The Mode of Action of Photosystem II-Specific Inhibitors in Herbicide-Resistant Weed Biotypes

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This report reviews studies which provide evidence defining the mode of action and site of action of photosystem II (PS II) herbicides; the involvement of the secondary electron carrier on the reducing side of PS II (called B) is indicated as the target site for these compounds. These studies of the action of PS II-inhibitors were performed in chloroplasts of various weed species in order to define the mechanism which is responsible for herbicide tolerance at the level of chloroplast membranes in newly discovered triazine-resistant weed biotypes.

Many species of triazine-resistant weed biotypes have been collected in North America and Europe. Where data is available, these plants have been found to share the following common features:

a) they were discovered in areas where triazine herbicides had been used repeatedly,
b) resistance to the triazines is extreme; it is not due to a minor shift in herbicidal response,
c) no changes in herbicide uptake, translocation or metabolism — as compared to susceptible biotypes — can be detected,
d) resistance is selective for only certain classes of photosynthetic herbicides, and,
e) chloroplasts isolated from triazine-resistant weeds display high preferential resistance to the triazines in assays of photosystem II partial reactions.

To focus on the mechanism which regulates preferential herbicide activity, we have characterized susceptible and resistant chloroplasts in the presence and absence of herbicides. Properties of the PS II complex of chloroplasts from several different triazine-resistant weed biotypes share the following traits:

a) the herbicide binding site (as measured by direct binding of radiolabeled herbicides or by inhibition experiments) is modified such that the affinity for triazines is dramatically reduced.
b) alterations in response to many PS II-herbicides occur such that the triazine-resistant chloroplasts are very strongly resistant to all symmetrical triazines, strongly resistant to assymmetrical triazinones, partially resistant to pyridazones and uracils, only slightly resistant to ureas or amides, and increasingly susceptible to nitrophenols, phenols and the herbicide bentazon (all as compared to susceptible chloroplasts),
c) there is a change in the reaction kinetics of the electron transport step between the primary and secondary electron acceptors (referred to as Q and B), and
d) in two examples, specific small changes in a membrane polypeptide can be detected in the resistant thylakoids.

We suggest that certain amino acids or segments of the apoprotein of B (the bound quinone which acts as the secondary electron carrier) are modified or deleted in these chloroplasts. Such a polypeptide change could affect both the redox poising of the Q^-/B reaction pair, and the specific binding of herbicides.

Introduction

A wide variety of chemicals are currently available for use as weed-controlling herbicides. The mode of action of these compounds varies according to their chemical family; current knowledge indicates that plant death can result from alterations in any of a large number of physiological or developmental pathways. Of major commercial importance, however, are the classes of chemicals whose primary mechanism of action is to block photosynthetic functions; these comprise more than half of all currently utilized herbicides [1–3]. Of major importance as photosynthesis inhibitors are the

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Abbreviations: B, secondary electron acceptor of photosystem II; DCMU, diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, dichlorophenolindophenol; PQ,plastoquinone pool; PS I, PS II, photosystem I or II respectively; Q, primary electron acceptor of photosystem II; (Quencher).

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chemical groups of ureas, amides, triazines, triazinones, pyridazinones, carbamates and nitrophenols.These compounds all share the common feature that they block photosystem II-dependent Hill-reactions (PS II-inhibitors).

In recent years, there have been several reports of the occurence of weeds which developed a high degree of resistance to certain PS II-inhibitors (i.e., the triazines). The purpose of this paper is to present evidence that the photosynthetic target-site for triazines has been strongly modified in the resistant plants. We will review data which describe the occurrence and characteristics of herbicide-tolerant weeds, the mode of action of PS II-inhibitors in isolated chloroplasts from susceptible weeds, and evidence for changes in the PS II-complex that are correlated with herbicide resistance in the tolerant plants. These data may be of value, not only in the applied sense of understanding an agricultural problem, but also in understanding the mechanisms of action of various photosynthetic inhibitors.

Materials and Methods

All weed species used in these studies were grown in soil in a constant environment chamber (18/6 h light/dark; 28 °C/24 °C). Broken stroma free chloroplast thylakoid membranes were isolated as previously described [4]. All experimental procedures for electron transport assays [5], fluorescence measurement [5], or herbicide binding studies [6] were as previously described, except where indicated in the text or in figure legends.

Results and Discussion

I. Characteristics of triazine-resistant weed biotypes

A. Herbicide specificity

In recent years there have been several reports of weed biotypes which have developed a high degree of resistance to triazine herbicides [see Table I]. The common aspect of all these plants is that they were found in areas of agricultural production where triazines were used repeatedly with little or no rotation with other, non-triazine herbicides. It is not yet clear whether the new weed biotypes are the result of recent mutation(s) conferring immediate selective advantage in the face of herbicide application or whether the resistant biotypes have existed in the weed population at a low frequency for a long period of time and have only recently become prevalent during the course of weed eradication programs [7 – 9]. It is of considerable interest that the herbicide-tolerant trait has been shown to be maternally inherited [10]; this suggests that the DNA-containing cytoplasmic organelles (chloroplasts or mitochondria) may be involved, and furthermore suggests various means by which genetic transfer of this trait to crop species can be attempted.

The ten different weed species reported in Table I were discovered independently under conditions of agricultural weed eradication programs in widely different locations. In all cases, it appeared that the biotypes with extreme resistance to triazines were not controlled by triazine herbicides applied at several times the normal rate [11, 12]. This was

Plant Species		Origin	References
Ambrosia artemisiifolia L.	common ragweed	C	56, 66, 67
Amaranthus retroflexus L.	redroot pigweed	U, C	5, 18, 56, 67
Brassica campestris	wild turnip	C	10, 56, 67
Bromus sp.		U	*
Chenopodium album	common lambsquarters	F, C, U	12, 18, 19, 21 56, 67, 68, 69
Chenopodium strictum Roth. var. *glaucophyllum* (Aellen) Wahl	late flowering goosefoot	C	67
Poa annua L.	bluegrass	F	68, 69
Poligonum lapathifolium	knotweed	F	69, 72
Senecio vulgaris	common groundsel	U, C	4, 6, 11, 13 56, 67, 70
Solanum nigrum	black nightshade	F	69, 72

Table I. Plant species which have developed triazine resistance. C = Canada, U = United States. F = France.

* Seed provided by Dr. Jerry Hensely, CIBA-GEIGY.

demonstrated in more detail in experiments with *Amaranthus retroflexus* seedlings grown hydroponically (see ref. [5]). It was necessary to add 100-fold higher concentrations of atrazine to the nutrient solutions of triazine-resistant plants to give similar control of growth as was achieved at the normal micromolar concentration range used for susceptible plants. In contrast diuron sensitivity was identical within the level of sensitivity of these experiments.

