Differential Effects of Herbicides upon Trypsin-Treated Chloroplasts

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Z. Naturforsch. **34 c**, 1015–1025 (1979); received June 15, 1979

Chloroplasts, Herbicides, Trypsin Treatment, Electron-Transport Inhibitor, Classification

With trypsin-treated spinach chloroplasts and the assay system $H_2O \rightarrow FeCy$, photosynthetic electron transport inhibitors could be subdivided into four groups according to their different inhibitory behaviour under the conditions used.

1. Inhibition exerted by ureas, triazines, triazinones and pyridazinones was reversed to 50–60% as against the untreated control.
2. Inhibition by dibromothymoquinone, trifluralin or the 2,4-dinitrophenylether of 2-iodo-4-nitrothymol was almost completely reversed.
3. Inhibition caused by higher concentrations of nitrofen or the analog RH 2512 could only be reversed to a small extent.
4. Phenol herbicides showed increased inhibitory activity during short-term assays.

Introduction

The binding site(s) of photosynthetic electron transport inhibitors is unknown. The replacement experiment of *e. g.* a substituted [^{14}C]labeled triazine by ureas, pyridazinones or biscarbamate herbicides, as originally shown by Tischer and Strotmann [1], suggested an identical binding component in the thylakoid membrane. This hypothesis was later extended to triazinone and phenol herbicides like ioxynil [2, 3]. There is agreement that all these inhibitors do not necessarily react directly with a redox component of the electron transport chain, but with a protein located between the primary quencher Q of photosystem II and plastoquinone. However, this protein – which may incorporate the electron carrier denoted B or R [4, 5] – may have several binding sites [6], which explains the affinity of chemically non-related compounds to the same thylakoid region. Possibly by cooperative effects mutual replacements of different herbicides are induced, and interference of electron transport from Q to B. Arntzen discusses a lowering of the midpoint redox potential of B induced by the binding of herbicides [7].

Differentiation into subgroups of photosynthetic electron transport inhibitors is limited by replacement experiments. We, therefore, resumed the trypsin studies of Regitz and Ohad [8] and Renger [9, 10]. Apparently, a striking effect of trypsin treatment of isolated chloroplasts is to modify the binding of herbicides or the accessibility to their binding sites. As shown in this paper, the response of trypsin-treated chloroplasts is quite different to certain herbicide groups, indicative of differential binding properties of photosynthetic electron transport inhibitors at the reducing side of photosystem II.

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Abbreviations: Chl, chlorophyll; FeCy, potassium ferricyanide; MV, methylviologen (gramoxone); Herbicides (comp. Table): DCMU (diuron), N-(3,4-dichlorophenyl)-N′,N′-dimethylurea (from Riedel de Haen); Fluometuron, N-(3-trifluoromethylphenyl) N′,N′-dimethylurea (from Ciba-Geigy AG); Atrazine, 2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine (from Ciba-Geigy AG); BAS 44521, chloro-5-methoxy-2-(3-trifluoromethylphenyl)-pyridazin-3-(2H)one (from BASF AG); Sencor (metribuzin), 4-amino-6-*tert*-butyl-3-(methylthio)-1,2,4-triazin-5-(4H)-one (from Bayer AG); DBMIB, dibromothymoquinone; KNJ-724, 2,4-dinitrophenol-2′-iodo-3′-methyl-4′-nitro-6′-isopropylphenyl ether (2,4-dinitrophenylether of 2-iodo-4-nitrothymol) (from Bayer AG); Trifluralin, N,N-dipropyl-2,6-dinitro-4-trifluoromethylaniline (from Riedel de Haen); Nitrofen, 2,4-dichlorophenyl-4′-nitrophenyl ether (from Rohm and Haas); RH 2512, 2-chloro-4-trifluoromethyl-4′-nitrophenyl ether (from Rohm and Haas); Ioxynil, 2,6-diiodo-4-cyano-phenol (from Riedel de Haen); Bromoxynil, 2,6-dibromo-4-cyano-phenol (from Riedel de Haen); Bentazon, 3-isopropyl-2,1,3-benzothiadiazinone-(4)-2,2-dioxide (from BASF AG).

