The Interaction of Quinones, Herbicides and Bicarbonate with their Binding Environment at the Acceptor Side of Photosystem II in Photosynthesis

De Interactie van Chinonen, Herbiciden en Bicarbonaat met hun Bindingsomgeving aan de Acceptorkant van Foto-systeem II in Fotosynthese

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Proefschrift

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Stellingen bij het proefschrift van W. F. J. Vermaas, getiteld „De Interactie van Chinonen, Herbiciden en Bicarbonaat met hun Bindingsomgeving aan de Acceptorkant van Fotosysteem II in Fotosynthese"

Propositions to the dissertation of W. F. J. Vermaas, entitled "The Interaction of Quinones, Herbicides and Bicarbonate with their Binding Environment at the Acceptor Side of Photosystem II in Photosynthesis"

1. De versnelde uitdoving van de oscillaties in de flits-geïnduceerde O_2-produktie door van te voren donkergeadapteerde thylakoiden in de aanwezigheid van *o*-phenanthroline is een gevolg van remming van het elektronentransport aan de acceptorkant van Fotosysteem II, en niet van invloeden aan de donorzijde zoals is gesuggereerd door Anan'ev en Zakrzhevskii.
The accelerated damping of the oscillations in the flash-induced O_2-production by previously dark-adapted thylakoids in the presence of o-phenanthroline is due to inhibition of electron transport at the acceptor side of Photosystem II rather than to effects at the donor side, as has been suggested by Anan'ev and Zakrzhevskii.

Dit proefschrift / *This dissertation*

Anan'ev, G.M. and Zakrzhevskii, D.A., Study of the oxygen formation stage in photosynthetic decomposition of water, Fiziol. Rast. 30 (1983) 23-29

2. Vanwege de beweeglijkheid van het door Fotosysteem II reduceerbaar plastochinon in het thylakoidmembraan is remming van Fotosysteem II-afhankelijk elektronentransport door herbiciden geenszins proportioneel met de vermindering van de oppervlakte boven de chlorofyl *a* fluorescentieinduktiecurve, in tegenstelling tot de veronderstelling van van Assche en Carles.
Since the Photosystem II-reducible plastoquinone is mobile within the thylakoid membrane, inhibition of Photosystem II-dependent electron transport by herbicides is not at all proportional to the decrease of the area over the chlorophyll a fluorescence induction curve, in contrast to the supposition of van Assche and Carles.

Siggel, U., Renger, G., Stiehl, H.H. and Rumberg, B., Evidence for electronic and ionic interaction between electron transport chains in chloroplasts, Biochim. Biophys. Acta 256 (1972) 328-335

van Assche, C.J. and Carles, P.M., Photosystem II inhibiting chemicals; molecular interaction between inhibitors and a common target, in: Biochemical Responses Induced by Herbicides, ACS Symposium Series 181 (D.E. Moreland, J.B. St. John and F.D. Hess, eds.) (1982), pp. 1-21, American Chemical Society, Washington DC

3. Een verhoging van de dissociatieconstante van het ^{14}C-atrazine/bindingsplaats-complex in het thylakoidmembraan, zoals gevonden bij continue belichting in de afwezigheid van een exogene elektronenacceptor, kan niet toegeschreven worden aan een verhoogde concentratie van Q_B^-, zoals werd gepostuleerd door Jursinic en Stemler.
An increase of the dissociation constant of the ^{14}C-atrazine/binding site complex in the thylakoid membrane, as found during continuous illumination in the absence of an exogenous electron acceptor, cannot be attributed to an increased Q_B^- concentration, as was postulated by Jursinic and Stemler.

Dit proefschrift / *This dissertation*

Jursinic, P. and Stemler, A., Changes in ^{14}C-atrazine binding associated with the oxidation-reduction state of the secondary quinone acceptor of Photosystem II, Plant Physiol. 73 (1983) 703-708

4. De conclusie van Oettmeier en medewerkers dat de binding van fenol-type herbiciden aan het Fotosysteem II complex non-competitief is met die van diuron--type herbiciden is onjuist.
The conclusion of Oettmeier and co-workers that the binding of phenol-type herbicides to the Photosystem II complex is non-competitive with respect to that of diuron-type inhibitors is incorrect.

Dit proefschrift / *This dissertation*

Oettmeier, W., Masson, K. and Johanningmeier, U., Evidence for two different herbicide-binding proteins at the reducing side of Photosystem II, Biochim. Biophys. Acta 679 (1982) 376-383

5. Uit de kritiek die Bockris en Tunuli geven op de chemi-osmotische hypothese van Mitchell blijkt een volledig onbegrip van die schrijvers omtrent de specifieke lokalisatie van chinonreduktie- en chinoloxidatiereakties in een biologisch membraan.
Bockris' and Tunuli's criticism of Mitchell's chemi-osmotic hypothesis reflects their complete incomprehension about the specific localization of quinone-reduction and quinol-oxidation reactions in a biological membrane.

Bockris, J.O'M. and Tunuli, M.S., An electrochemical model of biological energy storage, J. Electroanal. Chem. 100 (1979) 7-12

6. De voor fenol-type herbiciden gebruikte omschrijving "inhibitory uncoupler", aangevend dat ze fotosynthese remmen en elektronentransport ontkoppelen van fosforylering, is misleidend, omdat in de plantecel het proces dat het meest gevoelig is voor fenol-type herbiciden de RNA-synthese is, en niet het fotosynthetisch elektronentransport of de ATP-synthese.
The description "inhibitory uncoupler" used for phenol-type herbicides, indicating that they inhibit photosynthesis and uncouple electron transport from phosphorylation, is misleading because the process in the plant cell that is most sensitive to phenol-type herbicides is RNA-synthesis, and not photosynthetic electron transport or ATP synthesis.

Moreland, D.E., Mechanisms of action of herbicides, Ann. Rev. Plant Physiol. 31 (1980) 597-638

Ashton, F.M., de Villiers, O.T., Glenn, R.K. and Duke, W.B., Localization of metabolic sites of action of herbicides, Pest. Biochem. Physiol. 7 (1977) 122-141

7. Uit metingen van de fluorescentielevensduur van het chlorofyl blijkt dat in Fotosysteem I de snelheidsbeperkende stap tussen fotonabsorptie en radikaalpaarvorming de energieoverdracht tussen antennepigmentmolekulen is, en niet het ladingsscheidingsproces in het reaktiecentrum.
Measurements of the chlorophyll fluorescence lifetime indicate that in Photosystem I the rate-limiting step between photon absorption and radical pair formation is the energy transfer between antenna pigment molecules, and not the charge separation process in the reaction center.

Beddard, G.S., Fleming, G.R., Porter, G., Searle, G.F.W. and Synowiec, J.A., The fluorescence decay kinetics of *in vivo* chlorophyll measured using low intensity excitation, Biochim. Biophys. Acta 545 (1979) 165-174

Gulotty, R.J., Fleming, G.R. and Alberte, R.S., Low-intensity picosecond fluorescence kinetics and excitation dynamics in barley chloroplasts, Biochim. Biophys. Acta 682 (1982) 322-331

Kamogawa, K., Morris, J.M., Takagi, Y., Nakashima, N., Yoshihara, K. and Ikegami, I., Picosecond fluorescence studies of P-700 enriched particles of spinach chloroplasts, Photochem. Photobiol. 37 (1983) 207-213

8. Het is niet te verdedigen dat twee fotonen nodig zijn voor een ladingsscheiding tussen P680 en feofytine, zoals aangenomen door Fong.
One cannot uphold the hypothesis that two photons are necessary for a charge separation between P680 and pheophytin, as proposed by Fong.

Fong, F.K., Light path of carbon reduction in photosynthesis, in: Light Reaction Path of Photosynthesis (F.K. Fong, ed.) (1982), pp. 277-321, Springer Verlag, Berlin

9. Ten onrechte wordt bij de interpretatie van de resultaten verkregen in het onderzoek naar de effekten van licht en CO_2 op de etheenproduktie in groene plantedelen geen rekening gehouden met de mogelijkheid dat een tussenprodukt in het CO_2-fixatieproces de omzetting van 1-aminocyclopropaan-1-carbonzuur in etheen reguleert.
It is incorrect that in the interpretation of the results obtained on the effects of light and CO_2 on the ethylene production in green parts of the plant, the possibility of regulation of the 1-aminocyclopropane-1-carboxylic acid conversion into ethylene by an intermediate in the CO_2-fixation process has not been taken into account.

Kao, C.H. and Yang, S.F., Light inhibition of the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene in leaves is mediated through carbon dioxide, Planta 155 (1982) 261-266

10. Bij de toekenning van waarden aan de resultaten van spektrofotometrisch fytochroomonderzoek wordt onvoldoende rekening gehouden met het bestaan van verschillende typen van fytochroom, en met de fytochroomcompartimentatie in de plant.
In the evaluation of the results of spectrophotometric phytochrome research, the existence of more than one type of phytochrome and the phytochrome compartimentation in the plant are not taken into account sufficiently.

Shimazaki, Y., Cordonnier, M.-M. and Pratt, L.H., Phytochrome quantitation in crude extracts of *Avena* by enzyme-linked immunosorbent assay with monoclonal antibodies, Planta 159 (1983) 534-544

Morgan, D.C., O'Brien, T. and Smith, H., Rapid photomodulation of stem extension in light-grown *Sinapis alba* L.; studies on kinetics, site of perception and photoreceptor, Planta 150 (1980) 95-101

11. Teneinde de toegankelijkheid van de stellingen bij een proefschrift tot buiten het Nederlands taalgebied uit te breiden, dienen de stellingen ook in een internationale omgangstaal te worden gesteld.
In order to increase the accessibility of the propositions of a dissertation to an audience beyond the Dutch linguistic frontier, the propositions should be additionally presented in a commonly used foreign language.

12. Het gevaar dat van conventionele en chemische strijdmiddelen uitgaat is te vergelijken met dat van kernwapens: met beide soorten bewapening kan men de wereldbevolking enige malen uitroeien en de levensvooruitzichten van de overlevenden beperken. Om deze reden is de toespitsing van de aandacht van politici en burgers op alleen de kernbewapening kortzichtig.
The danger of conventional and chemical weapons is comparable to that of nuclear weapons: with both types of armament the world population can be annihilated, and the prospects of life for the survivors can be seriously limited. For this reason, the attention of politicians and citizens focused on nuclear weapons alone, is short-sighted.

13. Het getuigt van een kronkel in het denkraam van volks- en regeringsvertegenwoordigers om enerzijds autogordels, valhelmen en fietsreflectoren verplicht te stellen, en anderzijds geen adequate maatregelen te nemen tegen handel in en gebruik van bijvoorbeeld rookwaar en verdovende middelen, die, in vergelijking met verkeersongevallen, een groter gevaar vormen voor menselijk welzijn
It is a feat of twisted logic in the mind of members of parliament and government that they compel, on the one hand, the use of seatbelts, crash-helmets and bicycle reflectors, and, on the other hand, refrain from taking adequate action against trade in and use of, for example, tobacco and drugs, which form, in comparison with traffic accidents, a greater danger to human welfare.

14. Het is met ontwapening net zo als met salarisvermindering: de meesten zijn ervan overtuigd dat het noodzakelijk is, maar men verwacht dat de ander de eerste stap zet.
Disarmament resembles salary cutting in that most people are convinced that it is necessary, but one group expects the other to make the first move.

15. Eén van de overeenkomsten tussen de wereld der wetenschap en der politiek is het veelvuldig gebruik van de kruiwagen: vooruitgang in werk en loopbaan wordt meer bepaald door kennissen dan door kennis.
One of the similarities between the world of science and politics is the use of the "old boys network": progress in work and career is determined more by who you know rather than by what you know.

Behorend bij proefschrift W.F.J. Vermaas
Wageningen, 25 april 1984

Dans les sciences, il n'y a rien de si simple
que ce qui a été trouvé hier,
mais rien de si difficile
que ce qui sera trouvé demain.

In science nothing is so simple
as that what has been found yesterday,
but nothing is so difficult
as that what will be found tomorrow.

In de wetenschap is niets zo eenvoudig
als dat wat gisteren gevonden is,
maar niets zo moeilijk
als dat wat morgen gevonden zal worden.

J.-B. Biot (1774-1862),
Physicien et astronome français
French physicist and astronomer
Frans natuur- en sterrenkundige

Contents

List of abbreviations, symbols and trivial names

ABP-32	azidoatrazine-binding 32,000 M_r thylakoid protein
atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine
azidoatrazine	2-azido-4-(ethylamino)-6-(isopropylamino)-*s*-triazine
6-azido-Q_0C_{10}	6-azido-5-decyl-2,3-dimethoxy-*p*-benzoquinone
BQ	benzoquinone
bromoxynil	3,5-dibromo-4-hydroxybenzonitrile
CBB	Coomassie Brilliant Blue
Chl	chlorophyll
cyt	cytochrome
D	one-electron donor to S_2 and S_3
DAD	diaminodurene
DBMIB	dibromothymoquinone
DCPIP	2,6-dichlorophenolindophenol
dinoseb	2,4-dinitro-6-*sec*-butylphenol
i-dinoseb	2,4-dinitro-6-isobutylphenol
diuron	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMSO	dimethylsulfoxide
DNOC	4,6-dinitro-*o*-cresol
DQH_2	tetramethyl-*p*-benzohydroquinone
EDTA	ethylenediaminetetraacetate
E_m	midpoint redox potential
E_n	Q_B/inhibitor exchange parameter ($1 \leq n \leq 4$)
EXAFS	extended X-ray absorption fine structure
FeCy	$K_3Fe(CN)_6$
H-subunit	"heavy" subunit of the reaction center protein complex from purple photosynthetic bacteria
I_{50}	inhibitor concentration causing 50 % inhibition of electron transport
Inh	inhibitor
ioxynil	3,5-diiodo-4-hydroxybenzonitrile
IRM	isolation / reaction medium
K_d	dissociation constant
kDa	kilodalton

L-subunit	"light" subunit of the reaction center protein complex from purple photosynthetic bacteria
LRP-32	lysine-rich Photosystem II-related 32,000 M_r thylakoid protein
M-subunit	"medium" subunit of the reaction center protein complex from purple photosynthetic bacteria
M_r	apparent relative molecular mass
MV	methyl viologen
P680	reaction center chlorophyll *a* of Photosystem II
P700	reaction center chlorophyll *a* of Photosystem I
o-phen	*o*-phenanthroline
Pheo	pheophytin
PQ	plastoquinone
PS	Photosystem
Q_A	primary electron-accepting quinone in Photosystem II
Q_B	secondary electron-accepting quinone in Photosystem II
RC	reaction center
Rubisco	ribulose-1,5-bisphophate carboxylase/oxygenase
S_n	water splitting enzyme system in state S_n $(0 \leq n \leq 4)$
SDS/PAGE	sodium dodecyl sulphate / polyacrylamide gel electrophoresis
UCE-32	a 32,000 M_r thylakoid protein that is extractable with urea or cholate
UQ	ubiquinone
Y_n	O_2 evolution by thylakoids at the n^{th} single-turnover flash
$Y_{n,c}$	O_2 evolution by control thylakoids at the n^{th} single-turnover flash
Y_{x-y}	average O_2 evolution by thylakoids in the x^{th} until y^{th} single-turnover flash
α	miss probability of net Photosystem II charge separation
β	double-hit probability of net Photosystem II charge separation in short flashes
$\Phi_{F,\ var}$	variable chlorophyll *a* fluorescence yield

Chapter 1, Introduction

1.1. General introduction on photosynthesis

The continuing existence of life on earth in its present diversity depends on photosynthesis; this is the process (taking place in, for example, green plants), in which CO_2 and H_2O are converted into sugars and O_2 in the light. In this way, light energy absorbed is converted partly into chemical energy that is used as a free energy source not only for both the photosynthetic and non-photosynthetic organisms, but also -in the form of fossile fuels- for most industries, heating, etc.

In higher plants and eukaryotic algae the process of photosynthesis takes place in a specialized cell organelle, the chloroplast. This organelle contains a complex membrane system, the thylakoids, in which the first part of the process, the photosynthetic electron transport coupled with ATP synthesis, occurs. This electron transport is initiated by light absorption by thylakoid pigments such as chlorophylls, which transfer their excitation energy to the reaction center pigments P680 and P700, chlorophylls in a special environment of pigment/protein complexes. P680, the reaction center pigment of Photosystem II (PS II), and P700, the reaction center pigment of PS I, are part of the photosynthetic electron transport chain that consists of numerous redox intermediates, often associated with thylakoid proteins in a very specific way.

After transfer of energy from an excited pigment molecule that is part of the antenna to the primary donor in the reaction center, the energy is available for photochemistry. The reaction center chlorophyll(s) in its excited state has a very high probability of transferring an electron to a neighboring pigment molecule, a chlorophyll *a* in PS I, or a pheophytin (or a chlorophyll *a* molecule) in PS II, which in turn is reoxidized rapidly (in the order of ps or ns) by other intermediates at a larger distance from the reaction center. In this way, a rapid and efficient charge separation between the reaction center pigment and an intermediate at more than 3 nm distance is possible. For recent reviews on this subject the reader is referred to Okamura *et al.* (1982a) and Parson and Ke (1982).

For PS II the first electron acceptor which, in its reduced form, is stable on a μs time scale is the primary electron-accepting quinone Q_A. This

quinone is thought to be embedded in a protein moiety in the thylakoid membrane. Reoxidation of reduced Q_A takes place via electron transfer to a plastoquinone (PQ) molecule that is non-covalently associated with a special binding site in the PS II protein complex (Section 1.2). This bound PQ is termed Q_B. Usually, PQ's are free in the lipid phase of the thylakoid membrane, and are termed the "PQ-pool".

The oxidized reaction centers are reduced by electron donors. For PS II the ultimate donor is H_2O; in the thylakoid two water molecules are oxidized to O_2, with concomitant production of $4H^+$ and 4 (bound) electrons; for a recent review, see Inoue *et al.* (1983). For PS I the electron donor is PS II: plastoquinol reduces $P700^+$ via several electron carriers. The ultimate electron acceptor is $NADP^+$ (nicotinamide adenine dinucleotide phosphate). In this way, a molecule of $NADP^+$ is reduced to $NADPH(+H^+)$ by H_2O with the help of two turnovers of both PS II and PS I. The $NADPH(+H^+)$ formed is utilized in enzymatic processes that take place in the "cytoplasm" of the chloroplast, the stroma. The net result of these stroma processes is that CO_2 and H_2O are converted into a sugar polymer, starch, at the expense of reducing power (in the form of $NADPH(+H^+)$) and energy (in the form of the energy-rich adenosine triphosphate, ATP). The details of this conversion are described by Bassham and Buchanan (1982).

The energy necessary for ATP formation, required for the CO_2 reduction, also comes ultimately from the light absorbed by the photosynthetic pigments. ATP is synthesized from adenosine diphosphate (ADP) and inorganic phosphate at the expense of an electrochemical gradient of protons across the thylakoid membrane generated by the photosynthetic electron transport processes. The thylakoids in the chloroplast are connected to each other and form a large complex in which the thylakoid membranes separate the stroma from a rather small compartment, the intrathylakoidal lumen. In intact systems this is not connected directly to the stroma. Parts of the thylakoids are often stacked in "piles" (grana). The localization of the intermediates of the photosynthetic electron transport is such that protons are released, upon oxidation of water or of plastoquinol (see Section 1.2), specifically into the inner space (the intrathylakoidal lumen) whereas proton uptake upon reduction of plastoquinone (see Section 1.2) is from the stroma. The resulting proton gradient over the thylakoid membrane is used for ATP synthesis by the thylakoid ATPase. Details of this process and of the regulation of ATP synthesis and hydrolysis can be found

in reviews by Junge and Jackson (1982), McCarty and Carmeli (1982), Ort and Melandri (1982), and in Strotmann and Schumann (1983).

The partial reactions of photosynthesis summarized above, involving water splitting, light-induced charge separation, electron transport, proton translocation, and enzymatic reactions in the stroma are very important and quite interesting, but are by far too complicated to be covered all together in any detail in a thesis. As a topic for the research described in this dissertation only one detail of the photosynthetic process was chosen: the electron transport through the two quinone molecules, Q_A and Q_B, which are bound to a protein complex, and which function at the electron acceptor side of PS II (the latter defined as the part of the electron transport chain between P680 and the PQ pool).

1.2. Electron transport on the acceptor side of Photosystem II

In this section, some recent developments concerning electron transport at the acceptor side of PS II (between P680 and the PQ pool) are summarized. Some other interesting aspects of this subject have been reviewed recently by Crofts and Wraight (1983).

An understanding of the electron transport processes at the acceptor side of PS II has come principally from studies on photosynthetic purple bacteria. It has become evident that the electron acceptors of photosynthetic bacteria are analogous to those of PS II in plants. Therefore, comparisons will often been made in this dissertation between green plants and purple photosynthetic bacteria. Knowledge of many processes in bacterial photosynthesis is far more detailed than that of comparable electron transport processes in the green plant. One of the reasons for this has been the ability to easily purify well-defined functional reaction center components from photosynthetic bacteria in contrast to green plants. In recent years, however, methods to isolate relatively undamaged subchloroplast particles, greatly enriched in PS II components, from green plants have been developed (see contributions to the book edited by Inoue *et al.* (1983)). It is generally assumed that the properties of the components in isolated form are identical to those in more intact systems. However, this assumption may be questioned (Verméglio, 1982).

1.2.1. Electron transport between P680 and Q_A

As described above, P680 forms the heart of PS II in green plants in that

it is the component that - upon excitation by light - "pumps" an electron from H_2O to the electron donor side of PS I. The first well-characterized acceptor for the electron from P680 is pheophytin (Pheo) (Klimov *et al.*, 1977), which is reduced in less than 2 ns by P680 (Shuvalov *et al.*, 1980). (By analogy with photosynthetic bacteria, this is expected to be in the ps range.) The reduced Pheo is oxidized in less than 200 ps by the quinone Q_A; this reaction has to be much faster than the back reaction between $P680^+$ and $Pheo^-$, which has a $t_{\frac{1}{2}}$ of about 4 ns when Q_A is reduced (Shuvalov *et al.*, 1980), since the efficiency of quantum absorption leading to a charge separation is more than 95 % (Thielen and van Gorkom, 1981). In purple bacteria, the distance between bacteriopheophytin and Q_A was estimated to be about 1 nm (Peters *et al.*, 1978; Okamura *et al.*, 1979; Gast and Hoff, 1979). In green plants, however, the distance between Pheo and Q_A may be much larger (van Gorkom *et al.*, 1983).

1.2.2. The Q_A/Fe^{2+} complex

In the green plant, Q_A is a PQ molecule (probably PQ-9, *i.e.*, with a side chain of 9 isoprenoid units) that is bound in a special protein environment and that has several unusual properties. First, Q_A can be reduced only to the semiquinone anion form (Witt, 1973; van Gorkom, 1974). The absorption spectrum indicates that on a relatively short timescale (ms to s) no protonation of Q_A^- is observed. After trypsin digestion of protein components around Q_A light-induced proton uptake does not occur either (Renger and Tiemann, 1979). However, the midpoint redox potential (E_m) of the Q_A/Q_A^- couple is pH-sensitive up to pH = 10 (n = 1) (Knaff, 1975), which is usually interpreted to indicate that at longer timescales (min) Q_A^- is protonated. This, however, may also be explained by a protonation occurring at a neighboring protein group rather than at Q_A^- itself (also see Section 1.2.5). When suspended in aqueous buffer, the PQ-9 semiquinone is estimated to show a pK_a of about 4.7 (Swallow, 1982). As a result of its special environment, the semiquinone Q_A^- may be stabilized considerably: the operative E_m of Q_A/Q_A^- at pH = 7 is about -130 mV (Crofts *et al.*, 1984), whereas the E_m at pH = 7 for the free PQ-9/$PQ\text{-}9^-$ couple is expected to be much lower.

In purple photosynthetic bacteria, an analogous Q_A appears to exist. For the species usually used (*Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides*), Q_A is a UQ-10 (ubiquinone-10) molecule. UQ is a plastoquinone analog, and is an electron carrier also in the respiratory chain in mitochondria. In the

purple bacteria, a large shift in E_m at pH = 7 of the Q_A/Q_A^- couple, as compared to that of the UQ-10/UQ-10$^-$ in solution, is also observed (Morrison *et al.*, 1982; Swallow, 1982).

It has often been observed that Q_A in green plants is heterogeneous in several of its properties, among which is its E_m. Since this topic is not related directly to the study described in this thesis, the interested reader is referred to the review of Vermaas and Govindjee (1981a) or Cramer and Crofts (1982).

Both in bacteria and in plants a Fe^{2+}-ion is located close to Q_A and its neighboring electron acceptor Q_B (Klimov *et al.*, 1980; Nugent *et al.*, 1981; Okamura *et al.*, 1982a; Petrouleas and Diner, 1982). Although it has been suggested earlier (Feher, 1971) that Fe^{2+} could be the primary electron acceptor or could facilitate electron transport from Q_A to Q_B (the iron-wire hypothesis (Okamura *et al.*, 1975)), and although depletion of Fe^{2+}, among other components, resulted in a blockage of electron transport at the quinone level (Blankenship and Parson, 1979), it is believed now that Fe^{2+} does not specifically participate in electron transport. The above-mentioned inhibition of electron transport by Fe^{2+} depletion was later found to be due to the concomitant depletion of the H-subunit (H stands for heavy) of the bacterial reaction center complex (Okamura *et al.*, 1982b). However, Fe^{2+} may play a role in the stabilization of the semiquinone forms of Q_A and Q_B (Wraight, 1982) from where the Fe^{2+} ion is probably equidistant (Wraight, 1978), and/or Fe^{2+} may prevent the formation of fully reduced Q_A (Dutton *et al.*, 1978). Indeed, a stabilization of semiquinone anions and anionic hydroquinones by monovalent and, especially, polyvalent cations has been demonstrated in solutions (Smith and Carrington, 1967; Jaworski and Kalinowski, 1977). However, a direct ionic interaction between Fe^{2+} and Q_A^- or Q_B^- is improbable as the exchange interaction between Fe^{2+} and the semiquinone spin has been found to be small (Butler *et al.*, 1980). EXAFS studies indicate that, in purple bacteria, neither Q_A nor Q_B belong to the primary ligand shell of the Fe^{2+} (Bunker *et al.*, 1982; Eisenberger *et al.*, 1982).

1.2.3. Q_A^- oxidation by Q_B

The reduced one-electron carrier Q_A^- is oxidized by a second quinone, Q_B (Pulles *et al.*, 1976). Q_B can be reduced twice by Q_A^-, forming a quinol (Mathis and Haveman, 1977). The existence of such a "two-electron gate", linking the

one-electron acceptor Q_A to the two-electron-accepting PQ pool, was postulated independently and simultaneously by Velthuys and Amesz (1974) and Bouges-Bocquet (1973).

In green plants the kinetics of electron transfer between Q_A and Q_B can be monitored, for example, by chlorophyll (Chl) *a* fluorescence (Q_A is a quencher of Chl *a* fluorescence whereas Q_A^- is not (Duysens and Sweers, 1963)) or by absorbance changes at 320 nm (the difference spectrum of the plastosemiquinone anion and plastoquinone or plastoquinol has a maximum near 320 nm (Bensasson and Land, 1973), which is slightly red-shifted in the thylakoid (Pulles *et al.*, 1974)). The advantage of the fluorescence method is that it monitors the redox state of Q_A and not of Q_B, but the disadvantage is that the variable Chl *a* fluorescence yield is not proportional to the Q_A^- concentration (Joliot and Joliot, 1964; Joliot *et al.*, 1973) because, most of the time, the pigment antenna serves more than one PS II center. It is clear that as long as not all PS II traps that are accessible to an excitation are closed, the excitation is likely to cause a charge separation rather than Chl fluorescence. This results in a relatively low variable fluorescence yield ($\Phi_{F,var}$) at a rather high Q_A^- concentration. On the other hand, the absorbance change measurements at 320 nm have the advantage of a linear relationship between the concentration difference of semiquinones and the absorbance change; however, one disadvantage of this method is that only the transition $Q_A^-.Q_B^- \rightarrow Q_A.Q_B^{2-}$ (disregarding possible protonation of the components) and not the transition $Q_A^-.Q_B \rightarrow Q_A.Q_B^-$ can be measured, since the latter does not show a net change in the semiquinone concentration (Mathis and Haveman, 1977). Another disadvantage of the 320 nm absorbance change measurements is that other components (for example, at the PS II donor side) also show light-induced absorbance changes at 320 nm (Renger and Weiss, 1982; Weiss and Renger, 1984). A third disadvantage is that protonation of the (semi)reduced forms of the quinone influences the difference spectra (Bensasson and Land, 1973; Morrison *et al.*, 1982). The absorbance change at 550 nm (Knaff and Arnon, 1969), due to a spectral shift of a neighboring pigment molecule, possibly Pheo (Klevanik *et al.*, 1977), upon reduction of Q_A, appears to be a better indicator of the redox state of Q_A than the 320 nm absorbance change.

In purple photosynthetic bacteria, Q_B (a ubiquinone in most organisms) also serves as a "two-electron gate": after dark adaptation, Q_B (as well as Q_A) is mainly oxidized, whereas odd flashes promote the formation of a ubisemiquinone, and after even flashes the semiquinone disappears again (the fully reduced quinone is formed) (Verméglio, 1977; Wraight, 1977). The electron

distribution between the two quinones in these bacteria can be monitored spectrophotometrically by following the kinetics of rereduction of the oxidized bacterial reaction center (RC) by Q_A^- after a flash in reaction center preparations (see, for example, Clayton and Yau (1972), Cogdell *et al.* (1974) and Wraight and Stein (1983)). Information on the electron transfer rate between primary and secondary quinones can be obtained by absorbance change measurements in the 730-780 nm region (Verméglio and Clayton, 1977; Verméglio, 1982). In this region, Q_A^- and Q_B^- induce distinct absorbance changes resulting from bandshifts due to local electrostatic effects on the bacteriopheophytin and/or the bacteriochlorophyll in the RC; thus, these absorbance changes are analogous to those at 550 nm in green plant systems.

Using fluorescence or absorbance measurements, Q_A^- oxidation by $Q_B^{(-)}$ was found to occur in the sub-ms range: 300-500 µs (Bowes and Crofts, 1980; Stiehl and Witt, 1969). Q_A^- oxidation by Q_B appears to be somewhat faster than that by Q_B^- (Bowes and Crofts, 1980; Weiss and Renger, 1984). Under certain conditions the Q_A^- oxidation by Q_B is known to be changed:

1. In triazine-resistant thylakoids (see Chapter 6) the Q_A^- decay is slower and incomplete. The rate of Q_B reduction is slower than that of Q_B^- in these triazine-resistant thylakoids (Bowes *et al.*, 1980).
2. In the absence of bicarbonate (HCO_3^-) and in the presence of formate ($HCOO^-$) the $t_{\frac{1}{2}}$ of Q_A^- oxidation is slowed down to 10 ms or more (see Jursinic *et al.* (1976) and Vermaas and Govindjee (1981b; 1982a)). Readdition of HCO_3^- restores the fast Q_A^- oxidation.

1.2.4. Q_B

Until recently, it was believed that reduced Q_B was oxidized by the PQ pool but did not exchange with molecules from the pool. However, Velthuys (1981) and Wraight (1981) independently proposed, for thylakoids and purple bacteria, respectively, Q_B to be readily exchangeable with the PQ pool when Q_B is fully oxidized or reduced, but to be bound tightly to its protein environment when it is in the semireduced form. In its protein-bound state the semiquinone appears to be highly stabilized. The semiquinone Q_B^- remains stable for about a minute or more, although the disproportionation constant $[QH\cdot]^2/[Q][QH_2]$ is estimated to be about 10^{-10} in a lipid environment (Mitchell, 1976). Furthermore, herbicides and other inhibitors of Q_A^- oxidation were suggested to act by displacing the quinone Q_B. This hypothesis readily explains the herbicide-induced increase of

$\Phi_{F,var}$ after illumination by one single-turnover flash. However, the experimental evidence supporting this hypothesis was rather limited. A large part of this thesis will be devoted to testing the hypothesis of a mobile Q_B which can be displaced by herbicides.

