The Effects of the Herbicide Sethoxydim on Transport Processes in Sensitive and Tolerant Grass Species

II. Effects on Membrane-Bound Redox Systems in Plant Cells

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Leaf slices of sethoxydim-sensitive (*Poa pratensis, Festuca ovina)* and tolerant *(Poa annua, Festuca rubra)* grass species were found to be able to reduce extracellular ferricyanide by electron transport across the plasmalemma. This reduction was inhibited by the herbicide sethoxydim, and the sensitive species were much more strongly affected than the tolerant ones. The inhibition was concentration dependent. Sethoxydim affected the electron transport in isolated *Poa* chloroplasts. Polarographic and photometric measurements of ferricyanide reduction suggested photosystem II to be the site of action. Inhibition occurred only in the sensitive species *Poa pratensis* and was linearly dependent on concentration. In isolated mitochondria from sensitive *Zea mays* coleoptiles sethoxydim also acted as an inhibitor of electron transport. Only slight inhibition was measured with mitochondria from tubers of tolerant potato (*Solanum tuberosum*).

It is concluded that sethoxydim generally acts as inhibitor of membrane-bound redox systems. The specific inhibition of the plasmalemma-bound redox system of sethoxydim-sensitive plants could contribute to a selective action of the herbicide.

Introduction

Our previous studies of the effect of the herbicide sethoxydim, a cyclohexene-1,3-dione derivative used to control grass weeds in broadleaf crops [1], on transport processes in grass leaves had shown that sethoxydim reversibly reduced the electrical membrane potential of leaf cells. Furthermore, it increased the transient depolarization that occurred at the onset of H^+-alanine-cotransport and simultaneously inhibited amino acid uptake [2]. These results indicated that sethoxydim interacted with primary active membrane transport mechanisms and first suggested the conclusion that the herbicide affected the H^+-translocating ATPase at the plasmalemma. The absence of an inhibition of the H^+-ATPase of plasmalemma vesicles *in vitro* [2], however, prompted us to examine the influence of sethoxydim on other mechanisms at the plasmalemma which possibly contribute to the generation of the electrochemical H^+-gradient.

Evidence has been presented that H^+-transport might result directly or indirectly from the activity of an electron transport system located at the plasmalemma of eucaryotic cells [3–6]. In fact, plasmalemma-bound redox systems have been found in various higher plant cells. It is suggested that plants in general have such a redox system containing chinones, flavoproteins and cytochromes of the b and probably c-type as redox carriers [3, 4, 7, 8]. Cytoplasmatic NADH or NADPH have been proposed as natural electron donors [9–13]. The natural acceptor might be oxygen [14, 15] or an oxidizing component of the soil [12]. For experimental studies extracellular electron acceptors such as the impermeable reagent ferricyanide were used to determine the activity of the plasmalemma redox system [9, 16]. There are already several hypotheses about the physiological function of the plasma membrane redox system, such as blue light perception, control of carrier transport by reducing SH groups or involvement in growth regulation [14, 17]. However, until now the only proven function of the redox system appears to be iron uptake [12, 18]. To date it is still debated if the activity of the redox system is closely associated with the activity of the plasmalemma H^+-ATPase,

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Abbreviations: asc., ascorbic acid; BSA, bovine serum albumine; DCMU, 3(-3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, dichlorophenol indophenol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; Hepes, (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid); MV, methylviologen; PS, photosystem; seth., sethoxydim.

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i.e. if protons are pumped by the H^+-ATPase for charge balance of transported electrons [19, 20], or if the proton efflux proceeds simultaneously by the redox system itself [4, 5].

In the present paper we present evidence for the existence of a redox system in the plasmalemma of *Poa* and *Festuca* leaf cells, and the inhibition of the redox system by the herbicide sethoxydim. Furthermore, we will demonstrate the effects of sethoxydim on other membrane-bound redox systems in plant cells, *i.e.* the photosynthetic electron transport chain in isolated chloroplasts and the respiratory redox system in isolated mitochondria, and will give further informations about the mode of herbicidal action.

