodesmus b.* after addition of DBMIB ($2.5 \times 10^{-5} M$), see Fig. 2. At the time indicated by an arrow following substances were added (final concentrations): (above) DBMIB ($2.5 \times 10^{-5} M$), ioxynil ($10^{-5} M$), DNT ($2 \times 10^{-5} M$), DINP ($5 \times 10^{-5} M$), BNT ($5 \times 10^{-7} M$); (middle) DCMU ($2.5 \times 10^{-7} M$), sencor ($5 \times 10^{-7} M$), aglypt ($4 \times 10^{-7} M$); (below) pyramin ($2 \times 10^{-5} M$), fenuron ($3 \times 10^{-5} M$). The columns show the inhibitory effect of some compounds used (representative for the other) in algae without pretreatment by DBMIB.

nil or with DBMIB in regard to potent and less potent photosynthetic inhibitors. The temporary resistance of DBMIB treated algal cells to DCMU, however, is also depending on the concentrations used (Fig. 6). In these algal cells the pI_{50} value of DCMU inhibition is decreased from 6.8 to 5.7 which means that about a ten fold higher concentration of DCMU is required to cause a similar inhibitory effect on photosynthesis than in untreated cells. The reason for this temporary resistance of algae to DCMU is not yet known. Further details about these effects as well as on tolerance of algal cells to phenol herbicides will be published elsewhere. Whether the recent discussion on a shielding protein at the thylakoid membrane with overlapping binding sites of different herbicides [4] can offer an explanation

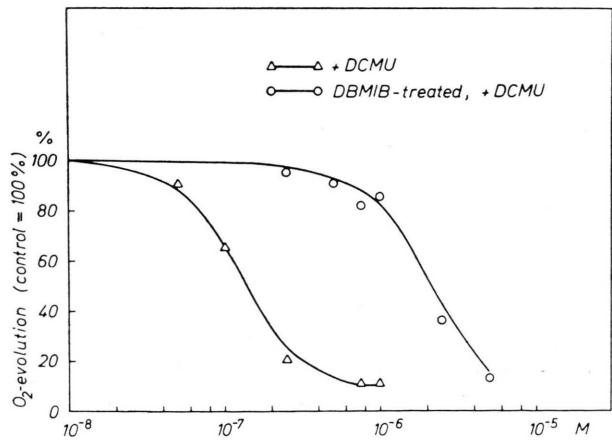


Fig. 6. Inhibition of photosynthetic O_2 evolution of untreated ($\triangle-\triangle$) and DBMIB pretreated ($\bigcirc-\bigcirc$) algae of *Ankistrodesmus b.*

for the effects in algae described above is still open. The alteration of the protein shield by trypsin treatment [26] or a possible modification of the herbicide target site in chloroplasts from herbicide resistant plants [27] have led to comparable effects as we observed with tolerant algae. But also a drastic change of the permeability of membranes within these cells as concluded from studies with resistant *Euglena* cells to 2,4-dinitrophenol [28] has to be taken into consideration.

Although we do not understand the mechanism of this type of herbicidal tolerance in algal cells yet,

these experiments may help to explain the mode of action of photosynthetic inhibitors *in vivo* and offer possibilities to investigate selectivity and resistance within herbicidal action.

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