B. Photosynthetic response to herbicides *in vivo*

Studies of CO_2 fixation in susceptible and triazine-resistant weed biotypes in the presence or absence of triazine herbicides were preformed by several investigators [13, 14]. It was discovered that the triazines caused an inhibition of photosynthesis only in the susceptible biotype weeds; this focused attention upon the site of action of the triazines *in vivo*.

A very direct test system for studying the photosynthetic response of intact leaves following application of a range of PS II-herbicides is the analysis of chlorophyll fluorescence transients. We have used this to characterize several of the newly discovered weed biotypes.

In a set of experiments (data not shown) using susceptible or resistant weed leaf samples, we have monitored the fluorescence induction curve before and at various times after application of atrazine or diuron (procedure as in [15]). A decrease of the variable fluorescence indicating inhibition of photosynthesis [16] was observed with atrazine only in the susceptible leaves, whereas diuron was active in both biotypes.

C. Physiological basis for herbicide resistance

For the last two decades, the rationale for use of individual herbicides in agricultural practice has been based on the selectivity of action of a particular herbicide which results in eradication of weed species but which does not affect crop plant growth or productivity. It has been found that the basis of this differential response is due to the ability of a crop species to exclude or metabolize into an inactive form those chemicals to which weed species are otherwise susceptible. The discovery of the triazine resistant weed biotypes led several investigators to study the uptake, translocation, accumulation, and metabolism of triazine herbicides in these plants. In all cases, differences in these parameters between resistant and susceptible biotypes were minor and could not account for the extreme tolerance of the resistant weeds [17 – 20].

The first indication that this resistance phenomenon was associated with a change in the target site for the herbicides came when it was observed that resistance of photosynthetic reactions persisted in preparations of isolated chloroplast membranes [13, 18, 21]. An example of the type of data obtained is shown in Fig. 1. When chloroplasts were isolated from the two biotypes of *Amaranthus retroflexus*, photosynthetic electron transport was inhibited in both samples by diuron with nearly the same herbicidal activity. In contrast, atrazine affected the electron transport only in the susceptible chloroplasts.

The parallel behavior of diuron and atrazine in both whole plants [5] and isolated chloroplasts [Fig. 1] suggests that an alteration of the target site for atrazine in the chloroplasts is directly related to herbicide tolerance in the intact plant. This now focuses our attention upon the mechanism(s) by which PS II-herbicides interact with their receptor site(s) at the chloroplast membrane. In the following sections we will discuss current concepts of the organization of PS II and specific effects of PS II-inhibitors on this PS II-complex.

II. The site of action of PS II-herbicides

A. Characterization of the PS II-complex

Photosynthetic electron transport is mediated by two reaction centers acting in series and connected by a chain of electron carriers [22]. The enzymatic

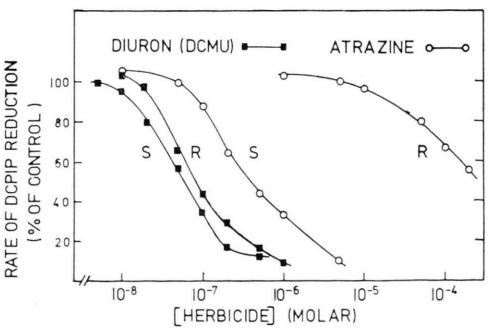

Fig. 1. Inhibition of DCPIP reduction in isolated susceptible and resistant *Amaranthus retroflexus* chloroplasts by atrazine and diuron (DCMU).

components, plus light-harvesting and reaction-center pigments associated with specific proteins, are localized within the chloroplast thylakoid membranes. Extensive evidence is available which demonstrates that these proteins are organized in structural complexes; functional activity of the complexes is dependent upon ordered interactions of proteins, chlorophylls and lipids. It is possible to solubilize chloroplasts with detergents by procedures which allow recovery of functionally active complexes. In this way, photosystem II sub-membrane preparations have been isolated and characterized [for further discussion and review, see ref. 23].

A schematic interpretation of the currently available evidence concerning the functional composition and possible organization of the photosystem II complex is shown in Fig. 2. We suggest that the core of this complex consists of polypeptides of approximately 44 – 50,000 molecular weight [24, 25]. These polypeptides are believed to be associated with functional reaction-centers (designated as P_{680}) activity [24], as well as with the binding of chlorophyll a molecules which serve as tightly associated light-harvesting antennae [26, 27]. More loosely associated with the PS II core proteins is a light-harvesting pigment-protein complex comprised of peptides averaging 25 – 30,000 molecular weight and containing both chlorophylls a and b. This complex, which has been purified in its native form [28], is functionally connected via a reversible, cation-mediated process to the PS II-complex [29].

Electron donation to the PS II reaction center is via an enzyme (protein as yet unidentified) or enzyme complex localized on the inner surface of the thylakoid membrane [30, 31] in which Mn^{2+} is

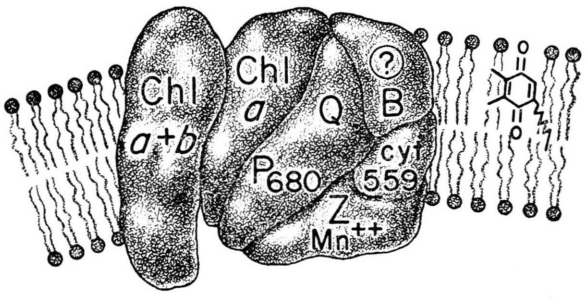

Fig. 2. Schematic representation of the organization features of the photosystem II complex. Details of the literature upon which this very generalized model was developed are explained in the text.

thought to function as a cofactor involved in water oxidation [32]. The PS II-complex also contains a tightly associated cytochrome b_{559} which does not appear to participate in a PS II-dependent linear electron transport [33].

The primary electron acceptor for the PS II reaction center chlorophyll is thought to be a special plastoquinone molecule (indicated as Q) which is probably also part of the reaction center complex. Several lines of evidence [34 – 36] indicate that this is a one-electron carrier forming a semiquinone in the reduced state. The second electron carrier on the reducing side of PS II is also thought to be a quinone molecule (indicated as B) which is presumably bound to a specific polypeptide (as yet not identified) in the reaction center complex. The component B is a two electron carrier which acts as a gating mechanism for the delivery of an electron pair into the pool of plastoquinone (PQ) molecules that serves to interconnect PS II and PS I [36 – 39]. In the semiquinone state, B^- is stable for many seconds to minutes [39]; in the fully reduced state, $B^=$ transfers electrons to PQ with a reaction time $<$ 1 msec [40].