Materials and Methods

Plants

Plants of *Poa annua* L., *Poa pratensis* L., *Festuca rubra* L. and *Festuca ovina* L. were grown as described in the concomitant communication [2].

Ferricyanide reduction of leaf slices

Cut leaf slices of *Poa* and *Festuca* species were washed for 16 to 24 h in standard experimental solution 1× (1 mM KCl, 1 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, 0.95 mM Na phosphate buffer pH 5.7) [21]. Trans-plasmalemma ferricyanide reduction was measured in 5 ml 1× standard solution, containing 0.25 g leaf slices and 1 mM $K_3Fe(CN)_6$, at 20 °C, in complete darkness. Various concentrations of sethoxydim (0.01–1.0 mM) dissolved in EtOH, or EtOH as control (0.01–1.0%) were added at the onset of the measurements. The rate of ferricyanide reduction was determined photometrically as the difference in absorbance between 420 and 500 nm.

Isolation of chloroplasts

Chloroplasts were isolated from leaves of 3 to 4 week old *Poa annua* and *Poa pratensis* plants. Apex and base of the leaves were discarded. Five g of leaves were cut into segments with razor blades and homogenized with an ultra turrax for 2–4 min at maximum speed in an ice-cold solution containing: 0.5 M sucrose, 50 mM Hepes-KOH (pH 7.6), 5 mM ascorbic acid, 3.6 mM cysteine and 0.6% (w/v) polyvinylpyrrolidone (PVP-40). The isolation procedure was carried out as described by Heldt and Sauer [22]. The homogenate was filtered through 2 layers of muslin and was then centrifuged three times: a) 4 min at 2900 × *g*, the pellet was resuspended in washing medium containing 0.33 M sucrose, 3 mM KCl, 10 mM Hepes-KOH (pH 7.6); b) 2 min at 200 × *g*; c) 4 min at 2900 × *g*. The final pellet was resuspended in washing medium.

Oxygen evolution

Photosynthetic oxygen evolution by isolated broken chloroplasts was measured in a Clark O_2-electrode (Rank Bros, Bottisham, U.K.). The reaction vessel was illuminated for 3 min with 2000 μmol quanta $m^{-2}s^{-1}$ at 400–700 nm. The oxygen concentration at 20 °C in air-saturated medium was taken as 276 μM [23]. The reaction medium consisted of 0.33 M sucrose, 3 mM KCl, 10 mM Hepes-KOH (pH 7.6), 5 mM NH_4Cl and chloroplasts giving 0.10–0.25 mg chlorophyll/ml. The photosystems were measured separately, *i.e.* PS I with 1 mM ascorbic acid, 60 μM dichlorophenol indophenol (DCPIP), 15 μM dichloromethylurea (DCMU), 0.45 mM methylviologen (MV) and 1.5 mM sodium azide, and PS II with 0.5 mM ferricyanide or 0.15 mM DCPIP. PS I and PS II together were assayed with 0.45 mM MV and 1.5 mM sodium azide. The final volume was 3 ml.

Chloroplast suspensions were preincubated for 30 min with varying concentrations of sethoxydim (0.01–0.5 mM) or EtOH (0.01–0.5%).

Ferricyanide reduction of isolated chloroplasts

Ferricyanide reduction of isolated broken chloroplasts was measured photometrically as the difference of absorbance between 420 and 500 nm. Reduction medium contained washing medium, 0.5 mM ferricyanide, chloroplasts giving 0.10–0.25 mg chlorophyll/ml and varying concentrations of sethoxydim or EtOH. Final volume was 3 ml. The absorbance of the solution was determined before adding the chloroplast suspension. After an incubation of 1 h in a shaking water bath (20 °C, 450 μmol quanta $m^{-2}s^{-1}$ illumination at 400–700 nm) chloroplasts were pelleted (4 min, 2900 × *g*) and the absorbance of the supernatant was measured.

Chlorophyll

The chlorophyll content of chloroplast suspensions was determined by the method of Arnon [24].