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Materials and Methods

Spinach (*Spinacia oleracea*, "Atlanta") was cultured in the greenhouse at about 18 °C on fertilized soil during the winter. Chloroplasts were obtained by homogenizing 60 g of deribbed leaves in a Sorvall Omnimixer four about 2–3 sec including 160 ml homogenization medium (50 mM tricine-NaOH, pH 8.0; 0.4 M sucrose; 10 mM NaCl, and

5 mM $MgCl_2$). After having removed the debris by filtering through four layers of cheese-cloth and one layer of nylon gauze, the homogenate was centrifuged for 1 min at 4500 × *g*. The pellet, suspended in about 7 ml of 50 mM tricine-NaOH, pH 8.0, including 10 mM NaCl and 5 mM $MgCl_2$, was centrifuged for 2 min at 8000 × *g*. Then, the pellet was resuspended in 4 – 5 ml homogenization medium; the final chlorophyll content being between 1.5 – 2.0 mg/ml.

For trypsin treatment, this chloroplast suspension was adjusted to 0.5 mg Chl/ml including 50 μg of trypsin per ml taken from a freshly prepared stock solution containing 5 mg trypsin/ml. Incubation was done at room temperature under casual shaking for 8 min (see Fig. 1) unless indicated otherwise. Incubation was stopped by placing the suspension into ice. Due to activity losses, trypsin-treated chloroplasts were used for 5 to 7 assays and the activity of the control extrapolated from the beginning through the end of one assay set. No trypsin inhibitor (see ref. [8]) was added. Photosynthetic electron transport was measured by oxygen evolution or uptake at the Clark electrode [11] at 20 °C, with saturating red light of 1.6 kW/m² (using a Schott RG 610 + KG 1 heat filter) with either the systems $H_2O \rightarrow$ FeCy or $H_2O \rightarrow$ MV. The reaction medium contained in a 2-ml volume: 0.1 M sucrose, 50 mM tricine-NaOH, pH 8.0, 5 mM $MgCl_2$, and either 1 mM ferricyanide + 0.75 mM KCN for the first, or 0.25 mM MV + 0.75 mM cyanide for the latter system. In all cases, chloroplast material was equivalent to 25 – 40 μg of chlorophyll. NH_4Cl was 0.5 mM when added as indicated. Reaction time was 40 sec unless mentioned otherwise.

Herbicides were dissolved in acetone or ethanol and kept in the deep freeze for 1 month. In the assays, the solvent was below 10 μl/ml.

All chemicals were from Merck, Darmstadt, including trypsin Nos. 8212 or 24579 from bovine. Another trypsin sample was type III from bovine pancreas, Sigma, Munich. Methylviologen was purchased from Serva, Heidelberg; for herbicides see under abbreviations.

Results and Discussion

Trypsin incubation leads to total decay of the complete linear electron transport as can be observed in the system $H_2O \rightarrow$ MV (Fig. 1 A), or

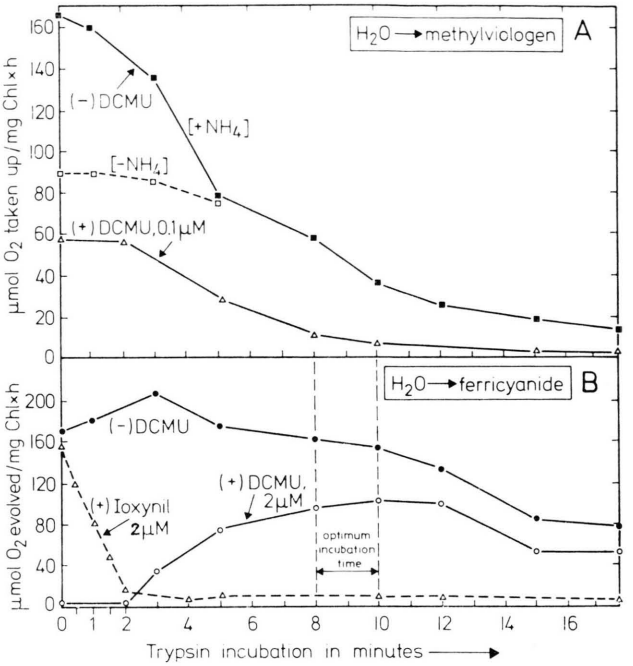

Fig. 1. Trypsin treatment of isolated spinach chloroplasts assayed by the system $H_2O \rightarrow$ methylviologen (A) and $H_2O \rightarrow$ ferricyanide (B). For details see Methods.