It has been suggested that diuron and atrazine interrupt photosynthetic electron transport by acting at the level of B [41]. The following sections will review evidence for this idea, and will expand upon this concept by presenting experimental data from studies with triazine susceptible and resistant weeds.

B. Effects of PS II-inhibitors on photosynthetic partial reactions and chlorophyll fluorescence transients

Photosynthetic light reactions can be studied with isolated chloroplast membranes using various spectrophotometric and polarographic (O_2) analysis systems. Specific steps (partial reactions) of the electron transport chain can be monitored by selecting various electron acceptor and/or donor couples [42]. It has been observed that diuron and other PS II-inhibitors block all classical "Hill" reactions (defined as electron transport which is PS II-dependent, is catalyzed by an artificial electron acceptor, and uses H_2O as an electron donor). The only exception is the Hill reaction mediated by silicomolybdate (SiMo), which indicates that the primary site of action of PS II-inhibitors is after Q

and is not at the reaction center *per se*, or on the oxidizing side of PS II [43, 44]. Electron transport reactions mediated only by PS I are insensitive to diuron and related compounds (except at very high concentrations where secondary inhibitor effects appear).

Analysis of the exact step where PS II-inhibitors interrupt the electron transport chain have been highly dependent upon the use of chlorophyll fluorescence measurements. Diuron dramatically stimulates the rate of fluorescence rise observed upon illumination of dark adapted chloroplasts. This lead to the conclusion that the herbicide blocks electron flow near the reducing side of photosystem II [45]. Comparisons of the area above the chlorophyll fluorescence induction curves in treated and untreated chloroplasts allow estimation of the electron acceptor pool sizes located before and after the inhibitor block; this has been discussed in detail elsewhere [46, 47]. It was concluded that diuron inhibits electron transport between Q and the remaining segment of the electron transport chain. Atrazine affects fluorescence transients in a pattern identical to that of diuron [Fig. 3], indicating that atrazine blocks electron transport at a mutual site.

C. Evidence for the action of PS II-inhibitors at "B"

The results discussed in the section above demonstrate that primary PS II photochemistry (including water oxidation and the reduction of Q) are not principal sites of action of PS II-inhibitors. There are now suggestions that the inhibitors act at the secondary PS II electron acceptor B.

The first line of evidence is based upon proteolytic enzyme modifications of the chloroplast membranes. Trypsin treatment selectively alters surface-exposed membrane polypeptides [48]. This results in the appearance of inhibitor-insensitive PS II-dependent ferricyanide reduction [49, 50]. Our recent studies [51, 52] indicate that the use of trypsin at low concentrations causes loss of PS II-inhibitor binding sites in parallel with interruption of electron flow at the level of B; in these chloroplasts, electron transport from water to ferricyanide remains active due to an exposure of Q to this electron acceptor. These observations are best interpreted as indicating that a surface-exposed polypeptide of the PS II-complex is essential for both the function of the bound plastoquinone called "B" and the binding of PS II-inhibitors.

The second line of evidence again depends on the use of fluorescence analysis of PS II photochemistry. As was shown in Fig. 3 above, Q is reduced (remains functional) in herbicide-treated chloroplasts. To determine the number of electron carriers functioning in the inhibited system, the reduction of PS II electron acceptors can be studied with single, short flashes. An experiment in which fluorescence increase was measured after short, saturating intensity flashes is shown in Fig. 4. Chlorophyll fluorescence was detected during a 2 msec weak measuring flash administered once every two seconds; this is considered "dark" fluorescence since Q remains in the oxidized state (F_0-level) even for extended periods of measurement. The upward arrow indicates the beginning of fluorescence measurement with weak measuring flashes (the trace shown before the arrow is the baseline in the absence of all illumination). At various times, indicated by downward arrows, the sample was illuminated by 8 μsec intense white actinic flash; this was given during the first half of the dark period between fluorescence measurements. With the suscepible chloroplasts, these actinic flashes did not increase the measured fluorescence intensity since electron transfer from Q^- to B was completed during the remaining 1 sec dark interval between fluorescence measurements. When diuron was added to a similar sample, almost no increase in fluorescence occured in the weak measuring beam. In contrast, a single 8 μsec actinic flash after diuron addition increased the fluorescence to a maximal value [Fig. 4 d]. These data indicate that a single flash fills the entire electron pool available before the site of action of the herbicide; *i.e.* a single flash totally reduced the Q pool. For comparison, it has been shown that bicarbonate depletion of isolated chloroplasts blocks electron flow between B and the PQ pool; in these chloroplasts three actinic flashes are needed to obtain maximal fluorescence yield [53], which means that the Q plus B pools can store a total of three charges. The data of Fig. 4 d could also be reproduced if atrazine was substituted for diuron. In total, these data demonstrate that Q is functional as an electron carrier in inhibited chloroplasts, but B is no longer active as a functional electron acceptor.

The third of evidence for B as the target site of PS II-inhibitors comes from experiments initiated by Velthuys [41]. In these studies, the effect of

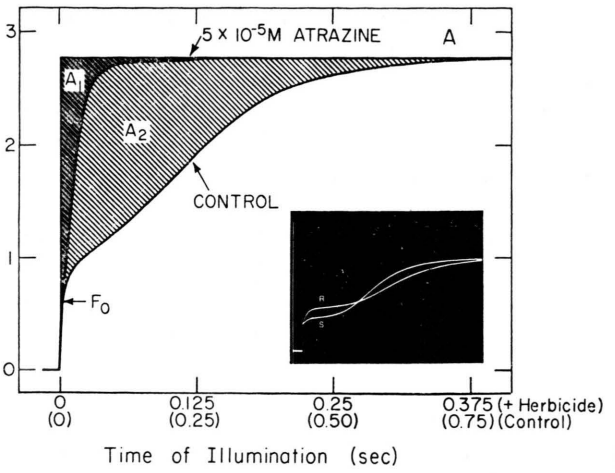

Fig. 3. Fluorescence induction curve of isolated susceptible chloroplasts of *Amaranthus* in presence and absence of atrazine. The areas (A_1, A_2) over the induction curves represent the pool size of the reducible primary and secondary electron acceptors. Calculations of pool size rations in these experiments gave identical values for diuron and atrazine ($A_1/A_2=0.08$). Note: Two different time scales are used. The transient in presence of inhibitor is recorded at twice the speed as the control.
Inset of Fig. 3. Fluorescence induction curves of dark-adapted chloroplasts isolated from susceptible and resistant *Chenopodium album* biotypes. S=susceptible, R=resistant chloroplasts.