Isolation of mitochondria

Mitochondria were isolated from 6 day old etiolated coleoptiles of *Zea mays* as described by Hampp [25]. Seedlings were grown in a dark room maintained at 25 °C and 80% relative humidity. Fifty g of corn coleoptiles were disrupted for 2×5 s with a Braun Starmix in an ice-cold solution, containing 0.3 M mannitol, 1 mM $MgSO_4$, 1 mM $NaNO_3$, 1 mM EDTA, 50 mM Hepes-NaOH (pH 7.3), 2 mM DTT and 0.2% (w/v) bovine serum albumine (BSA). The homogenate was then filtered through 3 layers of muslin and centrifuged for 7 min at 1000×*g*. The pellets obtained were resuspended in washing medium without DTT and centrifuged for 10 min at 8000×*g*. The final pellet was resuspended in a small amount of washing medium. All steps of the isolation procedure were carried out at 0–5 °C.

Mitochondria from *Solanum tuberosum* tubers were isolated as described previously [26].

Oxygen consumption

Oxygen uptake by mitochondrial suspensions was measured in a Clark O_2-electrode (Rank Bros, Bottisham, U.K.) at 20 °C. The reaction medium contained: 0.3 M mannitol, 1 mM $MgCl_2$, 8 mM KH_2PO_4, 0.2% bovine serum albumine (BSA), 50 mM Hepes-NaOH (pH 7.3), 15 mM glutamate and mitochondria giving 0.4–0.8 mg of protein.

Structural integrity of isolated mitochondria was determined as cytochrome *c*-dependent oxygen consumption. To the reaction medium 8 mM ascorbic acid and 25 μM cytochrome *c* were added. Mitochondrial membranes were disrupted with 0.1% triton X-100. With the addition of 0.2 mM KCN nonenzymatic cytochrome *c*-oxidation by molecular oxygen was observed.

Enzyme essay

The activity of succinate-ferricyanide oxidoreductase was measured by following the reduction of ferricyanide at 420 nm and at 500 nm in a dual wavelength photometer (Sigma ZWS II). The assay medium was the reaction medium (see above) plus 1 mM KCN, 0.2 mM ATP and 1 mM ferricyanide. Reaction was started with 10 mM succinate; 50 μM ADP and 5 μM antimycin A were added as indicated. Final volume was 1 ml. The measurements were carried out at 20 °C, in the dark. Mitochondria were preincubated in the reaction medium for 3 min in the presence of sethoxydim or EtOH before the assays were started.

Protein

The protein content of mitochondria suspensions was measured by the method of Peterson [27] with crystalline bovine serum albumine as the standard.

Results

Leaf slices

Fig. 1 shows the reduction of ferricyanide by *Poa* leaf segments in relation to time. Rates of ferricyanide reduction were constant for a period of 4 h. If reducing agents leaking out of the damaged cells of the cut surfaces of the leaf slices were responsible for the extracellular ferricyanide reduction, high initial rates and a subsequent decline of ferricyanide reduction would be expected, so that this artifact is ruled out.

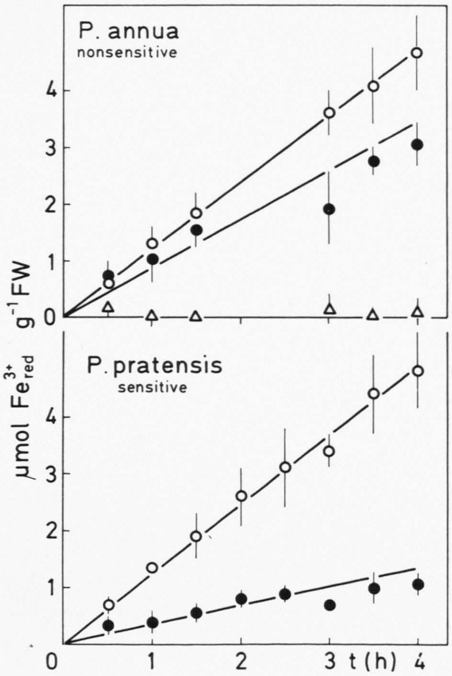

Fig. 1. Time course of ferricyanide reduction by sethoxydim treated leaf slices of the nonsensitive grass *Poa annua* and the sensitive grass *Poa pratensis*. The leaves were incubated with 0.8 mM sethoxydim in the dark, at 20 °C, pH 5.7. Open circles: 1 mM ferricyanide and 0.8% EtOH; closed circles: 1 mM ferricyanide and 0.8 mM sethoxydim; open triangles: 1 mM ferricyanide and 0.8 mM sethoxydim without plant material.