1.2.5. Protonation of the quinone/protein complex

The ability of Q_A^- and Q_B^- to become protonated is expected to be highly dependent on the characteristics of the microenvironment surrounding these protein-bound quinones. As protonation is critical to the function of these electron transport mediators, their protonation properties should be mentioned. For photosynthetic bacteria, Wraight (1979) reported that the H^+ binding to the RC system was pH-dependent: at pH < 6, $2H^+$ were taken up only at even flashes, whereas above pH = 6 there is a progressive increase in H^+ binding on the 1st flash and an equivalent decrease in H^+ binding on the 2nd flash, until, at about pH = 9.5, the extent of H^+ binding is the same on all flashes. It was suggested that an acid-base group in a protein undergoes a pK_a shift from about 6 to 9 in response to the appearance of the anionic semiquinone, and that this protein group rather than the quinone itself is the site of protonation on the 1st flash. No protonation of semiquinones after odd flashes was observed by absorption measurements. At high pH (> 8) the kinetics of $Q_A^- \rightarrow Q_B^-$ electron transfer were found to become progressively slower, suggesting a rate-limiting involvement of a protonation step: first a proton has to be bound to a protein group in the environment of $Q_A^- . Q_B^-$ before transfer of the electron from Q_A^- to Q_B^- can occur (Wraight, 1979). However, it should be noted that the results may vary greatly with the organism and with the type of preparation used (Verméglio, 1982).

Recently, a similar protonation model has been proposed for thylakoids (Crofts *et al.*, 1984): the pK_a for H^+ dissociation from a protein group near the quinones is about 6.4 when Q_A and Q_B are oxidized, and approximately 7.9 when Q_A or Q_B is semireduced. Furthermore, also in thylakoids a protein group in the vicinity of Q_A and Q_B has to be protonated before Q_A^- can be oxidized by Q_B^-: after two flashes Q_A^- oxidation by Q_B^- was found to be slowed down at higher pH, and a biphasic behavior of Q_A^- decay appeared whereas there was no appreciable pH dependence of Q_A^- oxidation by Q_B (Robinson and Crofts, 1984). It is still unclear why the quinone environment shows this peculiar protonation behavior. It should be noted, however, that Haehnel (1976) has observed an increase in the rate of Q_A^- oxidation by Q_B^- at increasing pH.

Wraight (1982) has hypothesized that a H^+ bound to the protein may have a hydrogen bonding interaction with the anionic semiquinone of Q_B and to a lesser extent with the quinone. Such an interaction is indeed expected to shift the pK_a of the proton-binding protein to higher pH values, and may cause a net stabilization of the semiquinone throughout the pH range.

1.2.6. Q_B/inhibitor interactions

The original hypothesis for the mode of action of PS II herbicides, like atrazine and diuron, which block the Q_A^- oxidation by Q_B, implied an allosteric interaction between inhibitor and Q_B without requiring a release of Q_B: the E_m of the Q_B/Q_B^- couple was proposed to be decreased dramatically upon inhibitor binding, resulting in a blockage of Q_A^- oxidation by Q_B and an inhibitor-induced oxidation of Q_B^- (Velthuys and Amesz, 1974). Using the "mobile-Q_B-model" (see 1.2.4.), the original Velthuys/Amesz hypothesis might still be valid for inhibitors that do not bind to the same domain as Q_B: the oxidation of Q_B^- leads to a large affinity decrease, and Q_B release is induced; however, this hypothesis cannot readily explain a quinone-induced release of inhibitor as described in this thesis without additional assumptions. We will argue in Chapter 4 that either a real Q_B/inhibitor binding competition or a sharp decrease in inhibitor or quinone affinity upon binding of quinone or inhibitor, respectively, is able to provide a much better explanation for the experimental inhibitor/quinone binding data. Most PS II inhibitors, perhaps with the exception of *o*-phenanthroline (*o*-phen) (Oettmeier *et al.*, 1981) (this exception may be artificial because of the relatively low *o*-phen concentration used), appear to act in a similar way because they show competitive binding (Chapter 4). In other words, only one inhibitor molecule can be bound simultaneously to a reaction chain (for example, Tischer and Strotmann (1977) and Laasch *et al.* (1982)).

At this time there are many experimental results that favor a (seemingly) competitive binding interaction of the native plastoquinone Q_B and inhibitors at the protein complex, although it should be kept in mind that allosteric quinone/inhibitor interactions may also behave as if they are competitive. Evidence for an apparently competitive quinone/inhibitor interaction is described in this thesis, but other published results also support this hypothesis. For example, Wraight and Stein (1980) showed that in RC's from *Rhodopseudomonas sphaeroides* *o*-phen bound very weakly and slowly to the acceptor com-

plex in the state $(Q_A.Q_B)^-$ compared to that in the $Q_A.Q_B$ state, and is, therefore, a poor inhibitor of electron transport when added to RC's in this semi-reduced state. Furthermore, the inhibition by *o*-phen was found to decrease at decreasing flash frequency. Although at that time the results were explained by an *o*-phen-induced shift in the redox equilibrium between $Q_A^-.Q_B$ and $Q_A.Q_B^-$, a much better explanation can be given now by a competitive quinone/*o*-phen binding interaction (Q_B^- having a higher affinity than Q_B) and a relatively short residence time of *o*-phen at its binding site (see Section 5.2). *o*-Phen binding is shown to have no preference for the redox state of Q_A below the pK_a of $Q_A^-(H^+)/Q_A^-$ (Wraight, 1982; Section 5.2). It should be noted, however, that in purple bacteria the redox state of Q_A may influence the affinity of herbicides such as terbutryn (Stein *et al.*, 1984; Wraight *et al.*, 1984). No significant effect of the Q_A redox state on the affinity of the terbutryn-related atrazine was observed in thylakoids (Section 4.1).

In support of the competitive inhibitor/quinone interaction, Velthuys (1981) interpreted the results obtained in the presence of the quinone analog DBMIB (dibromothymoquinone) as competition of DBMIB and the native quinone for binding. This was supported by results of Bowes and Crofts (1981) showing that reduced DBMIB is bound better to complexes in state $Q_A.Q_B$ than to those in state $(Q_A.Q_B)^-$. Lavergne (1982b) observed that diuron binding to complexes in state $Q_A.Q_B$ is faster than to those in state $(Q_A.Q_B)^-$, whereas the apparent diuron / binding site association constant was also reported to be diminished upon formation of a semiquinone. This concept is consistent with the newer observations from Laasch *et al.* (1983, 1984) indicating that less diuron is bound under equilibrium conditions when a semiquinone is present. These data will be discussed in more detail in Section 4.1. The affinity of inhibitors is found to be decreased upon increasing the concentration of native quinone, thus supporting the binding competition between inhibitors and quinones, not only at the Q_B site (Oettmeier and Soll, 1983; Vermaas *et al.*, 1984a; Soll and Oettmeier, 1984; Chapter 4), but also at other sites involving bound quinones (Matsuura *et al.*, 1983).

It should be stressed that electron transport through Q_B is also possible when Q_B is not a native quinone. For example, p-BQ^- can oxidize Q_A^- very effectively (Lavergne, 1982a), and other synthetic quinones can also serve as good PQ analogs (Section 4.1). In bacterial RC's most ubiquinone homologs can accept electrons from Q_A^- effectively (Baccarini-Melandri *et al.*, 1980, 1982). In these preparations the specificity of Q_A is much less even: Q_A can be replaced by

many synthetic benzoquinones, naphthoquinones and anthraquinones (Gunner *et al.*, 1982). In thylakoids a Q_A substitution by artificial quinones has not been achieved thus far.

1.2.7. Q_B/inhibitor binding proteins

Because of the interest in the mechanism of binding of both the inhibitor and the quinone, experiments have been performed to elucidate the identity of the protein responsible for binding of a herbicide and, possibly, the quinone Q_B. One powerful technique which has been utilized to identify herbicide binding domains has been the application of photoaffinity probes. A radioactively labelled herbicide analog containing an azido (N_3) group was added to a thylakoid membrane suspension. Upon UV-illumination at wavelengths absorbed by the azido group (around 310 nm), a highly reactive nitrene radical is formed (Bayley and Knowles, 1977), which reacts unspecifically with any molecule in its vicinity, forming a covalent bond with this molecule. Using an atrazine analog with an azido group, a protein with an apparent molecular weight of 32 kDa (kilodaltons) was specifically tagged (Pfister *et al.*, 1981a; Gardner, 1981). In this thesis, this 32 kDa protein will be referred to as ABP-32 (azidoatrazine-binding 32,000 M_r protein (M_r is relative molecular mass)). Using the azido--analog of a phenolic inhibitor of electron transport, *i*-dinoseb, a 40,000 M_r protein was inferred to be labelled specifically (Oettmeier *et al.*, 1980). In more refined experiments, the phenolic inhibitor was found to be bound to, among other components, two polypeptides that are associated with the PS II RC, and that have apparent molecular weights of 43-53 kDa, depending on the plant species (Johanningmeier *et al.*, 1983). However, in *Amaranthus hybridus* the labelling of proteins in the 43-53 kDa region was not very significant (Oettmeier *et al.*, 1982a), but a close examination of the data shows a relatively large amount of inhibitor bound to a protein in the 32,000 - 33,000 M_r region. One might argue that the direct interaction between phenolic inhibitors and the ABP-32 may play a larger role than assumed by Oettmeier and co-workers, since at low concentration of the phenolic inhibitor, *i*-dinoseb, much of the inhibitor binding does not seem to be related to inhibition of electron transport (Vermaas and Govindjee, 1981a), and, furthermore, since labelling in the 32-33 kDa region is often also observed besides that in the 43-53 kDa region. Moreover, it is recognized that photolysis leading to covalent labelling may occur at a point well-removed from the active site (Hixson and Hixson, 1975)

even when the dissociation constant K_d of the ligand/binding site complex is low (Chowdhry and Westheimer, 1979). Gressel (1982) has proposed that this may also occur for the case of azidoatrazine binding. He concluded that it is hard to conceive that the ABP-32 contains the active site for atrazine binding, since he observed that upon depletion of this protein by 70% (by growth in the presence of a protein synthesis inhibitor) no decrease in atrazine sensitivity of photosynthesis occurred whereas the protein depletion led to a loss of only 15-20% of net photosynthesis. However, it is difficult to quantitatively correlate herbicide binding and loss of electron transport, unless the light intensity is well-defined (Siggel *et al.*, 1972). Moreover, it is highly probable that the ABP-32 is necessary for electron transport (Mattoo *et al.*, 1981; Steinback *et al.*, 1981b; Kyle *et al.*, 1983) and this implies that the control measurements made by Gressel (1982) were not done under conditions where PS II turnover is rate limiting. Thus, a large shift in atrazine sensitivity is not expected under these conditions even when the ABP-32 does bind atrazine. At the present time, most of the evidence favors the identity of the 32 kDa protein with the atrazine binding protein, although, as is pointed out in Section 3.3, another protein also plays a role in determining the atrazine binding affinity. Arntzen *et al.* (1983) have provided evidence that PS II RC polypeptides contribute structural features to the herbicide binding environment.

Recently, experiments with azidolabelled quinones have also been performed: using ^{3}H-labelled 6-azido-5-decyl-2,3-dimethoxy-*p*-benzoquinone (6-azido-Q_0C_{10}), it was shown that the quinone mainly bound to two PS II-RC-related polypeptides, (43-47 kDa), a protein in the 32 kDa region, the light harvesting complex (25-28 kDa; very abundant in the thylakoid membrane) and to two or three lower molecular weight polypeptides of unknown function (W. Vermaas, C. Arntzen, L.-Q. Gu and C.-A. Yu, unpublished observations). Most of this quinone was found to be associated with the free pigment/lipid fraction. Similar results have been obtained independently by Oettmeier *et al.* (1984a): attachment of an azido-derivative of PQ (Oettmeier *et al.*, 1982b) to thylakoid proteins resulted in an identical preferential labelling of polypeptides as observed for 6-azido-Q_0C_{10}. The azido-PQ, however, was found to bind more specifically to a 32,000 M_r component than to 43-53 kDa PS II RC polypeptides. This difference was less pronounced in the case of 6-azido-Q_0C_{10}. These results indicate that the ABP-32 is probably also involved in Q_B binding. However, see Oettmeier *et al.* (1984b). Moreover, the PS II RC complex may also be involved in binding inhibi-

tors and/or quinones.

Until recently, there has been considerable confusion about PS II polypeptides in the 32 kDa region because it was not clear that there was more than one PS II protein in this region. In this thesis it is shown (Chapter 3) that the ABP-32, which is poorly stainable with Coomassie Brilliant Blue (CBB), is different from a 32-33 kDa protein that is easily stainable with CBB and that appears to be related primarily to oxygen evolution (Kuwabara and Murata, 1982c; Murata *et al.*, 1983) rather than to inhibitor binding (Croze *et al.*, 1979; Mullet and Arntzen, 1981; Metz and Miles, 1982; Arntzen *et al.*, 1982).

In the last several years much has become known about the ABP-32. This protein is encoded on the chloroplast-DNA (Driesel *et al.*, 1980; Keller *et al.*, 1982) and the appearance of both the protein and the m-RNA which encodes for it is greatly enhanced during the greening of the plant (Rosner *et al.*, 1975; Reisfeld *et al.*, 1978; Weinbaum *et al.*, 1979). Since the transcription of the gene encoding for the ABP-32 is light dependent, this gene has often been called a "photogene" (Bogorad *et al.*, 1980). As expected (Ellis, 1969) the synthesis of this protein is inhibited by chloramphenicol (Weinbaum *et al.*, 1979). The ABP-32 appears to be highly conserved in both the position of the gene on the chloroplast DNA (Bedbrook *et al.*, 1978; Malnoe *et al.*, 1979; Driesel *et al.*, 1980) and in the primary structure (Zurawski *et al.*, 1982). The primary product of translation is a 33,500-34,000 M_r protein (Grebanier *et al.*, 1978) which is inserted into the thylakoid membrane and processed rapidly afterwards (Reisfeld *et al.*, 1982). The turnover time of the ABP-32 is very short. Trypsin treatment, which digests a proteinaceous component covering Q_A and regulating electron transport from Q_A to the PQ pool (Renger, 1976), also attacks the ABP-32 protein and its precursor (Steinback *et al.*, 1981a); azidoatrazine labelling and subsequent trypsinization have revealed that the azidoatrazine is bound to an inner 16 kDa fragment of the trypsin-digested ABP-32 (Steinback *et al.*, 1982). Some herbicides appear to change the conformation of the ABP-32 such that the rate of trypsin digestion is modified. Mattoo *et al.* (1981) reported that diuron slowed down trypsin digestion considerably. Phenisopham and metribuzin appear to inhibit trypsin action to a lesser extent (Renger, 1979) whereas azidoatrazine does not seem to protect the protein against trypsin digestion (Steinback *et al.*, 1981a).

Recently, a model for the secondary structure of the ABP-32 has been proposed (Rao *et al.*, 1983): the ABP-32 is suggested to span the thylakoid mem-

brane several times by transmembrane helices connected by turn segments on the outer or the inner side of the thylakoid membrane. Shipman (1981, 1982) has proposed that an α-helix, which may generate a strong local electric field, may be involved in binding herbicides and/or stabilizing Q_B^-. These proposals seem to agree rather well with each other, but at this stage they remain as speculations.

The conformation of the ABP-32 is very important in determining the affinity of herbicides and quinones. For example, mild protein extraction with cholate or urea does not extract the ABP-32, but changes the herbicide binding dramatically (Section 3.1; Vermaas *et al.*, 1984b). In line with these results, Neumann *et al.* (1984) reported that *Chlamydomonas reinhardtii* subchloroplast particles enriched in PS II did not bind diuron-type inhibitors to any significant extent, but still retained the ABP-32. A very nice example of how important the protein conformation of the ABP-32 is for herbicide binding is presented in triazine-resistant biotypes of many plants. A single change from a serine residue into glycine (in *Amaranthus hybridus* or in *Solanum nigrum*) (L. McIntosh, personal communication) or into alanine (in *Chlamydomonas reinhardtii*) in the ABP-32 causes a decrease in atrazine affinity by about 3 orders of magnitude (see, for example, Pfister and Arntzen, 1979) and also a changed affinity of many other inhibitors and quinones (Pfister and Arntzen, 1979; Oettmeier *et al.*, 1982a; Vermaas and Arntzen, 1983; Section 6.2). It is almost certain that a change in only the ABP-32 is responsible for these changes in herbicide affinity, since the triazine resistance as well as changed electron transport kinetics involving Q_A and Q_B (Bowes *et al.*, 1980) is inherited maternally (Souza Machado *et al.*, 1978; Darr *et al.*, 1981; Arntzen *et al.*, 1982): maternally inherited effects are expected only from changes in a plastid-encoded protein as the ABP-32 is. Triazine resistance does not only lead to a large change of herbicide affinity and quinone-related electron transfer thermodynamics (Section 6.1) and kinetics, but also to a changed lipid composition, a lower Chl *a*/*b* ratio and increased grana stacking in the thylakoids (Burke *et al.*, 1982), although the latter changes may be considered to be secondary effects. In addition, a lower rate of light-saturated CO_2-reduction on a chlorophyll basis has been reported in triazine-resistant organisms (Ort *et al.*, 1983), although this appears to depend on the species (van Oorschot and van Leeuwen, 1984).

In the last few years many herbicide-resistant mutants, mainly from algae, have been developed artificially (Astier *et al.*, 1979; Galloway and Mets, 1982;

Janatkova and Wildner, 1982; Tellenbach *et al.*, 1983; Pucheu *et al.*, 1984; L. Sherman, personal communication). Unfortunately, extensive data on the biochemical and biophysical properties of these mutants are not available yet (see, however, Astier *et al.* (1981) and Astier and Joset-Espardellier (1981) for a preliminary examination of their mutants). Most herbicide-resistant mutants are expected to have a changed ABP-32. Indeed, diuron-resistance in *Chlamydomonas reinhardtii* is reported to be inherited uniparentally (Tellenbach *et al.*, 1983). Recently, a triazine-resistant mutant from the purple bacterium *Rhodopseudomonas sphaeroides* was also reported (Stein *et al.*, 1984). The modified binding site diminishes the affinity for triazines (such as terbutryn, which is very active in purple bacteria) as well as for ubiquinone. The decreased terbutryn affinity was reported to be due primarily to an increase in the herbicide release rate (Stein *et al.*, 1984).

From the analogies in electron transport through Q_A and Q_B in plants and purple bacteria one might expect strong parallels between the highly conserved ABP-32 and one of the proteins from the purple bacteria required for binding of Q_A or Q_B. Although the medium-sized (M) subunit from bacterial RC's is assumed to bind Q_A (Marinetti *et al.*, 1979), and the M-subunit (Debus *et al.*, 1982) or possibly both the M and H subunits (Okamura *et al.*, 1982b) is/are involved in creating the binding environment for Q_B, no homology between the N-terminal end sequence of either M or H subunit (Okamura *et al.*, 1982a) and that of the ABP-32 (Zurawski *et al.*, 1982) can be observed, although there is some structural homology between other parts of the M-subunit and the ABP-32 (Hearst and Sauer, 1984). However, it is questionable how much value one should attach to such structural homology studies. It should be noted that the estimated molecular weight of the M subunit is about 32 kDa (Okamura *et al.*, 1982a). Very recently, Williams *et al.* (1983) have reported the primary structure of the M-subunit. This subunit is composed of 307 amino acid residues corresponding to a M_r of 34,265. The azidoatrazine was reported to label mainly the third subunit of the bacterial RC complex, the L (low M_r) subunit (D. Kyle, personal communication), which is speculated to be also homologous to the ABP-32 (Hearst and Sauer, 1984). However, rather high herbicide concentrations are needed for electron transport inhibition in bacterial RC's, and it is not yet certain whether the labelling of the L-subunit by azidoatrazine is related to inhibition of electron transport.

1.2.8. Regulation of electron transport

There are several mechanisms by which electron transport can be regulated to insure a balance between the two photosystems. One example of how the rate of electron transport through PS II can be regulated *in vivo* is protein phosphorylation. As soon as the PQ pool is reduced, a kinase is activated, which phosphorylates, among other components, the light harvesting protein complex linked to PS II, resulting in a decreased flow of light quanta into the PS II RC, and thus in a slower rate of PQ pool reduction (see Haworth *et al.* (1982) for a review). Also the ABP-32 can be phosphorylated (Owens and Ohad, 1981) leading to decreased diuron binding affinity (Shochat *et al.*, 1982).

Another mechanism for *in vivo* regulation of electron transport through PS II is by photoinhibition: exposure of leaves that were grown under low light intensity to high light intensities leads to specific damage of PS II activity (Powles and Critchley, 1980; Critchley, 1981); moreover, the same damage can be induced in plants grown under high light intensity by illumination under low CO_2 and O_2 tension (Powles and Osmond, 1978; Powles *et al.*, 1979). Recently, it has been shown that this photoinhibition is likely to be caused by breakdown of the ABP-32 in the thylakoid membrane (Kyle *et al.*, 1983; I. Ohad, personal communication). It was suggested that the rapid turnover of the ABP-32 was possibly caused by the continuous generation of the semiquinone anion radical Q_B^- in the light that might lead to a rapid destruction of the surrounding ABP-32 (Arntzen *et al.*, 1983). Indeed, this hypothesis would explain the photoinhibition phenomenon at high light intensity in the presence of CO_2, but does not provide an explanation for why, by the absence of CO_2, photoinhibition is increased as shown by Powles and Critchley (1980) and Whitelam and Codd (1983). This observation suggests that other factors besides the generation of Q_B^- are also involved in the breakdown of the 32 kDa protein and in photoinhibition. One candidate for this may be the bicarbonate effect on photosynthetic electron transport. Without HCO_3^- and in the presence of formate ($HCOO^-$) electron transport between Q_A and PQ is impaired severely (see Vermaas and Govindjee (1982b)). The HCO_3^- binding to the PS II complex is known to influence the binding properties of PS II herbicides (Khanna *et al.*, 1981; Vermaas *et al.*, 1982; Section 7.2) and therefore it is considered as probable that HCO_3^- binds to the ABP-32 or a neighboring protein. Furthermore, there are indications that HCO_3^- binding may respond to the redox state of the $Q_A.Q_B$ complex (Stemler, 1979).

Another indication that the binding environments for quinones or herbicides and for HCO_3^- are closely related is found in the observation that diuron decreases the exchange rate of HCO_3^- (Stemler, 1977; Snel and van Rensen, 1983). Recently, Stemler and Murphy (1983) and Snel and van Rensen (1984) have estimated the K_d of HCO_3^- from a binding site to be about 80 μM in the absence of formate, and they have drawn the preliminary conclusion that the bicarbonate effect may not play a role in the regulation of electron transport *in vivo* since under physiological conditions the HCO_3^- binding site will be mainly unoccupied while electron transfer occurs normally. However, great care should be taken in interpreting the results because in the stroma the pH under *in vivo* conditions on illumination is expected to be much higher than 6.5, the value used in most experiments. Note that at higher pH the HCO_3^- concentration is increased ($pK_a(H_2O+CO_2)/HCO_3^- = 6.4$ at 25° C) and that possibly the HCO_3^- affinity for the binding site is increased as well (HCO_3^- depletion requires a low pH). There are preliminary indications of the existence of the bicarbonate effect *in vivo* (Gerbaud and Andrē, 1980; Garab *et al.*, 1983; Govindjee, personal communication) but more work on this subject is required to further investigate the role of HCO_3^- under physiological conditions as well as its possible link to the photoinhibition phenomenon.

HCO_3^- not only seems to influence the rate of Q_A^- oxidation by both Q_B and Q_B^- (see Vermaas and Govindjee (1982a)), but may also regulate the exchange between Q_BH_2 and the PQ pool (Govindjee *et al.*, 1976; Vermaas and Govindjee, 1982b; Farineau and Mathis, 1983). In view of the protonation characteristics of Q_B as described in subsection 1.2.5, one might speculate, because of a sharp pH dependence of the bicarbonate effect (Vermaas and van Rensen, 1981) that HCO_3^- may be regulating the rate(s) of the protonation reaction(s) (see Section 7.1).

1.2.9. The PQ pool; heterogeneity

Whatever the role of HCO_3^- in exchange between Q_BH_2 and PQ may be, under normal (*i.e.*, non-inhibited) conditions this exchange is fast (15 ms or less) (Diner, 1974; van Best and Duysens, 1975). Although there is about 1 PQ per 10 Chl molecules in the chloroplast (Crane, 1965), which is equivalent to about 40 PQ's per PS II RC, not more than about 7 per RC are reduced by PS II (Stiehl and Witt, 1969). The other PQ molecules may be located in the plastoglobuli

(Hauska and Hurt, 1982), lipid-containing particles in the chloroplast (Lichtenthaler, 1969; Bailey and Whyborn, 1963). Of the few PQ molecules that are reduced by PS II, only Q_A, Q_B and one more PQ molecule per PS II RC appear to be reduced within 3 ms (Bouges-Bocquet, 1981,1982). Bouges-Bocquet hypothesized that the third PS II-reducible PQ molecule could be connected to the cytochrome (cyt) b_6/f complex. If this were correct, it would imply that in a significant portion of the PS II RC's the RC complex is in close proximity to the cyt b_6/f complex. The same suggestion was made very recently on the basis of reconstitution experiments (Lam and Malkin, 1983). In this respect the interpretation of data obtained using intact chromatophores from *Rhodopseudomonas sphaeroides* by O'Keefe *et al.* (1982) is noteworthy: 70% of Q_B^- was oxidized by the cyt b_{560} of the quinol:cyt c_2 oxidoreductase (which may be considered to be analogous to the cyt b_6/f complex in thylakoids) whereas the other 30% of the RC's was not connected directly to the quinol:cyt c_2 oxidoreductase. This would predict that in certain centers Q_B^- is stable, whereas in others it is not. Indeed, in the thylakoid membrane some of the Q_B^- does disappear rapidly in the dark ($t_{\frac{1}{2}} \sim 1$ s) (Boussac and Etienne, 1982). The RC's that do not show a stable Q_B^- are generally called "non-B-type centers" (Lavergne, 1982c) and often amount up to about 40% of the total RC's. In spite of this considerable heterogeneity in Q_B^- stability, no significant biphasic behavior of, for example, herbicide binding in the dark is observed (see Chapter 4), suggesting that the herbicide/quinone binding environment is not changed. The data discussed above favor the hypothesis of either a cooperation of reaction centers resulting in a dismutation of two semiquinones (Stiehl and Witt, 1969; Crofts and Wraight, 1983), or a possible link between the RC complex and the cyt b_6/f complex in certain centers rather than the existence of two different types of Q_B. However, recently Crofts *et al.* (1983) showed the reduction kinetics of cytochrome b_{561} by ubiquinol to be second order at low ubiquinol concentrations in chromatophores of *Rhodopseudomonas sphaeroides*. This indicates that, in this system, there is no significant direct connection between the reaction center complex and the quinol:cyt c_2 oxidoreductase. Thus, the extent of connection between these two protein complexes seems to depend on, perhaps, the preparation used. Obviously, the clarification of the process of Q_B^- and PQH_2 oxidation needs further research.

In these models both the free PQH_2 and the cyt b_6/f complex (with or without bound PQ) may be the diffusing electron-carrying species from PS II (loca-

ted mainly in the grana region) to PS I (which is in the stroma thylakoids, the thylakoid parts that do not form grana). Also in the mitochondrial inner membrane both the quinone and the cyt b-c_1 complex (analogous to the cyt b_6/f complex) are suggested to function as diffusible redox carriers (Schneider *et al.*, 1982).

At this point (the PQH_2/cyt b_6/f complex interaction) we enter the region of processes that are no longer related directly to the topic of this thesis. For detailed information on the processes in photosynthetic electron transport following those discussed above, the reader is referred to Cramer and Crofts (1982) and Parson and Ke (1982).

In conclusion, it is clear that much is known about the acceptor side of PS II. However, many questions still remain to be answered. This thesis has solved some questions (see the Summary for a brief overview), but enough challenging problems are left for further research in this fascinating area of membrane biology.

1.3. General scope

The work described in Chapters 2-7 was directed towards a further clarification of processes occurring at the acceptor side of PS II as a function of the presence of quinones, inhibitors and bicarbonate in both triazine-sensitive and triazine-resistant thylakoids. The methods used include both biophysical and biochemical techniques. The ideas developed may be applied also in herbicidal and physiological studies, whereas the results obtained are of use for certain industrial purposes as well (for example, with respect to the development of safeners, herbicides and herbicide-resistant biotypes).

Most of the work described in the following chapters has already been published previously or will be published elsewhere soon (see Reference List under Arntzen, Hagemann, van Rensen or Vermaas as main authors). In the original publications often more details are provided than are included here.

Because of the complex character of the subject, a separation of results and discussion would be rather confusing: results obtained have to be discussed thoroughly before the rationale for the next experiments and the meaning of the following results can be appreciated. Because of the wide variety of experimental approaches used, I have chosen for including the discussion in the sections

that describe the experimental results and omitted a general discussion. In this way this thesis could be kept concise, logical and, therefore, relatively easy to read.

Chapter 2, Materials and Methods

Due to the fact that the research described in this thesis has been carried out in different laboratories, the materials and methods have been standardized less than would be expected in a thesis.

2.1. Thylakoid isolation

Most experiments have been carried out with pea (*Pisum sativum* L.) thylakoids. These were isolated from leaves of 2-3 week old pea seedlings. The leaves were washed in ice-cold water, ground for a few seconds in a homogenizer in the isolation/reaction medium (IRM), which consisted of 50 mM tricine, 10 mM NaCl, 5 mM $MgCl_2$ and 0.3 M sorbitol, and which had been brought to pH=7.6 or 7.8 by the addition of NaOH. In some experiments, the IRM was slightly different (for example, mannitol was used instead of sorbitol), but this did not affect the results reported. The homogenate was filtered through 4-8 layers of cheesecloth, and centrifuged at 3,000 x *g* for 5 min. The pellet was resuspended in 10 mM Tricine/NaOH, 10 mM NaCl and 5 mM $MgCl_2$ (pH = 7.6 or 7.8) in order to break the chloroplast envelope membrane, and the resulting stroma-free thylakoids were collected by centrifugation (3,000 x *g*, 5 min) and resuspended in IRM.

Isolation of thylakoids from triazine-resistant and -susceptible biotypes of *Amaranthus hybridus* L. or *Brassica napus* L. was carried out as described above. Seeds of *Brassica napus* were a kind gift from Dr. W.D. Beversdorf (Guelph, Ont., Canada) obtained via Pat Fuerst (MSU/DOE Plant Research Laboratory, East Lansing MI, USA). Chl concentrations were determined from spectrophotometric determination in 80% acetone at 652 nm (Bruinsma, 1963) or at 645 and 663 nm (Arnon, 1949). All isolation procedures were carried out at 0-4° C.

2.2. CO_2-depletion

a. method I (used from 1980 until the end of 1981).
Thylakoids were suspended to 50 µg Chl.ml^{-1} in CO_2-free medium (obtained by bubbling with N_2 filtered through a soda-lime or ascarite column) containing 50 mM sodium phosphate, 100 mM HCOONa and 5 mM $MgCl_2$ (final pH=5.3) in a capped, CO_2-free tube (Stemler and Govindjee, 1973). The low pH stimulates

the CO_2-depletion. Then, the thylakoids were incubated in the dark for 15 min, and subsequently pelleted by centrifugation. The supernatant was discarded and the thylakoids were resuspended under N_2 atmosphere in CO_2-free buffer of the same composition at pH = 5.3 or 6.5. In the first case, the thylakoids were again incubated in the dark for 15 min, and after centrifugation the thylakoids were resuspended in a medium of the same composition at pH = 6.5 (50 µg Chl.ml^{-1}). This procedure yields thylakoids that are more effectively depleted of CO_2 than those obtained by washing only once at pH = 5.3 (Vermaas and Govindjee, 1982b). The intersystem electron transport as assayed by the ferricyanide Hill reaction at saturating light intensity was increased by a factor of 10 or more upon readdition of 10 mM $NaHCO_3$, but the maximal electron transport yield was only 20 - 60 % of the original electron transport activity.

b. method II (used from 1982)

Because of the irreversible inhibition of electron transport by the CO_2-depletion procedure described above, a new method of CO_2-depletion was developed, in which the incubation at pH = 5.3 was circumvented. The thylakoids were incubated in a CO_2-free medium at pH = 6.0 (containing 50 mM MES (2[N-morpholino]ethane sulfonic acid)/ NaOH, 25 mM HCOONa, 10 mM NaCl, 5 mM $MgCl_2$ and 0.2 M sorbitol) at 50 µg Chl.ml^{-1} for one hour in the dark at room temperature. The same medium was used for the experiments, so that centrifugation was not necessary. Using this method, a low rate of electron transport was observed in the absence of CO_2, whereas after addition of 5 mM $NaHCO_3$ a return to the control rate of electron transport was observed, which usually was a factor of 10 - 20 higher than the rate before HCO_3^- addition.

