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MODE OF ACTION OF PHOTOSYSTEM II HERBICIDES STUDIED BY THERMOLUMINESCENCE

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The mode of action of chemically different herbicides (ureas, pyridazinones, phenylcarbamates, triazines, hydroxyquinolines, hydroxybenzonitriles and dinitrophenols) on photosynthetic electron transport was investigated by measurements of oxygen evolution and thermoluminescence. Depending on the particular herbicide used the thermoluminescence band related to Q (the primary acceptor of Photosystem II) appears at +5, 0 or −14°C. It was shown that these three different peak positions can be ascribed to various redox states of Q, the shifts being due to the binding of herbicides to the chloroplast membrane. Both displacement experiments and additive inhibition of herbicide pairs measured by thermoluminescence and oxygen evolution suggested that the sites of action of these herbicides are on the same protein. However, herbicide treatment of trypsinized chloroplasts showed that there were three different binding sites on the same protein, in agreement with the classification of herbicides into three groups based on thermoluminescence measurements. Our results suggest that the primary and secondary acceptors of Photosystem II (Q and B, respectively) are in close proximity and form a common complex with the herbicide-binding protein within the chloroplast membrane.

Introduction

Many herbicides of different chemical structure interrupt the electron-transport chain on the reducing side of PS II between the primary acceptor Q and the plastoquinone pool [1]. There are now suggestions that these inhibitors act on the secondary acceptor B of PS II [2–4] which is a bound plastoquinone molecule enclosed in a proteinaceous environment [3,5], and that herbicides bind to this protein shield. The nature of this binding has been extensively studied. Experiments on the replacement of radioactively labelled triazine by ureas, pyridazinones and biscarbamates have shown that these herbicides have an identical binding site in the thylakoid membrane [6]. On the other hand, experiments with herbicide-resistant weeds and algae led to the conclusion that several overlapping binding sites exist on the same protein [3,7]. The gradual removal of the herbicide-binding sites by trypsin treatment also support the concept of different specific subreceptor sites for various types of herbicides [8]. According to the latest proposal, the herbicide bound to the protein constitutes an allosteric blockage of the electron transport, probably an effect due to the lowering of the redox potential of the B component (thermodynamic inhibition) [3,5,9].

In the present study, we introduced thermolumin-

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Abbreviations: DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DSPD, disalicylidenepropanediamine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; HQNO, 2-(*n*-heptyl)-4-hydroxyquinoline-*N*-oxide; Chl, chlorophyll; PS, photosystem; *P*-680, reaction center of PS II; Q, primary acceptor of PS II; B, secondary acceptor of PS II. Trivial names: monuron, 1-(*p*-chlorophenyl)-3,3-dimethylurea; simazine, 4,6-bis(ethylamino)-2-chloro-*s*-triazine; bromoxynil, 3,5-dibromo-4-hydroxybenzonitrile.

escence as a new technique to investigate the mode of action of herbicides acting at the binding site of DCMU. It was shown that the herbicide treatment shifts the peak position of the thermoluminescence band related to Q, suggesting that the herbicide binding affects the midpoint redox potential not only of B, but also that of Q. Thus, we assume that the primary and secondary acceptors of PS II are in close proximity in the thylakoid membrane. On the basis of the shift in the midpoint potential of Q the herbicides of various chemical structure can be classified into three thermodynamically different groups. We further assume that the three groups are distinguished by the binding of herbicides to three different though overlapping domains of the same protein.

Materials and Methods

Isolation of chloroplasts. Intact chloroplasts were isolated from mesophyll protoplasts prepared by enzymatic digestion of the first leaves of maize (*Zea mays* KSC 360) according to a method described previously [10]. Chloroplasts were resuspended in a medium containing 0.4 M (+)-sorbitol, 10 mM NaCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM EDTA, 0.4% bovine serum albumin and 50 mM Hepes at pH 7.5 [11].

Trypsin treatment of chloroplasts. For trypsin treatment the chloroplasts were resuspended in a medium containing 0.4 M (+)-sorbitol, 10 mM NaCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM EDTA and 50 mM Hepes at pH 7.0. The trypsin digestion (1 μg trypsin/1 μg Chl) was carried out at room temperature in the dark and at various time intervals samples were taken for thermoluminescence and oxygen measurements.