$H_2O \rightarrow$ benzoquinone [9]. The degree of DCMU inhibition, being 60 – 70% under the conditions used, remained the same during incubation time while coupling was completely abolished after 4 – 5 min. Ferricyanide reduction, however, is very much preserved at least during the first 10 min, with a concurrent disappearance of DCMU inhibition (Fig. 1 B, comp. refs [8 – 10]). After an 8 – 10 min incubation time, generally 60% of the control rate was achieved in the presence of 2 μM DCMU, a concentration which completely inhibits electron transport in untreated chloroplast material. Addition of NH_4Cl had no effect. An increase of ferricyanide to 5 mM stimulated the rate by 30% in the trypsin-treated sample. The different inhibition patterns described below could be observed better using only 1 mM ferricyanide. The trypsin-treated chloroplasts, kept in ice, lost about 30% of their activity within 30 min, which could not be prevented by adding a trypsin inhibitor (*e. g.* Sigma, type IS from soybean in 10-fold excess *vs.* trypsin). Therefore, generally several trypsin incubations of chloroplasts had to be made for one set of experiments, which naturally increased the average error (see

Table. Classification of photosynthetic electron transport inhibitors as referred to their activity upon trypsin-treated and preilluminated chloroplasts.

Herbicides	Concentration	Pretreatment of chloroplasts:		
		(a) (−) Trypsin	(b) (+) Trypsin	(c) (+) Preillumination, − trypsin
		Percent inhibition of control *		
A.)				
(1) DCMU (Diuron)	0.3 μM	58	29	64
	2 μM	90	40	—
	5 μM	95	47	—
(2) Fluometuron	5 μM	93	47	—
(3) Atrazin	2 μM	56	28	59
	25 μM	88	49	—
(4) BAS 44521	30 μM	50	24	60
	100 μM	83	42	—
(5) Sencor (Metribuzin)	0.1 μM	63	25	—
	0.5 μM	78	35	—
B.)				
(6) DBMIB	0.2 μM	52	8	—
	0.4 μM	66	8	—
(7) KNJ-724	0.05 μM	50	5	40
	1 μM	75	10	—
(8) Trifluralin	6 μM	52	8	49
	13 μM	65	20	—
	50 μM	90	21	—
C.)				
(9) Nitrofen (Tok)	25 μM (+NH_4Cl)	53	45	—
	0.5 mM (+NH_4Cl)	85	76	83
(10) RH 2512	50 μM (+NH_4Cl)	90	65	—
D.)				
(11) Ioxynil	0.1 μM	5 [45]	40	40
	1 μM	10 [77]	70	78
	1 μM (+NH_4Cl)	27 [87]	70	87
	1 μM (H_2O→MV +NH_4Cl)	10 [65]	—	92
	10 μM	76 [90]	70	80
(12) Bromoxynil	5 μM	10 [49]	60	55
	100 μM	90	70	—
(13) 2,6-Diiodo-4-nitrophenol	0.5 μM	16 [70]	72	70
	1.3 μM	77	78	—
(14) 2,4-Dinitrothymol	12.5 μM	11 [80]	77	70
	50 μM	75	80	—
(15) 2-Bromo-4-nitrothymol	0.5 μM	15 [70]	73	66
	2.5 μM	88	72	—
(16) Bentazon	250 μM	13 [80]	62	72
	250 μM (H_2O→MV +NH_4Cl)	5 [67]	55	95
	1 mM	79 [100]	70	80

* System H_2O → FeCy, minus NH_4Cl unless mentioned otherwise. Control rates were 70−100 μmol O_2 evolved/mg Chl×h. With 0.5 mM NH_4Cl present rates were 2.5 to 3 times higher except for the trypsin-treated samples in which NH_4Cl had no influence. Rates for the H_2O → MV system: see Fig. 1. Rates were determined during the first 30 or 40 sec of reaction time, a lag phase of about 10 sec was excluded (see Fig. 2). Data in [ ] brackets of col. a, section D, however, represent inhibition *after* the first 40 sec reaction time. For preillumination see legend of Fig. 2 and Methods. See there also for trypsin treatment of column b. Data are means of 4−5 expts., error ±15%.

Table). Although most of the experiments were performed with trypsin, Merck Nr. 8212, other trypsins showed the same effect, the incubation time could be somewhat shortened. Other spinach varieties, *e. g.* "Montaku" did not change the general observations. However, spinach grown in the open and harvested in spring had higher rates and approximately 20% better recovery from DCMU inhibition after trypsin treatment. Incubation at pH 6.0 instead of pH 8.0 did not improve the inhibition reversal.

