High- and Low-Affinity Binding of Photosystem II Herbicides to Isolated Thylakoid Membranes and Intact Algal Cells

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High- and low-affinity binding of photosystem II herbicides to isolated thylakoids of *Spinacia oleracea* and to intact cells of the unicellular green alga *Ankistrodesmus braunii* were investigated.

Complete mutual displacement of bound diuron-type herbicides (*e.g.* diuron, atrazine, terbutryn) by either diuron- or phenol-type herbicides (*e.g.* ioxynil, dinoseb) in thylakoids as well as in intact algal cells was found for herbicide concentrations (< 4 nmol bound herbicide/mg Chl) which gave almost saturated high-affinity binding. This demonstrates a high degree of specific binding of these herbicides towards their receptor sites even in intact algal cells. In contrast, phenol-type herbicides are largely unspecifically bound in algal cells.

The mechanism of binding of all photosystem II herbicides at the high-affinity (specific) binding site was found to be competitive. Within the group of diuron-type and of phenol-type herbicides as well as between these two groups, graphical and quantitative analysis of the Lineweaver-Burk plot and of the Dixon plot indicated competitive binding. From this a common binding site for both types of herbicides was concluded. The involvement of two different herbicide binding-proteins is discussed. Low-affinity (unspecific) binding was found to be irreversible in contrast to the easily reversible high-affinity binding. Irreversibility was indicated by a lack of displacement. It is proposed that low-affinity binding represents either a partitioning of the herbicides into the lipophilic parts of the membranes or an attachment to distinct receptor sites. Unspecifically bound herbicides might be responsible for several high concentration effects of the photosystem II herbicides, which are described in the literature. Evidences for the possible existence of a second binding site of these herbicides are presented.

Introduction

The mechanism of action of many herbicides is the inhibition of photosystem II (PS II) reactions at the secondary acceptor B [1]. An important line of evidence for the fact that these compounds share a common binding site, is given by their mutual displacement from thylakoid membranes [2 – 6]. The binding of photoaffinity-labelled herbicides [7 – 9] contributed to the location of the binding sites at a 32 Kd and a 41 Kd protein of the PS II complex. The 32 Kd protein is considered to be the apoprotein of B [7] and contains the binding site for diuron-type herbicides. Binding of the phenol-type herbicides seems to require the 41 Kd protein [8, 9].

Herbicide binding to chloroplast membranes is not exclusively restricted to the 32 Kd or the 41 Kd proteins [2, 7 – 10]. While binding to these proteins shows a high binding affinity (small binding constant K_b), additional low-affinity binding to other structures is indicated in several binding studies. For high-affinity binding, a direct correlation between binding and inhibition of photosynthetic electron flow has been shown [2, 10, 11]. The binding constant (K_b) and the inhibition constant (K_i) were shown to be well comparable in thylakoids and intact algae for several PS II herbicides [2, 10, 11]. High-affinity binding is therefore also called 'specific' binding [2, 3]. All other cases of herbicide binding, which are not directly correlated with inhibition were called 'unspecific'.

More recently binding and displacing properties of phenol-type herbicides (*e.g.* dinoseb [3, 10], ioxynil [10, 12] and bromonitrothymol [12]) have become subject of herbicide studies. With increasing concentrations of bound dinoseb, Oettmeier and Masson [3] found a decreasing degree of dinoseb dis-

Abbreviations: Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine; Chl/I, chlorophyll molecules per bound herbicide molecule; DCPIP, dichlorophenolindophenol; dinoseb, 2-*sec*-butyl-4,6-dinitrophenol; DNSJ, 2-iodo-4-nitro-6-isobutylphenol; diuron, DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediamintetraacetic acid; HEPES, *n*-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; K_b, equilibrium constant of specific herbicide binding; K_{bb}, equilibrium constant of specific herbicide binding, evaluated with unlabelled herbicides; K_i, equilibrium constant of inhibition of photosynthesis; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; MES, 2(N-morpholino)-ethanesulfonic acid; PS II, photosystem II; X_g, concentration of herbicide binding sites.

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placement by metribuzin or diuron. This rises the question on the nature of low-affinity herbicide binding. For the binding of monuron to intact chloroplasts and isolated thylakoids, the existence of a biphasic binding process has already been described [13, 14]. The second binding process, taking place at higher concentrations, was not correlated with inhibition of photosynthesis and showed only slow removal of bound monuron by successive washings [13].

Previously we reported about herbicide binding to isolated thylakoids and to intact algal cells [10]. Binding constants (K_b) and the concentrations of binding sites (X_g) were found to be well comparable, especially for diuron-type herbicides. In this paper, the results of displacement experiments with isolated thylakoids and intact algal cells are presented. The results are analysed in order to characterize the special nature of phenol-type herbicide binding and to describe low-affinity binding of both, diuron- and phenol-type herbicides.

The analysis of binding experiments in the presence of a second unlabelled herbicide enables to distinguish between competitive and non-competitive binding. Tischer and Strotmann [2] found a competitive mechanism of binding for metribuzine and diuron. In contrast, non-competitive binding was detected by Oettmeier and Masson [3, 8] for mutual displacement of the phenol dinoseb and the phenylurea diuron. This could indicate the existence of two different binding sites for the two classes of herbicides. The results of extensive studies on the mechanism of competition of phenol- and diuron- type herbicides are presented.

Materials and Methods

The green alga *Ankistrodesmus braunii* (Naegeli) was grown in a synchroneous culture as described [10]. Thylakoids were isolated from freshly harvested, greenhouse grown spinach (*Spinacia oleracea* var. Monatol) in a medium containing 0.33 M sorbitol, 2 mM $MgCl_2$, 2 mM EDTA, 2 mM $MnCl_2$ and 50 mM MES-KOH (pH 6.5). Osmotic disrupture of the chloroplasts was done in a medium without sorbitol, containing only 5 mM HEPES-KOH (pH 7.6), 2 mM $MgCl_2$, 2 mM EDTA and 2 mM $MnCl_2$. Isomolarity was finally restored by addition of the same volume of 95 mM HEPES-KOH, 0.66 M Sorbitol and 2 mM of $MgCl_2$, $MnCl_2$ and EDTA. For further detail of the isolation procedure see [10].

Radioactively labelled herbicides from different chemical classes were used for the binding studies. As diuron-type herbicides, the phenylurea [^{14}C]-diuron (5.3 μCi/mg) and the s-triazines [^{14}C]atrazine (27.2 μCi/mg) and [^{14}C]terbutryn (7.9 μCi/mg) were used. As phenol-type herbicides, the benzonitrile [^{14}C]ioxynil (33.5 μCi/mg) and the nitrophenols [^{3}H]-i-dinoseb (1.31 mCi/mg) and [3H]-2-iodo-4-nitro-6-isobutylphenol (DNSJ, 1.037 mCi/mg) were applied.