The low pH required for CO_2-depletion may suggest that CO_2 rather than HCO_3^- leaves the binding site. On the other hand, it could also indicate that formic acid rather than formate competes with HCO_3^- or CO_2 for binding (H. Robinson, personal communication). A third possibility is that a protein group, or HCO_3^- itself, has to be protonated before HCO_3^- can exchange with $HCOO^-$ (the pK_a for an acidic group in the protein environment near Q_B has been estimated to be 6.4 when Q_A and Q_B are oxidized (Crofts *et al.*, 1984)).

2.3. Herbicide binding

Contrary to most of the experiments described in the earlier literature, the binding of radioactively labelled herbicides to thylakoid membranes was done under well-defined conditions of light intensity. The binding experiments were done virtually in the dark unless indicated otherwise. Usually, to different samples of a few mls of IRM, 5-10 different amounts of ^{14}C-labelled herbicides (specific activity 30-70 $\mu Ci.mg^{-1}$) were added. The final concentration of radioactive herbicide was between 10^{-8} and 5.10^{-7}M. The final concentration of ethanol or methanol (in which the herbicides were dissolved) in the IRM did not exceed 1 % (v/v). The IRM/herbicide mixtures were divided into 1 ml samples. To some samples a small volume of a concentrated thylakoid suspension was added to a final concentration of 50-100 $\mu g\ Chl.ml^{-1}$; when appropriate, other additions were also made. To the other samples, buffer was usually added to make up the same total volume.

Subsequently, the samples were incubated until completely equilibrated (for atrazine binding studies, 5 min were sufficient to obtain full equilibration, whereas for bromoxynil and, especially, ioxynil about 15 min were required, in our hands, in order to reach equilibration) and centrifuged in a microfuge (at 5,000 - 12,000 x *g*) in order to pellet the thylakoids. From the supernatant 800 µl was transferred to 8-12 ml of scintillation fluid, and counted in a scintillation counter.

Using the known specific activity of the labelled herbicide, the amount of radioactive herbicide bound to the thylakoids as well as the free herbicide concentration was calculated from the measured radioactivity in the fraction of the supernatant from samples with and without thylakoids. From these data a double-reciprocal plot of the free herbicide concentration (X-axis) *vs.* the amount of bound herbicide on a chlorophyll basis (Y-axis) was constructed (Tischer and Strotmann, 1977). Such a double-reciprocal plot should yield a straight line and this gives information on the K_d of the herbicide and its binding site (the K_d is the concentration of free herbicide necessary to give 50% occupation of the available binding sites) as well as the number of herbicide binding sites on a Chl basis. The K_d is equal to the negative reciprocal of the intersect on the X-axis, whereas the number of Chl molecules per herbicide binding site is equal to the ratio of Chl to bound herbicide at "infinite" herbicide concentration (intersect with the Y-axis). This ratio is determined by extrapolation in order to avoid artefacts due to "unspecific" herbicide partition into the thylakoid membrane. For more details on the analysis of herbicide binding data, the rea-

der is referred, for example, to Tischer and Strotmann (1977) and Tischer (1978).

2.4. Electron transport

Electron transport assays were carried out at saturating light intensity in the IRM. In order to uncouple electron transport from photosynthetic phosphorylation, 2-5 mM NH_4Cl was added. As artificial electron acceptors we used potassium ferricyanide (FeCy; 0.5 mM), *p*-benzoquinone (*p*-BQ; 0.25 mM), 2,6-dichlorophenolindophenol (DCPIP; 80 µM), methylviologen (MV; 80 µM), or a combination of diaminodurene (DAD; 0.1 mM) and FeCy (0.5 mM). In order to measure PS I electron transport, MV-mediated O_2 consumption was monitored in the presence of 1-5 µM diuron to prevent linear electron flow from PS II. Durohydroquinone (DQH_2; 0.25 mM; for the preparation procedure, see Izawa and Pan (1978)) or a mixture of sodium ascorbate (2 mM) and tetramethylphenylenediamine (TMPD; 0.5 mM) was used as electron donor to PS I. All electron transport assays were carried out at 20-25° C.

2.5. Fluorescence induction and decay

For measuring Chl *a* fluorescence induction kinetics, a dark-adapted thylakoid suspension (10-50 µg Chl.ml^{-1}) in IRM was illuminated with blue light from a tungsten lamp (filtered through a Corning CS 4-96, or 4-76 and 3-73, or through a Schott BG12 and BG18 filter) on opening a shutter (Uniblitz 26L2AOX5 or equivalent). The Chl *a* fluorescence was detected by a photodiode or photomultiplier after filtering out the scattered actinic light with a Corning 2-64 or a Schott RG665 filter. The fluorescence was recorded on a storage oscilloscope or, when the induction was slow, directly on an Esterline-Angus recorder.

In order to monitor the Q_A^- oxidation by, presumably, the water splitting complex in the S_2 state (see, *e.g.*, Robinson and Crofts, 1983), the Chl *a* fluorescence yield, elicited by weak non-actinic light pulses from a yellow light-emitting diode, was measured in the presence of 5 µM diuron. Fluorescence was monitored as a function of time after an actinic flash in a previously dark-adapted thylakoid suspension.

2.6. Flash-induced O_2-production

Oxygen evolution patterns induced by a series of single-turnover flashes were measured with a Joliot-type O_2-electrode (Joliot, 1972). The thylakoids were dark-adapted for more than two hours before they were applied to the Pt-electrode surface in very dim light. The Chl concentration varied between 0.6 and 1.1 $mg.ml^{-1}$. No significant PS II turnover appeared to occur upon thylakoid transfer to the electrode, since the flash-induced O_2-evolution pattern measured 0.5 min after transfer was found to be almost identical to that monitored 5 min after transfer. In the standard procedure the thylakoids were dark-adapted for 5 min after thylakoid application to the electrode surface. The O_2-evolution patterns induced by the first 10 or 12 flashes after dark adaptation were fitted to O_2-evolution values calculated from the classical model developed by Kok *et al.* (1970) using varying values for miss- and double hit probabilities (α and β, respectively) and the apparent $S_0/(S_0+S_1)$ ratio after dark adaptation.

When PS II inhibitors were present, possible exchange between Q_B and the inhibitor had to be considered. Then, additional calculations, described in Chapter 5, were necessary.

For measuring the lifetime of the S_2 and S_3 states of the water splitting complex, 1 and 2 preflashes, respectively, were fired at 4 Hz, and after a variable dark time a flash train was started (also 4 Hz flashes). The relative amount of water splitting complexes in S_2 state after one preflash was measured by the O_2 evolution at the second flash of the flash train (Y_2), corrected for the O_2 evolution at the second flash without preflashes ($Y_{2,c}$), and divided by the average O_2 evolution after the 2nd to 5th flash of the flash train after one preflash ($\bar{Y}_{2-5}$). Analogously, the relative amount of water splitting complexes in S_3 state after 2 preflashes is approximated by $Y_1/\bar{Y}_{1-4}$.

2.7. Gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) was carried out at 5° C using a 10-17.5 % linear polyacrylamide gradient slab gel with a 5 % stacking gel (1 cm) incorporating the buffer system of Laemmli (1970). Thylakoid membrane solubilization was carried out as described by Steinback *et al.* (1979). However, in order to minimize protein aggregation artefacts, most of the samples were not boiled, but incubated with SDS and β-mercap-

toethanol at room temperature. Electrophoresis was carried out at a constant current of 20 mA through the stacking gel and at 30 mA through the running gel. The technique used for staining of proteins in the gel has been described by Steinback *et al.* (1979). The gels were destained in 20 % methanol, 7 % acetic acid and 3 % glycerol. Apparent molecular weight determinations were made on the basis of electrophoretic mobility (Weber and Osborn, 1969). Protein standards used included bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa).

2.8. Photoaffinity labelling

^{14}C-azidoatrazine or 6-azido-5-decyl-2,3-dimethoxy-*p*-benzoquinone (6-azido-Q_0C_{10}), synthesized as described by Gardner (1981) and Yu *et al.* (1982), respectively, were added to a thylakoid suspension in IRM (100 µg Chl.ml^{-1}). Part of the suspension was transferred to a petri dish so that it formed a thin layer of liquid (less than 2,5 mm). The petri dish was covered subsequently by a material that transmits light with wavelengths longer than 300 nm, but absorbs shorter wavelength light in order to avoid major changes in the protein upon UV-treatment. A few layers of plastic film or a plastic petri dish cover are often suitable as a "far-UV" filter. The azido groups in the molecules to be linked covalently to their binding environment show an absorption peak at about 315 nm. The petri dish was embedded in ice and irradiated for 30-60 min with a germicidal lamp (Sylvania G30T8) at 5-10 cm distance.

In some experiments with 6-azido-Q_0C_{10}, Tris-washed (0.8 M; pH=8.0) thylakoids (Yamashita and Butler, 1968), in which the water splitting system has been blocked and in which only one electron can be transferred from PS II to PS I in the absence of electron donors, were used in order to prevent possible photoreduction of the azidoquinone.

After the UV-treatment the thylakoids were pelleted by centrifugation (3,000 x *g* for 5 min) and resuspended in fresh IRM.

2.9. Protease treatment

For the trypsin digestion experiments described in Section 3.2, 0.4 µg trypsin per ml suspension (216 units per mg; treated with L-(tosylamido-2--phenyl)-ethylchloromethylketone to inhibit contaminant chymotryptic activity; Worthington, Freehold NJ) was added to 50 µg Chl per ml IRM. The thylakoid/

trypsin mixture was incubated at room temperature in the dark. At certain times after trypsin addition, a sample was removed, 10 µg trypsin inhibitor (Sigma) per ml was added, and thoroughly mixed. The samples were divided for ^{14}C-herbicide binding measurements and for analysis of membrane proteins by electrophoresis. Prior to SDS/PAGE analysis, samples were centrifuged (3,000 x *g* for 5 min) and the pellet was resuspended in reaction buffer to a Chl concentration of 0.5 mg.ml^{-1}.

For the protease digestion experiments described in Section 3.3, 0.25 units of trypsin or lysine-specific protease (both from Boehringer, Mannheim, Germany) were added to 1 ml of a thylakoid suspension (50 µg Chl.ml^{-1}) in IRM. The thylakoids were incubated in the dark with the protease at room temperature. After a certain incubation time, the samples were centrifuged and, for herbicide binding experiments, the ^{14}C-herbicide concentration in the supernatant was measured (see Section 2.3). If thylakoids had been allowed to incorporate ^{35}S (see Section 2.10), they were processed further for SDS/PAGE (see Section 2.7) after treatment with a protease.

2.10. *In vivo* protein labelling and autoradiography

Pea leaves were painted with ^{35}S-methionine *in situ* and were allowed to incorporate this amino acid for 3 hrs under room light. Subsequently, thylakoids were prepared as described in Section 2.1. After various treatments (incubation with proteolytic enzymes, or incubation with 4 M urea or 1 % sodium cholate), SDS/PAGE was performed. After Coomassie Brilliant Blue (CBB) staining and destaining, the gels were dehydrated by incubation in dry DMSO (dimethylsulfoxide) for 2 x 45 min. Subsequently, the gels were incubated with DMSO/20% PPO (diphenyloxazole), and usually dried down after rinsing with water. X-ray films were exposed to the gels for one week (at - 80° C), and densitometer scans of the film were recorded. The ^{14}C-azidoatrazine-labelled gels were treated analogously. However, the necessary film exposure time was 3 months.

For further details on the precise procedures used for specific experiments, the reader is referred to the reference(s), if any, at the beginning of the Section in which these experiments are described.

Chapter 3, Proteins involved in herbicide and quinone binding

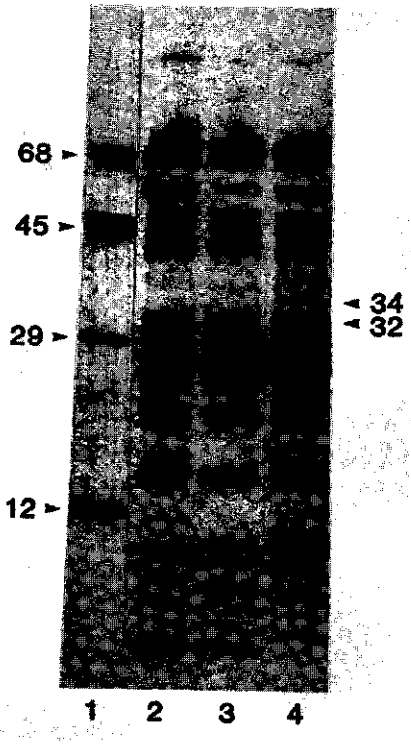
As has been pointed out in the Introduction (Section 1.2.7), a 32,000 M_r protein is involved in the binding of herbicides like atrazine, since azido-atrazine can be covalently bound to a thylakoid protein in this size class (Gardner, 1981; Pfister *et al.*, 1981a), and since a minor modification in this protein leads to a loss of atrazine sensitivity (L. McIntosh, personal communication). There has been much confusion in the literature about the identity of this protein and of another PS II-related protein involved in the water splitting process (see Section 3.1); therefore, we have determined some properties of the two proteins in this chapter.

Recently, even a third 32,000 M_r protein, which would be involved in quinone binding at the PS II acceptor side, was postulated (Oettmeier *et al.*, 1984b), but at this moment the evidence for the non-identity between the azido-atrazine- and quinone-binding 32,000 M_r protein is still weak. It will be pointed out in Chapter 4 that the interaction of binding of herbicides and quinones to the acceptor side of PS II is (seemingly) competitive, suggesting that herbicides and quinones are binding to the same polypeptide. However, in Section 3.3 it will be shown that the herbicide/quinone binding environment is also modified by another polypeptide than the ABP-32, indicating that more than one protein is involved in herbicide and quinone binding to the PS II acceptor complex.

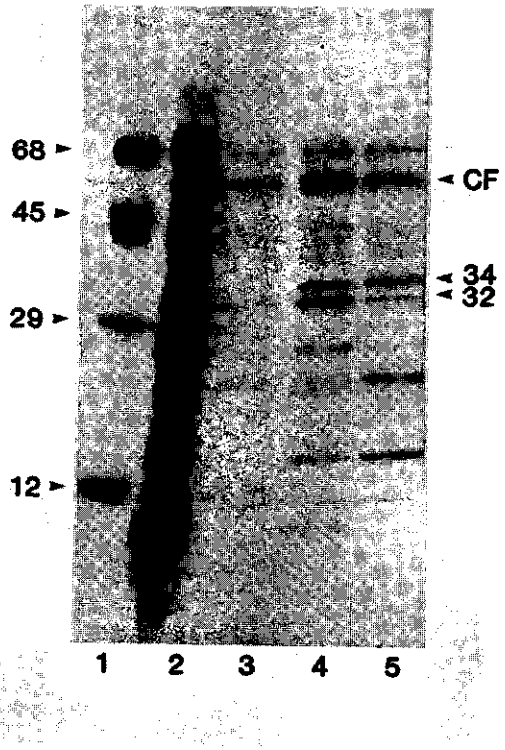
3.1. Properties of the ABP-32 and another polypeptide with approximately the same molecular weight

See also Vermaas *et al.* (1984b).

Certain procedures, such as treatment with the divalent-cation chelator EDTA (ethylenediaminetetraacetate) (Kuwabura and Murata, 1979), result in the selective removal of several polypeptides from the thylakoid membrane, including a polypeptide in the 32 kDa region, which has been implicated as a functional component of PS II (Kuwabura and Murata, 1979, 1982c). Kuwabura and Murata (1982b) suggested that the 32-33 kDa polypeptide which they isolated and purified was not the atrazine-binding polypeptide since the inhibitory activity of atrazine on electron transport was found to be unaffected by the



3.1



3.2

Fig. 3.1. (left) Coomassie Brilliant Blue stained SDS polyacrylamide gel of thylakoid membrane polypeptides (lane 4) and of the thylakoid fraction obtained after treatment with 4 M urea (lane 3) or 1 % cholate (lane 2) for 2.5 hrs at 4° C in the dark. Lane 1 contains molecular weight standards.

Fig. 3.2. (right) Coomassie Brilliant Blue stained SDS polyacrylamide gel of the supernatant extract obtained by incubating thylakoid membranes with IRM without $MgCl_2$ (lane 3) containing 4 M urea (lane 4) or 1 % cholate (lane 5) for 2.5 hrs at 4° C followed by centrifugation (5,000 x *g*; 5 min). Lanes 1 and 2 contain molecular weight standards and unextracted thylakoid membranes, respectively.

addition of purified 32 kDa polypeptide to the assay system. These workers, however, could not rule out the possibility that the isolated 32 kDa polypeptide, once extracted, no longer retained atrazine binding capacity. Electron transport at the acceptor side of PS II was found to be independent of this 32 kDa protein (Kuwabura and Murata, 1982b), suggesting again that it did not participate directly in electron transfer processes at the acceptor side of PS II. Kuwabura and Murata (1982c) suggested that this 32-33 kDa protein might be associated with the oxygen evolving system.

In possible contradiction to the results of Kuwabura and Murata, Mullet and Arntzen (1981) suggested that the protein ($\sim$ 32,000 M_r) they isolated, of similar molecular weight to the protein described by Kuwabura and Murata, was involved in herbicide binding since, upon extraction of this protein by urea and/or cholate from PS II particles, large changes in herbicide susceptibility occurred. In order to resolve the identity of the specific polypeptide(s) which bind herbicides, such as atrazine, we have developed a selective extraction procedure to differentiate the functions of thylakoid polypeptides in the molecular weight range of 32 kDa.

Extraction of a 32,000 M_r polypeptide from thylakoid membranes could be achieved by a method similar to that used by Mullet and Arntzen (1981); thylakoids (200 µg Chl.ml^{-1}) were incubated for 2.5 hrs in the dark at 4° C in the IRM, to which 4 M urea or 1 % purified sodium cholate was added, but in which the 5 mM $MgCl_2$, added normally to the IRM, was left out (in order to induce unstacking); subsequently, the thylakoids were centrifuged and resuspended into IRM, resulting in a release of several extrinsic proteins from the thylakoid membrane, including a thylakoid-bound protein of about 32,000 M_r. Extraction by urea treatment was much more effective than cholate treatment in removing the 32 kDa polypeptide (Fig. 3.1). As was expected, the supernatant fraction obtained upon centrifugation after incubation with urea or cholate contained the thylakoid protein complement (Fig. 3.2).

In order to investigate the possible correspondence between the extractable 32,000 M_r polypeptide and the rapidly turning-over, herbicide-binding protein (ABP-32), thylakoid membranes were labelled by ^{35}S-methionine incorporation *in vivo* (Steinback *et al.*, 1981a) or by covalent linkage with ^{14}C-azidoatrazine *in vitro* (Pfister *et al.*, 1981a; Gardner, 1981). Subsequently, thylakoids were extracted with 4 M urea or 1 % cholate as above. As shown in Fig. 3.3, the ^{14}C-labelled herbicide-binding protein remains completely associated

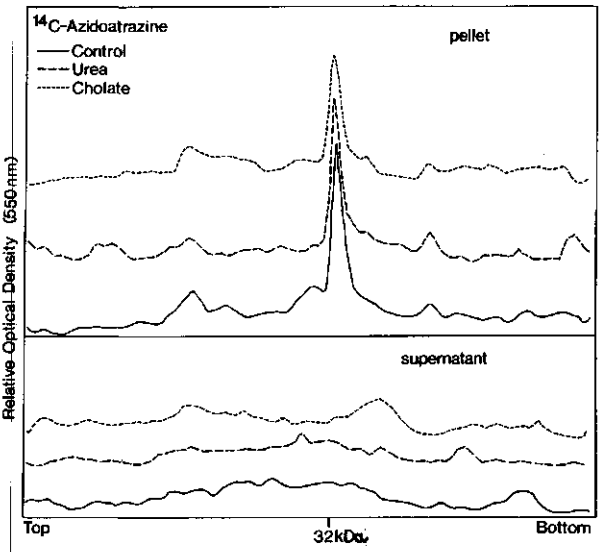


Fig. 3.3. Densitometer scan of autoradiograms of thylakoid membrane polypeptides (pellet) and proteins extracted in IRM (supernatant) without Mg^{2+} (control) in the presence of 4 M urea or 1 % cholate following covalent linkage of ^{14}C-azidoatrazine (see text).

with the thylakoid membrane upon treatment with urea or cholate. Likewise, the majority of ^{35}S-methionine label remains associated with the membranes (Fig. 3.4).

A diffuse band in the 32,000 M_r region, weakly stained by CBB (best visible in Fig. 3.1, lane 3), was found to comigrate with the ^{14}C and ^{35}S bands in the 32 kDa region. This confirms that a weakly stained and diffuse polypeptide band in the 32 kDa region is responsible for herbicide binding and turns over rapidly, whereas the protein in the 32 kDa region that stains intensely with CBB is not directly involved in atrazine binding (no ^{14}C-azidoatrazine is extracted together with the CBB-stainable 32 kDa protein) and may not be turning over rapidly (there is no significant ^{35}S-extraction in the 32 kDa region upon urea treatment; however, in the intensely CBB-staining protein only a trace (< 0.5 mole %) of methionine is present (Table 3.I)).

Recently, another point of distinction between the intensely-staining and herbicide-binding polypeptides was reported: the 32,000 M_r protein staining in-

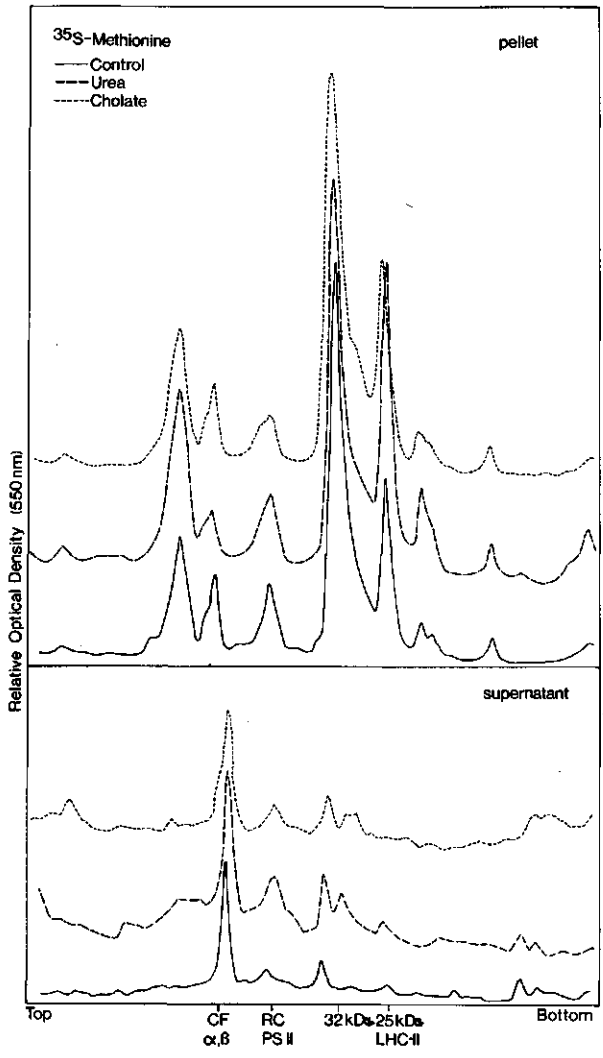


Fig. 3.4. Densitometer scan of autoradiograms of thylakoid membrane polypeptides (pellet) and proteins extracted (supernatant) following *in vivo* incorporation of ^{35}S-methionine, isolation of thylakoids and extraction in IRM without Mg^{2+} (control), after addition of 4 M urea or 1% cholate (see text).

TABLE 3.I, Properties of 32,000 M_r PS II proteins

Comparison of the properties of the protein labelled by azidoatrazine and involved in herbicide binding (ABP-32) and of the urea-extractable polypeptide in the 32 kDa region (UCE-32) with literature data on the lysine-rich, intensely CBB-stained 32,000 M_r polypeptide (LRP-32).

Properties	ABP-32	UCE-32	LRP-32
staining with CBB	diffuse	intense	intense
extractable [a]	-	+	+
lysine content (mole %)	0[b]	9.2	9.6[c]
methionine content (mole %)	4.6[b]	trace	0.3[c]
polarity index	41[b]	49.6	49[c]
^{14}C-azidoatrazine labelling	++	-	
^{35}S-methionine labelling	++	trace	

a): extractable with urea, and to a lesser extent with EDTA or cholate
b): data from Zurawski *et al.* (1982) and from L. McIntosh (personal communication)
c): data from Kuwabura and Murata (1982a)

tensely with CBB is lysine-rich and low in methionine (Kuwabura and Murata, 1982a) whereas the ABP-32 appears to be relatively methionine-rich and deficient in lysine (Zurawski *et al.*, 1982). For this reason, we will refer to the 32,000 M_r protein, which is staining intensely with CBB, and is not involved directly with herbicide binding, as the lysine-rich, 32,000 M_r polypeptide (LRP-32).

The results reported here are at variance with the interpretation by Mullet and Arntzen (1981) and Arntzen *et al.* (1982), since these authors assumed the polypeptide that was extractable from PS II particles (by methods similar to our extraction procedure for intact thylakoids) to be the herbicide-binding protein ABP-32. In order to identify the 32 kDa polypeptide extracted by their urea/cholate extraction procedures (we will refer to this protein as UCE-32), we analyzed the lysine and methionine content of the purified protein, and calculated its polar index (Capaldi and Vanderkooi, 1972). The UCE-32 was found

to contain 9.2 mole % of lysine and a trace of methionine. The polar index was 49.6. Comparing these data on the UCE-32 to the characteristics of ABP-32 and LRP-32 as shown in Table 3.I, we conclude that the protein extracted by Mullet and Arntzen (1981) was not the ABP-32, but the LRP-32.

Mullet and Arntzen identified the extractable 32,000 M_r polypeptide as the ABP-32 in their particles based on the observation that atrazine sensitivity was reduced after extraction. However, diuron and dinoseb were still rather active inhibitors (Mullet and Arntzen, 1981). Since our data indicate that the ABP-32 is not extracted by urea or cholate (Figs. 3.3 and 3.4), one might propose that the extraction procedure leads to a major conformational change in the herbicide binding environment of the PS II particles inducing altered herbicide binding properties. In order to check this possibility, binding of ^{14}C-atrazine and ^{14}C-ioxynil to thylakoids after treatment with 4 M urea or 1 % cholate was compared to herbicide binding to control thylakoids. The results are shown in a double-reciprocal fashion in Fig. 3.5. A slight loss in the number of herbicide binding sites upon urea treatment is observed (1 per 650 instead of 1 per 430 Chl molecules). Note that urea treatment causes an almost complete loss of LRP-32 (Fig. 3.1). Although the atrazine affinity does not change significantly, the K_d for ioxynil decreases upon urea treatment (K_d = 9.5 nM in control and 3.5 nM after urea treatment; Fig. 3.5B) indicating an increase in ioxynil affinity. Cholate treatment has no significant effect on ioxynil affinity, but decreases the atrazine affinity (K_d = 95 nM in control; K_d = 168 nM after cholate treatment). The observed differences in herbicide binding behaviour between ioxynil and atrazine after treatment of thylakoids with urea and cholate indicate that these two herbicides do not interact with exactly identical binding determinants at the herbicide binding domain.

Probably the change in herbicide binding characteristics caused by urea and cholate reflects a change in microenvironment at the herbicide binding domain. This change may have been brought about by a partial denaturation of the ABP-32 as well as by extraction or denaturation of neighboring proteins that are also involved in herbicide binding (see Section 3.3.).

Recently, support for the observation of loss of herbicide binding sites and/or a changed herbicide affinity without extraction of the protein primarily responsible for binding has been reported by Neumann *et al.* (1984): PS II particles prepared from *Chlamydomonas reinhardtii* are relatively diuron-insensitive, but the rapidly turning-over ABP-32 is still present in the PS II particles.

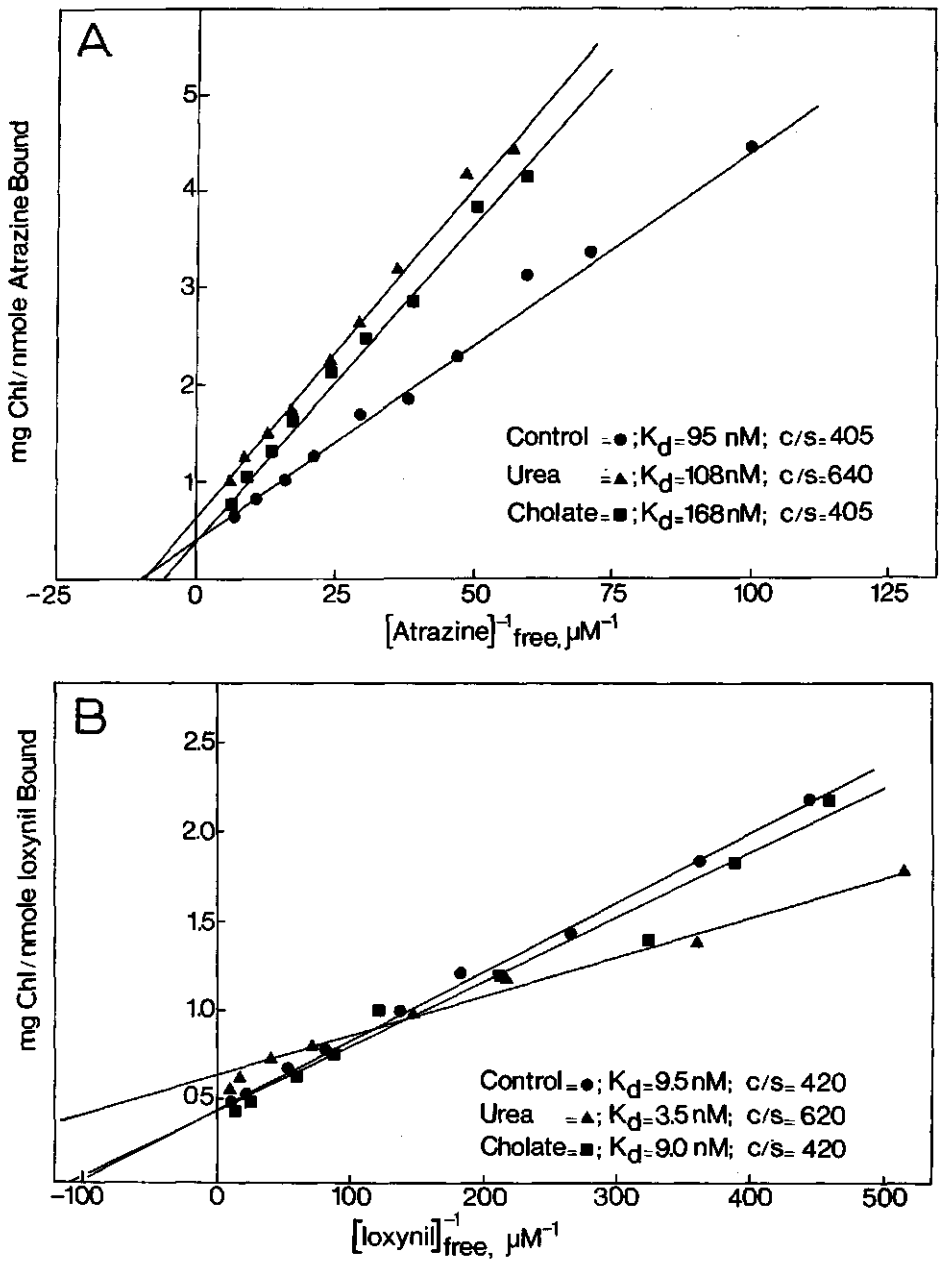


Fig. 3.5. Double-reciprocal plots of free and thylakoid-bound ^{14}C-atrazine (A) and ^{14}C-ioxynil (B) following extraction of thylakoids in IRM without $MgCl_2$ (control) in the presence of 4 M urea or 1 % sodium cholate for 2.5 hrs at 4° C in the dark.

In conclusion, we can say that the ABP-32, which turns over rapidly and which stains poorly with CBB is not identical to the LRP-32, which is also PS II-related (Mullet and Arntzen, 1981) but stains readily with CBB. Many researchers have assumed the CBB-stainable 32,000 M_r protein to be the ABP-32 (among others, Grebanier *et al.*, 1978; Croze *et al.*, 1979; Astier and Joset-Espardellier, 1981; Mullet and Arntzen, 1981; Arntzen *et al.*, 1982; Metz and Miles, 1982). However, the results reported here indicate that this assumption has to be revised. The best methods to "visualize" the ABP-32 include azidoatrazine labelling, and short *in vivo* incubation with ^{35}S-methionine or ^{14}C-acetate rather than CBB staining.