Table I. Rates of ferricyanide reduction by leaf slices of tolerant (t) and sensitive (s) grass species. The reaction was measured for 4 h in the dark, at 20 °C. Ferricyanide concentration was 1 mM. The effect of 0.8 mM sethoxydim on ferricyanide reduction was expressed as % inhibition. Mean values ± SD, number of experiments in brackets.

		μmol Fe^{3+}/g FW/h	% of inhibition
Poa annua	(t)	1.12 ± 0.25 (4)	33 ± 9 (4)
Poa pratensis	(s)	1.16 ± 0.20 (3)	78 ± 1 (3)
Festuca rubra	(t)	1.43 ± 0.36 (3)	55 ± 6 (3)
Festuca ovina	(s)	1.30 ± 0.29 (3)	73 ± 3 (3)

Leaf slices of *Poa* and *Festuca* species reduced extracellular ferricyanide (1 mM) at rates of 0.9 to 1.8 μmol g^{-1} FW h^{-1}. No differences in reduction rates occurred between sensitive and nonsensitive grass species although both *Festuca* species reduced ferricyanide somewhat more strongly than the two *Poa* species (Table I).

In the presence of sethoxydim (0.8 mM) the ferricyanide reduction was inhibited in sensitive and tolerant grass species. However, the effect was much stronger in the sensitive grasses than in the nonsensitive ones (Fig. 1; Table I).

No reduction of ferricyanide was observed in the presence of 0.8 mM sethoxydim without leaf slices in the reduction solution (Fig. 1). Consequently, no chemical reaction occurred between sethoxydim and ferricyanide.

Fig. 2 shows the relation between the extracellular ferricyanide reduction by *Poa* leaf segments and the sethoxydim concentration of the test medium. The reduction rates with leaf cells of both, sensitive and tolerant species, were equally inhibited by the presence of sethoxydim at concentrations up to 0.2 mM. Increasing the herbicide concentration resulted in a further depression of the ferricyanide reduction to about 20% of the control in leaf segments of the sensitive *P. pratensis* whereas the reduction rates of leaf cells of the tolerant *P. annua* remained unchanged at 60% of the control.

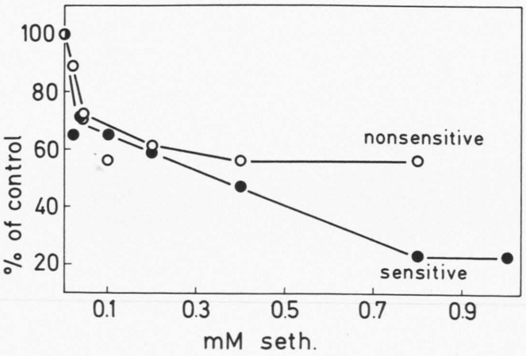

Fig. 2. Dependence of ferricyanide reduction by leaf slices of the nonsensitive grass *Poa annua* (open circles) and the sensitive grass *Poa pratensis* (closed circles) on sethoxydim concentration. The reduction was measured over a time period of 2 h in the dark, at 20 °C, pH 5.7.