Binding experiments were performed as previously described [10]. Computation of the bound herbicide concentration was done by substracting the free herbicide concentration from the total concentration. The total concentration was determined from a separate calibration. Competition or displacement experiments were performed by incubating 13 ml of a suspension of isolated thylakoids or intact algal cells with a defined concentration of radioactively labelled herbicide in the dark. The chlorophyll concentration was 40 – 70 μg/ml in experiments using labelled diuron, atrazine, terbutryn, ioxynil or DNSJ. 150 μg Chl/ml were used for experiments with dinoseb. Incubation took place in isolation medium at pH 7.6 (thylakoids) or in nutrient medium at pH 6.3 (algae). For equilibrium binding, thylakoids were incubated with labelled herbicide for 10 min, algae were incubated for 30 min [10]. The displacing, unlabelled herbicide was added to samples (1 ml) of thylakoid or algal suspension previously incubated with the labelled herbicide. The incubation time for the displacing herbicide was similar to that of the previously bound radioactive herbicide. Finally the suspension was centrifuged for 45 sec at 8000×g in a Beckman Minifuge B. Radioactivity in the supernatant was determined in a Berthold BF 8000 liquid scintillation counter as described before [10].

Graphical evaluation of the experiments was done by plotting the concentration of bound, labelled herbicide on a chlorophyll basis *versus* the total concentration of the displacing herbicide. That point of the resulting graph, that indicates equal concentration of both herbicides has been named isomolar point. A method of computing the equilibrium binding constants for unlabelled herbicides (K_{bb})was applied by using an equation of Tischer [11]. Based on this equation, a displacement curve was calculated

by a least square approximation, which fits best to the measured data. Alternatively K_{bb} was computed from the Lineweaver-Burk plot of binding experiments in the presence of the unlabelled herbicide. When the slope of the straightlines (*c.f.* Fig. 5) is plotted *versus* the total concentration of the unlabelled herbicide, a new straightline could be fitted to these data. The resulting abscissa intersection revealed the K_{bb}-value [15]. Both methods, the analysis of displacement experiments with the equation of Tischer [11] and the analysis of the Lineweaver-Burk plots gave identical results.

Experiments on the elucidation of the mode of competition of two mutually displacing herbicides were performed with binding experiments [10] in the presence of an unlabelled herbicide [2, 3, 11]. After equilibrium of binding of a constant concentration of unlabelled herbicide, increasing concentrations of labelled herbicide were added. Incubation times for these herbicides were the same as described above. Analysis of the mechanism of competition was done, using the Lineweaver-Burk plot and the Dixon plot [16] of the binding data. In the double reciprocal Lineweaver-Burk plot competitive binding of two compounds is indicated by a common intersection of the straightlines on the ordinate (constant concentration of binding sites, X_g) whereas the abscissa intersection approaches zero (decreasing binding affinity, K_b) with increasing concentrations of the competing herbicide.

Non-competitive binding leads to a decreasing number of X_g and a constant K_b. In the Dixon plot [16] competitive binding is indicated by an intersection of the straightlines different from the abscissa and by a slope of the straightlines approaching zero.

All herbicides used in these experiments were dissolved in pure methanol. The final methanol concentration never exceeded 1%. Chlorophyll determinations were performed as described [17].

Results

1. High- and low-affinity binding of [^{14}C]terbutryn to thylakoids and intact algal cells

In a preceeding paper high-affinity binding of diuron and atrazine to isolated thylakoids and intact algal cells has been shown to saturate at a concentration of 3–4 nmol bound herbicide/mg Chl, which corresponds to 250–330 Chl molecules per bound herbicide molecule (Chl/I-ratio) [10]. This stoichiometry of chlorophyll and herbicide suggests, that one herbicide molecule is bound per photosynthetic unit [18, 19].

For investigation of high- and low-affinity herbicide binding, the highly active s-triazine [^{14}C]terbutryn, also a PS II herbicide (20) was used. The binding of [^{14}C]terbutryn to isolated thylakoids and to intact algae was found to be biphasic with a very distinct high- and low-affinity binding phase (Fig. 1). With equilibrium constants of specific binding of $K_b = 3.0 \times 10^{-8}$ M (thylakoids) and $K_b = 4.6 \times 10^{-8}$ M (algae) specific terbutryn binding was similar in both cases. The concentration of specific binding sites (X_g) was also almost identical with 230 Chl/I (4.3 nmol binding sites/mg Chl) in thylakoids and 242 Chl/I (4.13 nmol binding sites/mg Chl) in algae. Up to a concentration of 20 nmol bound [^{14}C]terbutryn/mg Chl, corresponding to a total [^{14}C]terbutryn concentration of 3.0×10^{-6} M predominantly one low-affinity binding phase in thylakoids and algae was found (Fig. 1 b, e). Also monophasic low-affinity binding is very clearly demonstrated in the Scatchard plot (Figs. 1 c, f).

When low-affinity binding took place at bound herbicide concentrations higher than 3–4 nmol/mg Chl, high-affinity binding was already saturated and could be taken as constant. By substracting the amount of specific binding (calculated from Figs. 1 b, e) from total binding, pure low-affinity binding was obtained. While the relationship between bound and free terbutryn was described by a hyperbola in case of high-affinity binding, for low-affinity binding this function seemed to be sigmoid (Fig. 1 a, d). The same type of function was found for low-affinity binding of the phenol-type herbicide DNSJ (data not shown). As a consequence of this sigmoidicity, the binding constant and the concentration of binding sites of low affinity-binding could not be computed from the Lineweaver-Burk plot. For characterization of low-affinity binding, however, an apparent K'_b was tentatively estimated to be 4.6×10^{-6} M (thylakoids) or 4.0×10^{-6} M (algae) respectively. It should be noted, however, that the 'apparent' K_b-value does not describe the actual dissociation constant of the herbicide-receptor complex.