3.2. Trypsin digestion effects on herbicide binding

The protease trypsin is known to make Q_A accessible to the exogenous oxidant FeCy (Renger, 1976). Furthermore, trypsin induces changes in the affinity of PS II inhibitors and leads to a loss in the number of binding sites (Tischer, 1978; Steinback *et al.*, 1981b; Oettmeier *et al.*, 1982c). This would indicate that trypsin attacks the protein components "shielding" Q_A^- from the outside of the thylakoid, and also changes the herbicide binding environment. As will be shown in Section 3.3, only part of the change in the herbicide binding environment can be attributed to proteolysis of ABP-32.

One might expect from the "classical" picture of PS II herbicide action (non-identical, but physically overlapping binding sites for different herbicide groups) (Pfister and Arntzen, 1979; Trebst and Draber, 1979) that proteolysis of the inhibitor binding environment would not cause differential effects on herbicide binding. The data presented by, for example, Steinback *et al.*, (1981b) and Oettmeier *et al.* (1982c), however, suggest that trypsin treatment does have differential effects on herbicide binding, but it is not always clear from the published data whether trypsin digestion was carried out in the absence or the presence of a herbicide. In the presence of high herbicide concentrations the kinetics of trypsin digestion (Mattoo *et al.*, 1981) as well as the proteolysis pattern may be changed. In order to be able to directly compare trypsin digestion effects (incubation with trypsin in the absence of inhibitors) on binding behavior of two types of herbicides, ^{14}C-atrazine and ^{14}C-ioxynil binding to samples from one large batch of trypsin-treated thylakoids was measured. Our ^{14}C-atrazine binding results (Fig. 3.6) with pea thylakoids are qualitatively comparable to the results of Steinback *et al.* (1981b) obtained with

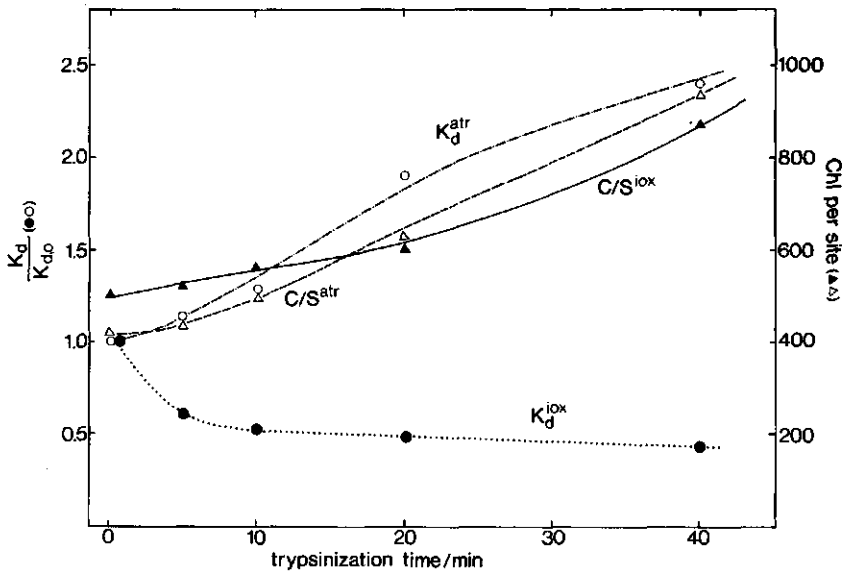


Fig. 3.6. The dissociation constant (K_d) and the number of Chl molecules per binding site (C/S) for atrazine and ioxynil as a function of mild trypsin digestion of thylakoid membranes. 8 µg trypsin per mg Chl; 50 µg Chl.ml^{-1}. $K_{d,o}$ represents the dissociation constant of the herbicide without trypsin treatment, and is 53 nM for atrazine and 6.3 nM for ioxynil. 0,-·-·-·-·-: $K_d/K_{d,o}$ for atrazine; ●, ...: $K_d/K_{d,o}$ for ioxynil; Δ, – – – – –: number of Chl molecules per atrazine binding site; ▲, ———: number of Chl molecules per ioxynil binding site.

spinach: there is a decrease in the number of binding sites together with a loss of atrazine affinity for the remaining sites upon trypsin treatment. However, ioxynil binding is affected differently by trypsin digestion (Fig. 3.6) : the ioxynil affinity increases (in agreement with Oettmeier *et al.* (1982c)) whereas the number of ioxynil binding sites appears to be affected less than that of atrazine binding sites. The number of binding sites for another PS II inhibitor, 2-iodo-4-nitro-6-isobutylphenol, is reported not to change at all upon trypsin treatment (Oettmeier *et al.* 1982c), but it is not clear whether the inhibitor was added before or after trypsin digestion.

These data indicate that large differences exist in trypsin effects on herbicide binding, depending on the herbicide structure. Although these results are not in direct disagreement with a postulated physically overlapping binding site of herbicides from two different classes, such as atrazine and 2-iodo--4-nitro-6-isobutylphenol, the data may be easier to interpret if the binding sites for these inhibitor molecules are spatially further apart, possibly so far apart that different types of inhibitors primarily interact with different polypeptides as suggested for nitrophenols and triazines by, *e.g.*, Johanningmeier *et al.* (1983).

The increase in ioxynil affinity observed upon trypsin treatment is paralleled by an increase in inhibitory activity with respect to electron transport. Inhibition of the DCPIP Hill reaction at saturating light intensity by 4×10^{-8} M ioxynil (after 30 min of preincubation of thylakoids with the herbicide) increases from 40 % after 0 min to 52 % after 2 min and about 60 % after 5-20 min of trypsin treatment (data not shown).

CBB-stained protein gels of the trypsinized thylakoids, obtained using the same batch of trypsin-treated thylakoids as used for the herbicide binding studies, reveal that the LRP-32 is also affected quite rapidly by trypsin (Fig. 3.7), suggesting that the LRP-32 may be -at least partially- exposed to the outside of the thylakoid membrane. After 40 min of mild trypsin treatment, the amount of this 32 kDa polypeptide is about half of that in the control. This agrees with results obtained by Croze *et al.* (1979) and Steinback *et al.* (1981b) showing a trypsin digestion of a CBB-stainable protein in the 32 kDa region (probably the LRP-32). However, in the former study PS II subchloroplast particles were used, and it is possible that the accessibility of the hydrophilic trypsin to proteins at the inside of the thylakoid membrane, which is normally negligible in intact thylakoids, is very good in these particles. We do not detect any significant change in the CBB-staining in the 43-53 kDa region upon tryptic digestion, indicating that the PS II RC proteins are not very accessible to trypsin. Fig. 3.7 shows that the apoproteins of the light-harvesting complex at 29 and 27 kDa were rapidly altered (within 5 min) to a slightly smaller size. This degradation causes an unstacking of the thylakoids because the segment of the light-harvesting complex which is altered by trypsin is necessary for cation--mediated grana stacking (Steinback *et al.*, 1979). Thus, the change in ioxynil affinity found might have been due to thylakoid unstacking. Therefore, the effect of stacking and unstacking, regulated by varying the divalent cation con-

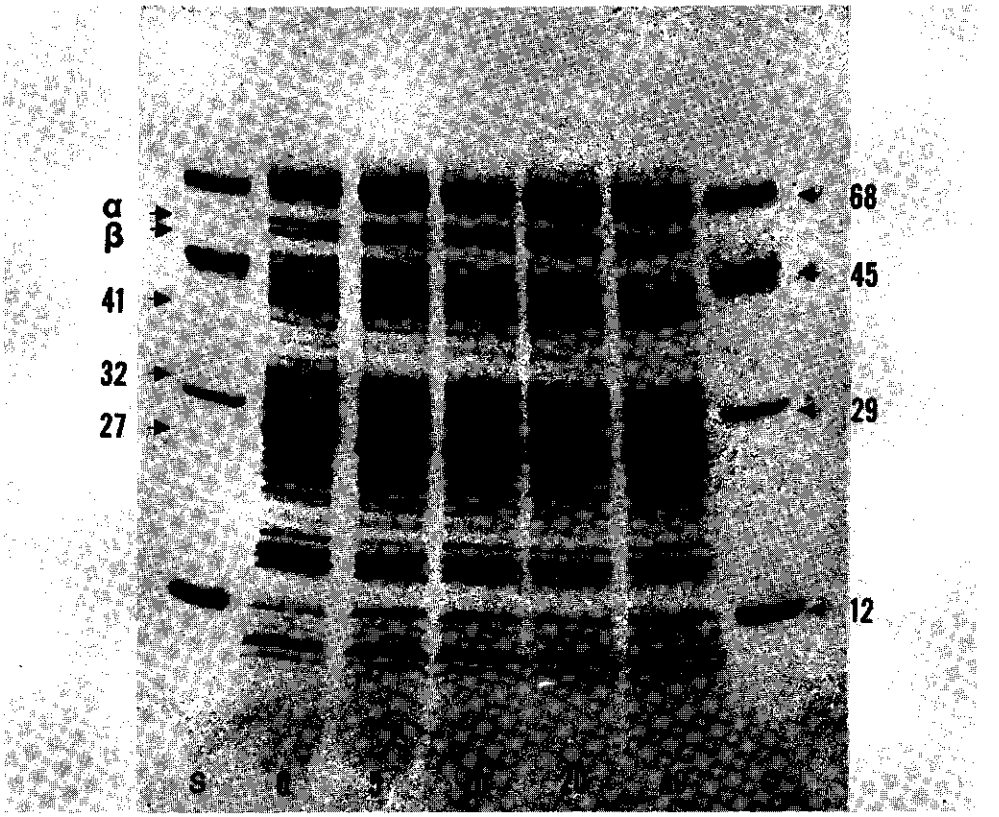


Fig. 3.7. Coomassie Brilliant Blue stained SDS/polyacrylamide gel of solubilized thylakoid membrane proteins after trypsin incubation of the thylakoid suspension for various times (0, 5, 10, 20 and 40 min from left to right). 8 µg trypsin per mg Chl. Protein standards (S) were applied to the two outside lanes.

centration at low concentrations of monovalent cations, on the affinity of ioxynil and atrazine was measured. Fig. 3.8 shows that the ioxynil affinity is decreased upon unstacking whereas the atrazine affinity is unchanged. The ioxynil affinity change caused by unstacking is qualitatively different from that caused by trypsin treatment (which induces an increase in ioxynil affinity) and, thus, it is likely that the trypsin-induced change in ioxynil affinity is not just an unstacking effect. Bromoxynil affinity is changed in a way similar to that of ioxynil upon unstacking (Vermaas *et al.*, 1984a).

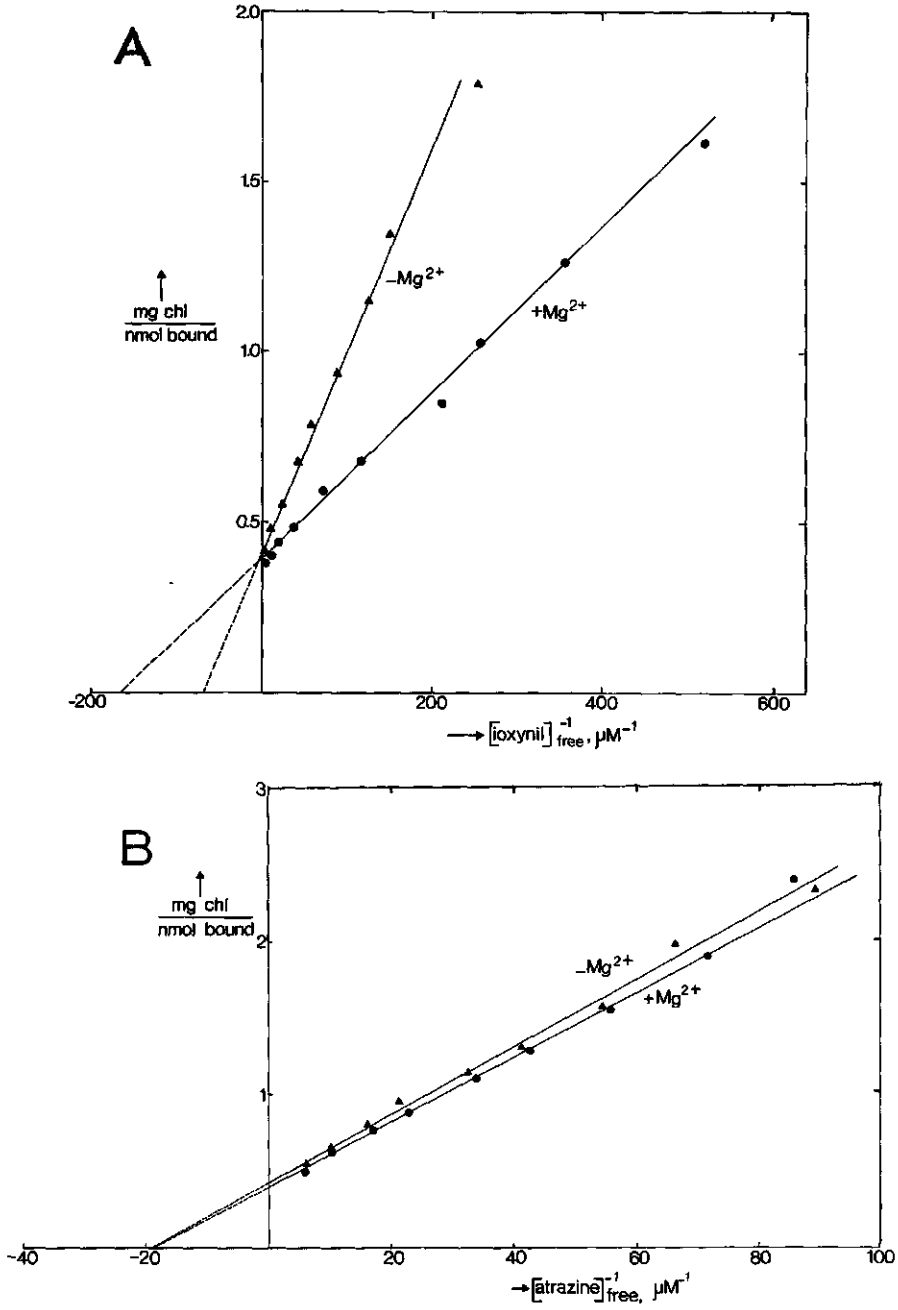


Fig. 3.8. Double-reciprocal plot of ^{14}C-ioxynil (A) and ^{14}C-atrazine (B) binding to thylakoid membranes in IRM without $MgCl_2$ before (▲) and after (●) addition of 5 mM $MgCl_2$. 50 μg Chl.ml^{-1}.

The data presented here cannot determine whether changes in the ABP-32 are primarily involved in the trypsin-induced herbicide affinity changes or not. Oettmeier and co-workers have suggested that RC proteins of 43,000 - 53,000 M_r may be primarily responsible for the binding of phenolic inhibitors (Oettmeier *et al.*, 1980). With some reservations, ioxynil may also be counted to belong to this group of phenolic inhibitors. However, it would be of interest to know whether or not the binding environment for other types of inhibitors, such as triazines, was created by the ABP-32 only. Since it is known that the ABP-32 does not contain any lysine residues (Zurawski *et al.*, 1982), the influence of a lysine-specific protease on herbicide binding was analyzed (see next section).

3.3. The influence of a lysine-specific protease on herbicide binding

See also Hagemann *et al.* (1984).

The lysine-specific endoproteinase (Boehringer, Mannheim, West Germany) is known to partially restore the FeCy Hill reaction in the presence of diuron-type inhibitors (Renger *et al.*, 1983) showing that the protein that "shields" Q_A^- from the environment can be attacked by this protease. It should be noted that Q_A is possibly embedded in the 47 kDa RC protein (H. Nakatani, personal communication), which might imply that the 47 kDa RC protein also serves as "shielding protein" for Q_A.

It appears that not only the accessibility of Q_A^- but also the PS II herbicide binding is changed upon treatment with the lysine-specific protease. The bromoxynil affinity is increased upon digestion with the lysine-specific protease, as observed for short trypsin treatment, whereas the atrazine affinity is decreased (Fig. 3.9). No significant change in the number of binding sites of either atrazine or bromoxynil was observed upon treatment with the lysine-specific protease; however, it cannot be excluded that upon treatment with higher concentrations of this protease such a change does occur. Due to limited financial resources, such an experiment has not been carried out yet.

The change in herbicide affinity upon treatment with the lysine-specific protease can be taken as evidence that proteins other than the ABP-32 are involved, directly or indirectly, in binding of atrazine as well as bromoxynil, unless the protease is not very specific after all, or is contaminated with other proteases. In order to check this possibility, digestion of the ABP-32

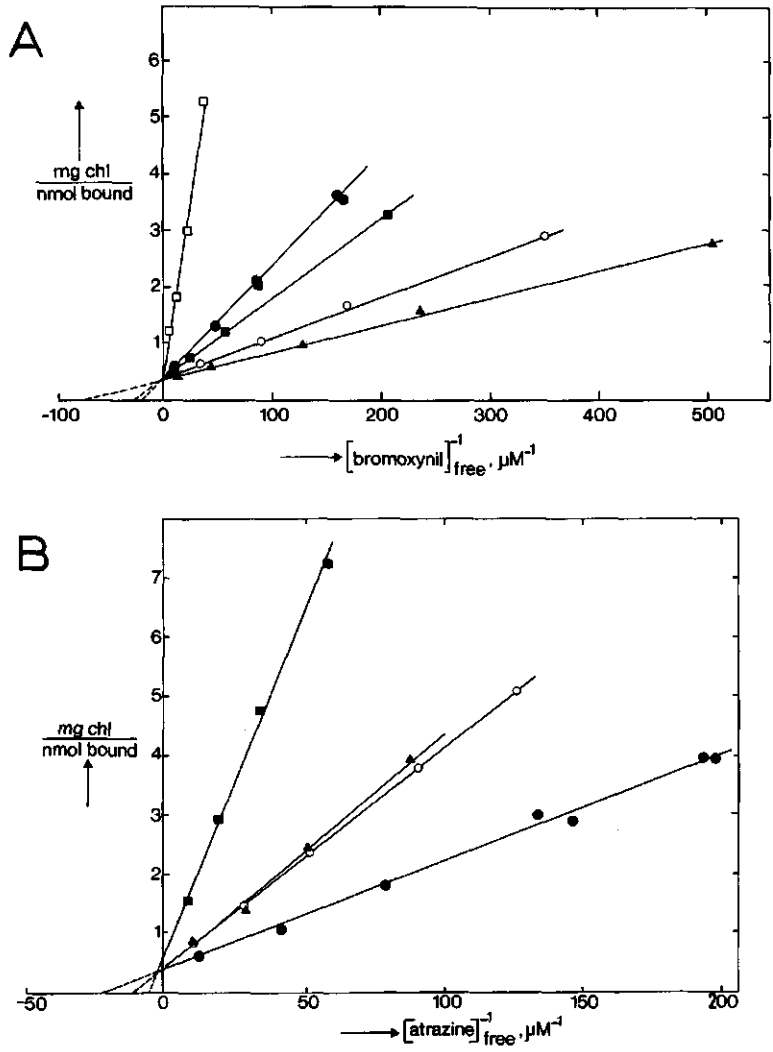


Fig. 3.9. Double reciprocal plot of ^{14}C-bromoxynil (A) and ^{14}C-atrazine (B) binding to thylakoids treated with proteolytic enzymes. ●: control; ○: 60 min lysine-specific protease; ▲: 5 min trypsin; ■: 20 min trypsin; □: 60 min trypsin (A only).
Incubation with the protease occurred in the presence of the herbicide. The source and activity of trypsin are different from those described in Section 3.2.

by the enzyme preparation was measured. Since the ABP-32 is not easily stainable by CBB, but is turned over very rapidly, ^{35}S-methionine pulse labelling *in vivo* was carried out, and after thylakoid isolation the lysine-specific protease was added. Densitograms of the autoradiogram (Fig. 3.10) show that the ABP-32 is attacked readily by trypsin, but is not digested by the lysine-specific protease. This indicates that the "lysine-specific" protease preparation does not contain significant contaminating activity which would digest the ABP-32. It should be noted that the polypeptides from the light-harvesting complex are readily accessible to both trypsin and the lysine-specific protease, as is also reflected by fluorescence measurements (Renger *et al.*, 1983).

The above data indicate that although only the rapidly turning-over 32 kDa protein (ABP-32) is labelled by azidoatrazine, another (lysine-containing) polypeptide is also involved in modifying the affinity of -among others- atrazine.

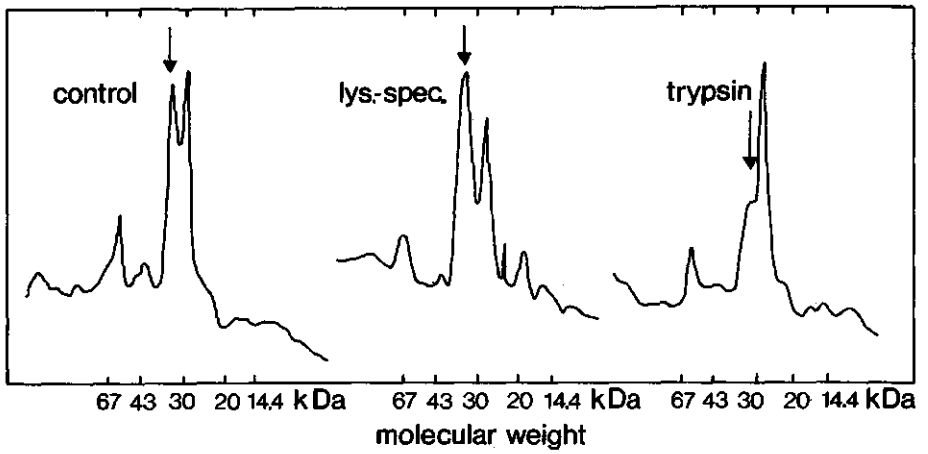


Fig. 3.10. Densitometer scan of an autoradiogram of a SDS-PAGE protein gel from thylakoids isolated from ^{35}S-methionine-pulselabelled pea leaves and treated subsequently with proteolytic enzymes for one hour as indicated in the figure. 50 μg Chl.ml^{-1}. Molecular weight indications on the abscissa correspond to the position of protein standards as determined by CBB staining. Arrows indicate the position of the ABP-32. The activity of trypsin was 0.025 units.ml^{-1}; that of the lysine-specific protease was 2.5 units.ml^{-1}, which is an order of magnitude higher than the amount used for the herbicide binding studies.

This shows how limited the method of azidolabelling is for identifying the protein(s) with which the azido-labelled ligand interacts. This limitation should be kept in mind when interpreting results obtained by photoaffinity labelling using, for example, azidoatrazine, azido-*i*-dinoseb or azidoquinones. For this reason, one should not refer to the rapidly turned-over 32,000 M_r protein labelled by azidoatrazine as "Herbicide-Binding Protein" or "Q_B protein".

The identity of the lysine-containing protein that is involved in influencing the herbicide affinity is currently under investigation. One might speculate that this protein is one of the two PS II RC proteins (Arntzen *et al.*, 1983), perhaps the 47-53 kDa protein, which is supposed to be involved in binding Q_A and phenolic inhibitors. In support of a Q_A-related protein being also important for the binding characteristics of not only phenolic inhibitors but also of "classical" herbicides, results from Rutherford *et al.* (1984) should be mentioned: an ESR-signal attributed to the Q_A^-. Fe^{2+} complex was changed in amplitude and/or linewidth, by addition of PS II inhibitors like *o*-phenanthroline, dinoseb or diuron.

On the other hand, the data reported here suggest that bromoxynil binding may also be sensitive to changes in the ABP-32 because bromoxynil affinity is decreased by longer times of trypsin digestion, just as atrazine affinity is (Hagemann *et al.*, 1984). The atrazine affinity decrease may be attributed to trypsin digestion of the ABP-32 (see Mattoo *et al.* (1981) and Steinback *et al.* (1981a,b)).

The results reported here favor the existence of an inhibitor binding environment, in which more than one polypeptide participates. The data cannot be explained readily in terms of some kind of a narrowly defined, inflexible area, to which all herbicides bind, and which is not easily affected by possible conformational changes; the role of neighboring polypeptides must be considered when discussing ligand binding to a certain part of the PS II complex.

Chapter 4, A common inhibitor/quinone binding environment

The hypothesis of competitive binding of the native quinone and inhibitors to the PS II acceptor complex (Velthuys, 1981; Wraight, 1981) implied that the sites of quinone and herbicide binding were closely related, if not identical. However, the precise nature of the interaction between herbicide and quinone was unknown, and, moreover, the hypothesis of competitive quinone/inhibitor binding lacked thorough experimental testing for a wide range of PS II inhibitors. Therefore, we performed experiments in order to elucidate the binding interaction between herbicides and synthetic or natural quinones after non-covalent (Section 4.1) and covalent (Section 4.3) binding of the quinone to the PS II acceptor complex. It is concluded that the herbicides tested are binding to closely related, but different sites compared to the quinones. Section 4.2 describes measurements of the binding interaction between two inhibitors. This binding interaction is found to be (seemingly) competitive for both diuron-type and other PS II inhibitors.

4.1. Herbicide/quinone competition

Also see Vermaas *et al.* (1983, 1984a) and Vermaas and Arntzen (1983).

The possibility of a herbicide-induced release of Q_B from the binding environment (and *vice versa*) (Velthuys, 1981; Wraight, 1981) was tested by the determination of herbicide binding in the presence of synthetic quinones. It was observed that some of the "simple"quinones (for example, *p*-benzoquinone (*p*-BQ), 5-hydroxy-1,4-naphthoquinone, 2,5-dimethyl-*p*-BQ, tetramethyl-*p*-BQ, anthraquinone-2,6-disulphonate, 2,5-dihydroxy-*p*-BQ and 3,6-dichloro-2,5-dihydroxy-*p*-BQ) did not decrease ^{14}C-atrazine binding to any significant extent when added in 0.5 mM concentration. However, many other quinones were found to be able to displace ^{14}C-atrazine from its binding site (Table 4.I). Other quinones with high affinity to the herbicide/quinone binding environment include 2-(3-cyclohexylpropyl)-3,6-dimethyl-5-hydroxy-*p*-BQ, 3,6-dimethyl-2-hydroxy-5-undecyl-*p*-BQ, 2-hydroxy-3-(3-methylbutyl)-1,4-naphthoquinone, 2-hydroxy-3-(11-oxododecyl)-1,4-naphthoquinone, 2-(10-bromodecyl)-3-hydroxy-1,4-naphthoquinone, 2-hydroxy-3-(9-hydroxy-9-pentyltetradecyl)-1,4-naphthoquinone, 2-hydroxy-3-nonyl-1,4-naphthoquinone and 2-hydroxy-3-(7-methyloctyl)-1,4-naphthoquinone.

TABLE 4.I, Herbicide/quinone competition

Final concentrations of synthetic quinones needed to increase the dissociation constant of ^{14}C-atrazine or ^{14}C-ioxynil by a factor of 2. Pea thylakoids at 50 µg Chl.ml^{-1} in isolation/reaction medium.

QUINONE	CONCENTRATION (µM)
2-hydroxy-1,4-napthoquinone	300
2,5-dichloro-*p*-benzoquinone	400
2,6-dichloro-*p*-benzoquinone	100
tetrachloro-*p*-benzoquinone	3

The apparent K_d for these quinones was less than 10 µM as determined by ^{14}C--ioxynil displacement studies under conditions as shown in Table 4.I (50 µg Chl.ml^{-1}) (not shown). However, the "real" K_d of the binding site/quinone complex (calculated on the basis of the local quinone concentration) may be much higher because of the expected partitioning of these rather hydrophobic quinones into the thylakoid membrane.

All the quinones that appeared to displace ^{14}C-labelled herbicides did so competitively: no change in the number of herbicide binding sites was observed, but the K_d of the herbicide was increased. However, as will be pointed out at the end of this section, this does not necessarily indicate that the binding sites for herbicides and quinones are identical. For the quinones tested no differences in activity between ^{14}C-atrazine and ^{14}C-ioxynil displacement were detected (Vermaas and Arntzen, 1983). Herbicide displacement by tetrachloro--*p*-benzohydroquinone, for example, is shown in Fig. 4.1. These data may support the hypothesis of a common binding environment for quinones and herbicides.

All quinones cited in Table 4.I were found to inhibit Q_A^- oxidation at saturating light intensity in the concentration range needed to displace herbicides such as atrazine and ioxynil. For example, Fig. 4.2 indicates that Q_A^- is accumulated initially and the filling of the PQ pool is slowed down in the presence of 2-hydroxy-1,4-naphthoquinone, pointing to an impaired Q_A^- oxidation by PQ.

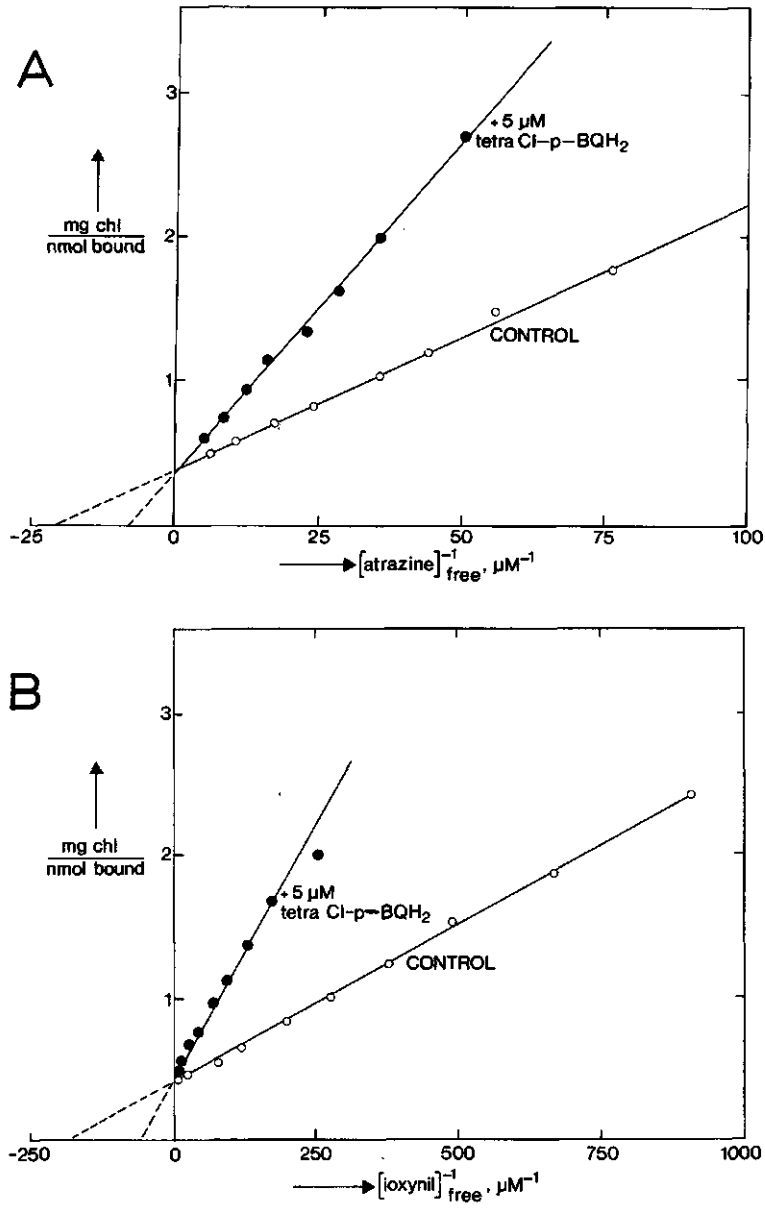


Fig. 4.1. Double-reciprocal ^{14}C-atrazine (A) and ^{14}C-ioxynil (B) binding curve in the absence (O) and the presence (●) of 5 µM tetrachloro--*p*-benzohydroquinone using pea thylakoids. 50 µg Chl.ml^{-1}.