After trypsin treatment most noteworthy is the very fast increase of inhibition by ioxynil (Fig. 1 B) or bromoxynil (data not shown). 1 μM ioxynil – which during the assay time of 40 sec used here barely affects electron transport – is drastically effective after a 2 min trypsin treatment. Because ioxynil had the opposite effect as compared to DCMU, it was decided to assay the inhibition pattern of herbicides of different chemical structure upon trypsin-treated chloroplasts.

Fig. 2 exhibits several traces of photosynthetic oxygen evolution (Nos. I to III) and uptake (IV). As demonstrated by Fig. 1, inhibition by ioxynil (and by the herbicides of group D of the Table) was increased drastically, *i. e.* from about 20% during the first 40 sec to about 75% after the reaction had proceeded for about 80 sec or longer. During preparation of this manuscript, a similar time lag was reported by [3]. Preillumination of a complete

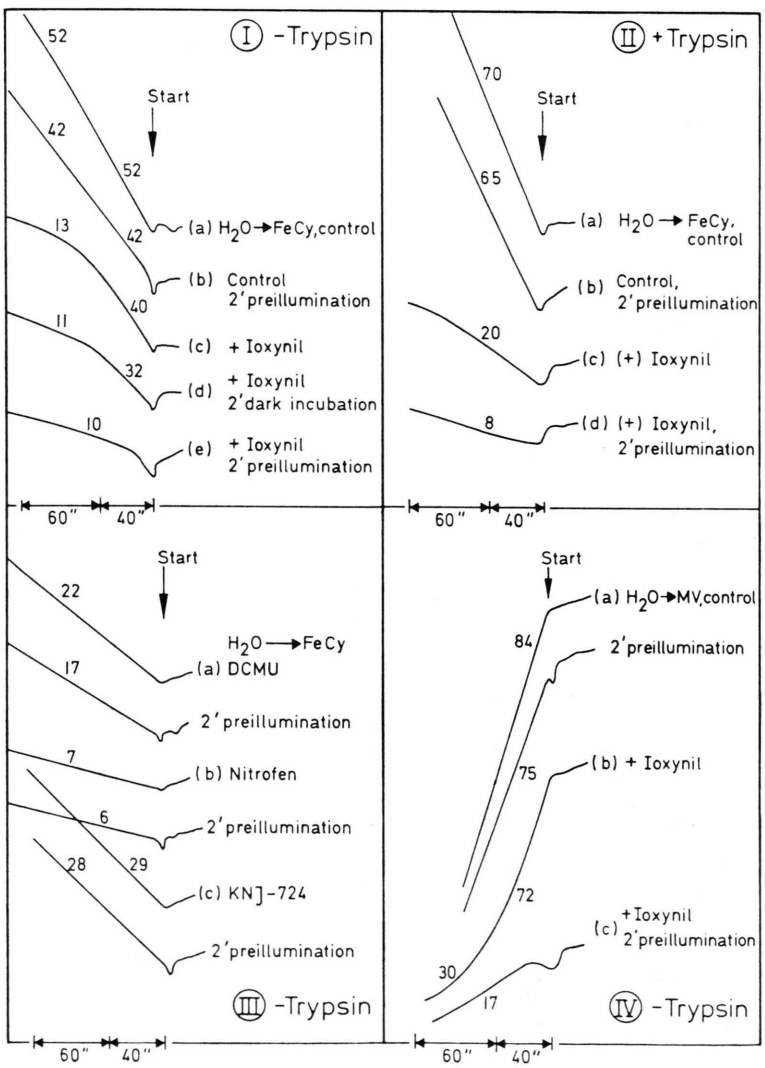

Fig. 2. Tracings of light-induced oxygen gas exchange with isolated spinach chloroplasts. The figures indicate the rate in μmol O_2 evolved or taken up/mg Chl×h during the first 40 sec reaction time or during a 1 min reaction time following the first 40–50 sec of reaction. Preillumination of the assay mixture was done under reaction conditions (see Methods) or for 2 min with the herbicide present and then the reaction started by adding the electron acceptor. Dark incubation was performed accordingly. Ioxynil, 1 μM; DCMU, 0.25 μM; nitrofen, 0.5 mM; KNJ-724, 0.05 μM.

reaction mixture except for the acceptor yielded the same strong inhibition right from the beginning of the assay (traces b and e of part I); a 2 min dark incubation had a similar, though much smaller effect (trace d). This treatment had some albeit low influence upon the control (b). The same effect was more or less clearly observed with other phenol herbicides mentioned in the Table and with bentazon (0.25 mM). It was also observed in the presence of NH_4Cl or when applying the $H_2O \rightarrow$ MV system (part IV). DCMU, nitrofen, KNJ-724 (documented in part III) and other ureas, atrazine or BAS 44521 did not show a substantial preillumination effect.