2. Displacement of bound herbicides in isolated thylakoids and intact algal cells

It is well known that specifically bound diuron-type herbicides easily displace each other from thy-

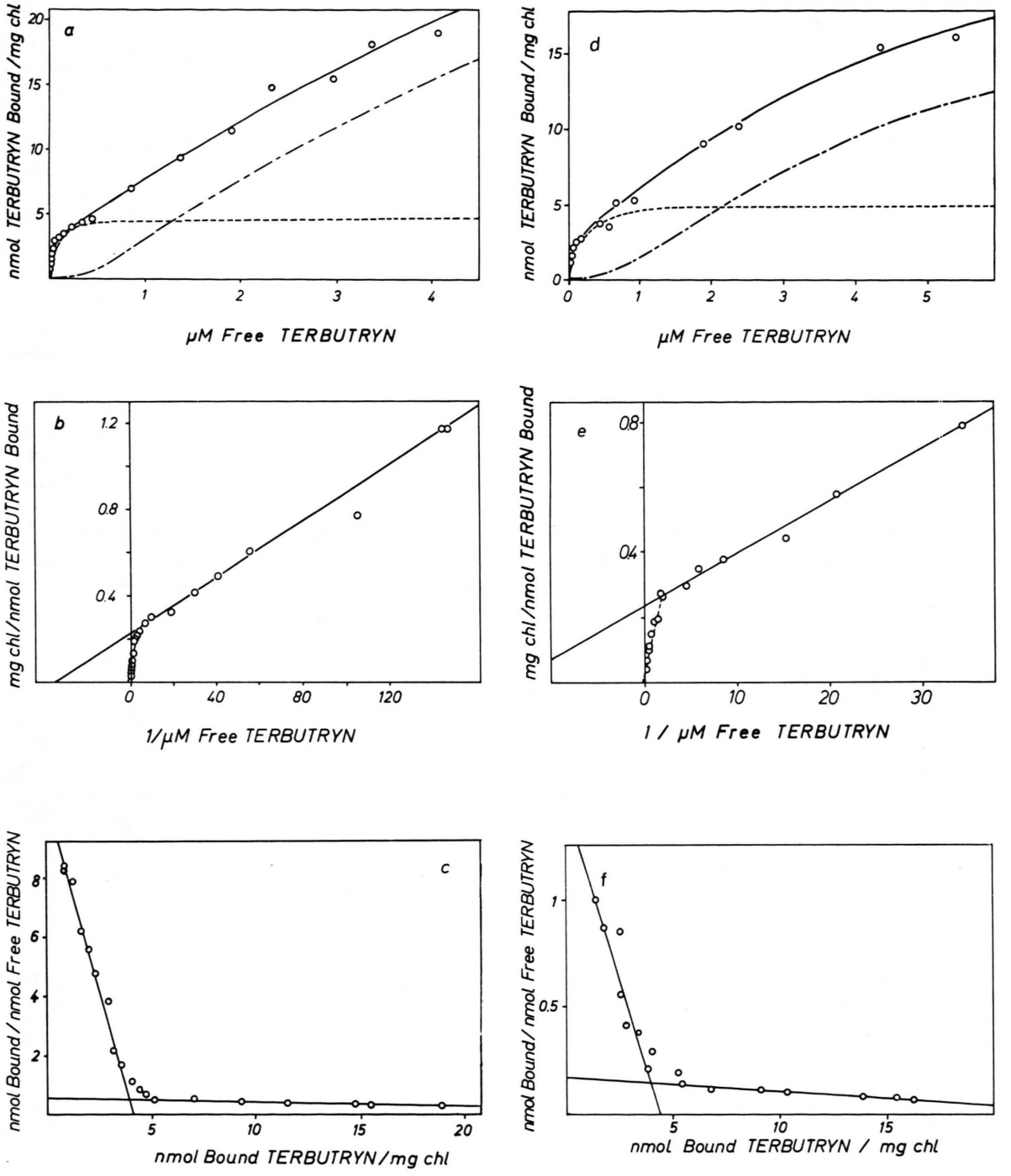

Fig. 1. Binding of [^{14}C]terbutryn to isolated thylakoids (a, b, c) and intact algal cells (d, e, f). Michaelis-Menten plot of [^{14}C]terbutryn binding (a, d). Pure high-affinity (– – – –) and low-affinity (– · –) binding were calculated from total binding (—). Lineweaver-Burk plot of binding (b, e). K_b and X_g are taken from the abscissa- and the ordinate intersections of the straightlines. Scatchard plot of bound over free [^{14}C]terbutryn versus bound [^{14}C]terbutryn on a chlorophyll basis (c, f).

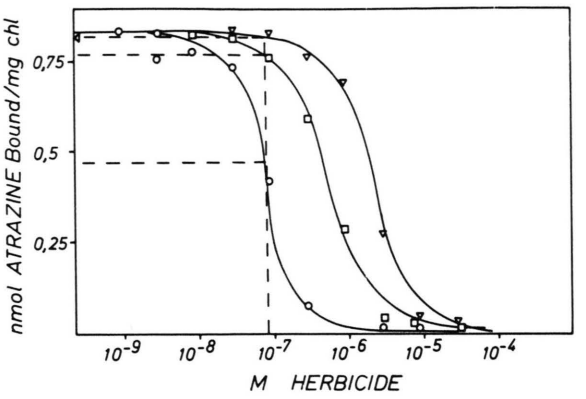

Fig. 2. Displacement of bound [^{14}C]atrazine in algae by increasing concentrations of diuron (○–○), ioxynil (□–□) and dinoseb (▽–▽). The concentration of [^{14}C]atrazine was 7.7×10^{-8} M. The broken lines show the points of isomolarity of both herbicides.

lakoid membranes [2, 4, 5, 11]. If these herbicides are bound in algae in concentrations where high-affinity binding is predominant, mutual displacement should also be expected. As can be seen in Fig. 2, in algal cells [^{14}C]atrazine was completely displaced by diuron at an initially bound [^{14}C]atrazine concentration of 0.8 nmol/mg Chl. In the presented experiments, about 40% of [^{14}C]atrazine was displaced by diuron at the isomolar point. Also the phenol-type herbicides ioxynil and dinoseb completely displace [^{14}C]atrazine from its binding sites in algal cells (Fig. 2). In these cases, however, less than 10% of the bound [^{14}C]atrazine were displaced at the isomolar point. These experiments demonstrate a high degree of specific binding of the PS II herbicides in intact algal cells.

The binding constants (K_{bb}) of unlabelled diuron, ioxynil and dinoseb could be computed from this type of displacement experiments. The K_{bb}-values, obtained by an approximation of a theoretically calculated competition curve to the experimental data [11], are in good agreement with the binding constants determined directly in binding experiments (Table I). For binding of diuron, dinoseb and ioxynil in algae, K_{bb}-values of 1.2×10^{-8} M, 2.5×10^{-7} M and 4.1×10^{-7} M were calculated.

For investigation of low-affinity binding, displacement experiments were performed with previously bound concentrations of labelled terbutryn, giving comparable amounts of high- and low-affinity binding (Fig. 3). The displacement of [^{14}C]terbutryn in a concentration up to about 10 nmol bound/mg Chl by terbutryn itself was tested in both, thylakoids and algal cells (Fig. 3). As expected, at low concentrations of 0.4 nmol (thylakoids) or 0.8 nmol (algae) bound herbicide/mg Chl, almost complete displacement was found. The K_{bb} of [^{12}C]terbutryn was calculated to be 2.9×10^{-8} M (thylakoids) and 3.9×10^{-8} M (algae), which again was in good agreement with those K_b-values obtained from binding experiments (Table I).