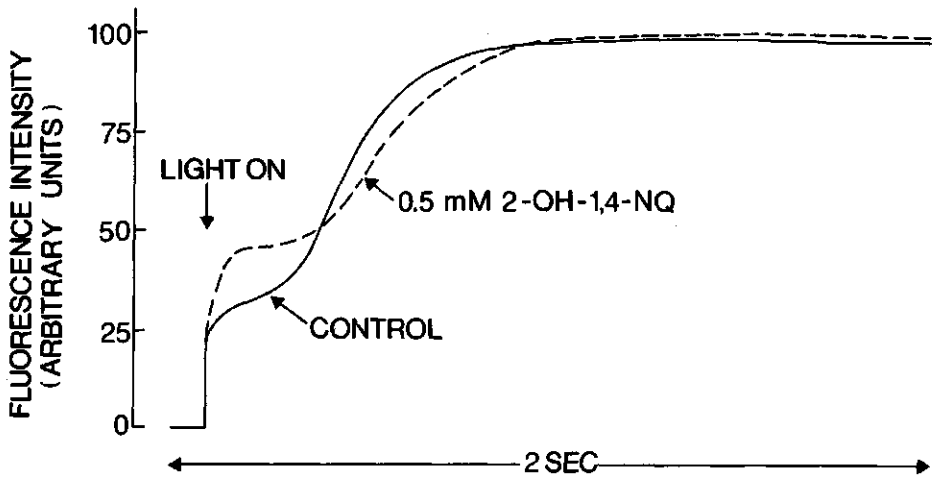


Fig. 4.2. Chl *a* fluorescence induction curve of pea thylakoids in isolation/reaction medium in the presence (---) and the absence (——) of 0.5 mM 2-hydroxy-1,4-naphthoquinone. Both samples contained 0.5 µM DBMIB and 2 mM ascorbate (to reduce DBMIB) to eliminate possible effects of PS I electron acceptance by the synthetic quinone and to decrease quenching.

These data indicate that the synthetic quinones are able to occupy a binding site such that herbicides and Q_B no longer bind effectively. The synthetic quinones are not reduced by Q_A^- rapidly (*i.e.*, on the time scale of about 100 ms; the $t_{\frac{1}{2}}$ of Q_A^- oxidation by $Q_B^{(-)}$ is about 0.5 ms) because an almost complete inhibition of electron transport at saturating light intensity (as measured by, *e.g.*, DCPIP reduction and fluorescence induction) can be obtained. At this time it is not clear whether this inhibition is caused by steric (the quinone does not occupy the binding site such that it is in the right position to be reduced by Q_A^-) or thermodynamic (the quinone/semiquinone midpoint redox potential of the bound synthetic quinone is lower than that of the Q_A/Q_A^- couple) reasons.

Quinones that allow electron transfer (*i.e.*, which are reduced by Q_A^-) and that are able to replace herbicides include 6-azido-5-decyl-2,3-dimethoxy-*p*-BQ (6-azido-Q_0C_{10}) and PQ-1 (a PQ molecule with 1 instead of the usual 9 isoprene units in the long side chain). 6-azido-Q_0C_{10} is an efficient displacer of atra-

zine and ioxynil (10 6-azido-Q_0C_{10} molecules per PS II chain increase the K_d of the herbicides by a factor of 2 - 3) (Fig. 4.3), and it does not affect the Hill reaction rates (Vermaas *et al.*, 1983). However, the related 2-azido-3--methoxy-5-geranyl-6-methyl-*p*-BQ (2-azido-Q_2) does not displace PS II herbicides effectively (Vermaas *et al.*, 1983). This indicates that the quinone structure (configuration of the side groups) is quite important in determining the affinity to the binding site.

A ratio of approximately 60 PQ-1 molecules per PS II RC are necessary to cause occupation of half of the Q_B binding sites as measured by competition studies with ^{14}C-atrazine (Vermaas *et al.*, 1984a), although Oettmeier and Soll (1983) reported that they could not observe herbicide displacement by PQ-1 addition without previous extraction of the native PQ. In any case, the amount of added PQ-1 per PS II RC needed to bring about occupation of half of the binding sites (≥ 60) is much higher than the size of the native PS II-reducible PQ pool (about 7 per PS II chain (Stiehl and Witt, 1969)). The relative inactivity of PQ-1 in herbicide displacement may be caused by the structural difference between PQ-1 and the natural PQ-9 resulting in different binding affinity and/or partitioning into the thylakoid membrane. Another possibility is that neither PQ-1 nor PQ-9 are effective in displacing herbicides, and that the quinone binding site is occupied by Q_B in only part of the electron transport chains (Robinson and Crofts, 1983). A third possibility is that part of the added PQ-1 is partitioned mainly into other hydrophobic domains such as plastoglobuli, rather than into the thylakoid. There are about 40 native PQ molecules per PS II reaction center (Crane, 1965) whereas only about 7 are active as PS II electron acceptor (Stiehl and Witt, 1969), indicating that there may be many PQ molecules in the chloroplast that are not in contact with the Q_B binding site in PS II. These three factors may thus induce an increase in the amount of synthetic quinones needed for herbicide displacement.

Most of the herbicide binding experiments were carried out in darkness in order to insure that, without further additions, only the oxidized form of the quinone was present. Reduction of the quinone to the hydroquinone does not appear to change the affinity dramatically (see below). However, the semiquinone has a greatly increased affinity for the site. This follows from the rather high equilibrium constant (~ 10) between $Q_A^-.Q_B$ and $Q_A.Q_B^-$ (Diner, 1974) and from diuron binding kinetics in the presence of Q_B and Q_B^- (Lavergne, 1982b).

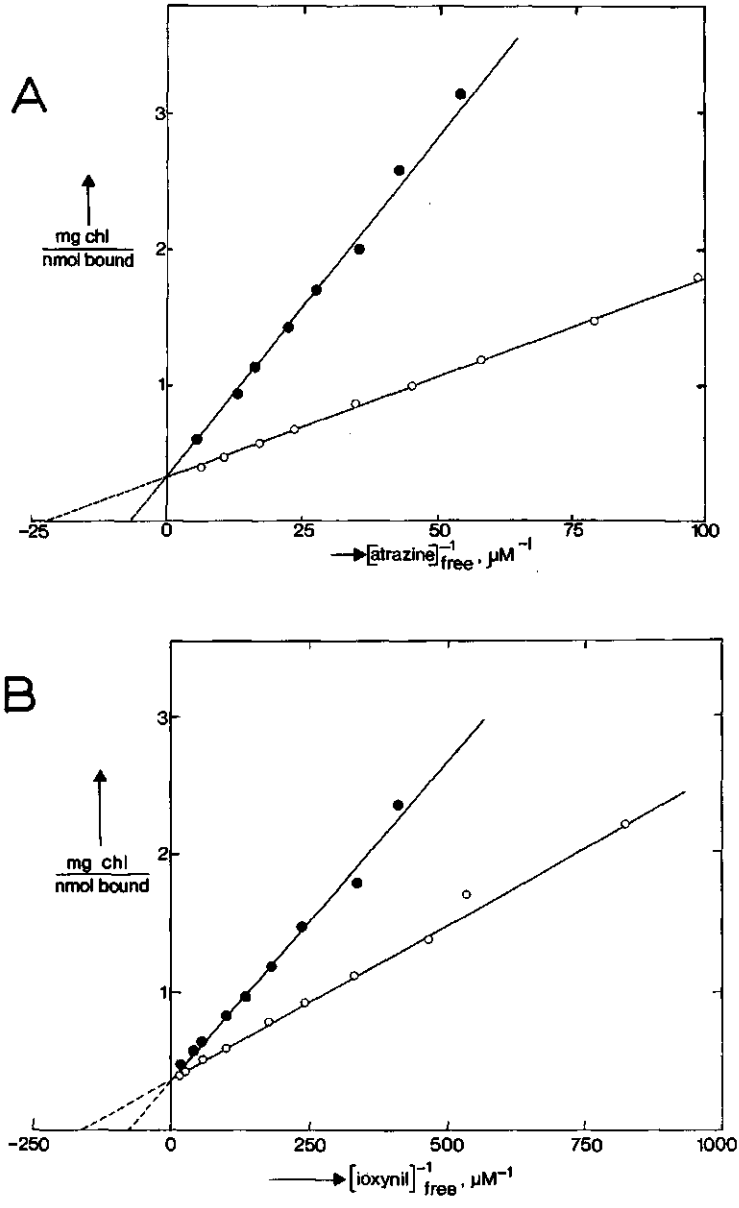


Fig. 4.3. Double-reciprocal plot of free and thylakoid-bound ^{14}C-atrazine (A) and ^{14}C-ioxynil (B) in the presence (●) and the absence (○) of 6-azido-Q_0C_{10}. The 6-azido-Q_0C_{10}/Chl ratio was 1/20 for A and 1/40 for B. Pea thylakoids.

Furthermore, recent results of Laasch *et al.* (1983, 1984) show that under certain conditions (pH ∿ 6) about 50% of the binding sites in thylakoids that have been illuminated by one flash (NH_2OH was present in order to prevent Q_A^- oxidation by the water splitting system) cannot be occupied by low concentrations of diuron. This can be interpreted to indicate occupation of the site by Q_B^-, which cannot be displaced by low concentrations of diuron because of its high affinity. This phenomenon is observed with a synthetic quinone (*p*-benzoquinone) as well (Lavergne, 1982a).

Laasch *et al.* (1984) explained their data using a model in which the conformation of the binding site changed upon formation of Q_B^-, inducing a shift in the K_d of diuron. However, from this model a greatly decreased diuron affinity would be expected upon Q_A reduction as diuron binding induces reversed electron flow from Q_B^- to Q_A, resulting in Q_A^- (Velthuys and Amesz, 1974). In this case, diuron would bind to the binding environment with a low affinity. However, as demonstrated below, the affinity of the diuron-type herbicide atrazine does not decrease significantly upon illumination. A simpler explanation for the data reported by Laasch *et al.* (1983, 1984) would be a biphasic diuron binding behavior with K_d = x nM for binding sites where Q_A and Q_B are both oxidized, and K_d = x(K+1) nM (in which K is the apparent equilibrium constant of $Q_A^-.Q_B \rightleftharpoons Q_A.Q_B^-$) for binding sites where a semiquinone is present. In this explanation Q_B^- is assumed to have a much higher affinity for the binding site than Q_B. Recently I noticed that Laasch, Urbach and coworkers have adopted this type of explanation (Urbach *et al.*, 1984). In Section 5.2 more evidence supporting this explanation is presented.

It is not yet clear why the oscillation in diuron binding behavior with a period of 2 only occurs at rather low pH (∿6) . A possible explanation may be that the fraction of flash-generated Q_B^- that is reoxidized rapidly ($t_{\frac{1}{2}}$ ∿ 1 s) (Boussac and Etienne, 1982) is pH-dependent, *i.e.*, that it is much larger at high pH than at low pH. Furthermore, the reoxidation rate of the Q_A^-.diuron complex in the presence of NH_2OH may be pH-dependent.

Full reduction of quinones to the corresponding quinol (=hydroquinone) does not appear to cause a dramatic shift in herbicide binding: the chlorinated *p*-BQ's tested did not show a large change in the efficiency of ^{14}C-atrazine displacement upon reduction (Vermaas and Arntzen, 1983). This might imply that quinols have the same (relatively low) affinity for the binding environment as the corresponding quinones. This can also be tested for PQ by monito-

ring herbicide binding in the dark, compared to that in the light in the absence of an electron acceptor (photoreduction of the PQ pool), or in the dark in the presence of a reductant (such as dithionite). The atrazine affinity is not sensitive to the reduction of the PQ pool and of Q_A either by illumination or chemical reduction, whereas bromoxynil, ioxynil and *i*-dinoseb show a reduced affinity when PQ and Q_A are reduced (Table 4.II). This reduction in the affinity is reversible (not shown). It should be noted that *i*-dinoseb affinity cannot be monitored in the dark in the presence of dithionite, since dithionite reduced *i*-dinoseb itself. In the case of ioxynil, the affinity decrease approximately parallels the reduction of the PQ pool: Chl *a* fluorescence induction measurements indicated that ascorbate addition does not reduce PQ appreciably whereas durohydroquinone addition under anaerobic conditions appeared to lead to reduction of PQ and possibly part of Q_A. This might be taken as evidence that the decreased ioxynil affinity is caused by an increased Q_B affinity upon reduction the PQ-pool. However, if that were the case, atrazine affinity

TABLE 4.II, Herbicide affinity as a function of the redox state of the thylakoids

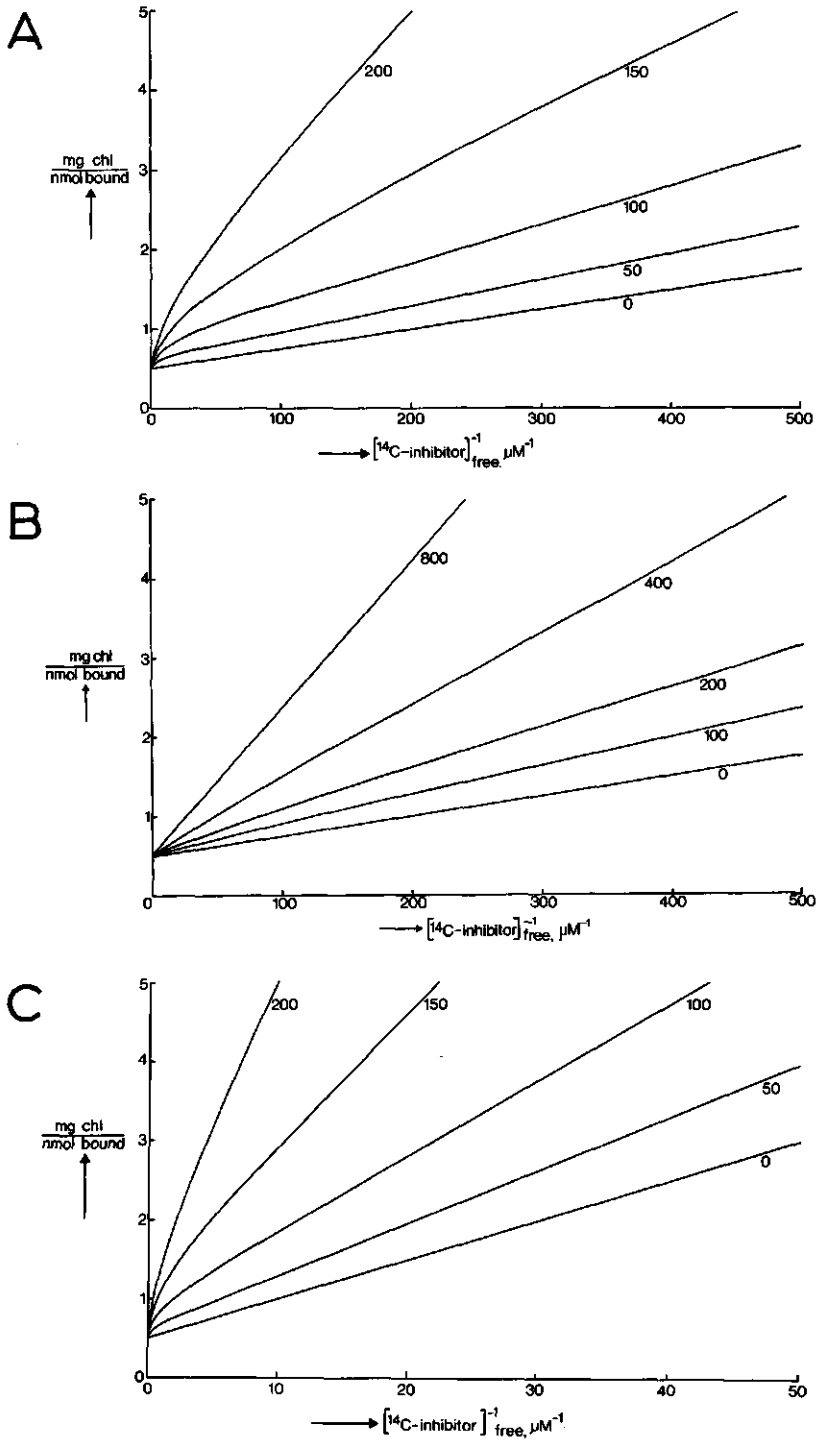
Dissociation constants of herbicides (nM) in the dark or in bright room light in pea thylakoids. The K_d was determined directly by using ^{14}C-labelled herbicides, except in the case of *i*-dinoseb: the K_d of *i*-dinoseb was measured by competition of ^{12}C-*i*-dinoseb with ^{14}C-atrazine (see Section 4.2) in order to avoid artefacts caused by unspecific binding of *i*-dinoseb. Where indicated, 0.25 mM FeCy, 2.5 mM sodium ascorbate, 0.25 mM durohydroquinone or 2.5 mM dithionite were added to the virtually O_2-free thylakoid suspension in isolation/reaction medium.

condition	K_d(nM) of atrazine	ioxynil	bromoxynil	*i*-dinoseb
dark	57	6	62	220
light	63	22	240	450
dark + dithionite	72	23	210	-
dark + durohydroquinone	65	27	-	-
dark + ascorbate	63	7	-	-
dark + FeCy	54	6	-	-

would also be expected to decrease upon Q_B reduction by the same factor as ioxynil affinity. This is not observed (Table 4.II). Another explanation is a reduction-induced alteration in the binding environment in a manner that changes the affinity of some (but not all) inhibitors of the Q_A^- oxidation. The proposed reduction-induced conformational change influences only the binding of some inhibitors but not that of others. Therefore, an allosteric interaction between ioxynil and atrazine binding, such that the binding affinity of one herbicide is decreased dramatically when the other is bound, may be more realistic than a true competitive interaction between atrazine and ioxynil, in which there would be a physical overlap if they were bound concomitantly.

4.2. Herbicide/herbicide interaction

In this thesis the concept of "competitive" herbicide binding is often used: all herbicides blocking Q_A^- oxidation are "competing" with each other for binding to the common inhibitor / Q_B binding environment in the sense that only one of these molecules can be bound to this environment with high affinity at any moment. Many experimental results support this assumption (Tischer and Strotmann, 1977; Laasch *et al.*, 1982, and others). However, some data were interpreted as indicating a non-competitive interaction between certain inhibitors of different chemical classes (Oettmeier *et al.*, 1982c). In order to establish an explanation for this discrepancy, an equation was derived which describes the binding of a labelled herbicide (the compound one directly measures) in the presence of an unlabelled herbicide under equilibrium conditions, assuming that only these two, and thus not PQ, compete for binding to the inhibitor/Q_B binding environment. Thus, it should be kept in mind that the equation derived below (Tischer and Strotmann, 1977) is a simplified treatment of these interactions. For the basic equations (4.1)-(4.4) (in which $K_{d,u}$ and $K_{d,l}$ is the dissociation constant of the unlabelled and labelled herbicide, respectively, [S] the concentration of unoccupied herbicide binding environments, [S.U] and [S.L] the concentration of binding environments occupied by unlabelled and labelled herbicide, respectively, and [U] and [L] the free concentration of unlabelled and labelled herbicide, respectively), equation (4.5), from which [S.L] can be calculated, can be derived by eliminating the other unknowns in this system (*i.e.*, [U], [S.U] and [S]) (Tischer and Strotmann, 1977).


A
mg chl / nmol bound
200
150
100
50
0
[14C-inhibitor]free.-1 μM-1
B
mg chl / nmol bound
800
400
200
100
0
[14C-inhibitor]free.-1 μM-1
C
mg chl / nmol bound
200
150
100
50
0
[14C-inhibitor]free.-1 μM-1

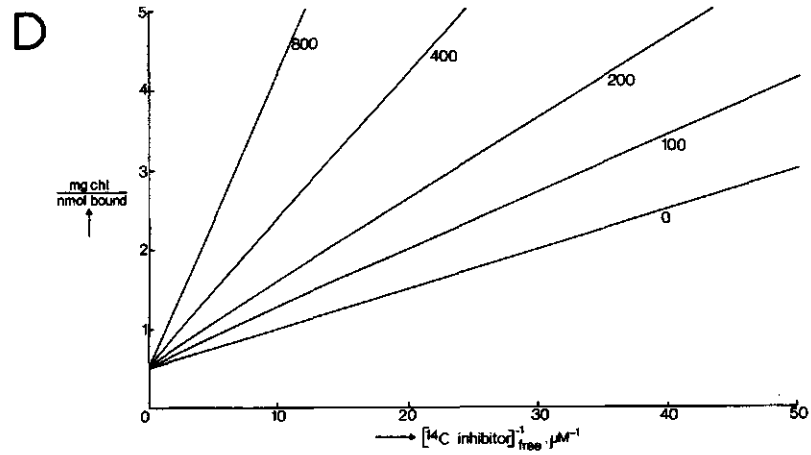


Fig. 4.4. Theoretical double-reciprocal plots of ^{14}C-herbicide binding in the presence of a ^{12}C-inhibitor that competes for binding. The concentration of binding sites is assumed to be 0.2 μM. K_d of the ^{14}C--labelled herbicide is 5 nM (A and B) or 100 nM (C and D); K_d of the ^{12}C-inhibitor is 5 nM (A and C) or 100 nM (B and D). The ^{12}C--inhibitor concentration (nM) is indicated in the figures.

$$K_{d,u} = \frac{[S][U]}{[S.U]} \tag{4.1}$$

$$K_{d,l} = \frac{[S][L]}{[S.L]} \tag{4.2}$$

$$[U]_{total} = [U] + [S.U] \tag{4.3}$$

$$[S]_{total} = [S] + [S.U] + [S.L] \tag{4.4}$$

$$[S.L]^2 + [S.L]\,\frac{K_{d,l}[L](K_{d,u} + [U]_{total} - [S]_{total}) + K_{d,u}[L]^2}{(K_{d,l})^2 + K_{d,l}[L]} - \frac{K_{d,u}[L]^2[S]_{total}}{(K_{d,l})^2 + K_{d,l}[L]} = 0 \tag{4.5}$$

With the use of equation (4.5) the concentrations [S.L] can be calculated for different concentrations of $[U]_{total}$ and [L]. In this way, double-reciprocal plots of free *vs.* bound concentrations of labelled herbicide in the presence of an unlabelled inhibitor, which binds competitively with respect to the labelled herbicide, can be constructed (Fig. 4.4). The apparent K_d of the la-

belled herbicide is increased, of course, by the addition of unlabelled inhibitor, whereas the total number of inhibitor binding sites is not changed. This is analogous to the traditional competitive inhibition of enzyme-catalyzed reactions as described in a Lineweaver-Burk plot. However, calculations using equation (4.5) show that the double-reciprocal plot remains linear only if the unlabelled free herbicide concentration remains almost constant at all labelled herbicide concentrations used. In many cases of herbicide/herbicide interactions, however, the concentration of free unlabelled herbicide is not constant, but increases upon increasing the labelled herbicide concentration: since the K_d of many inhibitors is comparable to or lower than the concentration of herbicide binding sites (about 2.10^{-7} M at 100 µg Chl.ml^{-1}), the concentration of free unlabelled inhibitor is not constant, but increases upon increasing the concentration of the labelled herbicide ($K_d \sim 10^{-8}$ M) from, for example, 10^{-8} to 10^{-7}M. Thus, in these cases linear double-reciprocal plots cannot be expected. Some theoretical examples of double-reciprocal plots of ^{14}C-herbicide binding in the presence of a ^{12}C-inhibitor (using equation (4.5)) are shown in Fig. 4.4. This theoretical simulation shows that significant curvatures in the double-reciprocal plots can be expected under experimental conditions.

The above considerations suggest an alternative explanation of the data reported by Oettmeier *et al.* (1982c), who concluded the ioxynil/diuron interaction to be non-competitive on the basis of double-reciprocal plots of ^{14}C-ioxynil binding at low concentrations of this herbicide in the presence of unlabelled diuron. The observations on ^{14}C-ioxynil binding ($K_d \sim 5$ nM) in the presence of ^{12}C-diuron ($K_d \sim 20$ nM) (Oettmeier *et al.*, 1982c) can be simulated (not shown) using equation (4.5) which assumes competitive rather than non-competitive herbicide/herbicide interaction. This shows that one should be very careful in interpreting double-reciprocal plots of herbicide binding to thylakoids.

In order to make sure that the theory as given by equation (4.5) (derived under the simplifying assumption that Q_B binding does not play a role in herbicide/herbicide interaction) is able to account for the experimental data rather well, the ^{14}C-ioxynil binding was measured in the presence of various concentrations of ^{12}C-atrazine, for which the K_d under our experimental conditions was known (53 nM). The experimental and theoretical results are shown in Fig. 4.5. Fig. 4.5A indicates that $[S]_{total}$ = 102 nM and K_d of ioxynil is 5.6 nM. These values were used for the theoretical plot. The fit between theo-

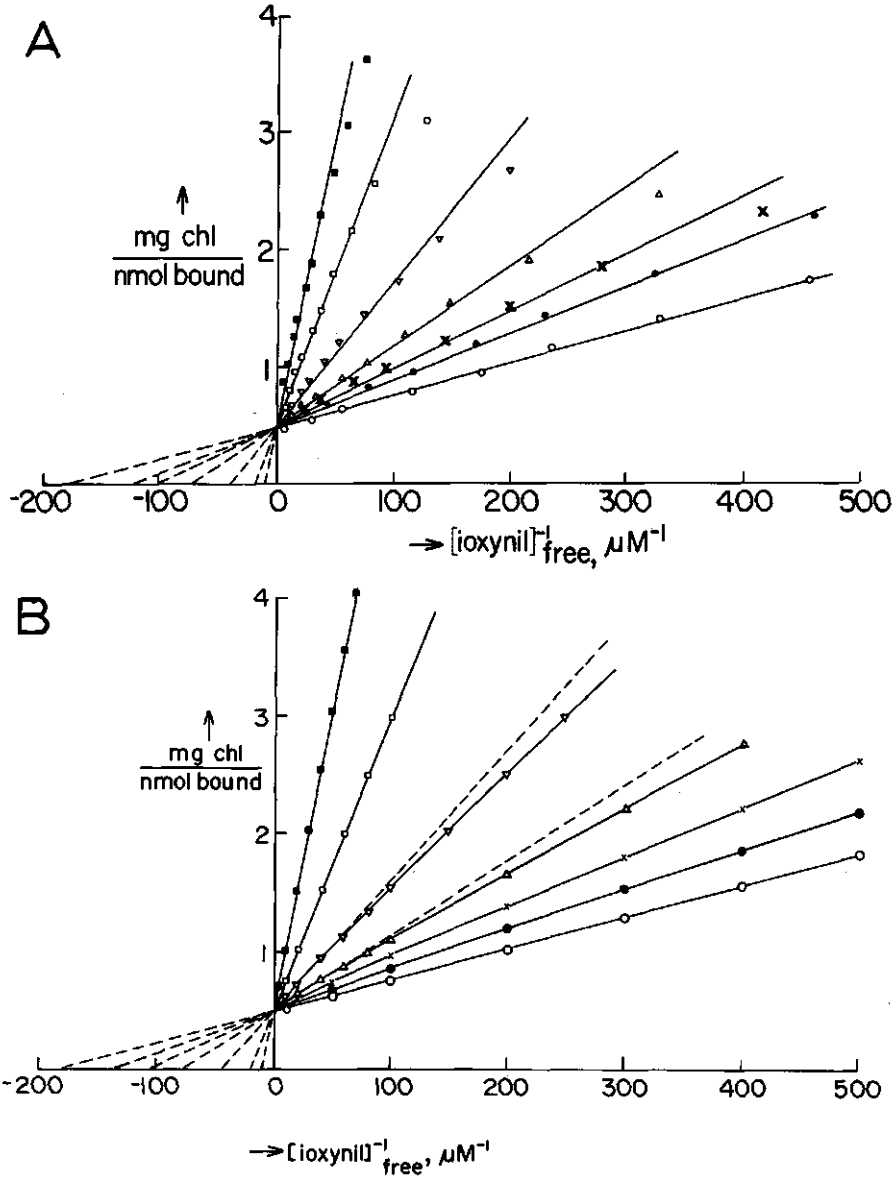
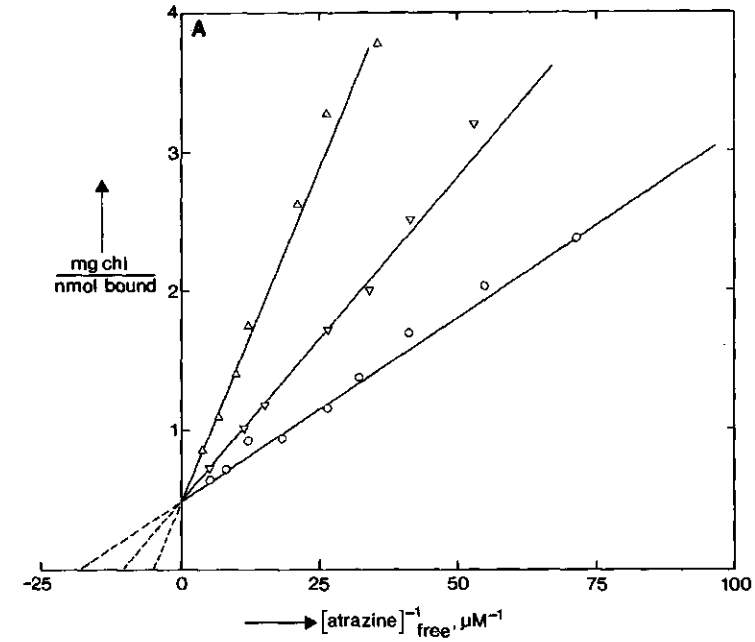
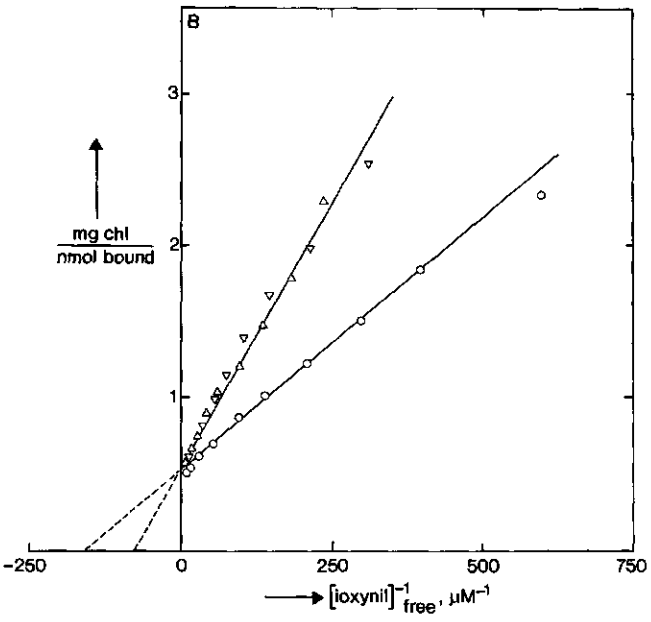


Fig. 4.5. Experimental (A) and theoretical (B) double-reciprocal plot of ^{14}C--ioxynil binding to pea thylakoids in IRM in the absence (0) and the presence of 30 (●), 60 (x), 100(Δ), 200 (▽), 500(□) and 1000(■)nM atrazine. Deviation from a straight line (---) at low ioxynil concentrations is shown for two theoretical curves (Δ and ▽). 50 μg Chl. ml^{-1}.


A
4
3
2
1
mg chl
nmol bound
-25
0
25
50
75
100
[atrazine]$^{-1}_{free}$, μM^{-1}


B
3
2
1
mg chl
nmol bound
-250
0
250
500
750
[ioxynil]$^{-1}_{free}$, μM^{-1}

retical and experimental results is reasonably good, and this supports the validity of equation (4.5) as well as the seemingly competitive ioxynil/atrazine interaction.

Thus, the data reported in the last sections support a (seemingly) competitive interaction between binding of herbicides and quinones or other herbicides.

4.3. Covalent attachment of 6-azido-Q_0C_{10}

Also see Vermaas *et al.* (1983).

As is shown in Section 4.1, 6-azido-Q_0C_{10} is a good PQ/Q_B analog in that it displaces PS II herbicides from their common binding environment. Furthermore, it does not block electron transfer (Vermaas *et al.*, 1983). As pointed out in the Introduction, the azidoquinone can be linked covalently to its binding environment by irradiation with UV-light. One might obtain more information on the mode of interaction between the quinone and the herbicide (*i.e.*, true competition *versus* an allosteric interaction) by measuring herbicide binding after covalent linkage of the quinone. In the case of allosteric interaction, herbicide binding affinity is expected to be decreased, whereas via a competitive mechanism covalent quinone binding would probably lead to a decrease in the number of herbicide binding sites.