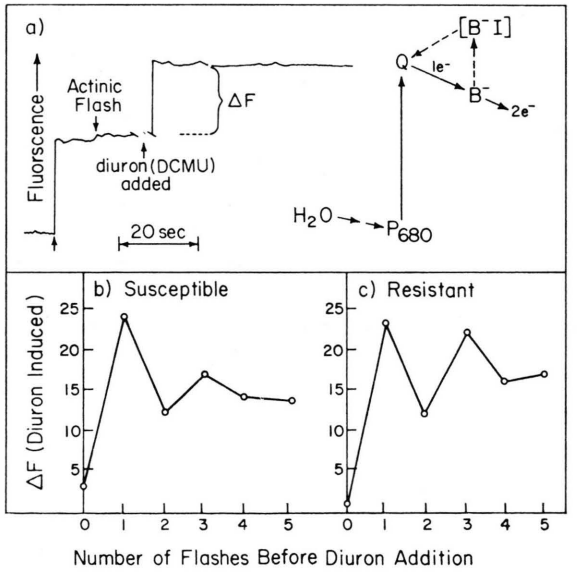

Fig. 5. Diuron (DCMU)-induced "dark" fluorescence increase in preflashed isolated *Amaranthus retroflexus* chloroplasts. a) *Experimental procedure:* Fluorescence measurements were made during weak blue flashes as described in Fig. 4 — onset of measuring was at the upward arrow. An 8 μsec saturating flash preceeded the addition of diuron to the sample. Fluorescence increase (ΔF) occuring as a result of "dark" herbicide addition is interpreted as indicating a change in the concentration of Q^-. This results from reversed electron flow from the modified secondary acceptor B to Q as described in the partial electron transport chain diagrammed on the right. b, c) *Experimental protocol:* was as in a) but using 1-5 preilluminating actinic flashes in different samples. The ΔF increase caused by addition of diuron in the dark is plotted as a function of the number of preilluminating flashes.

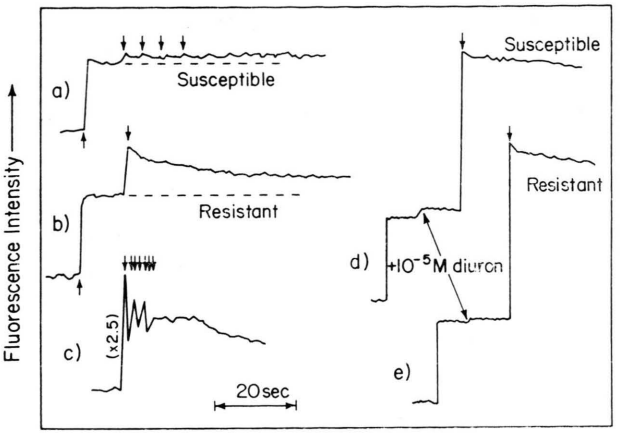

Fig. 4. Change in chlorophyll fluorescence level induced by one or more saturating flashes and monitored in weak measuring light. Experimental procedures: (see Text). *Material: Amaranthus retroflexus* chloroplasts. All measurements were done in the presence of 10 mM hydroxylamine. a) *Susceptible chloroplasts:* The fluorescence decay was completed during one measuring interval and left little remaining Q^-. Actinic flashes were spaced 6 sec apart. b) *Resistant chloroplasts:* The reduction of Q induced by a single flash is not fully reversible during approximately one minute. Compared to a) this indicates a rate limitation after the Q-pool which delays the exit of charges to B and PQ. c) *Resistant chloroplasts:* Fluorescence oscillations induced by a series of actinic flashes spaced 2 sec apart. (Note: Only the flash-induced fluorescence increase is shown at 2.5× magnification as compared to other traces presented at equal measurement scale.) d, e) *Reduction of the Q-pool in the presence of diuron (DCMU) induced by a single saturating flash:* Diuron (10^{-5} M) was added in the dark and induced only a small fluorescence increase in the susceptible chloroplasts immediately after addition.

inhibitors on the "dark" fluorescence level dependent on the number of charges stored at the reducing side of PS II was investigated. Similar experiments using chloroplasts from *Amaranthus retroflexus* biotypes are described in Fig. 5. With chloroplasts illuminated by a very weak ("dark") measuring beam, the addition of diuron can cause an increase in chlorophyll fluorescence that is dependent

on preillumination treatments. Dark-adapted chloroplasts show a very small increase in fluorescence [Fig. 4 a] whereas plastids subjected to one intense 8 μsec flash shortly before diuron addition respond to the herbicide with a marked increase in fluorescence [Fig. 5 a]. The diuron stimulated "dark" fluorescence (ΔF) increase shows a binary oscillation with respect to the number of prior actinic flashes [Fig. 5 b]. These data obtained with *Amaranthus* chloroplasts lead to the same conclusions as previous data [39, 41] using diuron or atrazine and spinach chloroplasts.

The interpretation of the "dark" effects of photosynthetic inhibitors are schematically indicated as part of Fig. 5 a. In dark-adapted chloroplasts, Q and the majority of B are in the oxidized state; diuron in the "dark" cannot affect the redox state of Q. However, when dark-adapted chloroplasts are exposed to one actinic flash, an electron is transferred from P_{680} to Q and then to B. This results in a continued low fluorescence level (oxidized Q), but a stored charge on the relatively stable semiquinone B^-. The increase of "dark" fluorescence induced by diuron results from reversed electron transfer from B^- to Q, giving Q^-, and correspondingly increasing fluorescence yield. We assume, in line with Velthuy's hypothesis [41], but without rigorous proof at this point, that the reversed electron flow occurs because inhibitor (I) binding to B results in a decrease of the redox potential of the bound quinone with respect to that of Q [see Fig. 5 a]. This, of course, would result in inhibition of electron flow to the electron transport chain.

D. Is the site of action of PS II-inhibitors the same in triazine-resistant chloroplasts?

The data described above indicate that both diuron and atrazine act at the same electron transport step and via the same mechanism of blocking photosynthetic electron transport in susceptible chloroplasts. Since atrazine was inactive as an inhibitor in chloroplasts from triazine-resistant plants, we have questioned whether or not the remaining activity of diuron was possible now at a new secondary location in these thylakoids. This idea proved not to be true. Fluorescence induction transients of diuron-treated susceptible and triazine-resistant chloroplasts were found to be nearly identical ([5, 21] and unpublished data with other weed biotypes). Calculations of pool size before and after the herbicide block gave nearly identical results for chloroplasts of both biotypes. In addition, a single 8 μsec actinic flash gave a maximal increase in the fluorescence level of dark-adapted chloroplasts containing diuron [Fig. 4 e], thus indicating that only one electron carrier, Q, functions before the block.