At a concentration of about 10 nmol initially bound [^{14}C]terbutryn/mg Chl, neither in thylakoids nor in algae complete displacement could be observed. In the thylakoid experiment (Fig. 3 a) at a total concentration of 2.6×10^{-6} M [^{14}C]terbutryn, corresponding to 8 nmol bound/mg Chl, only about 4 nmol/mg Chl [^{14}C]terbutryn was displaced. This corresponds well to the amount of saturated high-affinity terbutryn binding (Fig. 1). In the algal experiment (Fig. 3 b), at a total concentration of

Table I. Equilibrium constants of binding (K_b, K_{bb}) and of inhibition of photosynthetic electron transport (K_i) of the herbicides diuron, atrazine, terbutryn, ioxynil and dinoseb. The K_b-values are calculated from binding experiments (a), K_{bb}-values from displacement experiments (b) and from experiments of competitive binding (c). The K_i-values for isolated thylakoids are measured photometrically with DCPIP-reduction ($H_2O \rightarrow$ DCPIP) (d) [10]. In algae experiments, K_i was determined from the inhibition of oxygen evolution (e) (10).

	Isolated thylakoids				Intact algal cells			
	K_b [M] (a)	K_{bb} [M] (b)	K_{bb} [M] (c)	K_i [M] (d)	K_b [M] (a)	K_{bb} [M] (b)	K_{bb} [M] (c)	K_i [M] (e)
Diuron	1.9×10^{-8}	2.0×10^{-8}	2.5×10^{-8}	3.8×10^{-8}	2.8×10^{-8}	1.2×10^{-8}	3.5×10^{-8}	3.0×10^{-8}
Atrazine	6.7×10^{-8}	7.5×10^{-8}	4.2×10^{-8}	4.5×10^{-8}	1.6×10^{-7}	2.4×10^{-7}	1.2×10^{-7}	–
Terbutryn	3.0×10^{-8}	2.9×10^{-8}	3.5×10^{-8}	3.2×10^{-8}	4.6×10^{-8}	3.9×10^{-8}	3.0×10^{-8}	–
Ioxynil	2.4×10^{-8}	3.6×10^{-8}	5.0×10^{-8}	4.2×10^{-8}	4.5×10^{-7}	4.1×10^{-7}	–	–
Dinoseb	3.6×10^{-7}	5.7×10^{-7}	3.5×10^{-7}	7.8×10^{-7}	1.4×10^{-7}	2.5×10^{-7}	–	–

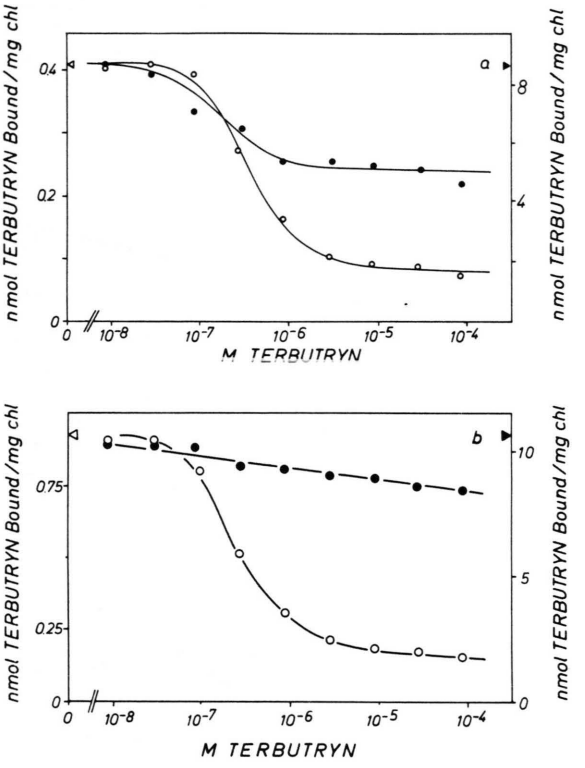

Fig. 3. Displacement of [^{14}C]terbutryn, bound with high (○–○) and low (●–●) binding affinity, by [^{12}C]terbutryn in thylakoids (a) and intact algae (b).

3.6×10^{-6} M [^{14}C]terbutryn, corresponding to 11 nmol bound/mg Chl, also about 3 nmol/mg Chl, but not the bulk of the bound [^{14}C]terbutryn could be displaced. In case of reversible high-affinity herbicide binding, however, displacement of terbutryn by itself was found to be complete (data not shown). Nevertheless, the presented results demonstrate, that low-affinity bound [^{14}C]terbutryn, although bound with a relatively low 'apparent' binding constant of 4×10^{-6} M, cannot be displaced by terbutryn itself. Low-affinity herbicide binding therefore seems to be an irreversible process.

Concerning their displacement the phenol-type herbicides show a similar behaviour in thylakoids as diuron-type herbicides. From thylakoid membranes, an initially bound concentration of 3 nmol [^{3}H]dinoseb/mg Chl was completely displaced by diuron and ioxynil (Fig. 4a). A calculation of the K_{bb}-value of diuron from this experiment revealed 8.8×10^{-8} M. At higher concentrations of bound [^{3}H]dinoseb, up to 14 nmol/mg Chl, also this phenolic herbicide could not be displaced effectively from thylakoids by diuron or even by dinoseb itself (Fig. 4a). Only ioxynil seemed to be a little more efficient in displacing dinoseb from thylakoids. In algae, the results of [^{3}H]dinoseb displacement experiments were found to be contrary (Fig. 4b). Even in a concentration as low as 0.007 nmol bound [^{3}H]dinoseb/mg Chl, corresponding to a total concentration of 7×10^{-9} M, only little displacement by diuron, atrazine and dinoseb itself was observed. Ioxynil proved to be somewhat more efficient in displacing dinoseb than the other tested herbicides. This lack of dinoseb displacement seemed to be typical for phenol-type herbicides bound in algal cells. It may be further indicated by the observation that [^{12}C]dinoseb could stimulate [^{14}C]ioxynil binding in algal cells up to fivefold but not in thylakoids (data not shown). Maximum stimulation was found near the isomolar point. These data will be presented elsewhere.