Isolated chloroplasts

Isolated broken chloroplasts of *Poa annua* (tolerant) and *P. pratensis* (sensitive) leaves showed oxygen evolution rates of 70 to 150 μmol O_2 h^{-1} mg^{-1} Chl, measured in the presence of different electron acceptors (see Materials and Methods). Control rates of oxygen evolution were similar in both *Poa* species. Fig. 3 shows the influence of several sethoxydim concentrations on the oxygen evolution of photosystems examined separately. No herbicidal effect was observed with chloroplasts of the nonsensitive species *P. annua*. Even at the highest concentration of sethoxydim, *i.e.* up to 0.5 mM, the electron transport rates of both, PS I and PS II corresponded to the control rates. Conversely, in chloroplasts of the sensitive *Poa pratensis* photosynthetic electron transport was reduced by increasing concentrations

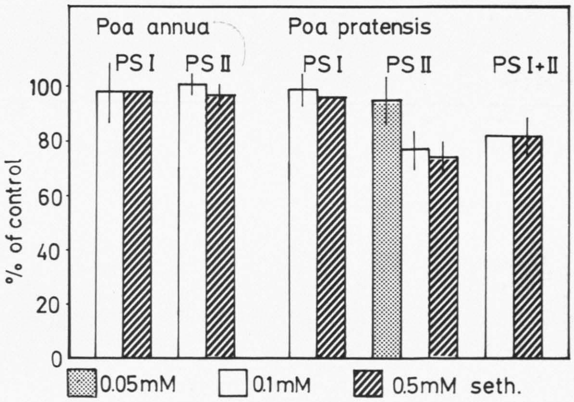

Fig. 3. Effects of different sethoxydim concentrations (0.05; 0.1; 0.5 mM) on the oxygen evolution of photosystem I and II of isolated chloroplasts from *Poa annua* (tolerant) and *Poa pratensis* (sensitive) leaves, as percentage of control. PS I: DCPIP/asc. → MV; PS II: $H_2O \rightarrow$ ferricyanide; PS I + II: $H_2O \rightarrow MV$; chloroplast suspensions were illuminated for 3 min with 2000 μmol quanta $m^{-2}s^{-1}$ at 400–700 nm, at 20 °C, pH 7.6.

of sethoxydim, *i.e.* up to 0.5 mM, to 70% of the control. However, only PS II was influenced by the herbicide whereas PS I remained unaffected. When both photosystems together were measured an inhibitory effect of sethoxydim was also observed.

The same results were obtained when ferricyanide reduction of isolated chloroplasts was measured photometrically. Reduction rates were similar in sensitive and nonsensitive *Poa* species. No ferricyanide reduction occurred without chloroplast suspensions or in darkness, the latter indicating that sethoxydim had no effect on ferricyanide-reductase activity. EtOH concentrations up to 0.5% also did not affect the reduction.

Fig. 4 shows that ferricyanide reduction of chloroplast suspensions of the tolerant *Poa annua* was not inhibited by sethoxydim. Even the tendency of a slight increase in reduction rates was observed. In contrast, sethoxydim inhibited the reduction of ferricyanide by chloroplasts of the sensitive *Poa pratensis*. This inhibition showed a linear concentration dependence.

Isolated mitochondria

Mitochondrial preparations of *Zea mays* coleoptiles showed respiratory activities of 20 to 30 nmol O_2 mg^{-1} protein h^{-1} with the substrate succinate (5 mM) and of 25 to 50 nmol O_2 mg^{-1} protein h^{-1} with ADP (0.1 mM). The mitochondrial electron transport appeared not to be coupled to ATP synthesis, but this was not essential for studying the effects of sethoxydim on the electron transport processes. The integrity of mitochondrial membranes was evaluated by the measurement of cytochrome *c*-dependent oxygen consumption. When the mitochondria have intact outer membranes, exogenous cytochrome *c* is unable to enter, and therefore is not oxidized [28]. Addition of the detergent triton X-100 disrupts the membrane and exogenous cytochrome *c* can be oxidized enzymatically. The comparison of the cytochrome *c*-dependent oxygen consumption before and after addition of triton showed a structural integrity of isolated mitochondria in the preparations obtained from corn coleoptiles of about 90%.