The mechanism of diuron action also appeared to be the same in the triazine-resistant chloroplasts to that of susceptible plastids. "Dark" diuron addition gave a fluorescence increase in pre-flashed chloroplasts [Fig. 5 c]. The binary dependence of the ΔF increase supports a diuron effect on the 2-electron carrier B, as was suggested for normal plastids [Fig. 5 b].

III. Binding of PS II-inhibitors

The fact that diuron and atrazine both act at the same electron transport step and via the same mechanism in normal chloroplasts (see sections above) appeared condradictory to the observation that only diuron was a potent inhibitor in the triazine-resistant plastids. Three explanations seemed possible: a) atrazine is selectively excluded from the membrane in the resistant chloroplasts, b) atrazine binds to membranes but is inactive, or c) atrazine binding sites are selectively lost. The first possibility was tested by measuring the inhibitory activity of various concentrations of atrazine or terbutryn added to chloroplasts with short or long (10 min) incubation times prior to assay. Terbutryn was used in these experiments because it is an extremely active triazine; this allowed the direct determination of the I_{50}-concentration even in the triazine-resistant chloroplasts. The data from these experiments were used to calculate I_{50} values for each herbicide [Table II]. The results show no large change in sensitivity of either sample after increasing incubation time. In a separate study, no increase in atrazine activity was found even after 3 h incubation of *Senecio vulgaris* chloroplasts with inhibitor [4]. As a further check on this point, detergent-derived PS II submembrane fragments were prepared. These particles, in which any penetration barriers should have been removed, showed the same extent of triazine-resistance as whole membranes [Table II]. Atrazine exclusion from PS II can therefore be ruled out as a mechanism of herbicide resistance [see also 21].

Sample	Preincubation time [min]	I_{50} Concentration [M]	
		Atrazine	Terbutryn
Susceptible:			
Chloroplasts	0	4.0×10^{-7}	3.2×10^{-8}
Chloroplasts	10	4.2×10^{-7}	1.9×10^{-8}
* PS II Particles	0	3.5×10^{-7}	2.5×10^{-8}
Resistant:			
Chloroplasts	0	$>10^{-4}$ (Est. 4×10^{-4})	5.6×10^{-6}
Chloroplasts	10	$>10^{-4}$ (Est. 4×10^{-4})	7.5×10^{-6}
* PS II Particles	0	$>10^{-4}$ (Est. 4×10^{-4})	7.0×10^{-6}

Table II. I_{50} concentrations for the inhibition of PS II assays (DCPIP photoreduction) in chloroplasts isolated from susceptible and resistant *Amaranthus retroflexus* biotypes. All preincubations were done at room temperature. Submembrane particles were prepared with digitonin [71] and their activity was assayed in the Diphenylcarbazide → DCPIP system.

Methodology for the measurement of binding of radioactively labeled herbicides to chloroplast membranes was reported by Tischer and Strotmann [54, 55]. An example of our binding studies with triazine susceptible *Amaranthus retroflexus* chloroplasts is shown in Fig. 6 a. Increased amounts of added atrazine results in increased binding of the inhibitor on a chlorophyll basis. When expressed in a double-reciprocal form, the data provide clear evidence for a high affinity binding site. Tischer and Strotmann convincingly demonstrated that the binding constant for this high affinity site is directly related to the inhibitor constant for any of the PS II-inhibitors studied. Secondary binding sites with lower affinity can also be detected in susceptible chloroplasts but these do not correlate with inhibition at the PS II-complex and are considered as non-specific binding to the chloroplast membranes. The ordinate intercept of the reciprocal herbicide binding plot [Fig. 6 b] can be used to calculate the number of binding sites on a chlorophyll basis. The value obtained (1 bound inhibitor/≅ 450 Chl) agrees very well with the photosynthetic unit size measurements for these plastids (unpublished data). We have previously reported Hill plots for diuron and atrazine inhibition of photosynthetic electron transport; these demonstrated that one inhibitor binds per active site [6].

In binding studies, Tischer and Strotmann [55] have found that a urea, several triazines, triazinone, and pyridazinone herbicides compete for the same binding site. These studies have been extended using weed chloroplasts to show that bentazon and phenols (DNOC, Ioxynil, Bromonitrothymophenol) also compete for the same active site [56]. With susceptible *Senecio vulgaris* chloroplasts, bound, radiolabeled diuron was found to be displaced by unlabeled atrazine, or *vice versa* [6], thus supporting the concept that these herbicide classes share at least a portion of the same binding site.

When triazine-resistant chloroplasts were analyzed for herbicide binding, diuron was found to have a slightly reduced affinity as compared to the affinity in susceptible chloroplasts [Fig. 7]. This is consistent with a small reduction in its inhibitory activity [Fig. 1]. In contrast, atrazine binding to the resistant chloroplasts [Fig. 6 a] could not be detected. Furthermore, atrazine could not displace radiolabeled diuron from these membranes. Similar experiments studying inhibitor binding to resistant *Senecio vulgaris* membranes are discussed in more detail in ref. [6]. The data described above can now be used to answer the question of the nature of the triazine resistance mechanism; the triazine binding site was strongly modified in resistant plastids, resulting in a very large decrease in binding affinity.