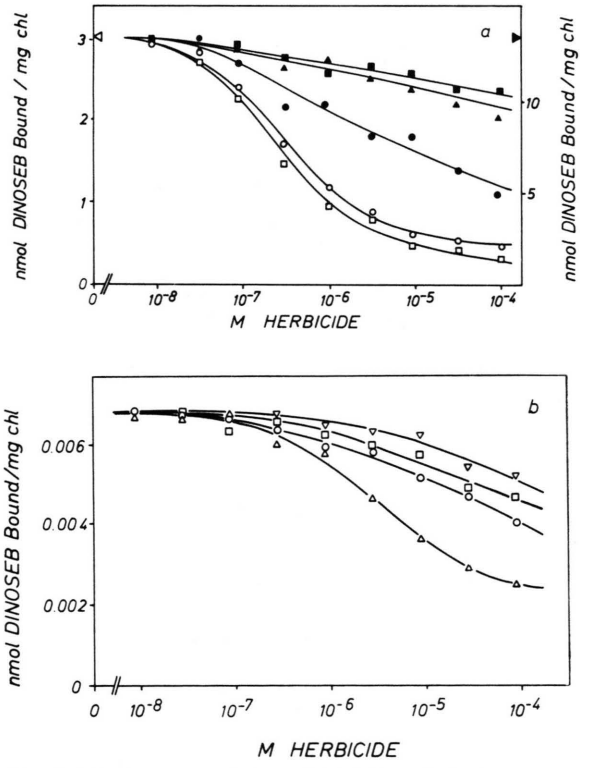

Fig. 4. Displacement of bound [^{3}H]dinoseb in thylakoids (a) by diuron (□–□ ■–■), ioxynil (○–○ ●–●), dinoseb (▲–▲) and in intact algae (b) by atrazine (▽–▽), diuron (□–□), dinoseb (○–○), ioxynil (△–△).

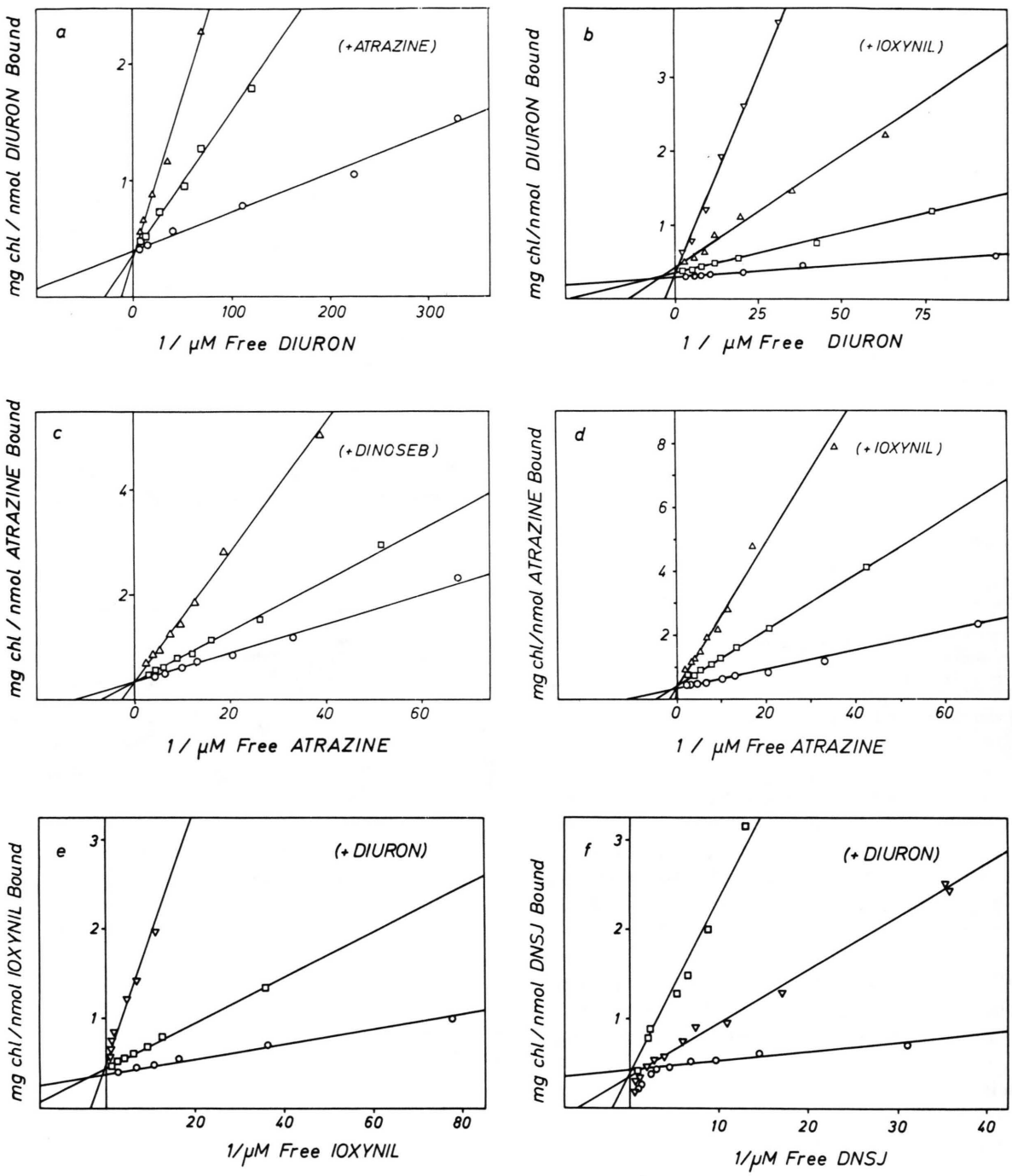

Fig. 5. a) Binding of [^{14}C]diuron to thylakoids in the presence of 4×10^{-7} M (□–□), 6×10^{-7} M (△–△) and without (○–○) [^{12}C]atrazine. b) Binding of [^{14}C]diuron to thylakoids in the presence of 1×10^{-7} M (□–□), 3×10^{-7} M (△–△), 8×10^{-7} M (▽–▽) and without (○–○ ioxynil. c) Binding of [^{14}C]atrazine to thylakoids in the presence if 2×10^{-7} M (□–□), 5×10^{-7} M (△–△) and without (○–○) dinoseb. d) Binding of [^{14}C]atrazine to thylakoids in the presence of 2×10^{-7} M (□–□), 5×10^{-7} M (△–△) and without (○–○) ioxynil. e) Binding of [^{14}C]ioxynil to thylakoids in the presence of 1×10^{-7} M (□–□), 3×10^{-7} M (△–△) and without (○–○) diuron. f) Binding of [^{3}H]DNSJ in the presence of 5×10^{-8} M (□–□), 2×10^{-7} M (△–△) and without (○–○) diuron. Only the data points of high-affinity DNSJ binding were used for K_b and X_g determinations. In all cases K_b and X_g were taken from the abscissa and the ordinate intersections of the straightlines.

3. Mechanism of competition of photosystem II herbicides

Diuron- and phenol-type herbicides have been shown to compete for their high-affinity binding site in thylakoids [2, 5] and in algae (Figs. 2, 3). The elucidation of the mechanism of competition may be a helpful tool for a further characterization of the diuron- and phenol-type herbicide binding sites.