Corn mitochondria reduced ferricyanide when the reaction was started by adding the substrate succinate (Fig. 5). The electron flow to the artificial electron acceptor ferricyanide was increased after addition of ADP but was completely inhibited by antimycin A (Fig. 5a). This indicates that ferricyanide did not penetrate the inner mitochondrial membrane and thus could only accept electrons from endogenous cytochrome *c* located on the outer face of the inner membrane [28]. No ferricyanide reduction could be measured without mitochondria in the presence of succinate or ADP.

After preincubation with sethoxydim for 3 min corn mitochondria showed a decrease in ferricyanide reduction (Fig. 5b, c; 6). In the range of 0.01 to 0.1 mM sethoxydim inhibition of ferricyanide reduction approached 50% of the control and it increased further to about 25% of the control at 0.5 mM sethoxydim (Fig. 6).

The sethoxydim effect on ferricyanide reduction of sensitive corn mitochondria was compared with the

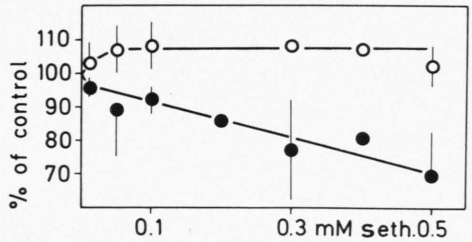

Fig. 4. Dependence of ferricyanide reduction by isolated chloroplasts of the tolerant species *Poa annua* (open circles) and the sensitive species *Poa pratensis* (closed circles) on the sethoxydim concentration. Reduction was determined over a period of 1 h in the light (450 µmol quanta $m^{-2}s^{-1}$ at 400–700 nm), 20 °C, pH 7.6.

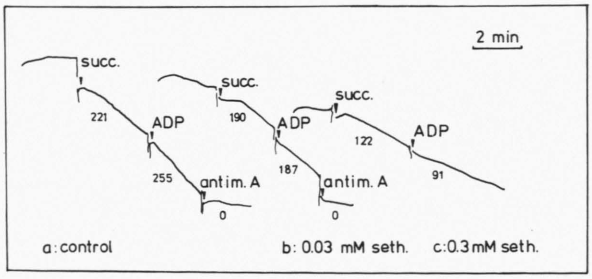

Fig. 5. Dependence of succinate-ferricyanide oxidoreductase activity of isolated mitochondria from *Zea mays* coleoptiles on sethoxydim concentration. The reaction was initiated with 10 mM succinate. ADP (0.05 mM) or antimycin A (5 µM) were added as indicated. The incubation temperature was 20 °C, pH 7.3. Mitochondrial suspensions were preincubated for 3 min in various herbicide concentrations. The numbers on the traces refer to nmol Fe^{3+} reduced mg^{-1} protein min^{-1}.

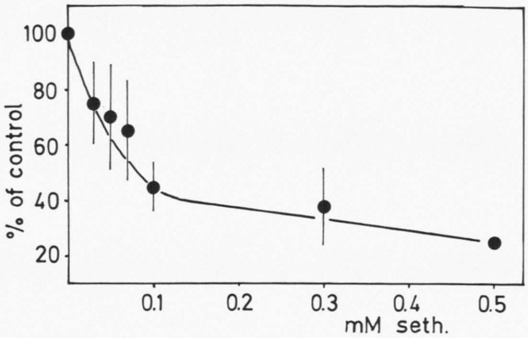

Fig. 6. Effect of sethoxydim (0.01–0.5 mM) on ferricyanide reduction of isolated corn mitochondria in the dark, at 20 °C, pH 7.3.

effect on mitochondria from tubers of tolerant potato (Table II). While 0.1 mM sethoxydim inhibited the ferricyanide reduction in corn mitochondria to about 30 to 40% no inhibition occurred in potato mitochondria. At the highest tested sethoxydim concentration of 0.5 mM the ferricyanide reduction of sensitive corn mitochondria was severely inhibited to 25% of the control whereas the reduction rate of tolerant potato mitochondria only decreased to 68% of the control (Table II).

Table II. Effect of sethoxydim on the ferricyanide reduction by isolated mitochondria from *Solanum tuberosum* tubers (tolerant) and *Zea mays* coleoptiles (sensitive), in the dark, at 20 °C. Data were expressed as % of control. Mean values ± SD, number of experiments in brackets.