The binding of ^{14}C-atrazine and ^{14}C-ioxynil to thylakoids to which 6-azido-Q_0C_{10} was added before and after UV-illumination (covalent and non-covalent binding of the quinone, respectively) was measured (Fig. 4.6). The atrazine affinity was decreased considerably upon covalent linkage of the quinone. There seems to be no change in the number of atrazine binding sites, but the scatter in the experimental data do not allow us to fully exclude the occurrence of such a change. The ioxynil affinity was, surprisingly, unaffected by covalent linkage of the quinone. These data cannot be explained by a simple competitive interaction between the quinone and the herbicides, but are more readily ac-

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Fig. 4.6. Double-reciprocal plot of free and bound ^{14}C-atrazine (A) and ^{14}C-ioxynil (B) in UV-treated thylakoids. Addition of 6-azido-Q_0C_{10}, if any, occurred before (Δ) or after (▽) UV treatment. Binding of the herbicide was measured subsequently in the absence (0) or presence (Δ,▽) of 6-azido-Q_0C_{10} (6-azido-Q_0C_{10}/Chl = 1:40).

counted for by allosteric models, assuming that no artefact (for example, reduction of the azido group by Q_A^-) has played an important role in the photoaffinity labelling process. In order to check for a possible reduction of the azido group by Q_A^- formed upon illumination, the experiments were also carried out using Tris-washed thylakoids, in which linear electron flow is inhibited due to an inactivation on the water splitting site. Photoreduction of 6-azido-$-Q_0C_{10}$ is prevented in these systems. The herbicide binding properties upon photoaffinity labelling were essentially the same as those obtained with thylakoids with an intact electron transport chain (data not shown), indicating that Q_A^- oxidation by the azido group indeed did not play a major role.

Unfortunately, azidoquinones that have the azido group directly attached to the quinone ring (which are, therefore, much more useful in studying quinone/inhibitor interactions than those that have an azido group somewhere in the rather mobile side chains) are rather susceptible to rearrangements and reactions other than nitrene formation (for example, see Germeraad and Moore (1974), Germeraad *et al.* (1974); Weyler *et al.* (1973)). However, I have not been able to find a report in the literature regarding internal rearrangement or a major side reaction that is likely to occur for 6-azido-Q_0C_{10} under the conditions used.

As another control, the Chl *a* fluorescence induction characteristics with and without covalent linkage of the azidoquinone were measured (Fig. 4.7). The control curve (1) shows the normal fluorescence induction behavior. When non-covalently bound 6-azido-Q_0C_{10} is present (no UV-treatment), the Chl *a* fluorescence induction takes a much longer time indicating that the quinone group of 6-azido-Q_0C_{10} can be photoreduced (curve 2). The F_0 value (as measured on an expanded time scale (not shown)) in curves 2 and 3 is decreased compared to the control because of the chemical quenching properties of the oxidized 6-azido-Q_0C_{10}. After covalent linkage of 6-azido-Q_0C_{10} to its binding site (curve 3), Q_A^- oxidation is greatly inhibited: the rise of Chl *a* fluorescence is fast (the same kinetics as that in the presence of diuron) upon turning on the light; furthermore, little of the 6-azido-Q_0C_{10} can be photoreduced (there is only a small additional rise after the large fast rise in curve 3; the maximal fluorescence level does not approach the other two due to the non-photochemical quenching effect of oxidized 6-azido-Q_0C_{10}). This is indeed in agreement with what would be expected from covalent binding of 6-azido-Q_0C_{10} to the inhibitor/quinone binding environment of a large proportion of the reaction

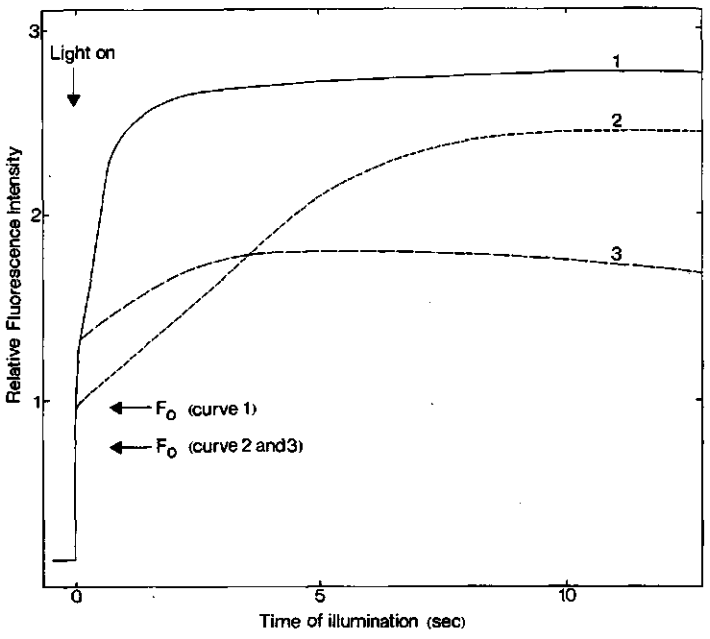

Fig. 4.7. Chl *a* fluorescence induction curves of UV-treated thylakoids in isolation/reaction medium. Curve 1 (——), no 6-azido-Q_0C_{10} addition; curve 2 (– – –), 6-azido-Q_0C_{10} added after UV-treatment; curve 3 (— —), 6-azido-Q_0C_{10} added before UV-treatment. The 6-azido-Q_0C_{10}/Chl ratio is 1 : 20 for curves 2 and 3. The initial fluorescence yield F_0 is indicated. The time resolution was 5 ms.

chains. The blockage of Q_A^- oxidation upon covalent linkage of 6-azido-Q_0C_{10} may have been caused by steric as well as thermodynamic changes caused by the covalent linkage.

These data again indicate that artefacts of the type of azido-reduction are unlikely: in particular, in the case that the azido group from 6-azido-Q_0C_{10} instead of the quinone group was preferentially reduced by Q_A^- during UV-treatment, inhibition of electron transport would not have been expected unless the quinone with the reduced N_3-group was inhibitory; in this case, however, only a fast fluorescence induction would have been expected since the quinone group could not have been reduced. Furthermore, curve 2 from Fig. 4.7 shows that the quinone group from 6-azido-Q_0C_{10} is reduced readily, even after many turnovers of the $Q_A.Q_B$ complex, rendering it improbable that any Q_A^- oxidation by the azido group occurs.

The data presented here indicate that a true competitive interaction between the herbicides and the quinones at a common binding site is not compatible with the experimental results. A specific allosteric interaction between binding of these compounds has to be assumed (binding of different types of molecules to different domains in the inhibitor/quinone binding environment, in which binding of a ligand to one domain greatly decreases the affinity of ligands for the other domains by changes in the conformation of the environment). In this mechanism, herbicide/herbicide and herbicide/quinone interactions appear to be competitive when displacement studies are done, but the different types of herbicides and quinones are not binding to physically overlapping sites.

Many details of herbicide/quinone interactions remain unsolved, however. For example, many quinones (with the exception of 2-bromo-3-methyl-1,4-naphthoquinone (Pfister *et al.*, 1981b)) are able to displace PS II herbicides in parallel with their binding to the herbicide/quinone binding environment (for example, Vermaas and Arntzen, 1983), which suggests that the K_d of the herbicide should increase more upon covalent binding of the quinone than is observed here. A reason for this discrepancy may be that the covalently bound quinone does not induce the same conformational change in the binding environment as does the non-covalently bound quinone. It is obvious that more work has to be done on this subject in order to clarify the very details.

Chapter 5, Kinetics of inhibitor/quinone interactions

In the previous Chapter, inhibitor/inhibitor and inhibitor/quinone interactions under equilibrium conditions have been described. Little is known about the kinetics of inhibitor binding and release in thylakoids, although recently some experimental data on this subject have been published (Bowes and Crofts, 1981; Lavergne, 1982b). Very recently, elegant measurements of redox-state-dependent inhibitor/plastoquinone exchange (using fluorescence techniques) were reported (Taoka *et al.*, 1983). Independently, we have developed an approach in which it is rather easy and straightforward to measure inhibitor exchange with Q_B on the seconds time scale. With the help of computer processing of data a quantitative analysis of inhibitor binding to and release from the inhibitor/Q_B binding environment as a function of the redox state of the $Q_A.Q_B$ complex can also be carried out. The approach involves measurements of single-turnover-flash-induced O_2-evolution as a function of flash number after previous dark adaptation of thylakoid membranes.

At this point some background information on O_2 evolution by previously dark-adapted thylakoids induced by single-turnover flashes (duration 10 - 20 μs) should be provided. The O_2 yield per flash has been found to oscillate with a period of four as a function of flash number, with a maximum after the third flash (Joliot *et al.*, 1969). The O_2 evolution oscillations have been described quite well by a model using five "S-states" ($S_0 - S_4$), as proposed by Kok *et al.* (1970), in which O_2 evolution occurs upon the spontaneous conversion of S_4 into S_0. S_n is converted into S_{n+1} ($0 \leq n \leq 3$) by electron transfer to the oxidized PS II electron donor (Z^+) formed during a flash. Thus, S_0 is the most reduced and S_4 the most oxidized S-state. Although it has often been assumed that after dark adaptation 25 - 30 % of the water splitting complexes is in S_0 state, recently it has been shown that, after a thorough dark adaptation, more than 90 % is in state S_1 (Vermaas *et al.*, 1984c). This was, in fact, already suggested by Velthuys and Visser (1975). The S-states are mere phenomenological descriptions of the state of the water splitting system. At this moment, neither the nature nor the number of components which act as electron carriers in the water splitting system is well-defined, although the specific involvement of Mn-ions is established (see, for example, Wydrzynski (1982), Amesz

(1983) and Govindjee (1984). A multi-line ESR signal, attributable to a specific redox state of a Mn-complex, has been assigned specifically to the S_2 state (Dismukes and Siderer, 1980; Brudvig *et al.*, 1983).

The oscillation of the flash-induced O_2-evolution is damped. This is interpreted to be due to "misses" (no turnover of the S-state system upon a flash; probability α) and "double hits" (two electrons are transferred from the water splitting system to components closer to the PS II reaction center upon one flash; this occurs with probability β). Jursinic (1979, 1981) observed that the probability of double hits cannot be reduced to zero by decreasing the duration of the flash; on the other hand, α does not reach a value lower than about 0.1 when increasing the light intensity. As will be discussed in Section 6.1, the equilibrium Q_A^- concentration contributes to the miss probability. An "intrinsic" double hit probability (*i.e.*, not induced by a flash that is long enough to allow PS II-turnover twice) (Jursinic, 1981) might be due to an interaction of the water splitting complex with more than one PS II RC complex within the lifetime of the oxidized electron donor as previously discussed by Renger (1978). Another possibility to explain an intrinsic double-hit probability larger than zero, calculated mainly from the O_2 evolution at the second flash (y_2), is that a small fraction of S_2 does not readily decay to S_1 upon dark adaptation. Indeed, in triazine-resistant thylakoids a rather large "stable" fraction of S_2 is observed (Section 6.1).

Usually, α and β are assumed to be independent of the flash number. This is not completely correct in the case of α, since the equilibrium Q_A^- concentration after a certain number of flashes after dark adaptation is expected to oscillate with a period of 2 (see Section 6.1). Delrieu (1983) assumes the occurrence of misses to be limited mainly to the $S_2 \rightarrow S_3$ conversion. However, it is difficult to discriminate between the occurrence of misses at only one transition and that at all transitions with a quarter of the probability.

5.1. Qualitative approach to estimate inhibitor/Q_B exchange

Also see Vermaas *et al.* (1984d,e).

The value of β (0.03 under the conditions used in this study) excludes significant interaction of the electron transport chains with each other between the water splitting event and the PS II charge separation. Furthermore, since the components between P680 and Q_B are embedded in or bound to a monomeric

complex, no direct interaction between reaction chains is expected between the water splitting system and Q_B. Therefore, there are two possibilities for the flash-induced O_2-evolution patterns of previously dark-adapted thylakoids in the presence of moderate concentrations of Q_A^- oxidation inhibitors (inhibiting 20-80% of the O_2 evolution) depending on the residence time of the inhibitor at the binding site (*i.e.*, the average time between binding and release of the inhibitor molecule:

1. Within the time of the flash train no displacement of the inhibitor by Q_B or *vice versa* occurs. This implies that the reaction chains that have not bound an inhibitor molecule will behave like the control, whereas the other chains will not make more than one net turnover (producing no O_2). Such a "static" situation results in an oscillation pattern with normal damping but with a decreased amplitude.
2. Within the time of the flash train the inhibitor molecule has a considerable probability to be displaced by Q_B and *vice versa*. After an inhibitor molecule that was already bound to the site before the first flash is replaced by Q_B during the flash train, the reaction chain will start out in state $(S_1.Q_A.Q_B)$ or $(S_2.(Q_A.Q_B)^-)$, irrespective of the number of flashes fired before the inhibitor molecule was released. This reaction chain may be "out-of-phase" with the oscillation of the permanently uninhibited chains. On the other hand, reaction chains in which Q_B is replaced by an inhibitor molecule will not make more than one net turnover and will remain in the $(S_n.Q_A^-.Inh)$ state (Inh is inhibitor) until the inhibitor molecule is released again and replaced by Q_B. Such centers may also be out-of-phase with the uninhibited centers when they bind Q_B. It is obvious that such a dynamic inhibitor/Q_B interaction not only leads to an inhibited O_2 evolution, but also to an increased damping of the oscillation.

For the case that the residence time of the inhibitor at the binding environment is in the same order of magnitude as the duration of the flash train, rather large differences in flash pattern are expected when comparing a flash pattern obtained at high flash frequency with one obtained at lower flash frequency. In the former case the inhibitor molecule will exchange less between flashes than in the latter case, resulting in an increase in damping at decreasing flash frequency. As the method is based on oxygen yield measurements, the time domain of herbicide exchange kinetics that can be analyzed is restricted by the internal lifetime of the states S_2 and S_3.

Based on these considerations, O_2 evolution measurements as a function of flash number in the presence of Q_A^- oxidation inhibitors have been carried out. Using inhibitor concentrations that blocked O_2 production by approximately 50%, large differences in O_2 evolution pattern were observed for different inhibitor groups. The "classical" PS II herbicides (diuron, atrazine) do not damp the O_2-evolution pattern (Table 5.I and Fig. 5.1), whereas many others (*o*-phenanthroline, *i*-dinoseb, quinone-type inhibitors) increase the damping (Table 5.I), especially at lower flash frequency (Fig. 5.1). This indicates that, in the case of *i*-dinoseb, there is a significant inhibitor/Q_B exchange when the time

TABLE 5.I, Inhibitor/Q_B exchange

Qualitative rates of inhibitor/Q_B exchange at the common binding environment near Q_A in pea thylakoids as measured by the Joliot-type O_2 electrode. In this Table, the exchange rates are defined as "fast" when at flash frequencies of 1 Hz or less considerably more damping of the O_2 yield oscillation occurs than in the control; in this case, the exchange rates are in the order of 0.1 - 1 s^{-1} or more. After inhibitor addition, the average O_2 evolution was 20 - 50 % of the control.

INHIBITOR	CLASS	APPROX. pI_{50}	DAMPING OF OSCILLATION	INHIBITOR/Q_B EXCHANGE RATE
diuron	urea	7.5	-	slow
atrazine	*s*-triazine	7.0	-	slow
phenisopham	biscarbamate	6.8	-	slow
metamitron	triazinone	6.0	+-	intermediate
ethoxyethyl-2-cyano-3-*n*-decylaminoacrylate	cyanoacrylate	7.3	+	fast
4,6-dinitro-*o*-cresol	nitrophenol	4.8	+	fast
i-dinoseb	nitrophenol	5.9	+	fast
bromoxynil	nitrile	6.0	-	slow
o-phenanthroline	-	5.8	+	fast
tetrachloro-*p*-benzoquinone	quinone	4.8	+	fast
2-hydroxy-3-(11-oxodo-decyl)-1,4-naphthoquinone	quinone	6-7	+	fast

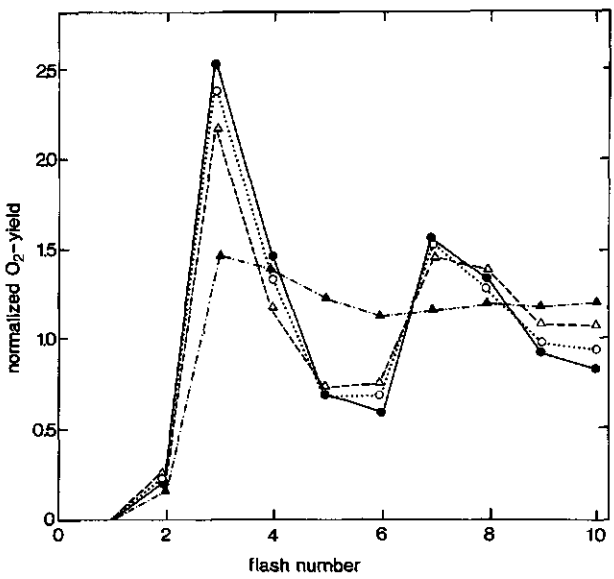

Fig. 5.1. Single-turnover flash-induced oxygen evolution pattern of previously dark-adapted pea thylakoids as a function of flash number. The flash frequency was 0.5 Hz. o: control; ●: + 0.1 μM atrazine; ▲: + 1 μM *i*-dinoseb; Δ: + 1 μM *i*-dinoseb but at 4 Hz flash frequency. The Chl concentration was 0.8 mg.ml^{-1}. The oxygen yields are normalized to an average of 1.0 over 10 flashes. The concentrations of inhibitors used inhibited the O_2-evolution by 50-70%.

between the flashes is 2 s, but that the exchange is much less at 4 Hz flash frequency. Therefore, the rate of exchange in the presence of 1 μM *i*-dinoseb seems to be in the order of 0.5 s^{-1}.

A qualitative inspection of the data shows that there is little relationship between the pI_{50} of the inhibitor (the negative logarithm of the inhibitor concentration needed to inhibit electron transport by 50%) and the exchange rate (Table 5.I). The inhibitor binding- and release kinetics appear to be governed primarily by the structure of the inhibitor. It is striking that bromoxynil -and also ioxynil (data not shown)- exchange slowly, and, thus, do not behave like the phenolic inhibitors (DNOC and *i*-dinoseb) that exchange faster. This supports the idea that bromoxynil and ioxynil do not belong to the group of the phenolic herbicides, but rather to a separate group: that of the nitriles. The quinone-type inhibitors exchange quite rapidly with Q_B, sup-

porting the hypothesis of a rapid binding and release of the analogous plastoquinone Q_B.

The results obtained with ethoxyethyl-2-cyano-3-*n*-decylaminoacrylate (a kind gift from Drs. J.N. Phillips and J.L. Huppatz, CSIRO, Canberra, Australia) are rather surprising. Table 5.I shows that all inhibitors that contain a $=C-\bar{N}-$group, a common feature of most PS II herbicides (diuron, atrazine, etc.) - which, therefore, is called the "essential element"; see, for example, Trebst and Draber (1979) - exchange slowly, except ethoxyethyl-2-cyano-3-*n*--decylaminoacrylate. This suggests that the cyanoacrylates, a group of Q_A^- oxidation inhibitors developed recently (Huppatz *et al.*, 1981; Phillips and Huppatz, 1984a,b), do not necessarily belong to the group of diuron-type herbicides, although they contain a $=C-\bar{N}-$ group. At this time, the cyanoacrylates appear to be a very interesting group of inhibitors: recently, a 200-fold difference in I_{50} for two stereoisomers of a cyanoacrylate was reported (Phillips and Huppatz, 1984c).

A relatively slow binding and release of certain inhibitors may be taken to suggest a high activation energy for binding and release of that inhibitor. Alternative explanations, however, are possible.

The data presented suggest that the binding mechanism of, for example, diuron-type inhibitors, nitrophenols, cyanoacrylates, *o*-phenanthroline, quinones and nitriles is different. This supports the suggestion that the binding interactions between inhibitors from different groups may be allosteric (see Chapters 3 and 4).

5.2. Calculation of exchange parameters

Also see Vermaas *et al.* (1984e).

In principle, flash-induced O_2-evolution patterns can also be used to make calculations of binding and release rates of inhibitors, even as a function of the redox state of the $Q_A.Q_B$ or Q_A.Inh complex. The best method to do these calculations is the fitting of theoretical predictions to the experimental data. Therefore, the theoretical O_2 evolution as a function of flash number was calculated for many different binding and release rates. The calculations and the theoretical O_2-evolution patterns are based on the following assumptions:

1. The classical Kok-model (Kok *et al.*, 1970) can describe the O_2 evolution as a function of flash number in the presence of inhibitors (α and β are as in thylakoid suspensions without inhibitor) with the extension that in state ($S_n.Q_A$.Inh) $\beta = 0$, whereas in state ($S_n.Q_A^-$.Inh) $\alpha = 1$ and $\beta = 0$.

2. After thorough dark adaptation of thylakoids, all reaction chains are in state ($S_1.Q_A.Q_B$) or ($S_1.Q_A.Inh$). Under the conditions used, S_0 is shown to be virtually absent (Vermaas *et al.*, 1984c). The Q_B^-/Q_B ratio in the presence of inhibitors was found to be less than 1/15 under our conditions (as measured by diuron-induced chlorophyll *a* fluorescence in the presence of NH_2OH; data not shown).
3. The following reactions can occur in the dark time between the flashes:

$$S_n.Q_A.Q_B + Inh \underset{E_1}{\overset{E_3}{\rightleftharpoons}} S_n.Q_A.Inh + PQ \qquad (5.1)$$

$$S_n.(Q_A.Q_B)^- + Inh \underset{E_2}{\overset{E_4}{\rightleftharpoons}} S_n.Q_A.Inh + PQ \qquad (5.2)$$

$$S_n.Q_A^-.Inh \xrightarrow{k} S_{n-1}.Q_A.Inh \qquad (5.3)$$

In reactions (5.1) and (5.2), $0 \leq n \leq 3$; for reaction (5.3), however, $n = 2$ or 3 (it is assumed here that a back reaction between Q_A^- and S_3 can also occur; at this time it is not clear whether this assumption is valid; however, the maximal $S_3.Q_A^-$ fraction is low because it can be formed only in the electron transport chains that are connected to a one-electron donor, which in turn is able to reduce S_2 or S_3 only once with a half time of about 1.4 s (Vermaas *et al.*, 1984c); after dark adaptation of thylakoids under our conditions (more than 2 hrs on ice) only about 20% of the PS II electron transport chains have this donor (Vermaas *et al.*, 1984c)). For these calculations, the back reaction of $(Q_A.Q_B)^-$ with S_2 or S_3 is neglected, because it is relatively slow ($t_{\frac{1}{2}} \geq 25$ s) (Vermaas *et al.*, 1984c). This, however, implies that this method cannot analyze the binding dynamics of inhibitors of Q_A^- oxidation that simultaneously react with certain S-states (*e.g.*, via the so-called ADRY effect (Renger, 1972)) as was found for certain diphenylamine derivatives (Oettmeier and Renger, 1980). The back reaction rate (k) of $Q_A^-.Inh$ oxidation by S_2 is 0.25 s^{-1} (Vermaas *et al.*, 1984c). It should be noted that in the calculations below the exchange parameters E_1-E_4 are assumed to remain constant during the series of flashes.

The aim of the derivation of the equations below is to calculate theoretical O_2-evolution oscillation patterns as a function of the exchange parameters E_n ($1 \leq n \leq 4$) as defined in reactions (5.1) and (5.2). The theoretical patterns were compared to the experimental ones, and the best-fitting set of

exchange parameters was selected. In order to calculate the O_2 evolution as a function of flash number and exchange parameters at a certain flash frequency, the relative concentration of the S-states as a function of the state at the PS II acceptor side before a flash is calculated. In this way, the concentrations of 32 states have to be considered before each flash: $(S_n.Q_A.Inh)$, $(S_n.Q_A.Q_B)$, $(S_n.Q_A^-.Inh)$ and $(S_n.(Q_A.Q_B)^-)$ $(0 \leq n \leq 3)$ in centers that have and do not have the fast one-electron donor to S_2 and S_3 in the reduced form. Subsequently, the effect of a single-turnover flash is calculated using assumption (1), and from this the O_2 evolution in that flash can also be determined. Then, we correct for the fast one-electron donation to S_2 and S_3 in some centers occurring not more than once during the flash train (assumption (3)) by calculating the amount of electron donation by this donor that will occur in the time until the next flash is fired, based on the concentration of centers that are in state S_2 or S_3 and contain a reduced one-electron donor immediately after the flash (see below). Subsequently, from the corrected relative concentrations of the S-states as a function of the state at the acceptor side the relative concentrations of these states just before the next flash are calculated, using equations (5.1)-(5.3). Then, the O_2 evolution at the next flash can be determined, etc.. This procedure is followed for the first 10 flashes after dark adaptation.

In order to simplify the calculations, the states that, directly or indirectly, can neither back-react nor be formed by a reaction of Q_A^- with S_2 or S_3 (*i.e.*, $(S_0.Q_A.Q_B)$, $(S_0.(Q_A.Q_B)^-)$, $(S_0.Q_A.Inh)$, $(S_0.Q_A^-.Inh)$, $(S_1.(Q_A.Q_B)^-)$, $(S_1.Q_A^-.Inh)$, $(S_3.Q_A.Q_B)$ and $(S_3.Q_A.Inh)$) are separated from the others.

A. A back reaction of Q_A^- with the S-states can occur.

The concentration changes of the different states can be calculated from equations (5.4)-(5.7) using the assumptions stated above.

$$\frac{d[S_n.Q_A.Q_B]}{dt} = E_3[S_n.Q_A.Inh]_t - E_1[S_n.Q_A.Q_B]_t \qquad (5.4)$$

$$\frac{d[S_n.Q_A.Inh]}{dt} = k[S_{n+1}.Q_A^-.Inh]_t + E_1[S_n.Q_A.Q_B]_t - E_3[S_n.Q_A.Inh]_t \qquad (5.5)$$

$$\frac{d[S_n.(Q_A.Q_B)^-]}{dt} = E_4[S_n.Q_A^-.Inh]_t - E_2[S_n.(Q_A.Q_B)^-]_t \qquad (5.6)$$

$$\frac{d[S_n.Q_A^-.Inh]}{dt} = E_2[S_n.(Q_A.Q_B)^-]_t - (k + E_4)[S_n.Q_A^-.Inh]_t \qquad (5.7)$$

Defining X_t^n as the quantity of $(S_n.Q_A^-.Inh)$ that decays to $(S_{n-1}.Q_A.Inh)$ in the time interval $\tau = 0$ (when the last flash was fired) until $\tau = t$,

$$X_t^n = k\int_0^t [S_n.Q_A^-.Inh]_\tau d\tau, \text{ or}$$

$$\frac{dX^n}{dt} = k[S_n.Q_A^-.Inh]_t \qquad (5.8).$$

Since

$$[S_n.(Q_A.Q_B)^-]_t = [S_n.(Q_A.Q_B)^-]_0 + [S_n.Q_A^-.Inh]_0 - [S_n.Q_A^-.Inh]_t - X_t^n,$$

equation (5.7) can be rewritten and differentiated, yielding

$$\frac{d^2[S_n.Q_A^-.Inh]}{dt^2} + (E_2 + E_4 + k)\frac{d[S_n.Q_A^-.Inh]}{dt} + E_2k[S_n.Q_A^-.Inh]_t = 0.$$

The solution for this equation is

$$[S_n.Q_A^-.Inh]_t = A_1e^{-\lambda_1 t} + A_2e^{-\lambda_2 t} \qquad (5.9),$$

where:

$$\lambda_1 = \frac{E_2 + E_4 + k + \sqrt{[(E_2 + E_4 + k)^2 - 4E_2k]}}{2}, \text{ and}$$

$$\lambda_2 = \frac{E_2 + E_4 + k - \sqrt{[(E_2 + E_4 + k)^2 - 4E_2k]}}{2},$$

and, using equation (5.7) for $t = 0$,

$$A_1 = \frac{(\lambda_2 - E_4 - k)[S_n.Q_A^-.Inh]_0 + E_2[S_n.(Q_A.Q_B)^-]_0}{\lambda_2 - \lambda_1}, \text{ and}$$

$$A_2 = [S_n.Q_A^-.Inh]_0 - A_1.$$

The differential equations for the concentrations of the other states (equations (5.4) - (5.6)) can be solved analogously. However, somewhat more mathematical conversions are necessary for these states than for $[S_n.Q_A^-.Inh]$. The concentration of $(S_n.Q_A.Inh)$ can be calculated as follows:

Combination of equations (5.5) and (5.8) yields:

$$\frac{d[S_n.Q_A.Inh]}{dt} = k[S_{n+1}.\bar{Q}_A.Inh]_t + E_1([S_n.Q_A.Inh]_0 + [S_n.Q_A.Q_B]_0 - [S_n.Q_A.Inh]_t + X_t^{n+1}) - E_3[S_n.Q_A.Inh]_t.$$

Differentiation of this equation and substitution of $[S_{n+1}.\bar{Q}_A.Inh]_t$ (equation (5.9)) and of $d[S_{n+1}.\bar{Q}_A.Inh]/dt$ (derivative of equation (5.9)) yields:

$$\frac{d^2[S_n.Q_A.Inh]}{dt^2} + (E_1 + E_3)\frac{d[S_n.Q_A.Inh]}{dt} = e^{-\lambda_1 t}(E_1 kA_1 - k\lambda_1 A_1) + e^{-\lambda_2 t}(E_1 kA_2 - k\lambda_2 A_2).$$

This equation is simplified by defining

$$\alpha_1 = E_1 kA_1 - k\lambda_1 A_1 \quad \text{and}$$

$$\alpha_2 = E_1 kA_2 - k\lambda_2 A_2 \quad \text{to}$$

$$\frac{d^2[S_n.Q_A.Inh]}{dt^2} + (E_1 + E_3)\frac{d[S_n.Q_A.Inh]}{dt} = \alpha_1 e^{-\lambda_1 t} + \alpha_2 e^{-\lambda_2 t} \qquad (5.10).$$

In order to solve equation (5.10), first the homogeneous equation

$$\frac{d^2[S_n.Q_A.Inh]}{dt^2} + (E_1 + E_3)\frac{d[S_n.Q_A.Inh]}{dt} = 0$$

is solved.

Assuming $[S_n.Q_A.Inh]_t = C^o e^{-\mu t}$, the homogeneous equation can be rewritten to:

$$C^o \mu^2 e^{-\mu t} - \mu(E_3 + E_1)C^o e^{-\mu t} = 0,$$

which yields $\mu = 0$ or $\mu = E_1 + E_3$, since C^o is not 0 at all times t.

Thus, a solution to the homogeneous equation

$$\frac{d^2[S_n.Q_A.Inh]}{dt^2} + (E_1 + E_3)\frac{d[S_n.Q_A.Inh]}{dt} = 0 \quad \text{is}$$

$$[S_n.Q_A.Inh]_{homogeneous} = C_1 e^{-(E_1 + E_3)t} + C_2 \qquad (5.11).$$

Now, the inhomogeneous part of equation (5.10) is solved.

$$[S_n.Q_A.Inh]_{inhomogeneous} = \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} \qquad (5.12)$$

Substitution into equation (5.10) yields:

$$\beta_1\lambda_1^2 e^{-\lambda_1 t} + \beta_2\lambda_2^2 e^{-\lambda_2 t} - (E_1 + E_3)(\beta_1\lambda_1 e^{-\lambda_1 t} + \beta_2\lambda_2 e^{-\lambda_2 t}) = \alpha_1 e^{-\lambda_1 t} + \alpha_2 e^{-\lambda_2 t} ,$$

which is equal to

$$(\beta_1\lambda_1^2 - E_1\beta_1\lambda_1 - E_3\beta_1\lambda_1 - \alpha_1)e^{-\lambda_1 t} + (\beta_2\lambda_2^2 - E_1\beta_2\lambda_2 - E_3\beta_2\lambda_2 - \alpha_2)e^{-\lambda_2 t} = 0.$$

Since $e^{-\lambda_1 t}$ and $e^{-\lambda_2 t}$ cannot be 0 at all times t,

$$\beta_1\lambda_1^2 - E_1\beta_1\lambda_1 - E_3\beta_1\lambda_1 - \alpha_1 = 0, \text{ and}$$

$$\beta_2\lambda_2^2 - E_1\beta_2\lambda_2 - E_3\beta_2\lambda_2 - \alpha_2 = 0.$$

Thus,

$$\beta_1 = \frac{\alpha_1}{\lambda_1(\lambda_1 - E_1 - E_3)} \quad \text{and} \quad \beta_2 = \frac{\alpha_2}{\lambda_2(\lambda_2 - E_1 - E_3)} \tag{5.13}.$$

Combination of the homogeneous and inhomogeneous parts (equations (5.11)-(5.13)) yields:

$$[S_n.Q_A.Inh]_t = C_1 e^{-(E_1 + E_3)t} + C_2 + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} \tag{5.14}.$$

Then, the constants C_1 and C_2 are determined.