In the binding analysis of ligand molecules (herbicides) to macromolecules (membrane receptors) the Lineweaver-Burk plot (Figs. 5, 6) as well as the Dixon plot (Fig. 7 b) of herbicide binding data allow to distinguish between competitive and non-competitive binding of the ligands [16]. According to the analysis of enzyme kinetics, in the Lineweaver-Burk plot a decreasing binding affinity (increasing K_b), together with a constant concentration of binding sites (X_g), indicates pure competitive binding, On the other hand, a constant K_b together with a decreasing X_g points out non-competitive binding. In our experiments, we studied the mechanism of competition within the group of diuron- or phenol-type herbicides, as well as between these two groups. In thylakoid experiments, the binding of [^{14}C]diuron and [^{12}C]atrazine proved to be competitive (Fig. 5 a). The K_b of [^{14}C]diuron increased from 1.3×10^{-8} M to 9.5×10^{-8} M in the presence of 6×10^{-7} M atrazine. The concentration of binding sites (X_g) was not changed. In the analogue experiment, [^{14}C]atrazine binding in the presence of [^{12}C]diuron was competitive as well (data not shown). Competition between diuron- and phenol- type herbicides is shown in Figs. 5 b – f.

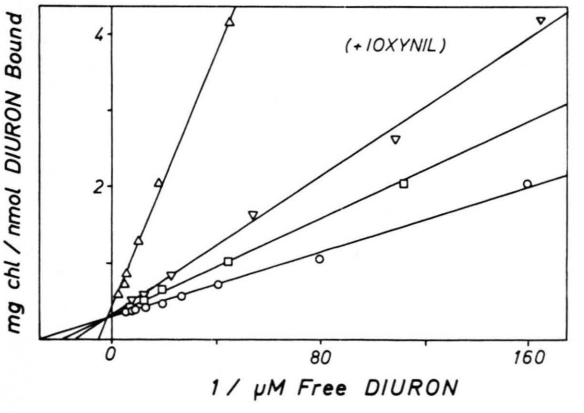

Fig. 6. Binding of [^{14}C]diuron to intact algal cells in the presence of 2×10^{-7} M (□–□), 5×10^{-7} M (△–△), 1×10^{-6} M (▽–▽) and without (○–○) ioxynil. K_b and X_g were determined as described in Fig. 5.

Again the K_b-values in all these experiments were effected considerably whereas the concentrations of binding sites kept nearly constant. The K_b of diuron raised about tenfold from 1.9×10^{-8} M to 2.8×10^{-7} M with 8.0×10^{-7} M ioxynil added to the assay, whereas the Chl/I-ratio was not affected (Fig. 5 b). Binding of [^{14}C]ioxynil and [^{12}C]diuron showed also competitive properties (Fig. 5 e). Hence the order of addition of the herbicides to the experimental assay was without influence on the mode of competition. The competitive mechanism of high-affinity herbicide binding was also found with [^{14}C]atrazine and [^{12}C]dinoseb (Fig. 5 c). With 2×10^{-6} M dinoseb added, the K_b [^{14}C]atrazine was raised to 4.2×10^{-7} M. Also [^{14}C]atrazine and [^{12}C]ioxynil showed competitive binding and the K_b of atrazine raised to 5.8×10^{-7} M when 5.0×10^{-7} M ioxynil was added (Fig. 5 d). In both cases, the Chl/I-ratio kept almost constant. DNSJ is another phenol-type herbicide [21], which showed highly specific binding in concentrations up to 4 nmol bound DNSJ/mg Chl. DNSJ and diuron also exhibit competitive specific binding (Fig. 5 f). As can be seen from the data, low-affinity DNSJ binding is not involved in the analysis of the binding mechanism. The K_b (DNSJ) is raised from 2.0×10^{-8} M to 5×10^{-7} M with 2×10^{-7} M diuron added. X_g was almost constant with a Chl/I-ratio of 444 (control) and 395 (with 2×10^{-7} M diuron).

Analogue experiments were performed using intact algal cells. As has been described for thylakoids, [^{14}C]diuron and [^{12}C]ioxynil displayed a competitive mechanism of binding in algae (Fig. 6). With 1.0×10^{-6} M ioxynil added, K_b (diuron) raised from 3.7×10^{-2} M to 2.2×10^{-7} M but the Chl/I-ratio was only slightly affected. Other herbicide combinations, *e.g.* atrazine and ioxynil, also showed a competitive mechanism of binding in algal cells.

The results of these binding experiments with two competing herbicides demonstrate in total, that in thylakoids and in algal cells, diuron-type herbicides as well as phenol-type herbicides show a competitive mechanism of binding within one group of the herbicides as well as between both groups.

4. Evaluation of the binding constants K_{bb} of unlabelled herbicides

Several methods are available for determination of the K_{bb} of specific binding for unlabelled herbicides. Tischer [11] described a mathematical analysis

of competition experiments as they are given in Fig. 2. The Lineweaver-Burk plot and the Dixon plot as well are suitable for K_{bb} determinations.

In the analysis of binding experiments in the presence of a second, unlabelled herbicide, a series of straightlines was obtained with the Lineweaver-Burk plot. By replotting the slopes of the straightlines taken from this plot versus the total concentration of unlabelled herbicide in the experiment, a new straightline is generated with an abscissa intersection, giving the K_{bb}-value of the unlabelled herbicide [15]. For competitive binding the slope of the straightlines is given by the equation:

$$\text{'slope'} = \frac{K_b}{\text{Chl/I-ratio}} + \frac{K_b}{\text{Chl/I-ratio} \cdot K_{bb}} \cdot \quad \text{(I)}$$

$$(y = \quad b \quad + \quad m \quad \cdot x)$$

(I) denotes the concentration of unlabelled herbicide in the experiment of competitive binding. A replotting of the individual 'slopes' *versus* the total concentration of the unlabelled herbicide (I) reveales at $y = 0$:

$$-\frac{K_b}{\text{Chl/I-ratio}} = \frac{K_b}{\text{Chl/I-ratio} \cdot K_{bb}} \cdot \quad \text{(I)}$$

which gives

$$\text{(I)} = -K_{bb}.$$

This method was applied for the K_{bb} determination of unlabelled dinoseb and ioxynil (Fig. 7 a). The binding data were taken from the experiments shown in Fig. 5. The K_{bb} of ioxynil was determined to be 5×10^{-8} M, a value which is in good agreement with the K_b taken from direct binding experiments (Table I). The same was true for dinoseb with a K_{bb} of 3.5×10^{-7} M (Table I).