Sethoxydim [mM]	*Solanum tuberosum* (% of control)	*Zea mays*
0.1	102 ± 11 (4)	45 ± 9 (4)
0.5	68 ± 11 (3)	25 (1)

Discussion

Evidence for plasmalemma redox activity of *Poa* and *Festuca* leaf segments was obtained by monitoring the reduction of ferricyanide used as an external electron acceptor. Ferricyanide reduction rates were comparable to those of other higher plant tissues (Table I, [10, 13, 20]).

The linear time course of ferricyanide reduction over at least 4 h rules out the involvement of reduction agents that may leak out rapidly from the cut edges of leaf slices. Hence, we conclude that ferricyanide reduction in *Poa* and *Festuca* leaves is a result of the transfer of electrons across the plasmalemma. As in other plant tissues endogenous electron donors to extracellular ferricyanide may be NADH or NADPH [9, 10, 12].

Application of 0.8 mM sethoxydim to leaf segments of sensitive and tolerant species of *Poa* and *Festuca* decreased ferricyanide reduction rates. The sensitive species were much more severely affected by the herbicide than the nonsensitive ones (Table I, Fig. 1). The inhibitory effect depended linearly on the herbicide concentration (Fig. 2).

It remained uncertain if sethoxydim interacted directly with the redox chain in the plasmalemma, or if it interfered indirectly by decreasing the pool of the endogenous electron donor NAD(P)H. The uptake of ^{14}C sethoxydim into leaf slices of *Poa* and *Festuca* species is nearly completed within 1 h [29]. It occurs predominantly at low pH indicating that the lipophilic protonated and electrically neutral molecule accumulates in the lipid phases of cell membranes [29]. It may be possible that sethoxydim also rapidly penetrates to and equilibrates with energy-generating cell organelles, *e.g.* chloroplasts and mitochondria, inhibiting NAD(P)H and/or ATP synthesis.

Our results show inhibition of ferricyanide reduction by isolated chloroplasts of sethoxydim sensitive *Poa pratensis* leaves and of the electron transport of the light reaction II by the herbicide (Fig. 3 and 4). In contrast, photosynthetic electron transport of isolated chloroplasts of the tolerant *Poa annua* appeared not to be affected by sethoxydim.

Sethoxydim also inhibited ferricyanide reduction of isolated mitochondria, and again the inhibition occurred predominantly in mitochondria from the sensitive corn (Fig. 5, Table II). Since sethoxydim inhibits electron transport processes not only at the plasma membrane but also in isolated chloroplasts and mitochondria we conclude that sethoxydim may affect membrane-bound redox systems in general. However, sethoxydim is not known to inhibit photosynthesis *in vivo* [30–32]. Effects of sethoxydim on respiration *in vivo* have been found only several days after application [33].

At this stage it is difficult to evaluate the role, which the specific inhibition of electron transport processes in isolated chloroplasts and mitochondria of sensitive species by sethoxydim may play in its phytotoxicity *in vivo*. Our results suggest that the inhibition of plant redox systems by sethoxydim was caused by a direct interaction of the herbicide with the electron transport chains and not indirectly by reducing the supply of coenzymes, since sethoxydim inhibited the electron transport system of both chloroplasts producing NADPH and mitochondria using NADH. In analogy we may also conclude, that the inhibition of the plasmalemma redox system by sethoxydim was the result of a direct interference.

The effect of sethoxydim was highly specific, in that the plasmalemma-bound redox system and the redox activities of isolated organelles of the sensitive species *Poa pratensis, Festuca ovina* and *Zea mays* were much more strongly affected than those of the tolerant species *Poa annua, Festuca rubra* and *Solanum tuberosum*. The selective inhibition of the plasmalemma redox system by sethoxydim could influence transport processes leading to a perturbation of cell metabolism especially in meristematic regions of plants which have a high turn-over of metabolic activity. Hence, we assume that the inhibition of plasmalemma-bound redox systems contributes to selective action of the herbicide.

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