At $t = 0$, $[S_n.Q_A.Inh]_t = [S_n.Q_A.Inh]_0$ and

$$\frac{d[S_n.Q_A.Inh]}{dt} = k[S_{n+1}.\bar{Q}_A.Inh]_0 + E_1[S_n.Q_A.Q_B]_0 - E_3[S_n.Q_A.Inh]_0 .$$

Differentiation of (5.14) yields (for $t = 0$):

$$\frac{d[S_n.Q_A.Inh]}{dt} = -(E_1 + E_3)C_1 - \beta_1\lambda_1 - \beta_2\lambda_2.$$

Thus,

$$-(E_1 + E_3)C_1 - \beta_1\lambda_1 - \beta_2\lambda_2 = k[S_{n+1}.\bar{Q}_A.Inh]_0 + E_1[S_n.Q_A.Q_B]_0 - E_3[S_n.Q_A.Inh]_0 ,$$

which is equal to:

$$C_1 = \frac{E_3[S_n.Q_A.Inh]_0 - k[S_{n+1}.\bar{Q}_A.Inh]_0 - E_1[S_n.Q_A.Q_B]_0 - \beta_1\lambda_1 - \beta_2\lambda_2}{(E_1 + E_3)} .$$

At $t = 0$, $C_2 = [S_n.Q_A.Inh]_0 - \beta_1 - \beta_2 - C_1$ (see equation (5.14)).

Now, the concentration of $(S_n.Q_A.Inh)$ as a function of time can be written as:

$$[S_n.Q_A.Inh]_t = C_1 e^{-(E_1+E_3)t} + C_2 + \beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t} \quad (5.15),$$

where

$$\beta_1 = \frac{E_1 kA_1 - k\lambda_1 A_1}{\lambda_1(\lambda_1 - E_1 - E_3)}$$

$$\beta_2 = \frac{E_2 kA_2 - k\lambda_2 A_2}{\lambda_2(\lambda_2 - E_1 - E_3)}$$

$$C_1 = \frac{E_3[S_n.Q_A.Inh]_0 - k[S_{n+1}.\bar{Q}_A.Inh]_0 - E_1[S_n.Q_A.Q_B]_0 - \beta_1\lambda_1 - \beta_2\lambda_2}{(E_1+E_3)}$$

$$C_2 = [S_n.Q_A.Inh]_0 - C_1 - \beta_1 - \beta_2 .$$

The time-dependent concentrations of $(S_n.(Q_A.Q_B)^-)$ and $(S_n.Q_A.Q_B)$ can be calculated analogously. For the sake of conciseness the elaborate calculations are not worked out in detail here. The results of the calculations are as follows:

from equation (5.6):

$$[S_n.(Q_A.Q_B)^-]_t = B_1 e^{-E_2 t} + \frac{E_4 A_1}{E_2 - \lambda_2} e^{-\lambda_1 t} + \frac{E_4 A_2}{E_2 - \lambda_2} e^{-\lambda_2 t} \quad (5.16),$$

where

$$B_1 = [S_n.(Q_A.Q_B)^-]_0 - \frac{E_4}{E_2 - \lambda_2}(A_1 + A_2) ;$$

from equation (5.4):

$$[S_n.Q_A.Q_B]_t = D_1 e^{-E_1 t} - C_1 e^{-(E_1+E_3)t} + \frac{E_3}{E_1} C_2 + \frac{E_3\beta_1}{E_1 - \lambda_1} e^{-\lambda_1 t} + \frac{E_3\beta_2}{E_1 - \lambda_2} e^{-\lambda_2 t} \quad (5.17),$$

where

$$D_1 = [S_n.Q_A.Q_B]_0 + C_1 - \frac{E_3}{E_1} C_2 - \frac{E_3\beta_1}{E_1 - \lambda_1} - \frac{E_3\beta_2}{E_1 - \lambda_2} .$$

B. A back reaction of Q_A^- with the S-states does not occur ($k = 0$)

In this case, the differential equations (5.4)-(5.7) can be solved by standard differential equation calculus:

$$[S_n.Q_A.Q_B]_t = E_3\nu_1 + ([S_n.Q_A.Q_B]_0 - E_3\nu_1)e^{-(E_1+E_3)t} \quad (5.18)$$

$$[S_n.Q_A.Inh]_t = E_1\nu_1 + ([S_n.Q_A.Inh]_0 - E_1\nu_1)e^{-(E_1+E_3)t} \quad (5.19)$$

$$[S_n.(Q_A.Q_B)^-]_t = E_4\nu_2 + ([S_n.(Q_A.Q_B)^-]_0 - E_4\nu_2)e^{-(E_2+E_4)t} \quad (5.20)$$

$$[S_n.Q_A^-.Inh]_t = E_2\nu_2 + ([S_n.Q_A^-.Inh]_0 - E_2\nu_2)e^{-(E_2+E_4)t} \quad (5.21),$$

where

$$\nu_1 = \frac{([S_n.Q_A.Q_B]_0 + [S_n.Q_A.Inh]_0)}{E_1 + E_3} \quad \text{and}$$

$$\nu_2 = \frac{([S_n.(Q_A.Q_B)^-]_0 + [S_n.Q_A^-.Inh]_0)}{E_2 + E_4} .$$

Of course, the same results can be obtained from equations (5.9), (5.15), (5.16) and (5.17), when $k = 0$.

According to assumption (3) the equations given above are simplified because the fraction of reaction chains, in which the unknown electron donor can donate one electron once during the flash train to S_2 or S_3 with $t_{\frac{1}{2}} = 1.4$ s, has not been included. In order not to make the calculations more complicated than strictly necessary, the calculations have been simplified using a reasonable approximation: although the one-electron donation occurs gradually during the dark time between the flashes, it is assumed that S_2 and S_3 reduction by the unknown donor takes place directly after the flash. In this way, the amount of, on this timescale irreversible, oxidation of the unknown donor D by S_2 or S_3 that would occur between the two flashes can be calculated (the rate constant of oxidation of D, k_2, is 0.5 s^{-1}) using the concentration of centers that have a reduced donor D and that are in state S_2 or S_3. This amount of interconversion from state $(D.S_n.(Q_A.Q_B/Inh)^{(-)})$ to $(D^+.S_{n-1}.(Q_A.Q_B/Inh)^{(-)})$ (n = 2 or 3) was added to the amount of the corresponding state $((D^+).S_{n-1}.(Q_A.Q_B/Inh)^{(-)})$ already present directly after the first of the two flashes, and subtracted from the amount of $(D.S_n.(Q_A.Q_B/Inh)^{(-)})$ present at the moment directly after that flash. These corrected concentrations are then used as the concentrations at t = 0 after the flash. It is clear that the concentration of D decreases with the number of flashes fired. This decrease is larger if the time between the flashes is increased. The approximation described above leads to a large simplification of the calculations because now only inhibitor exchange and S-state-dependent Q_A^- oxidation occurring in the time interval between the two flashes has to be considered, and not, in addition, the S-state-dependent oxidation of D. Thus, for example, the differential equation

$$\frac{d[D.S_2.Q_A^-.Inh]}{dt} = E_2[D.S_2.(Q_A.Q_B)^-]_t - (k + k_2 + E_4)[D.S_2.Q_A^-.Inh]_t$$

is simplified to

$$\frac{d[D.S_2.Q_A^-.Inh]}{dt} = E_2[D.S_2.(Q_A.Q_B)^-]_t - (k+E_4)[D.S_2.Q_A^-.Inh]_t \ ,$$

in which $[D.S_2.(Q_A.Q_B)^-]_t$ and $[D.S_2.Q_A^-.Inh]_t$ are already corrected for the oxidation of D that would occur in the dark period between the two flashes.

Using the equations derived above, and using the Kok model (Kok *et al.*, 1970), including the dependence of the miss- and double hit parameters on the redox state of Q_A and on whether Q_B or Inh is bound to the inhibitor/quinone binding environment (see assumption (1)), not only the distribution of S-states linked to the state of the $Q_A.Q_B$/Inh complex before and after each flash, but also the O_2 production in each flash can be calculated as a function of exchange parameters and flash frequency. The O_2 production in the n^{th} flash (Y_n) can be calculated from the S-state distribution by:

$$Y_n = \beta([S_2.Q_A.Q_B]_n + [S_2.(Q_A.Q_B)^-]_n) + (1-\alpha)([S_3.Q_A.Q_B]_n + [S_3.(Q_A.Q_B)^-]_n + [S_3.Q_A.Inh]_n) \qquad (5.22),$$

where $[S_2.Q_A.Q_B]_n$ represents the $(S_2.Q_A.Q_B)$ concentration just before the n^{th} flash. The other concentrations are defined analogously. The values calculated from equation (5.22) were fitted to experimental values by varying the values of the exchange parameters.

As can be seen from reactions (5.1) and (5.2), E_1 and E_2 are dependent on the concentration of free inhibitor whereas E_3 and E_4 are not. In principle, E_3 and E_4 are dependent on the concentration of free plastoquinone in the membrane, but since the values of E_3 and E_4 are generally found to be much smaller than 10^3 s^{-1} (see below), the value that would be expected for PQ binding to the Q_B site (the time for $Q_A \rightarrow PQ$ electron transport is in the order of 1 - 10 ms),

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TABLE 5.II, *o*-Phenanthroline / Q_B exchange →→

Experimental (E) and calculated (C) O_2 evolution by pea thylakoids in the 2^{nd} - 10^{th} flash after dark adaptation as a function of flash frequency in the presence of 1, 2 or 4 μM *o*-phenanthroline. In the first flash, the O_2 evolution was zero. All flash patterns are normalized to an average O_2 evolution of 1.00 in the first 10 flashes. The calculated fraction (F) of O_2 evolution in the first ten flashes in the presence of *o*-phenanthroline as compared to the control is also indicated in the Table. The control values for α and β were 0.13 and 0.04, respectively. Exchange parameter values as in text. →→

flash frequency (Hz)		0.5		1		2		4	
[o-phenanthroline], μM	flash number								
		E	C	E	C	E	C	E	C
1	2	0.11	0.20	0.18	0.24	0.19	0.27	0.21	0.29
	3	2.00	1.94	2.24	2.17	2.48	2.43	2.79	2.64
	4	1.56	1.48	1.43	1.40	1.35	1.32	1.15	1.25
	5	1.03	1.03	0.85	0.84	0.72	0.67	0.57	0.54
	6	0.85	0.91	0.77	0.80	0.69	0.70	0.65	0.64
	7	1.13	1.13	1.29	1.23	1.47	1.36	1.66	1.49
	8	1.18	1.17	1.26	1.24	1.29	1.31	1.30	1.36
	9	1.11	1.10	1.03	1.07	0.95	1.02	0.86	0.94
	10	1.02	1.05	0.95	1.00	0.86	0.93	0.81	0.86
	F		0.54		0.48		0.44		0.41
2	2	0.09	0.17	0.13	0.21	0.19	0.24	0.25	0.27
	3	1.76	1.75	1.94	1.98	2.21	2.26	2.48	2.50
	4	1.51	1.49	1.44	1.42	1.36	1.35	1.21	1.27
	5	1.19	1.20	1.07	1.02	0.89	0.82	0.71	0.64
	6	1.01	1.06	0.96	0.95	0.86	0.81	0.75	0.71
	7	1.09	1.09	1.17	1.14	1.28	1.24	1.45	1.39
	8	1.12	1.10	1.17	1.15	1.23	1.22	1.27	1.30
	9	1.11	1.08	1.10	1.09	1.02	1.06	0.98	0.99
	10	1.12	1.06	1.04	1.05	0.96	1.00	0.90	0.93
	F		0.43		0.36		0.31		0.27
4	2	0.08	0.14	0.10	0.17	0.16	0.21	0.20	0.24
	3	1.55	1.55	1.70	1.76	2.00	2.03	2.37	2.30
	4	1.47	1.48	1.47	1.45	1.40	1.40	1.28	1.32
	5	1.29	1.30	1.22	1.19	1.03	1.01	0.87	0.80
	6	1.12	1.18	1.08	1.10	0.96	0.96	0.78	0.82
	7	1.10	1.13	1.09	1.11	1.17	1.14	1.32	1.26
	8	1.12	1.09	1.12	1.10	1.15	1.14	1.20	1.21
	9	1.15	1.07	1.11	1.08	1.10	1.07	1.00	1.04
	10	1.11	1.06	1.11	1.05	1.03	1.04	0.97	1.00
	F		0.34		0.27		0.22		0.18

E_3 and E_4 are probably limited by the release of the inhibitor from the site. Since the Q_B/inhibitor exchange is time-dependent (see the previous equations), and since E_1 and E_2 are dependent on the concentration of free inhibitor, one can obtain more reliable estimations of $E_1 - E_4$ by measuring the O_2 production patterns at varying inhibitor concentration and flash frequency. In this way, many flash patterns are obtained that have to be fitted by four parameters.

The O_2 production as a function of flash number in the first ten flashes after dark adaptation was measured at four different flash frequencies (4, 2, 1 and 0.5 Hz) and in the presence of three different concentrations (1, 2 and 4 μM of free *o*-phenanthroline and *i*-dinoseb. In this way, for one experiment 120 data points were obtained, and a unique and very reproducible solution for the values of the best-fitting exchange parameters could be obtained. The calculated values were fitted to the experimental values by means of a least--squares fit. It was checked that the amount of inhibition for the best-fitting set of exchange parameters was in approximate agreement with the actual average inhibition of O_2 evolution in the first ten flashes. Since the absolute amplitude of total O_2 evolution is not very quantitative using a Joliot-type O_2-electrode, a difference of about 20 % between calculated and experimental values of the average O_2 evolution ("fraction F", see Table 5.II and 5.III) was often observed.

For *o*-phenanthroline, the best-fitting set of exchange parameters is found to be:

$$E_1 = 0.32 \ \mu M^{-1}.s^{-1}$$
$$E_2 = 0.034 \ \mu M^{-1}.s^{-1}$$
$$E_3 = 0.19 \ s^{-1}$$
$$E_4 = 0.11 \ s^{-1} .$$

TABLE 5.III, *i*-Dinoseb / Q_B exchange →→→

Experimental (E) and calculated (C) O_2 evolution by pea thylakoids in the 2^{nd} - 10^{th} flash after dark adaptation as a function of flash frequency in the presence of 1, 2 or 4 μM *i*-dinoseb. In the first flash, the O_2 evolution was zero. All flash patterns are normalized to an average O_2 evolution of 1.00 in the first ten flashes. The control values of α and β were 0.13 and 0.04, respectively. The calculated fraction F of O_2 evolution in the first ten flashes in the presence of *i*-dinoseb as compared to the control is indicated. The exchange parameter values are listed in the text. →→→

flash frequency (Hz)		0.5		1		2		4	
[i-dinoseb], μM	flash number								
		E	C	E	C	E	C	E	C
1	2	0.10	0.18	0.14	0.19	0.23	0.22	0.26	0.24
	3	1.47	1.48	1.57	1.67	1.83	1.91	2.17	2.18
	4	1.39	1.37	1.36	1.32	1.27	1.25	1.17	1.19
	5	1.23	1.21	1.09	1.05	0.90	0.86	0.72	0.69
	6	1.13	1.14	1.00	1.01	0.87	0.88	0.74	0.77
	7	1.15	1.16	1.22	1.20	1.32	1.29	1.46	1.41
	8	1.19	1.17	1.23	1.23	1.32	1.30	1.37	1.36
	9	1.16	1.15	1.21	1.18	1.15	1.17	1.06	1.11
	10	1.19	1.14	1.18	1.15	1.11	1.14	1.05	1.07
	F		0.30		0.32		0.34		0.33
2	2	0.08	0.16	0.16	0.16	0.19	0.18	0.35	0.21
	3	1.32	1.28	1.39	1.44	1.66	1.63	1.95	1.88
	4	1.30	1.34	1.33	1.34	1.27	1.27	1.24	1.20
	5	1.30	1.28	1.19	1.21	1.03	1.04	0.86	0.85
	6	1.23	1.24	1.17	1.16	0.96	1.04	0.85	0.90
	7	1.19	1.21	1.16	1.19	1.23	1.22	1.31	1.31
	8	1.20	1.19	1.23	1.19	1.28	1.24	1.31	1.31
	9	1.19	1.17	1.19	1.17	1.17	1.20	1.09	1.19
	10	1.20	1.15	1.19	1.15	1.19	1.18	1.05	1.17
	F		0.20		0.22		0.22		0.19
4	2	0.11	0.15	0.16	0.14	0.30	0.16	0.36	0.17
	3	1.21	1.15	1.26	1.26	1.45	1.40	1.75	1.58
	4	1.11	1.29	1.22	1.34	1.24	1.32	1.22	1.23
	5	1.26	1.28	1.22	1.29	1.16	1.21	1.06	1.04
	6	1.29	1.26	1.23	1.24	1.14	1.18	0.99	1.05
	7	1.26	1.24	1.22	1.22	1.16	1.21	1.18	1.24
	8	1.24	1.22	1.22	1.19	1.21	1.20	1.27	1.26
	9	1.25	1.21	1.28	1.17	1.20	1.18	1.09	1.22
	10	1.28	1.19	1.20	1.15	1.14	1.16	1.08	1.21
	F		0.11		0.13		0.13		0.13

A comparison of calculated and experimental data is given in Table 5.II. The decreasing O_2 evolution at increasing flash frequency indicates that *o*-phenanthroline is a better inhibitor at higher flash frequency. This is confirmed by measuring flash-induced steady-state O_2 evolution and DCPIP reduction in the presence of 1 - 4 μM *o*-phenanthroline (data not shown).

For *i*-dinoseb, the best fit between theory and experimental data is obtained for:

$$E_1 = 0.032\ \mu M^{-1}.s^{-1}$$
$$E_2 = 0.54\ \mu M^{-1}.s^{-1}$$
$$E_3 = 0.009\ s^{-1}$$
$$E_4 = 0.18\ s^{-1}\ .$$

In Table 5.III the experimental and calculated values of O_2 evolution are listed for three different *i*-dinoseb concentrations and four flash frequencies.

The exchange parameters obtained for *o*-phen show that the affinity of *o*-phen for the inhibitor / Q_B binding environment is much higher when the quinone complex is oxidized than when it is semi-reduced. This is in good agreement with the hypothesis of Q_B having a low and Q_B^- having a high affinity for the inhibitor / Q_B binding environment. The approximately tenfold difference in *o*-phen binding rate to the oxidized and semireduced quinone complex may reflect the apparent equilibrium constant of the $Q_A^-.Q_B \rightleftarrows Q_A.Q_B^-$ equilibrium, which is in this order of magnitude (see, for example, Robinson and Crofts (1983)). The displacement of *o*-phen by Q_B appears to be relatively insensitive to the redox state of Q_A ($E_3 \sim E_4$). This indicates that the release rate of *o*-phen is not modified dramatically by reduction of Q_A because the rate-limiting step in this displacement is the release of *o*-phen. A model of *o*-phen binding is shown in Fig. 5.2.

The binding and release kinetics of *i*-dinoseb, however, do not seem to behave like those of *o*-phen. The values of E_2 and E_4 are high, and of E_1 and E_3 are low, indicating that the inhibitor / quinone exchange is faster in the presence of a semiquinone than when the quinones are oxidized. Since $E_1 / E_3 \sim E_2 / E_4$, the *i*-dinoseb affinity seems to be just as large for the semiquinone complex as for the complex in oxidized state. Since the Q_B^- affinity is assumed to be much higher than that of Q_B, this would indicate that the *i*-dinoseb affinity is also increased upon formation of Q_A^-. However, we cannot exclude the possibility that in the case of *i*-dinoseb one of the starting assumptions used above is incorrect. For example, the exchange parameters may change during the flash train. Another possibility is that binding of *i*-dinoseb and Q_B to one

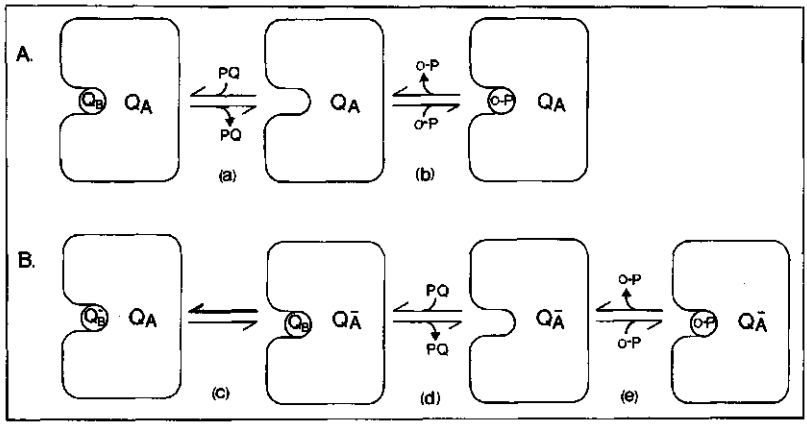

Fig. 5.2. A model for the interaction between *o*-phenanthroline (*o*-P) and plastoquinone (PQ) for binding to the inhibitor / Q_B binding environment in the oxidized (A) and semireduced (B) form of the $Q_A.Q_B$ or Q_A.Inh complex. It should be stressed that the moiety creating the binding environment for Q_A^-, and for *o*-phenanthroline and plastoquinone (Q_B) may contain more than one protein. In order to minimize complexity, a true competition for binding of *o*-phenanthroline and plastoquinone is suggested here. However, the inhibitor / quinone interaction may also be allosteric (see Vermaas *et al.* (1984a)).

binding environment can occur simultaneously. It should be stressed that the minimum in O_2 evolution always observed at the 4^{th} flash in the presence of rather high *i*-dinoseb concentrations at low flash frequency (Table 5.III) cannot be accounted for by the model used. Furthermore, *i*-dinoseb may have additional effects on the donor side of PS II (K. Pfister, personal communication; Rutherford *et al.*, 1984). Another reason for the rather anomalous results obtained for *i*-dinoseb may be that the model has been simplified too much. For example, it does not include "non-B-type centers" (Lavergne, 1982c), in which the semiquinone is less stable.

Every combination of exchange parameters appears to yield its unique set of predicted O_2 yields at different flash frequencies and inhibitor concentrations. The method used here is able to measure straightforwardly whether or not an inhibitor molecule exchanges with Q_B on the 0.1 - 10 s timescale. Although the quantitative determination of the exchange parameters is somewhat elaborate with

our method, yet it can be done in a relatively short time with the help of a computer.

5.3. Kinetics of bromoxynil binding

If the herbicide binding kinetics are sufficiently slow, a direct detection thereof is possible. Laasch *et al.* (1981) have reported that binding of low concentrations of, for example, diuron and ioxynil equilibrates within two min in the dark. In order to measure more precisely the binding kinetics of herbicides that exchange slowly with Q_B at the inhibitor / quinone binding environment, binding of ^{14}C-atrazine and ^{14}C-bromoxynil was monitored after different incubation times. The herbicide was incubated with the thylakoids for a certain time, and then the thylakoid / herbicide mixture was centrifuged for 30 s, which led to a pelleting of the thylakoids. The actual time of contact between the thylakoids and free herbicide molecules in the bulk solution was somewhat longer than the incubation time because centrifugation does not lead to instantaneous pelleting of the thylakoids. ^{14}C-atrazine appeared to bind quite fast to the thylakoids ($t_{\frac{1}{2}} < 15$ s at 50 nM), so that the atrazine binding kinetics could not be well-resolved with the method used (data not shown). However, ^{14}C-bromoxynil binding in the dark was somewhat slower, especially in the presence of dithionite (Fig. 5.3). Unfortunately, the experimental conditions did not allow measurements of bromoxynil binding kinetics in the presence of high concentrations of $(Q_A.Q_B)^-$. The first part of the kinetics of bromoxynil binding to the thylakoid membrane is not resolved because of the time necessary to separate thylakoids from free herbicide. From Fig. 5.3 a $t_{\frac{1}{2}}$ for bromoxynil binding of about 20 and 40 s (at 100 nM) is determined when the quinones are oxidized and fully reduced, respectively. The decreased rate of binding in the presence of dithionite might indicate either that plastoquinol affinity is higher than that of plastoquinone, or that the vicinity of the bromoxynil binding site is less accessible under reducing conditions, or that the rate constant for reaction of bromoxynil with the binding site is decreased. The latter possibility also offers an explanation for the increased K_d of bromoxynil in the presence of dithionite (see Section 4.1) provided that the bromoxynil release rate is relatively insensitive to the redox state of the plastoquinones.

The advantage of monitoring herbicide binding by the method described here is that a rather slow binding process can be measured. However, estimations of

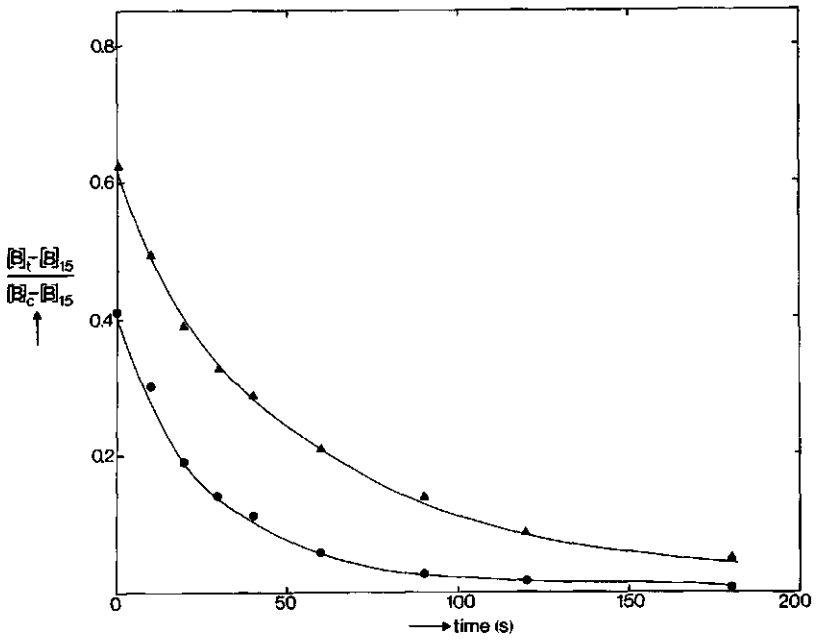

Fig. 5.3. ^{14}C-Bromoxynil binding to pea thylakoids in isolation / reaction medium in the dark as a function of time in the presence of 1 mM ferricyanide (●) or 3 mM sodium dithionite (▲). 0.1 µM bromoxynil; 50 µg Chl.ml^{-1}. In vertical direction the difference between the free bromoxynil concentration at time t and that at 15 min after bromoxynil addition (*i.e.*, under equilibrium conditions) ($[B]_t-[B]_{15}$) divided by the difference between the free bromoxynil concentration in the absence of thylakoids and that at 15 min after bromoxynil addition in the presence of thylakoids ($[B]_c-[B]_{15}$) is plotted.

herbicide binding rates do not necessarily reflect the rate of the actual association reaction of the herbicide molecule with the binding site, since there may be extra barriers which the herbicide has to cross before reaching the binding site.

In this chapter, methods have been described that can provide details of the dynamics of inhibitor binding and release. Very recently, the inhibitor / Q_B exchange kinetics have also been estimated by means of fluorescence methods (Taoka *et al.*, 1983). Thus, at this moment the redox-state-dependent inhibitor /

Q_B exchange kinetics can be monitored in two independent ways. The data reported in this chapter show a differential binding behavior of different groups of inhibitors: the kinetics of binding and release of the classical "diuron-type" inhibitors (with a $-\overset{|}{\underset{}{\bar{N}}}-C=$ group), as well as bromoxynil and ioxynil are slow, whereas phenolic inhibitors, quinones, *o*-phenanthroline and cyanoacrylates exchange much faster with the native PQ. This suggests that different groups of inhibitors interact with different parts of the inhibitor / quinone binding environment (see Chapters 3 and 4), which is also supported by many results described in the literature (see Chapter 1).

Chapter 6, Properties of triazine-resistant thylakoids

In fields where continuous application of triazine herbicides occurs, triazine-resistant weed biotypes have a chance of preferential multiplication. Many different weed species are now known for which a triazine-resistant biotype exists. For comprehensive overviews on this topic the reader is referred to LeBaron and Gressel (1982), Gressel (1984) or van Rensen (1984).

The triazine-resistant biotypes show a dramatic decrease in triazine susceptibility (3 orders of magnitude). This resistance is due to a change in the herbicide binding environment in the thylakoid membrane (Pfister and Arntzen, 1979; Pfister *et al.*, 1979). The thylakoids from triazine-resistant weeds also often show a changed affinity for other PS II inhibitors (Pfister and Arntzen, 1979; Oettmeier *et al.*, 1982a). This would suggest that the binding environment is changed. Indeed, as mentioned in the Introduction, one amino acid in the ABP-32 is changed in the triazine-resistant thylakoids. The observation that most compounds from all classes of Q_A^- oxidation inhibitors have a different affinity for triazine-resistant compared to triazine-susceptible thylakoids indicates that the ABP-32 is involved, directly or indirectly, in the binding of all classes of Q_A^- oxidation inhibitors.

Although it is known that Q_A^- oxidation by $Q_B^{(-)}$ is slower in triazine-resistant thylakoids, the Q_B properties in triazine-resistant thylakoids are still largely unknown. In the following sections, data on the $Q_A^-.Q_B \rightleftarrows Q_A.Q_B^-$ equilibrium as well as on herbicide/quinone interactions in triazine-resistant thylakoids will be presented.

6.1. The redox equilibrium between Q_A and Q_B

Also see Vermaas and Arntzen (1983) and Vermaas *et al.* (1984c).

The Chl *a* fluorescence induction curve (Pfister and Arntzen, 1979) as well as the flash-induced O_2-evolution pattern (Holt *et al.*, 1981, 1983) are quite different in triazine-resistant compared to triazine-susceptible thylakoids. Pfister and Arntzen (1979) suggested that the changed fluorescence induction curve (indicating a higher initial Q_A^- accumulation after turning on the light) was due to slower Q_A^- oxidation by Q_B. In order to check this, the fluorescence induction kinetics at high and low light intensities were measured (Fig. 6.1).

At low light intensity better equilibration of the negative charge between Q_A and Q_B can occur than at higher light intensity because of a longer time between two consecutive "hits" of PS II at low light intensity. The shape of the fluorescence induction curves at the two light intensities is almost identical (Fig. 6.1), indicating that the changed Chl *a* fluorescence induction kinetics are not due to slow Q_A^- oxidation as suggested previously (Pfister and Arntzen, 1979). The initial equilibrium Q_A^- concentration after turning on the light appears to be increased in triazine-resistant compared to triazine-susceptible thylakoids. This might indicate that the $Q_A^-.Q_B \rightleftharpoons Q_A.Q_B^-$ equilibrium is shifted to the left. Since Q_B^{2-} is protonated and likely to leave the site within a few ms, it is impossible to make statements about a possible shift in the $Q_A^-.Q_B^- \rightleftharpoons Q_A.Q_B^{2-}$ equilibrium (disregarding protonation reactions) on the basis of these data, since a real equilibration is not likely to take place. It should be noted that in many places in this thesis the notation "Q_B^-" indicates the semiquinone form of Q_B, regardless of possible protonation of Q_B or of a neighboring protein group (see Crofts *et al.*, 1984).