For increasing concentrations of a labelled herbicide in the assay, the Dixon plot of mg Chl over nmol bound labelled herbicide *versus* the total concentration of unlabelled herbicide generated a series of straightlines. In case of competitive binding of both herbicides, the slope of the straightlines approaches zero and the straightlines do not converge on the abscissa [16]. The intersection of the vertical axis from the point of the converging straightlines to the abscissa revealed the binding constant K_{bb} of the unlabelled herbicide. In case of non-competitive binding, the intersection of the straightlines would be located on the abscissa and the slope would not reach zero at infinite concentrations of labelled herbicide.

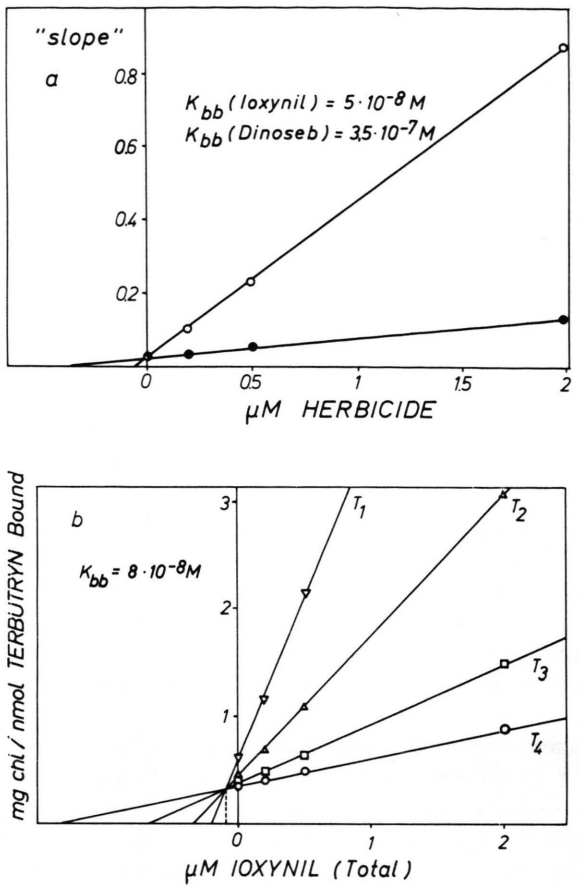

Fig. 7. Determinations of the binding constants of unlabelled herbicides. a) The slope of the straightlines from the Lineweaver-Burk plots of competitive herbicide binding is plotted versus the total concentrations of unlabelled herbicide. K_{bb} is given by the intersections of the straightlines with the abscissa. Ioxynil (○–○), dinoseb (●–●). b) Dixon plot of [^{14}C]terbutryn and [^{12}C]ioxynil binding. The straightlines represent increasing concentrations of [^{14}C]terbutryn ($T_1 - T_4$). K_{bb} is determined by the vertical axis from the intersection of the straightlines to the abscissa. The [^{14}C]terbutryn concentrations were 1×10^{-7} M (T_1), 2.5×10^{-7} M (T_2), 8×10^{-7} M (T_3) and 1.5×10^{-6} M (T_4).

The Dixon-plot of [^{14}C]terbutryn binding in the presence of [^{12}C]ioxynil is shown in Fig. 7 b. In this case the K_{bb} of ioxynil was determined to be 8×10^{-8} M. As has been discussed above, competitive binding of [^{14}C]terbutryn and [^{12}C]ioxynil was indicated. The Dixon plot revealed similar results for the analysis of binding data given in Figs. 5 and 6.

Discussion

For a characterization of PS II herbicide binding, mutual displacement of these compounds is an important tool. In displacement studies it was shown that diuron-type herbicides such as diuron, atrazine or terbutryn are completely displaced from their thylakoid binding sites by themselves (Fig. 3a) and by other diuron- and phenol-type herbicides [2, 5]. Complete displacement was also observed in algal cells (Fig. 2). For obtaining complete displacement the initially bound herbicide concentration should not exceed 3–4 nmol/mg Chl. This concentration gives almost saturated high-affinity binding [2, 10, 11]. The stoichiometry between bound herbicide and chlorophyll at these concentrations suggested the binding of one herbicide molecule per photosynthetic unit which corresponds with 250 to 500 Chl/inhibitor binding site [2, 4, 18, 19]. It was also shown that phenol-type herbicides like ioxynil, dinoseb or DNSJ in thylakoids are almost completely displaced at similar bound concentrations by phenol- as well as by diuron-type herbicides (Fig. 4a). These results demonstrate, that the PS II herbicides in low concentration (< 3–4 nmol bound/mg Chl) bind highly specific to a common receptor site. A common binding site for PS II herbicides has been proposed in several studies [2–5, 8].

The existence of a common binding site and of two different binding proteins, a 32 Kd and a 41 Kd protein for diuron-type and for phenol-type herbicides respectively [7, 9] rises the question for the mechanism of binding of two types of herbicides. In our experiments, competitive binding of two herbicides was found within as well as between representatives of the two herbicidal classes. The competitive binding mechanism was indicated by the Lineweaver-Burk plot of the binding data (Fig. 5, 6). With increasing concentrations of the unlabelled herbicide, the binding affinity (K_b) of the labelled herbicide decreased. The concentration of binding sites (X_g), however, confirmed by the ordinate intersection of the straightline in the Lineweaver-Burk plot, kept constant. The Dixon plot of herbicide binding also allowed discrimination between competitive and non-competitive binding (Fig. 7b). In case of competitive binding, the slopes of the generated straightlines approached zero and the straightlines did not converge on the abscissa. The following herbicide combinations were tested with isolated thylakoid membranes for their mechanism of competition: Diuron to atrazine and diuron to ioxynil (Fig. 5a, b), atrazine to ioxynil and atrazine to dinoseb (Fig. 5c, d), ioxynil to diuron and DNSJ to diuron (Fig. 5e, f). In all cases, competitive binding was found.

Also with algal cells a competitive binding mechanism between both types of herbicide was shown for the combinations diuron to ioxynil (Fig. 6) and atrazine to ioxynil (data not shown). As a special feature, stimulation of ioxynil binding (lowering of K_b) by added dinoseb was found in algal cells but not in thylakoid membranes (data not shown). This phenomenon will be subject for further investigations.

Concerning the mechanism of binding, our studies are in contrast to results of Oettmeier *et al.* [3, 9]. These authors observed competitive binding of two phenol-type herbicides but non-competitive binding between phenol- and diuron-type herbicides. These results served as support for their suggestion that there are two different binding sites for diuron- and phenol-type herbicides at the 32 Kd and at the 41 Kd thylakoid membrane protein. Their binding data, however, have been obtained by substraction of an amount of low-affinity (unspecific) binding from the measured total binding, assuming a linear dependence of unspecifically bound from the corresponding free herbicide. In our experiments, no linear function of these parameters was found for the s-triazine terbutryn (Fig. 1a, d) as well as for the phenol-type herbicide DNSJ (data not shown). In all our studies we calculated the high-affinity binding from the linear branch of the double reciprocal plot. The difference of the total observed binding and of high-affinity binding neither gave a straightline nor a hyperbola. Instead, a sigmoid dependence was found (Fig. 1a, d). The reason for this non-linearity propably is the competition of the low-affinity receptors and the high-affinity binding sites for free substrate before high affinity binding becomes saturated at 3–4 nmol bound herbicide/mg Chl. Our studies of competitive binding were performed at concentrations where only high-affinity binding took place. Therfore no correction of the original data before graphical analysis was necessary.