IV. Specificity of PS II-herbicides in susceptible and triazine-resistant chloroplasts

Since there was variability between diuron and atrazine in affecting photosynthetic electron transport in both the isolated chloroplasts and intact leaves of the susceptible and resistant biotypes, we began to analyze the comparative activities of other classes of PS II-inhibitors. This has been completed for five different weed species and for a large number of inhibitors [56]. As a brief example, some data obtained with *Amaranthus retroflexus* are shown in Table III. For each herbicide, a range of concentrations was used to test inhibitory activity. These data were used to calculate the I_{50} values for both susceptible and resistant chloroplasts. For easy comparison of the different responses, we have calculated the ratio:

$$\frac{I_{50}\,(\text{resistant chloroplasts})}{I_{50}\,(\text{susceptible chloroplasts})} = \text{R/S}.$$

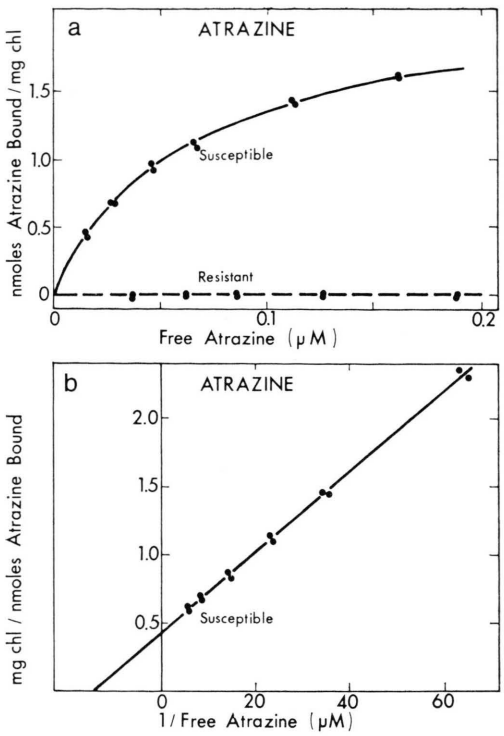

Fig. 6. Binding of [^{14}C]atrazine to isolated susceptible and resistant *Amaranthus retroflexus* chloroplasts. Chloroplasts were incubated with various concentrations of radioactively labeled atrazine. After centrifugation, the amount of inhibitor bound to the pelleted chloroplast membranes was calculated. For details see [6]. a) Amount of bound inhibitor on a chlorophyll basis depending on the concentration of the free inhibitor. b) Double reciprocal plot of the data shown in a).

For all symmetrical triazines the degree of resistance was very large (R/S values $\cong 10^3$). The resistance to the triazinone metribuzin was less dramatic. The tolerance of the new resistant biotype chloro-

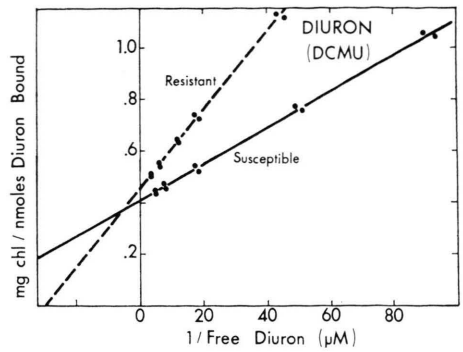

Fig. 7. Binding of [^{14}C]diuron (DCMU) to isolated susceptible and resistant *Amaranthus retroflexus* chloroplasts. Data are given in the double reciprocal form (see Fig. 6).

plasts to uracils and pyridazinones (R/S values of 20 – 80) was much less than to the triazines, but significantly more than that to for the amides and ureas (R/S $\cong$ 2 – 4). Of perhaps the greatest interest was the finding that chloroplasts which are highly resistant to triazines are actually more susceptible to certain phenols, nitrophenols, and the herbicide bentazon (R/S values of 0.1 to 0.6). Field studies in which bentazon was applied to whole plants of the resistant *Amaranthus* biotype seem to indicate that this is also the case *in vivo* (personal communication, Edward Stoller and John Bandeen).

V. A model for the binding site of PS II-herbicides

Based upon the review of published and current data presented above, we conclude: a) there is one herbicide binding site for PS II-inhibitors per electron transport chain (Figs. 6 and 7, ref. [6, 55]), b) a single herbicide molecule binds at each site [6], c) inhibitory activity of PS II herbicides

Inhibitor Chemical group	Common name	I_{50} conc. [M] susceptible chloroplasts	I_{50} conc. [M] resistant chloroplasts	R/S
s-Triazines	atrazine	3.6×10^{-7}	$\cong 3\times10^{-4}$	$\cong 10^3$
	atratone	6.0×10^{-7}	$\cong 6\times10^{-4}$	$\cong 10^3$
	ametryne	4.3×10^{-8}	2×10^{-5}	4.6×10^2
Triazinone	metribuzin	2.1×10^{-7}	5.4×10^{-5}	2.6×10^2
Urea	diuron (DCMU)	6.0×10^{-8}	8.1×10^{-8}	1.4
Amide	SWEP	8.0×10^{-7}	1.7×10^{-6}	2.1
Pyridazinone	pyrazon	6.1×10^{-6}	$\cong 4\times10^{-4}$	$\cong 65$
Uracil	bromacil	2.5×10^{-7}	5.0×10^{-6}	20
Phenols	DNOC	3.5×10^{-5}	5.0×10^{-6}	0.14
	ioxynil	7.0×10^{-7}	4.5×10^{-7}	0.64
Nitrophenol	bromonitro-thymophenol	2.2×10^{-7}	4.8×10^{-8}	0.22
Benzothiadiazinone	bentazon	5.0×10^{-5}	3.4×10^{-5}	0.6

Table III. I_{50} concentrations for inhibition of DCPIP reduction ($H_2O \rightarrow$ DCPIP) in isolated chloroplast from resistant and susceptible *Amaranthus retroflexus* biotypes. Resistance ratio (R/S) = I_{50} concentration resistant/I_{50} concentration susceptible.

is directly related to binding affinity [54, 55], d) inhibition of electron transport occurs due to an herbicide binding-induced alteration in the redox properties of the Q/B complex (Fig. 5, ref. [41], and g) mild trypsin treatment of chloroplast membranes selectively removes surface-exposed portions of some thylakoid polypeptides and concomitantly removes the binding sites for PS II-inhibitors [51, 52, 57]. We conclude from the trypsin studies that a membrane bound protein of the PS II-complex determines the herbicide binding site and is probably the apoprotein of the secondary electron carrier, B.

Based upon these conclusions, we can state that the weed biotypes which have developed triazine-resistance via a chloroplast membrane alteration must contain a thylakoid component with a subtle alteration responsible for reduced triazine binding affinity. We stress that the herbicide binding component of the membrane can not be expected to be absent, or even highly disordered since diuron and several other herbicides are still effective inhibitors [Table III] and the PS II-complex, *per se*, is photochemically active. This view leads us to suggest a model for the binding of inhibitors to the PS II-complex. Such a model must contain a mechanism for similar inhibitory action but allow variability among chemical classes of inhibitors in determining the actual binding process.

Photosynthetic herbicide research has traditionally had important emphasis on structure/activity analysis of the many chemical families that block PS II electron transport. It is recognized that there is a common "essential element" to all of the PS II-inhibitors (an electron deficient sp^2-carbon adjacent to a nitrogen with a lone electron pair) [58]. In addition to the essential element, comparisons of biological activity with chemical structure have shown that various hydrophobic side chains determine efficiency of action within various chemical classes [58 – 60]. These two chemical features – an essential element plus specificity-determining hydrophobic substituents – are recognized in our model of the inhibitor binding site [Fig. 8].