Measurement of photosynthetic oxygen evolution and uptake. The rate of photosynthetic oxygen evolution and uptake was measured by using a Clark-type electrode (Rank Brothers, Cambridge, U.K.) in a temperature-controlled cuvette at +25°C under saturating white light. The assay medium contained 0.1 M (+)-sorbitol, 10 mM K_2HPO_4, 20 mM NaCl, 4 mM $MgCl_2$, 2 mM EDTA, 50 mM Hepes, pH 7.5, and chloroplasts containing 50 μg Chl in a final volume of 3.0 ml [11].

Different parts of the electron-transport chain were studied by addition of electron acceptors and donors: 2 mM ferricyanide or 0.1 mM methyl viologen (PS II + PS I), 0.25 mM *p*-benzoquinone (PS II). The assay medium for the reaction $H_2O \rightarrow$ methyl viologen was supplemented with 0.1 mM NaN_3.

Measurements of thermoluminescence. The chloroplast suspension was diluted to a final chlorophyll concentration of 170 μg/ml. Aliquots (0.6 ml) of the suspension were used for measurement of thermoluminescence.

Measurement of glow curves was carried out in the temperature range from −80 to +80°C using an apparatus similar to that described by Tatake et al. [12]. The light emission of the samples was measured by a red-sensitive photomultiplier (EMI 9558 B) and the signal was amplified through a home-made differential amplifier and fed to a Philips PM 8120 *X-Y* recorder. The temperature of the sample holder was monitored using a platinum resistor thermometer placed below the samples. Samples were illuminated for 5 min by white light from a 650 W NARVA halogen lamp during continuous cooling from +20 to −80°C. The excitation light was passed through a heat-absorbing water filter (thickness 10 cm) and a Balzers neutral density filter giving an illumination intensity of 10 W/m^2. For the best resolution of peaks in the glow curve the rate of heating was 10°C/min, as used by Sane et al. [13].

Determination of the activation energies of thermoluminescence bands. A computer program was used to fit a single Randall and Willkins band [14] to the main thermoluminescence band of the glow curve of herbicide-treated chloroplasts and the activation energy was calculated as previously described [14].

Results and Discussion

Thermoluminescence originates from PS II and the bands of the glow curve can be related to the charge recombination between positively charged donors and negatively charged acceptors [15–19]. It has been previously shown that the glow curve of isolated chloroplasts is strongly influenced by the addition of PS II herbicides [13,16,19]. Thus, thermoluminescence seems to be a useful technique for studying the mechanism and sites of action of herbicides that inhibit the electron transport of PS II. As shown in Fig. 1, the treatment of chloroplasts with different herbicides acting at the level of DCMU causes charac-

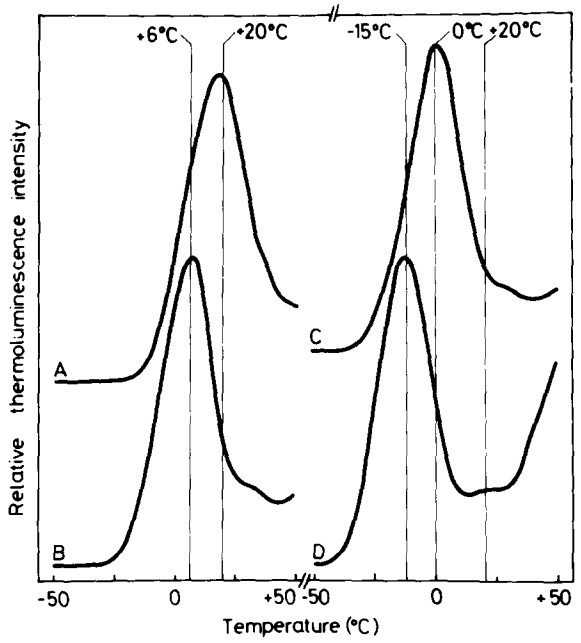

Fig. 1. Effect of different herbicides on the thermoluminescence of isolated chloroplasts. A, control; B, 63 μM monuron; C, 35 μM simazine; D, 180 μM bromoxynil. Chlorophyll concentration was 170 μg/ml. The suspension contained 65% glycerol. Excitation of the samples was performed at −80°C for 5 min. For more details see Materials and Methods.