Furthermore, a direct correlation of herbicide binding and inhibition of photosynthetic electron flow has been found for diuron- and phenol-type herbicides ([2, 11] Table I). This indicates that a sub-

straction of low-affinity binding in a concentration range of predominant high-affinity binding is not allowed. From the Dixon plot (Fig. 7 b) and from a plot of the straightlines in the corresponding Lineweaver-Burk plot versus the total concentration of unlabelled herbicides (Fig. 7 a), binding constants of unlabelled herbicides (K_{bb}) were evaluated. Both methods revealed K_{bb}-values which are well comparable with K_b-values directly obtained from binding experiments (Table I). We take this as further evidence, that only 'specific' binding is involved in our analysis of the binding mechanism. As we tested the binding of diuron and ioxynil (Fig. 5 b) as well as vice versa, binding of ioxynil and diuron (Fig. 5 e), there is no influence of the order of herbicide addition to the experimental assay.

The displacement of diuron-type herbicides in intact algae by diuron- and phenol-type herbicides (Fig. 2) was similarly complete as compared to displacement in thylakoids ([2, 4], Fig. 3). On the other hand, there was only little displacement of the phenol-type herbicide dinoseb in algae, even when dinoseb is previously bound in concentrations about 200 times lower than required for saturated high-affinity binding (Fig. 4 b). Thus dinoseb seemed to exhibit special binding properties in algae, which are not common to diuron-type herbicides. The different behaviour of phenol-type herbicides bound in algal cells are not yet understood and will be further studied. Using the s-triazine terbutryn, high-affinity (specific) and low-affinity (unspecific) binding could clearly be separated (Fig. 1). As discussed above, unspecific binding does not exhibit a hyperbolic or linear dependence of bound and free herbicide. From this we conclude, that a binding constant (K_b') of low-affinity binding cannot be determined from the Lineweaver-Burk plot of terbutryn binding. An 'apparent' K_b-value can arbitrarily be used for characterization of low-affinity binding but does not describe the real association of the herbicide towards its receptors. Also the Scatchard plot of terbutryn binding (Fig. 1 c, f) gave clear distinction between high- and low-affinity binding for both thylakoids and algae. In both cases, however, low-affinity binding seemed to be monophasic.

For further characterization of low-affinity binding, displacement experiments were performed. In isolated thylakoids as well as in intact algae, terbutryn concentrations of 8 nmol (thylakoids) and 11 nmol (algae) bound/mg Chl were not completely displaced by terbutryn itself (Fig. 3). Only as much labelled terbutryn was displaced as corresponded to specific binding. This lack of exchange of labelled bound and unlabelled, free terbutryn points out, that low-affinity binding is an irreversible process. But as most of the total amount of terbutryn in the experimental assay remained free and as there was a rather large capacity for low-affinity binding, this lack of exchange could not be due to very tight herbicide binding to membrane receptors, even if the 'apparent' K_b seemed to suggest highly reversible binding. The observed lack of displacement of unspecifically bound herbicides (< 5 nmol bound/mg Chl) at high herbicide concentrations might be explained to some extend by a large capacity for low-affinity binding.

Irreversible herbicide binding has alread described by Izawa and Good [14] utilizing a bioassay method and by Lee and Fang [13], using radioactively labelled monuron. Lee annd Fang [13] found that monuron could not be removed completely from isolated thylakoids or intact chloroplasts by successive washings. In binding experiments with photoaffinity labelled phenol-type herbicides, Oettmeier *et al.* [8, 9] found dinoseb binding taking place to a large extent in low molecular fractions of the thylakoids (free lipids and pigments). This suggests a distribution of phenol-type herbicides into the lipid phase of the membranes. To a small degree, several thylakoid polypeptides are labelled by azido-dinoseb [8, 9] and by azido-atrazine [7]. The diuron-type herbicides used in our experiments (Fig. 1) as well as the phenol-type herbicide DNSJ (Fig. 5 f) exhibited distinct low-affinity binding in thylakoids in concentrations higher than 3–4 nmol bound herbicide/mg Chl. Also the diuron-type herbicides, *e.g.* terbutryn, may be distributed in the lipid phase of the membranes. A possible existence of a second distinct binding site for diuron-type herbicides has recently been discussed by Ramanujam *et al.* [22]. These authors found a stimulation of photosystem I electron flow in uncoupled chloroplasts by diuron and other diuron-type herbicides. The total herbicide concentration in this case was higher than 10^{-5} M. Also Schreiber and Pfister [23] recently reported about high concentration effects of diuron on PS II reactions. Phenol-type herbicides and also diuron, in concentrations higher than 10^{-5} M may act as inhibitory uncouplers or as inhibitors of photo-

phosphorylation, probably by altering the lipid structure of the thylakoid membrane [24–26]. Uncoupling of photophosphorylation can be accomplished either by shutteling protons through the membrane or by increasing membrane permeability for protons. For both mechanisms no distinct binding sites seem to be required. It has been demonstrated, however, that the uncoupler 2-azido-4-nitrophenol binds to specific receptors at the mitochondrial membrane [27]. Looking at the Scatchard plot of terbutryn binding, our experiments seem to support the existence of a second distinct binding site for diuron-type herbicides in thylakoids as well as in algae (Fig. 1 c, f). In these cases low-affinity binding was shown to be monophasic. Monophasic low-affinity binding has not been found for the phenol-type herbicide dinoseb in algae [10]. The present information from our experiments and from the literature does not yet allow a final conclusion whether distinct low-affinity receptor sites exist or whether the observed low-affinity binding represents a partitioning of the herbicides into the lipophilic membranes. Studies on this subject are in progress.

In respect to specific binding the following conclusions are drawn from our experiments. In order to reconcile the involvement of at least two membrane proteins, a 32 Kd- and a 41 Kd-protein [7, 9], in high-affinity herbicide binding with the presented results, we propose that binding of the diuron- and phenol-type herbicides takes place as follows: The two receptor proteins are arranged close together in the thylakoid membrane. For both types of herbicides, the 32 Kd- and 41 Kd-protein create a common binding site which is essential for inhibition of photosynthetic electron flow and for setting the competitive mechanism of binding. Specific subreceptors for both types of herbicides are located on the adjacent proteins.

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