The essential element is a property of the small molecule which is conserved in all PS II-inhibitors. We suggest that this element interacts specifically with a special domain of the herbicide binding site – perhaps a part of the prosthetic group of B, which is essential for blocking electron flow. The hydrophobic side chains surrounding the "essential element" display elaborate diversity among the many different PS II-inhibitor classes. No common portion of these is consistently required for binding or activity. We suggest that a variety of amino acids in the herbicide-binding protein create domains surrounding the "active site" which specify the selectivity for these hydrophobic regions of the inhibitor. Successful inhibition of electron transport requires interaction of the herbicides with both binding site domains.

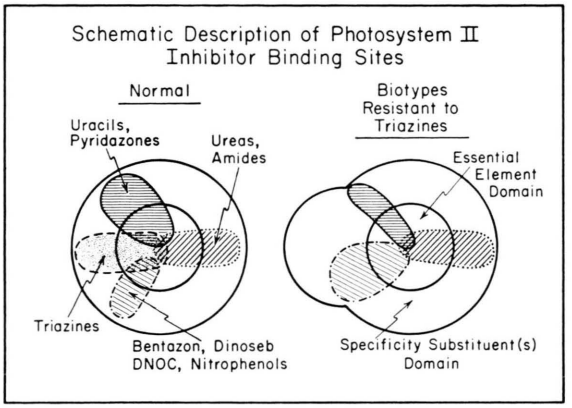

Fig. 8. Model of the binding sites for PS II-inhibitors. The existance of two domains determining binding properties is emphasized; one domain is responsible for binding of the "essential element", the other for binding of the "specificity determining substituents".

With the model of Fig. 8 in mind, the experimental usefulness of susceptible and herbicide-resistant weeds becomes apparent. By genetically modifying selective regions of the "specificity determining" domains of the herbicide binding site, we can analyze herbicides in both pairs of biological test systems and describe their degree of similarity with respect to occupying common regions of this domain. For example, diuron and atrazine behaved very differently [Table III] – we interpret this in the model as indicating that they recognize different segments (presumably several amino acids) of the specificity domain. After a small alteration occurred in this domain in herbicide-resistant plants, binding of only one compound was strongly affected. The R/S values of pyrazon were not as large as the triazines, but higher than the ureas. We interpret this as indicating that pyrazon occupies only part of the "triazine portion" of the domain. Similarly, increased activity of the nitrophenols in the triazine resistant plants indicates that alterations of the "specificity domain", which cause limited atrazine

binding, actually create a more favorable microenvironment for the nitrophenols. The model is at this stage very preliminary, but it does suggest a general approach for more detailed three-dimensional steric analysis of the herbicide binding compartment of the PS II-complex. It should be noted that the model is consistent with a multiple-site interaction hypothesis for the PS II binding site which has been proposed by Trebst and Draber [61] based mainly on structure/activity relationship analyses.

VI. Alterations in the native PS II-complex of triazine-resistant chloroplasts

We have described above the data which demonstrate that the binding site for PS II herbicides is modified in the triazine-resistant plants. This leads us to ask the question of whether or not the alteration of the herbicide receptor was accompanied by a detectable change in the kinetic or compositional characteristics of the native membranes even in the absence of all herbicides.

A. Kinetic analyses of PS II electron transport

Evaluation of chlorophyll fluorescence induction transients was the first measurement which suggested *in situ* differences in the PS II-complex of susceptible and triazine-resistant chloroplasts (Fig. 3, inset, see also [5, 21]). This analysis is based on the idea that the variable part of the induction curve reflects the oxidation-reduction state of Q [45, see reviews 62, 63]. Onset of illumination of dark-adapted chloroplasts results in an immediate rise of fluorescence to F_0 (zero-time fluorescence level). This is followed by a time-dependent increase in fluorescence intensity to an intermediate (F_I) level, and then another, slower rise to a maximal fluorescence intensity (F_M). Though the F_0 and F_M levels of both susceptible and resistant chloroplasts were identical (on samples of identical chlorophyll content), the resistant chloroplasts showed an initially faster $F_0 \rightarrow F_I$ increase. Although data for only one weed species are shown in Fig. 3 (inset), this characteristic feature of the transients has been observed for every triazine susceptible/resistant biotype pair examined. It should be noted that diuron-inhibited susceptible/resistant chloroplasts showed identical rates of fluorescence rise, indicating that the rate of primary photochemistry of the PS II complexes was not different [see also 5, 21]. The difference in

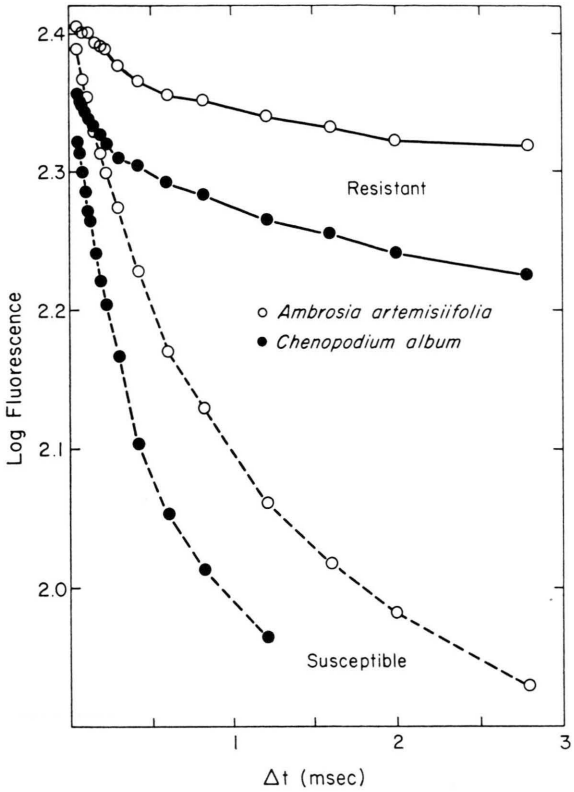

Fig. 9. Reoxidation of Q^- in susceptible and resistant chloroplasts for *Ambrosia artemisiifolia* and *Chenopodium album*. The chloroplasts (5 μg chlorophyll/ml) were illuminated with a very short saturating laser flash (20 ns, λ=430 nm) to reduce the Q pool completely. After various dark times (Δt between 50 μs and 3 ms) the remaining Q^- concentration, monitored as a function of the fluorescence level, was detected with a short, weak measuring flash (8 μs). A measuring cycle (actinic flash followed by a dark time Δt and the measuring flash) was repeated every 15 sec. Note: all measurements were done in *absence* of inhibitors.