teristic changes in the glow curve. The glow curve of untreated chloroplasts suspended in 65% glycerol and excited at −80°C exhibits a main band at +20°C originating from the charge recombination of an S state of the water-splitting enzyme and plastoquinone [19]. After the addition of monuron, simazine and bromoxynil this band completely disappears and a new band appears at +6, 0 and −15°C, respectively (Fig. 1).

The effect of some other herbicides on the thermoluminescence of isolated chloroplasts was also compared. The peak positions and activation energies of the thermoluminescence bands are listed in Table I. It can be seen that on the basis of the peak position of the main thermoluminescence band the herbicides investigated, representing very different chemical structures, can be classified into three groups. After addition of ureas, pyridazinones, phenylcarbamates and disalicyclidenepropandiamines, the glow curve exhibits the main band at about +5°C. Treatment of chloroplasts with triazines and hydroxyquinolines gives a band at about 0°C. In the presence of hydroxybenzonitriles and dinitrophenols the main band was shifted to −14°C. On the basis of the activation energies of thermoluminescence bands the herbicides can be divided into the same three groups. It is generally accepted that all of these herbicides inhibit electron transport at the level of B by interrupting the electron flow between Q and plastoquinone [2–4]. The mechanism of inhibition was explained by a lowering of the redox potential of the B component resulting in a thermodynamic blockage of electron transfer from Q to B [4,9]. Thus, we can conclude that in the presence of these herbicides electrons can only be accumulated on Q [3,19] and the thermoluminescence bands appearing at +5, 0 and −14°C represent the charge recombination between an S state and Q. From the theory of thermoluminescence it follows that the peak position and activation energy of a thermoluminescence band are determined by the redox span between the donor and acceptor molecules participating in the recombination [14,20]. Since it can be assumed that the S states are not influenced by the different herbicides acting at the level of DCMU, the shift in the peak position and activation energy of the main thermoluminescence band reflects a change in the redox potential of Q. The shift in the peak position of the thermoluminescence band characterizes the redox changes only qualitatively. The differences in the activation energies, however, show the exact value of the shift in the redox potential of Q. On the basis of our thermoluminescence measurements we emphasize that herbicide binding to the proteinaceous component of B causes a shift not only in the redox potential of the secondary acceptor B, as was proposed in the literature earlier [9], but also changes the redox state of the primary acceptor, Q. The different shifts in the peak position of the main thermoluminescence band can be explained in two ways:

(i) Besides Q two more acceptors may exist in the electron-transport chain between *P*-680 and B. The herbicides of the three groups inhibit the electron transport at the sites of these acceptors and thus the peaks appearing at +5, 0 and −14°C are related to Q and two other acceptors, respectively.

(ii) Although electrons for charge recombination originate only in Q, herbicide binding to the different domains of the protein component of B results in

TABLE I

THE EFFECT OF DIFFERENT HERBICIDES ON THE THERMOLUMINESCENCE OF ISOLATED CHLOROPLASTS

T_{max}, temperature at the maximum of the thermoluminescence band; E, activation energy; $\bar{X}$, mean values – significantly different at the level of at least 99% in all cases. DNOC, 4-6-dinitro-*o*-cresol.

Herbicide	T_{max} (°C)	E (eV)
Ureas		
Diuron (DCMU)	+6	0.745
Monuron	+5	0.705
Fenuron	+5	0.715
Pyridazinones		
Pyrazon	+4	0.710
Phenylcarbamates		
Phenmedipham	+5	0.725
Disalicylidenepropandiamines		
DSPD	+6	0.750
$\bar{X} = +5.2 \pm 0.7 \approx$	5	$\bar{X} = 0.730 \pm 0.019$
Triazines		
Atrazine	0	0.660
Simazine	0	0.650
Hydroxyquinolines		
HQNO	+1	0.710
$\bar{X} = 0.3 \pm 0.6 \approx$	0	$\bar{X} = 0.673 \pm 0.032$
Hydroxybenzonitriles		
Bromoxynil	−15	0.550
Ioxynil	−13	0.530
Dinitrophenols		
DNOC	−13	0.550
$\bar{X} = -13.7 \pm 1.2 \approx$	−14	$\bar{X} = 0.543 \pm 0.012$

different shifts in the redox state of Q, and in the position of the related thermoluminescence band as well.