Trypsin more or less abolished this influence of preillumination (part II). A 70% inhibition (*i.e.* the rate going down from 70 to 20 μmol O_2/mg Chl × h (trace a, c) by 1 μM ioxynil became apparent immediately after the light was turned on, and the rate was quite stable. Preillumination still had little effect increasing the inhibition to about 80% (traces b, c). It should be pointed out that by using higher herbicide concentrations (see Table, part D) an 8 min trypsin treatment although increasing inhibition in the 40 sec short-term assay, somewhat decreased the overall inhibition as compared to the untreated chloroplasts during *e.g.* a 1.5-min long-term assay. This became particularly apparent with bentazon (No. 16). In addition, higher concentrations like 1 mM bentazon exhibit complete inhibition almost immediately after start of the Hill reaction. Apparently, trypsin treatment like preillumination remove an accessibility barrier which can be circumvented in the untreated control by higher herbicide concentrations. Since overall inhibition in the long-time assay is lowered after trypsin treatment, also the binding is seemingly decreased for phenol herbicides and bentazon although less than observed for ureas and others of group A of the Table. This matches the observation of Pallett and Dodge [12] who found a higher I_{50} figure for ioxynil in trypsin-treated pea chloroplasts, but a complete inhibition was still possible by higher ioxynil concentrations in contrast to DCMU or metribuzin.

The Table extends these inhibition studies to other herbicides allowing 4 groups to be tentatively assembled. The first one (A) comprises the "classical" electron transport inhibitors. Applied in concentrations yielding either 80 – 90% or 50% inhibition (col. a) trypsin treatment achieves a 50 – 60% reversal in the $H_2O \rightarrow$ FeCy system with all herbicides mentioned. Preillumination had no effect; presence of ammonia did not change the pattern. It is also noteworthy that high concentrations giving 100% inhibition of the control could not completely inhibit the $H_2O \rightarrow$ FeCy system after trypsin treatment. These compounds are thought to bind at sites which are very similarly affected by trypsin.

Group (B) are herbicides acting at or close to the plastoquinone site (see [6] for KNJ-724 and [13] for trifluralin). Their inhibition is almost completely reversed by trypsin treatment. No preillumination effect is observable.

The third group (C) lists two diphenyl ethers which markedly differ from KNJ-724 in their substitution and were found to inhibit energy transfer and photosynthetic electron transport [14]. In the presence of ammonia, nitrofen and RH 2512 in comparatively high concentrations which yielded about the same degree of inhibition as the compounds of group A, inhibition was almost as effective in the treated chloroplasts as in the control.

The fourth group (D) we were able to compile, lists the phenol derivatives and bentazon. Low concentrations of these herbicides cause substantial inhibition during a long assay time (data in brackets of col. a) or immediately after preillumination (col. c). Trypsin treatment leads to immediate expression of inhibition although somewhat lower than in the controls when checked for longer assay times.

The details of the action of trypsin upon thylakoids are not yet understood. Besides (1) affecting the binding of herbicides [15, 16], trypsin (2) decreases linear electron flow (as shown in Fig. 1 A), and (3) increases the accessibility of ferricyanide to photosystem II [9, 10]. The interactions of these effects have to be clarified. For the moment, we just take trypsin treatment as a tool and simply assume that the different inhibitory activities of herbicides have their bearing on different binding properties of the inhibitors assayed to the quencher/plastoquinone region, which become apparent after trypsin treatment or preillumination. Binding studies have to be carried out to obtain further insight. Our methods allow the rather coarse but quick classification of herbicides which leads to groups that apparently match with some properties concluded from their inhibitory behaviour in partial photosynthetic redox reactions as far as reported.

Acknowledgement

This work has been supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. The authors are grateful for the extremely competent assistance of Miss R. Miller and to the companies mentioned for the supply of pure herbicides. The compounds Nos. 13 – 15 (see Table) were a gift from Dr. A. Trebst, Bochum.

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