the fluorescence rise in untreated chloroplasts therefore indicates that the Q^- concentration is higher in the early times of illumination of the dark adapted resistant chloroplasts. This suggested that the rate constant for Q^- reoxidation was altered. This was tested by directly measuring the rate of Q^- reoxidation by following the decay of fluorescence which occurred after an intense actinic flash illumination of isolated chloroplasts. As was shown in Fig. 4 a, this decay is completed in less than 1 sec in susceptible chloroplasts, but a portion of the decay persists for many seconds in the resistant sample [Fig. 4 b]. The decay in the fluorescence yield in the latter showed a binary oscillation after the first few

flashes [Fig. 4c]; this phenomenon is currently being investigated in more detail by Bowes, Crofts, and Arntzen.

A characterization of the fluorescence decay over a much shorter time scale (50 μsec to 3 msec) was achieved using a laser-flashed, computer-assisted fluorometer (available through the coutesy of Dr. A. Crofts, University of Illinois). In this system a sample was repeatedly illuminated using a 15 sec dark time between actinic flashes. At variable times (Δt) after the actinic flash, a weak, 8 μsec measuring flash was used to monitor the level of Q^-. The half-time of Q^- decay under these conditions for susceptible chloroplasts of *Ambrosia artemisiifolia* and *Chenopodium album* varied between 300 and 700 μsec [Fig. 9]. In contrast, the decay half-time for triazine-resistant chloroplasts of the same species was $\geqq 10$ fold longer. These data provide direct evidence for an alteration in the rate of $Q^- \rightarrow B$ electron transport which is an inherent feature of the triazine-resistant chloroplasts.

B. Polypeptide changes in triazine-resistant chloroplasts

In the sections above we have summarized evidence indicating that PS II-herbicides interact with a protein of the PS II-complex. Since this protein is apparently modified in resistant chloroplasts, we have analyzed the polypeptide composition of susceptible and triazine-resistant chloroplasts of *Amaranthus retroflexus* [5 and (Fig. 10)] and *Brassica campestris* [Fig. 10]. In both cases an integral membrane polypeptide with apparent molecular weight of 18 – 20,000 varied in mobility in the paired samples (see arrows). (It should be noted that species specific polypeptide differences do not allow direct comparison across all sample pairs.) A polypetide in the 18 – 20,00 Kdalton size class range is present in highly purified, diuron-sensitive PS II particles [J. Mullet and C. J. Arntzen, unpublished data]. We have also noted alterations in the relative mobility of polypeptides in this molecular weight range in trypsin-treated chloroplast membranes which have lost most of the PS II-inhibitor binding sites [52]. While we can not yet prove the possibility that the proteins which are altered in the chloroplast of Fig. 10 are directly related to the phenomenon of herbicide resistance, the presence of an altered polypeptide is at least consistent with the idea that a subtle change has occurred in a specific PS II constituent.

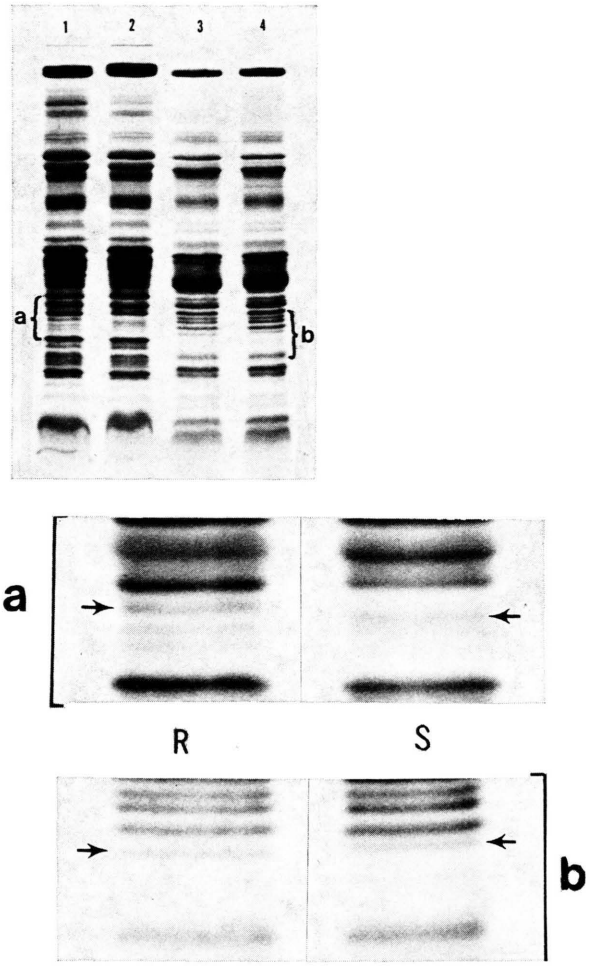

Fig. 10. Polypeptide composition of integral chloroplast membranes of *Amaranthus retroflexus* (1=resistant, 2=susceptible) and *Brassica campestris* (3=resistant, 4=susceptible). Procedures for sample preparation were as in ref. 5; 10–17% polyacrylamide was used in the separating gel. Enlargements of the approximate 15–24 Kdalton range of the gel (a, b, bottom) indicate the polypeptide which shows altered mobility in the respective paired samples.

Summary

The availability of plant material containing chloroplasts which show modified selectivity in response to PS II-inhibitors has opened a new pathway by which the "active site" analysis for these

inhibitors can be pursued at a basic level. Also the use of artificially induced algae mutants seems promising [64, 65].

Our studies, as well as previously published reports, lead us to focus on polypeptides of the PS II-complex for the specific herbicide binding component. It now seems likely that the bound quinone B is the candidate for the herbicide target site. It seems worth noting that a focus on the protein chemistry of the herbicide binding site would be of immediate value to applied as well as basic research. Selection of herbicidally active chemicals has been largely empirical to date. Knowledge of the amino-acid residues and/or protein microenvironmental features that regulate the binding efficiency for specific herbicide side chains within the chloroplast membrane could lead to a better understanding of inhibitor selectivity. In addition, crop-specific herbicide antidotes or safeners (to block herbicide action) might be devised through chemical manipulation to create a compound which does not contain the herbicidally active "essential element", but which does occupy the specificity binding domains of the herbicide target site.

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