Experiments were carried out to test the validity of these two hypotheses.

The inhibition of electron transport by parallel addition of different herbicides together with DCMU was investigated. If the inhibitory sites of these herbicides are the same as that of DCMU, the inhibition caused by both herbicides should be additive [21]. Table II shows the percentage of the inhibition by DCMU and other herbicides on the Hill reaction separately and in combination. The concentrations of herbicides were chosen so as to give an inhibition of approx. 30%. Additive inhibition of two herbicides thus should yield about 60% inhibition. As shown in Table II, in contrast to the low percentage of inhibition upon simultaneous application of DCMU and DBMIB (known to act at the level of plastoquinone [22]), the inhibition obtained with other herbicide pairs is indeed quite additive. Addition of these herbicides in reverse order (not shown in the Table) gave the same results confirming the assumption that the inhibitory site of these herbicides is similar to that of DCMU [2,23]. These data are in agreement with the results obtained earlier with radioactively labelled compounds, indicating that herbicides used in our experiments are in competition with DCMU for the same binding site [6].

Since the herbicides investigated can be classified into three groups on the basis of thermoluminescence measurements, we tried to use the thermoluminescence technique to follow the displacement of herbicides exhibiting thermodynamically different properties. Fig. 2 shows the time dependence of the displacement of bromoxynil by DCMU. Bromoxynil treatment gives a thermoluminescence band at −15°C. The addition of DCMU to the bromoxynil-

TABLE II

ADDITIONAL INHIBITION OF PHOTOSYNTHETIC ELECTRON FLOW BY VARIOUS HERBICIDES IN THE PRESENCE OF DCMU

The rate of electron transport was measured from H_2O to methyl viologen. Inhibition is expressed as a percentage of the control.

Herbicide (μM)	Inhibition (%)
0.08 DCMU	32
0.80 Monuron	28 } 60
0.08 DCMU + 0.80 monuron	52
0.08 DCMU	30
4.00 HQNO	24 } 54
0.08 DCMU + 4.00 DQNO	51
0.08 DCMU	31
0.06 Phenmedipham	21 } 52
0.08 DCMU + 0.06 phenmedipham	52
0.08 DCMU	33
7.00 Pyrazon	29 } 62
0.08 DCMU + 7.00 pyrazon	55
0.08 DCMU	30
0.40 Atrazine	34 } 64
0.08 DCMU + 0.40 atrazine	54
0.08 DCMU	29
7.00 Bromoxynil	32 } 61
0.08 DCMU + 7.00 bromoxynil	55
0.08 DCMU	34
2.00 Ioxynil	28 } 62
0.08 DCMU + 2.00 ioxynil	56
0.08 DCMU	29
0.10 DBMIB	33 } 62
0.08 DCMU + 0.10 DBMIB	39

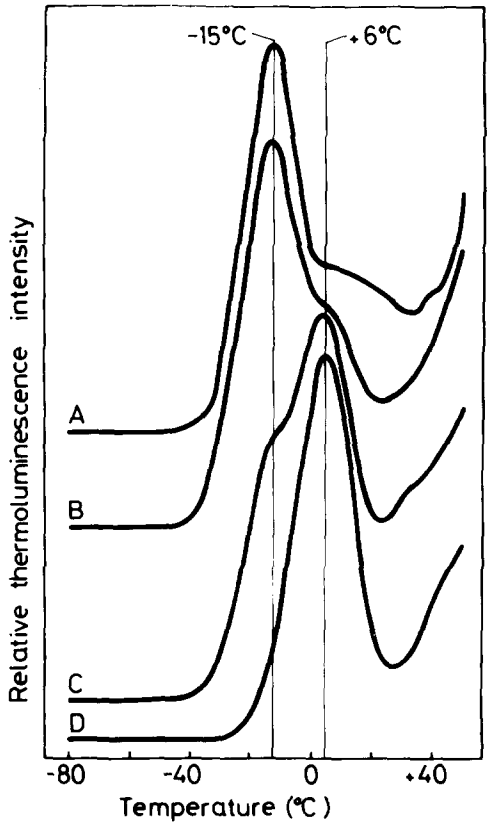

Fig. 2. Thermoluminescence of herbicide-treated chloroplasts during displacement of bromoxynil by DCMU. After 5 min incubation of the samples with 180 μM bromoxynil, 10 μM DCMU was added and thermoluminescence was recorded at different times. A, 1 min; B, 2 min; C, 10 min; D, 15 min. Measuring conditions as given in Fig. 1 except that the samples were excited for 5 min during continuous cooling from +20 to −80°C.

treated sample causes a gradual disappearance of the band at −15°C. Simultaneously, a thermoluminescence band at +6°C emerged and increased in intensity. The appearance of a thermoluminescence band at +6°C is a characteristic result of DCMU treatment. Similar results were obtained from the displacement of atrazine by DCMU, i.e., the thermoluminescence band at 0°C was replaced by a band appearing at +6°C (not shown in the figure).

Thus, we suggest that thermoluminescence can be used as a simple technique to measure the displacements of herbicides belonging to thermodynamically different groups. The displacement experiments (as measured by thermoluminescence) confirm the results obtained by oxygen evolution measurements (Table II), namely, that the target of herbicides belonging to the three thermodynamically different groups is similar to that of DCMU. The fact that all of these herbicides have a similar action site argues against the validity of hypothesis i.

It is generally accepted that the common target of these herbicides is the proteinaceous component of B [2,3]. Regitz and Ohad [24] and later Renger [4] reported that the proteolytic enzyme trypsin can be used to modify this proteinaceous component of B. More recently, it has been shown that trypsinization removes the herbicide-binding sites and concomitantly blocks the electron-transport chain between Q

and plastoquinone [3,5]. As shown in Fig. 3, after trypsin treatment the thermoluminescence band at +20°C disappears and a new band appears at −6°C (Fig. 3A–C). Since trypsinization interrupts the electron transport between Q and B, the new band at −6°C can be related to Q. As reported, the redox state of Q can change owing to the modification of the chloroplast membrane [25,26]. The trypsin treatment modifies the surface-exposed polypeptides [27] and thus it may cause a change in the surroundings of Q, which in turn determines the peak position of the thermoluminescence band after trypsin treatment. Addition of DCMU and simazine to the chloroplast suspension has no effect on the thermoluminescence of trypsin-treated chloroplasts. Our results obtained by DCMU and simazine addition are consistent with the data published recently by Steinback et al. [27], i.e., trypsin treatment causes a removal of the atrazine-binding site. The lack of any effect of DCMU and simazine on the thermoluminescence suggests that trypsinization modifies or removes the binding sites of these herbicides. However, bromoxynil shifts the peak position of the band at −6 to −15°C, suggesting that bromoxynil can affect the redox state of Q even after trypsin treatment.

To explain the inhibitory effect of bromoxynil after trypsin digestion, the electron-transport chain was investigated by oxygen evolution measurements. The results shown in Fig. 4 are in agreement with the data available in the literature [27]. It can be seen that a short trypsin treatment (10 min) suspended the inhibitory effect of simazine and DCMU while the inhibitory effect of bromoxynil was not influenced to any significant extent. After prolonged incubation (15 min) the inhibition of bromoxynil begins to decrease only gradually.

The linear electron transport from H_2O to *p*-benzoquinone was practically abolished after 10 min trypsinization and at the same time bromoxynil inhibits the electron transport from H_2O to ferricyanide. This confirms the results obtained by thermoluminescence (Fig. 3F); namely, that bromoxynil influences the state of Q after short trypsin treatment.

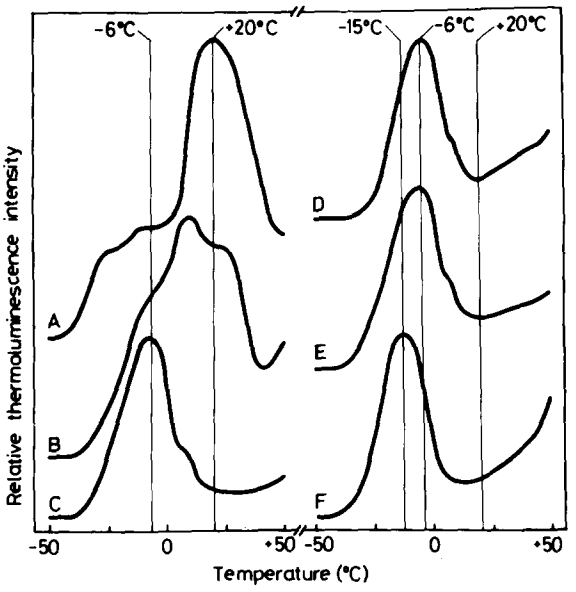

Fig. 3. Effect of herbicides on the thermoluminescence of trypsin-treated chloroplasts. A, control; B, 5 min trypsin digestion; C, 10 min trypsin digestion. After 10 min trypsin treatment the herbicides was added. D, 10 μM DCMU; E, 35 μM simazine; F, 180 μM bromoxynil. For more details see Materials and Methods.

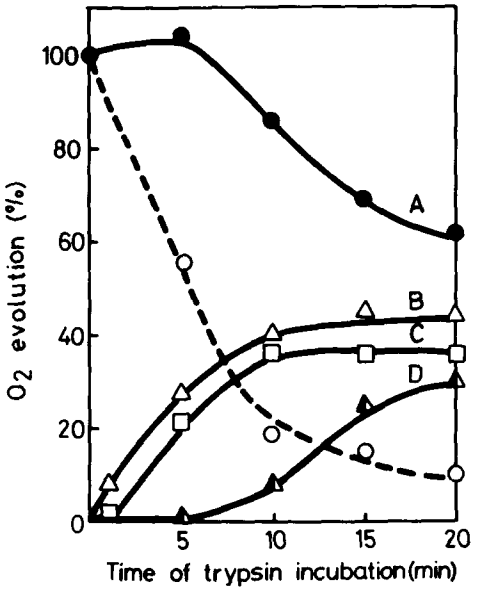

Fig. 4. Loss of the herbicide sensitivity of the photosynthetic electron transport after trypsin treatment of the thylakoid membrane. (———) Electron transport from H_2O to ferricyanide; (- - - - -) electron transport from H_2O to *p*-benzoquinone. A, no addition; B, 7 μM simazine; C, 1 μM DCMU; D, 36 μM bromoxynil. Other conditions as given in Materials and Methods.

It was shown earlier that trypsin treatment of isolated thylakoids resulted in a step-wise loss of the binding site of herbicides on the protein [27]. It can be seen in Fig. 4 that during trypsin digestion the time dependence of the inhibition of the effect of simazine, DCMU and bromoxynil is different. The inhibitory effect of simazine is most sensitive and that of bromoxynil is most resistant to trypsin treatment. These differences in the time course of the trypsin challenge to the inhibitory effect of herbicides suggest that the binding sites of these herbicides may be different. However, the data presented in Table II and Fig. 2 led to the conclusion that all of these herbicides have a common action site. This contradiction can be resolved by assuming that these herbicides act on the same protein and within the protein they have different, but probably overlapping binding sites.

This is in agreement with those results which proposed that several different overlapping binding sites of herbicides can exist on the proteinaceous component of B [2,3,8]. Summarizing our results, we conclude that herbicide binding changes not only the redox state of B, as stated previously [3,9], but also lowers the redox potential of Q. Since herbicides bind to the different domains of the same protein [3] and the binding has a strong influence on the redox states of both B and Q components of the electron-transport chain, we suggest that B and Q are in close proximity and that they form a common complex with the herbicide-binding protein shield.

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