The hypothesis that the $Q_A^-.Q_B \rightleftharpoons Q_A.Q_B^-$ equilibrium is shifted to the left in triazine-resistant thylakoids is supported by measurements of the decay of the S_2 state of the water splitting system. This S_2 decay was monitored by flash-induced O_2-evolution measurements of thylakoids that were preilluminated with one flash at varying times before the flash train. The preilluminating flash converts most centers into the S_2 state, which is rereduced by Q_A^- to the S_1 state (Diner, 1977; Robinson and Crofts, 1983). The rate of S_2 reduction is assumed to be proportional to the equilibrium Q_A^- concentration after a flash

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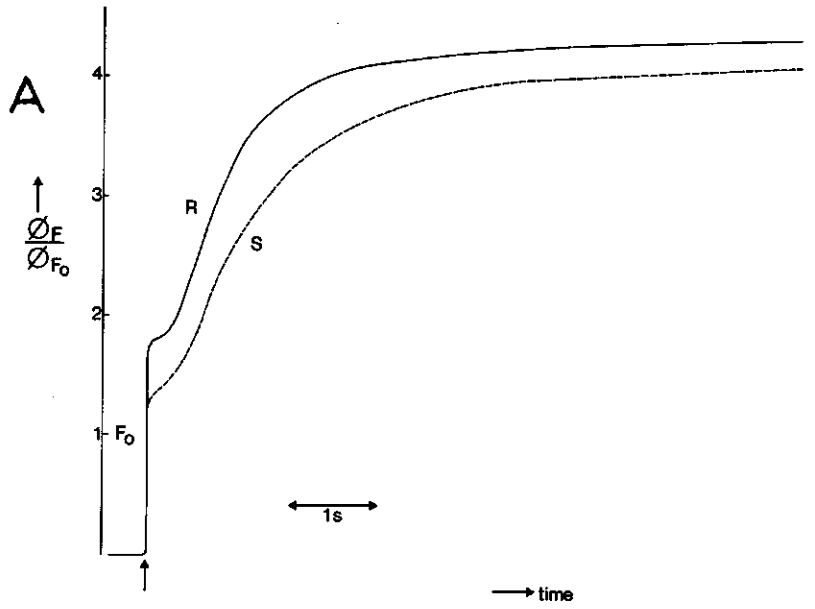
Fig. 6.1. Chl *a* fluorescence induction curve of triazine-susceptible (S) and -resistant (R) thylakoids from *Amaranthus hybridus* at full light intensity (100%; A; upper figure) and in the presence of a neutral density filter in the actinic light beam, which transmits 20% of the light (20%; B; lower figure). 15 μg Chl.ml^{-1} in isolation/reaction medium. At the arrow, the illumination was started. The constant fluorescence yield F_0, which was the same for resistant and susceptible thylakoids, is indicated. In our hands, triazine-resistant thylakoids consistently showed a slightly higher maximal fluorescence yield. →→→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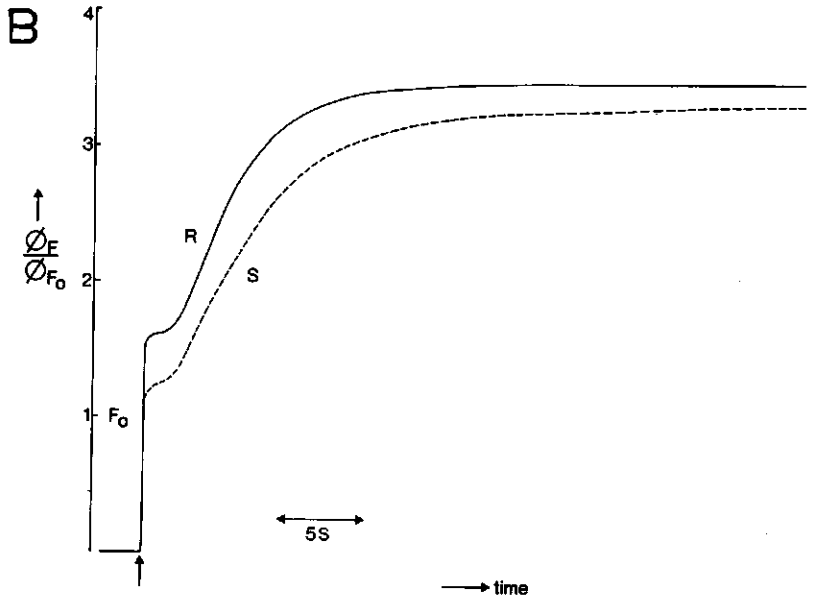
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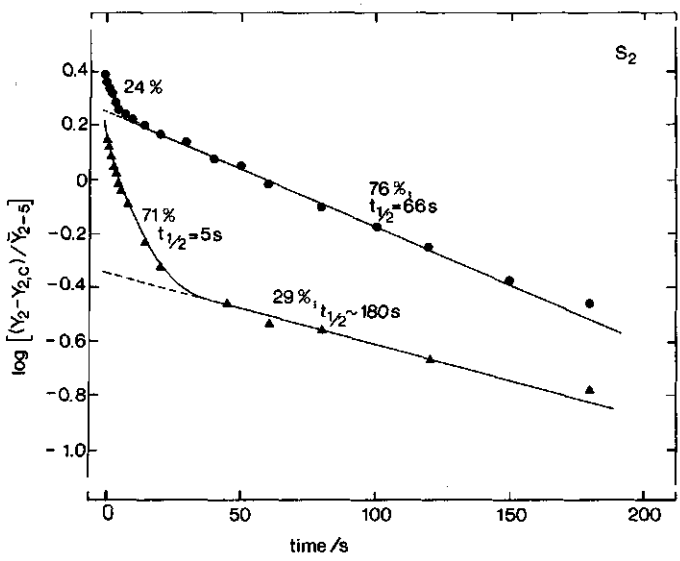

Fig. 6.2. Decay kinetics of the S_2 state in triazine-susceptible (●) and -resistant (▲) thylakoids from *Brassica napus* in isolation/reaction medium.

(Robinson and Crofts, 1983). When Q_A^- oxidation by Q_B is blocked, the Q_A^- oxidation by S_2 has a halftime of ∿ 2.8 s (Vermaas *et al.*, 1984c). In the absence of an inhibitor of Q_A^- oxidation, $t_{\frac{1}{2}}$ of the major phase of the S_2 decay is approximately 30 s in pea thylakoids (Robinson and Crofts, 1983; Vermaas *et al.*, 1984c), and about 60 s in triazine-susceptible *Brassica napus* (Vermaas *et al.*, 1984c; Fig. 6.2). A small fast phase in S_2 reduction is due to a one-electron donor, probably a quinol that gives rise, in semi-oxidized form, to ESR signal II_s (Velthuys and Visser, 1975), that is present in about 20 % of the electron transport chains. However, in triazine-resistant *Brassica napus* thylakoids most of the S_2 decay is much faster than $t_{\frac{1}{2}}$ = 30-60 s: the major component (about 75 %) has a halftime of about 5 s (Fig. 6.2). The very fast S_2 decay in these thylakoids indicates a high Q_A^- equilibrium concentration after one flash subsequent to dark adaptation. The equilibrium constant between $Q_A^-.Q_B$ and $Q_A.Q_B^-$ appears to be approximately 1 in triazine-resistant thylakoids because the rate of S_2 decay is about half compared to that when Q_A^- oxidation

by Q_B is blocked. In pea thylakoids the apparent $Q_A^-.Q_B/Q_A.Q_B^-$ equilibrium constant is about 10 ($t_{\frac{1}{2}}$ of S_2 decay + and - diuron is 2.8 and 30 s, respectively) whereas in triazine-susceptible *Brassica napus* thylakoids this equilibrium constant is even about a factor of 2 higher. For a more thorough treatment of this problem the reader is referred to Vermaas *et al.* (1984c). In triazine-resistant thylakoids there is a very slow component in the S_2 decay (Fig. 6.2). The cause of this slow phase is still unknown. It may be associated with the existence of "non-B-type centers" in which $(Q_A.Q_B)^-$ is reoxidized rapidly by components other than the water splitting complex. This slow-decaying component of S_2 may have caused the abnormally high O_2 evolution in the second flash after a rather short dark adaptation as was observed by Holt *et al.* (1981, 1983) and also by the author of this dissertation. Thorough dark adaptation leads to the disappearance of the high O_2 yield in the second flash (data not shown).

The results reported above indicate that in triazine-resistant thylakoids under conditions where there is one "free" electron in the $Q_A.Q_B$ complex, the probability of finding Q_A in the reduced form (thus, the PS II trap closed) is about 50%. Assuming that Q_BH_2 exchanges readily with PQ, much fewer PS II traps are closed when 0 or 2 electrons are on $Q_A.Q_B$. This has consequences for, for example, the analysis of the flash pattern of O_2 evolution as measured by the Joliot-type O_2-electrode. For the triazine-resistant thylakoids the miss probability α is greatly dependent on the redox state of the $Q_A.Q_B$ complex. When the complex is oxidized, α is low (and probably equal to that in triazine-susceptible thylakoids) whereas when the complex is semi-reduced α is expected to be about 0.5. This means that for triazine-resistant thylakoids one should not assume α to be independent of flash number. The miss parameter α is, in this system, expected to show a large (damped) oscillation with a periodicity of 2. Although in triazine-susceptible systems α is also expected to oscillate with flash number with a period of 2, the amplitude of these oscillations is much less because the maximal equilibrium Q_A^- concentration is markedly lower.

The question arises of what could be the reason for the shifted $Q_A^-.Q_B/Q_A.Q_B^-$ redox equilibrium. One possibility could be that the Q_B affinity is greatly decreased in triazine-resistant thylakoids; another possibility could be that the E_m of the Q_B/Q_B^- couple is decreased with respect to that of the Q_A/Q_A^- couple. In order to distinguish between these possibilities, the amount of PQ-1,

TABLE 6.I, Herbicide/quinone competition in triazine-resistant and -susceptible thylakoids

Concentration of electron-transporting quinones needed to increase the dissociation constant of ^{14}C-bromoxynil in the dark by a factor of 2 in triazine-susceptible (S) and triazine-resistant (R) thylakoids. Because most quinone partitions into the thylakoid, the local quinone concentration in the thylakoids is expected to be much higher. Thylakoids from *Brassica napus* at 100 µg Chl . ml^{-1} in isolation/reaction medium.

QUINONE	S-THYLAKOIDS	R-THYLAKOIDS
PQ-1	15 µM	20 µM
6-azido-5-decyl-2,3-dimethoxy--*p*-benzoquinone	2.5 µM	5 µM

or an analog, needed to increase the K_d of a herbicide by a factor of 2 was determined (Table 6.I). The affinity of electron-transporting quinones appears to be slightly higher in triazine-susceptible than in triazine-resistant thylakoids, but this small effect is expected to be insufficient to explain the changed $Q_A^-.Q_B/Q_A.Q_B^-$ equilibrium. Therefore, the changed protein environment probably induces a smaller Q_B^- stabilization (a lower Q_B/Q_B^- midpoint redox potential) in triazine-resistant thylakoids, causing a large shift in the semiquinone equilibrium between Q_A and Q_B.

6.2. Ligand binding to the inhibitor / quinone binding environment

The inhibitory activity of a range of Q_A^- oxidation inhibitors has been determined in triazine-resistant thylakoids relative to that in triazine-susceptible thylakoids (for example, Pfister and Arntzen, 1979; Oettmeier *et al.*, 1982a). However, the properties of electron-transport-inhibiting quinones in triazine-resistant thylakoids is still largely unknown. As is shown in Section

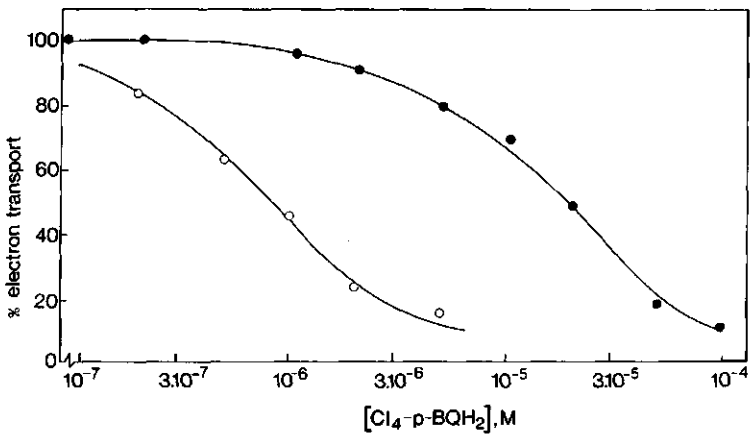

Fig. 6.3. Inhibition curve of the p-benzoquinone Hill reaction of tetrachloro-*p*-benzohydroquinone in triazine-susceptible (•) and triazine-resistant (o) *Amaranthus hybridus* thylakoids in isolation/reaction medium. The *p*-benzoquinone concentration was 0.25 mM. For the experiment shown, the control Hill reaction rate was 148 µmol O_2.mg Chl^{-1}.h^{-1} for susceptible and 128 µmol O_2.mg Chl^{-1}.h^{-1} for resistant thylakoids. The light intensity was saturating.

6.1, the Q_B affinity is likely to be decreased slightly in triazine-resistant thylakoids. However, tetrachloro-*p*-benzoquinone, a quinone that inhibits electron transport by blocking Q_A^- oxidation, was found to be about 20 times as effective in triazine-resistant as in triazine-susceptible thylakoids at saturating light intensity (Fig. 6.3; Vermaas and Arntzen, 1983). The dissociation constant was found to be 0.4 µM in resistant and 3 µM in susceptible thylakoids (Vermaas and Arntzen, 1983). The discrepancy in the difference in susceptibility to tetrachloro-*p*-benzoquinone between results obtained on inhibition of electron transport at saturating light intensity and those obtained from binding studies in the dark (a factor of 20 and 8, respectively) probably originates from the relative inefficiency of electron transport at the acceptor side of PS II in triazine-resistant thylakoids. This causes the rate of the rate-limiting step in electron transfer at the acceptor side of PS II to be closer to that of the overall rate-limiting step in photosynthetic electron transport, the oxidation of PQH_2, than in triazine-susceptible thylakoids. Therefore, fewer PS II chains can be blocked in triazine-resistant thylakoids before a signifi-

TABLE 6.II, Effects of reduction of thylakoid components on ioxynil affinity in triazine-resistant and -susceptible thylakoids

Dissociation constants (K_d) of ^{14}C-ioxynil in the dark or in bright room light using triazine-resistant (R) or -susceptible (S) thylakoids from *Brassica napus* in isolation/reaction medium (100 µg Chl.ml^{-1}). Where indicated, 2.5 mM dithionite was added.

CONDITION	K_d(nM) in S-thylakoids	K_d(nM) in R-thylakoids
dark	11	17
light	21	33
dark + dithionite	25	31

cant inhibition at saturating light intensity occurs than is the case in triazine-susceptible thylakoids. The same discrepancy in susceptibility ratios of triazine-resistant and -susceptible thylakoids between inhibition of electron transport at saturating light intensity and binding affinity in the dark is observed for bromoxynil and ioxynil. For example, for ioxynil the I_{50} for the DCPIP-Hill reaction has been found to be 4.5×10^{-8} M for triazine-resistant, and 6.5×10^{-8} M for -susceptible thylakoids from *Amaranthus hybridus*, whereas the K_d of ioxynil is 1.25×10^{-8} M and 0.6×10^{-8} M for triazine-resistant and -susceptible *Amaranthus hybridus* thylakoids in the dark (Vermaas and Arntzen, 1983). For bromoxynil similar differences have been observed (data not shown). The discrepancy between the ratio of the I_{50}'s and that of the K_d's in resistant and susceptible thylakoids is not caused by a larger change in herbicide affinity upon photo- or chemical reduction (as discussed in Section 4.1) in triazine-susceptible than in -resistant thylakoids: reduction of the PQ pool both in triazine-resistant and -susceptible *Brassica napus* thylakoids led to a twofold reduction of ioxynil affinity (Table 6.II). Note that the reduction-induced decrease in ioxynil affinity is less in *Brassica napus* than in pea

thylakoids (see Table 4.II). Thus, the discrepancy between the ratio of the I_{50}'s and that of the K_d's in triazine-resistant and -susceptible thylakoids appears to be due only to less effective electron transport at the acceptor side of PS II in triazine-resistant thylakoids.

When discussing differences between resistant/susceptible ratios of I_{50} and K_d, it should also be pointed out that the values of I_{50} as measured at saturating light intensity and K_d as measured in the light are not identical, as was previously assumed (Tischer and Strotmann, 1977; Tischer, 1978). The I_{50} is expected in fact to be higher than the K_d (which is also observed experimentally, data not shown) because at saturating light intensity the rate--determining step is after collection of electrons in the PQ pool; this causes inhibition of 50 % of the PS II chains to lead to less than 50 % inhibition of the Hill reaction. At subsaturating light intensities I_{50} should be equal to K_d when measured under identical redox conditions.

Ioxynil and bromoxynil which both have a phenol group do not behave like the other phenol-type inhibitors in that the K_d in triazine-resistant thylakoids is increased compared to the triazine-susceptible thylakoids. The other phenol-type inhibitors are more active in triazine-resistant than in -susceptible systems. Because of this, and because of the low binding and release rates for these herbicides compared to the phenolic inhibitors, it is better to treat ioxynil and bromoxynil as representatives of a separate group (the nitriles) rather than as phenol-type inhibitors.

As reported above, tetrachloro-*p*-benzoquinone is a better inhibitor in triazine-resistant than in -susceptible thylakoids. In this respect, this synthetic quinone behaves like phenol-type inhibitors (dinoseb, 4,6-dinitro-*o*--cresol) that are known to be more active in resistant thylakoids (Pfister and Arntzen, 1979). Indeed, the phenol-type inhibitors may function as quinol analogs (see Vermaas and Arntzen (1983)). It would be interesting to know why the tetrachloro-*p*-benzoquinone affinity is significantly increased in resistant thylakoids, whereas the affinity of the native quinone is decreased. One might speculate that although the binding affinity of the quinone head group of PQ is increased, the long side chain cannot be accommodated very well in the changed environment, which can result in a net decrease of affinity. It remains to be established whether the possible change in the side chain environment which caused the affinity decrease is in some way directly related to the huge affinity decrease of atrazine.

The results reported in this section indicate that a small change in the ABP-32 results in a large effect on the semiquinone equilibrium between Q_A and Q_B, and in an affinity change of Q_A^- oxidation inhibitors, indicating that the conformation of the ABP-32 plays a very important role in determining the characteristics of Q_B and the affinity of inhibitors. This indicates that the ABP-32 is a part of the binding environment for Q_B and for all PS II herbicides and other Q_A^- oxidation inhibitors. It should be noted that the properties of Q_A do not seem to be considerably changed. For example, the rate of Q_A^- oxidation by S_2 in the presence of diuron is not changed (Vermaas *et al.*, 1984c). This would be expected if Q_A is assumed to be located in the 47 kDa PS II RC polypeptide (H. Nakatani, personal communication), as there are no indications that this polypeptide is changed in triazine-resistant thylakoids.

Chapter 7, The bicarbonate effect

It is well-known that CO_2 is fixed in the photosynthetic process. Stated more precisely, CO_2 is a substrate for ribulose-1,5-bisphosphate carboxylase/oxygenase (abbreviated as Rubisco). However, CO_2 (or HCO_3^-) also has other functions in the photosynthetic process. For example,

1. CO_2 is required as an activator of Rubisco (Lorimer *et al.*, 1976; Lorimer and Miziorko, 1980);
2. HCO_3^- enhances photophosphorylation at suboptimal pH (Punnett and Iyer, 1964) through a conformational change in the thylakoid coupling factor (Nelson *et al.*, 1972; Cohen and McPeek, 1980), and
3. HCO_3^- or CO_2 is necessary for efficient photosynthetic electron transport (Warburg and Krippahl, 1958; see a review by Vermaas and Govindjee (1981b)).

In this chapter the third effect, the CO_2/HCO_3^- effect on photosynthetic electron transport, often referred to as the "bicarbonate effect", will be considered. In the absence of CO_2 and HCO_3^- and in the presence of $HCOO^-$ (formate; probably a competitive inhibitor of HCO_3^- binding) electron transport is severely inhibited. The site of this inhibition is reduction and reoxidation of Q_B. Readdition of HCO_3^- restores efficient electron transport (Vermaas and Govindjee, 1981b).

7.1. The bicarbonate effect in the dark and in continuous light; the formate effect

See also Vermaas and van Rensen (1981).

At pH = 6.5 and in the presence of 100 mM formate, 1 mM $NaHCO_3$ is required to restore 50 % of the maximal electron transport in CO_2-depleted thylakoid membranes (Khanna *et al.*, 1977). Formate and some other organic anions are likely to remove HCO_3^- from its binding site in the thylakoid membrane, thus inhibiting electron transport (Good, 1963). A dark period of about 2 min after $NaHCO_3$ addition is necessary to reactivate electron transport in the CO_2-depleted thylakoids. No restoration is observed in the light (Stemler, 1979; Stemler and Govindjee, 1973). However, at much lower formate concentrations ($\leq$ 1 mM), HCO_3^--induced reactivation of the Hill reaction is possible in the light, and formate decelerates this rate of reactivation (Vermaas and van Ren-

sen, 1981). Furthermore, it has been shown that HCO_3^- depletion can also occur in the light in the presence of formate, without a CO_2-depletion procedure (Stemler, 1979). Therefore, it is likely that the HCO_3^- affinity as compared to the formate affinity is higher in the dark than in the light. At this moment, it is not known whether the HCO_3^- affinity, the $HCOO^-$ affinity, or both are significantly modified in the light. Furthermore, also the mechanism of the affinity change(s) is not known; reduction of the PQ pool by tetramethyl-*p*-benzohydroquinone in the dark did not appear to alter the formate and HCO_3^- affinity compared to normal dark conditions (PQ pool oxidized) (W. Vermaas and J. van Rensen, unpublished observations). Therefore, the redox state of the PQ pool does not seem to be involved directly in the HCO_3^-/formate affinity regulation.

It would be tempting to speculate on a role of HCO_3^-/CO_2 in protonation reactions near Q_B (cf. Govindjee and van Rensen, 1978). At this moment the only indication for such a mechanism may be that the pK_a of (de)protonation of a group near Q_B is 6.4 when Q_A and Q_B are oxidized (Crofts *et al.*, 1984). The pK_a of ($CO_2 + H_2O$) is 6.4 at 25° C. However, the pK_a value for (de)protonation of a Q_B-related acid/base group in the presence of a semiquinone is estimated to be 7.9 (Crofts *et al.*, 1984), whereas the pK_a of HCO_3^- is 10.2. It should be noted, though, that the local pH in the protein near Q_B may be entirely different from the pH in the solution, and it is possible that shifts may take place in the pK_a values of acid/base groups when ligated to a protein compared to when free in solution.

Such an involvement of $CO_2/HCO_3^-/CO_3^{2-}$ in protonation processes, which resembles a HCO_3^- model presented by Stemler (1979) in that it assumes a close interaction between Q_B and HCO_3^- (or CO_2 or CO_3^{2-}), might explain why redox turnovers of Q_B accelerate the exchange of bound HCO_3^-/CO_2 with, for example, exogeneous formate. Furthermore, it explains why HCO_3^- is necessary for efficient electron transport. As mentioned in Chapter 1, protonation has to occur before Q_B^- can be reduced by Q_A^-, and $Q_B^{2-}.H^+$ will have to be protonated before being able to leave the binding environment. Moreover, the mechanism described might explain why formate ($pK_{a,\,HCOOH} = 3.8$) is not able to function well in supplying $(Q_A.Q_B)^{(2(-))}$ with a proton. However, this is mere speculation, and experiments will have to be performed in order to (dis)prove these suggestions.

7.2. Bicarbonate effects on herbicide binding

See also Vermaas *et al.* (1982; 1984a).

As noted above, absence of HCO_3^- leads to a dramatic decrease in the rate of electron transport through Q_A and Q_B. Since the site of the HCO_3^- effect seems to be functionally close to that of PS II herbicides, herbicide binding was measured in the absence and presence of HCO_3^- in previously CO_2-depleted thylakoids (Table 7.I). The binding affinity of most inhibitors is decreased in the absence of HCO_3^- (in agreement with the data of Khanna *et al.* (1981)).

TABLE 7.I, HCO_3^- effects on herbicide affinity

The K_d of various herbicides in CO_2-depleted pea thylakoids (prepared by method II; see Section 2.2) in the medium at pH= 6.0 (Section 2.2) was determined with or without the addition of 5 mM $NaHCO_3$. Where indicated, 2.5 mM dithionite was added, 5 mM $MgCl_2$ was left out from the reaction medium, or bright room light was on. In all cases, the maximal number of herbicide binding sites was 1 per 350-500 Chl molecules. 50-100 μg Chl.ml^{-1}. The *i*-dinoseb affinity was determined from competition experiments using unlabelled *i*-dinoseb competing with ^{14}C-ioxynil in order to exclude *i*-dinoseb binding that is not related to inhibition of photosynthetic electron transport.

HERBICIDE	CONDITIONS	$K_{d,-HCO_3^-}$ (nM)	$K_{d,+HCO_3^-}$ (nM)
atrazine	dark	225	58
bromoxynil	dark	120	58
	dark-$MgCl_2$	130	80
	dark +dithionite	250	190
i-dinoseb	dark	30	105
ioxynil	dark	9	7
	light	20	17

However, the affinity of phenolic herbicides seems to be increased when no HCO_3^- is present as was already suggested by the results of van Rensen and Vermaas (1981) and Snel and van Rensen (1983). For bromoxynil binding, we checked whether the decreased affinity in the absence of HCO_3^- could be attributed to some kind of an unstacking effect or to reduction of thylakoid components (see Chapter 4 for such effects on inhibitor binding). As can be seen from Table 7.I, this is not likely: after unstacking ($-Mg^{2+}$) or chemical reduction a HCO_3^- effect on bromoxynil binding can still be observed (although it is somewhat less than in the control). Therefore, HCO_3^- appears to cause a conformational change in the inhibitor/Q_B binding environment such that the herbicide affinity is changed, implying that the HCO_3^- binding site is indeed located closely to that of Q_A^--oxidation inhibitors.

Diuron and *i*-dinoseb are known to increase the time needed for HCO_3^- binding to thylakoids (Snel and van Rensen, 1983). Furthermore, diuron is able to prevent release of HCO_3^- from thylakoids on silicomolybdate washing (Stemler, 1977). We checked whether the kinetics of ^{14}C-bromoxynil binding were HCO_3^---sensitive. The results are shown in Fig. 7.1. No significant differences in kinetics of bromoxynil binding to the thylakoid membrane were observed. It is concluded that either the bromoxynil release rate is HCO_3^- sensitive (the bromoxynil affinity is decreased upon HCO_3^--depletion), or that there is a HCO_3^---insensitive barrier in the thylakoid membrane that bromoxynil has to cross before it reaches the binding site.

7.3. The influence of bicarbonate on other PS II reactions

See also Vermaas *et al.* (1984c) and Vermaas and Govindjee (1982b).

Originally, the HCO_3^- effect was assumed to be related to the water splitting process (Warburg, 1964). Later, some results were presented that might be interpreted as suggesting a bicarbonate effect on the water splitting side (Stemler, 1982). However, many experiments did not show any involvement of HCO_3^- on the water splitting side of PS II (see Vermaas and Govindjee (1982a)). Although at this moment there is no direct evidence for a function of HCO_3^- in water splitting in the photosynthetic electron transport chain, some pathways of PS II other than the $Q_A^- \rightarrow PQ$ electron transport seem to be blocked or slowed down in the absence of HCO_3^- and in the presence of formate. For example, the Q_A^- oxidation by an accessory electron acceptor, C400 (Bowes and Crofts, 1980), was suggested to be inhibited by HCO_3^- removal (Radmer and Ollinger, 1980). Furthermore, the Q_A^- oxidation by S_2 in the presence of diuron

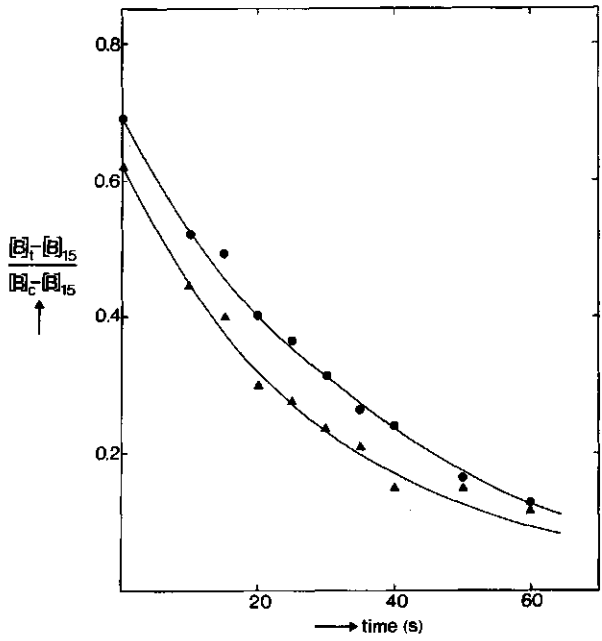

Fig. 7.1. ^{14}C-bromoxynil binding kinetics to pea thylakoids in CO_2-depleted thylakoids (method II; in medium at pH = 6.0; see Section 2.2) in the dark with (●) and without (▲) addition of 5 mM $NaHCO_3$. 0.1 μM bromoxynil; 100 μg Chl.ml^{-1}. In vertical direction the difference between the free bromoxynil concentration at time t and at 15 min after bromoxynil addition (*i.e.*, under equilibrium conditions) ($[B]_t - [B]_{15}$) divided by the difference between the free bromoxynil concentration in the absence of thylakoids and that in the presence of thylakoids at 15 min after bromoxynil addition ($[B]_c - [B]_{15}$) is plotted.

was found to be slowed down upon HCO_3^- depletion: the $t_{\frac{1}{2}}$ of decay of variable Chl *a* fluorescence was found to be 2.3 s in the absence and 1.0 s in the presence of HCO_3^- (Vermaas *et al.*, 1984c). This also provides a good explanation for the observation that the Chl *a* fluorescence induction curve on the 1 s timescale in the presence of diuron was faster in the absence of bound HCO_3^- than in its presence (Vermaas and Govindjee, 1982b): in the presence of HCO_3^- the back reaction to S_2 may play an important role, whereas this is not the

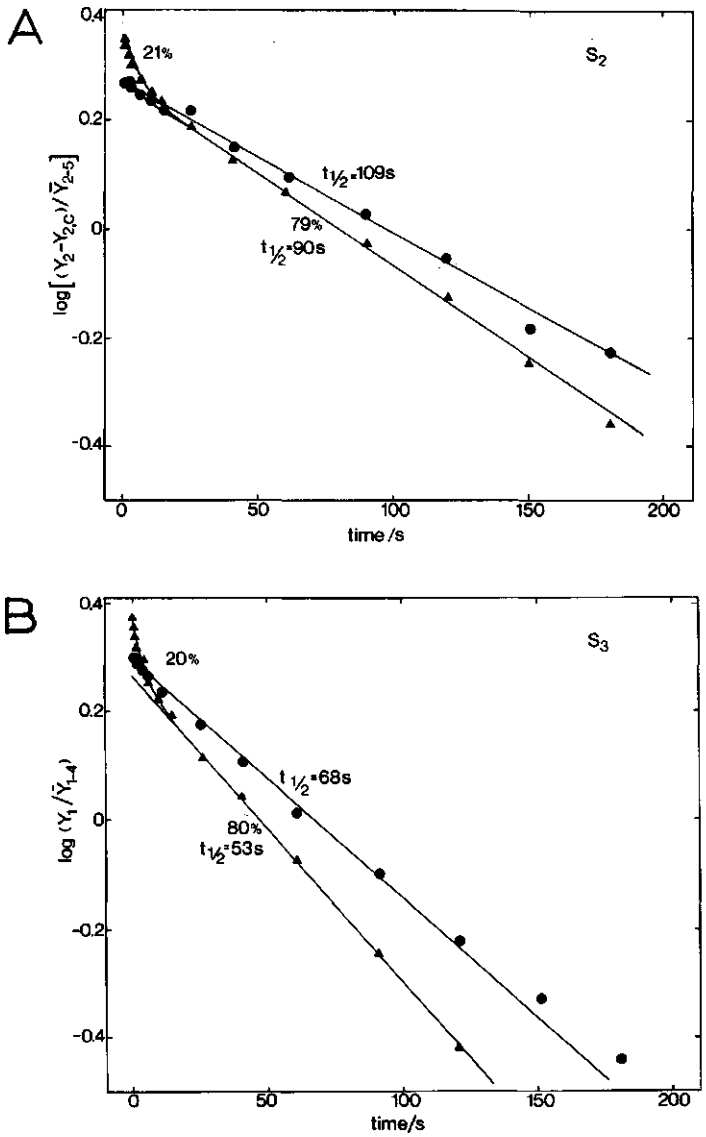

Fig. 7.2. Decay kinetics of the S_2 and S_3 states (A and B, respectively) in CO_2-depleted pea thylakoids (method II; in medium at pH = 6.0; see Section 2.2) with (▲) and without (●) the addition of 5 mM $NaHCO_3$ to both thylakoid suspension and electrode buffer.

case in the absence of HCO_3^-. These data might suggest that in the absence of HCO_3^- the properties of Q_A may have changed.

In order to investigate whether the bicarbonate effect may be related to changes in equilibria (for example, that between Q_A and Q_B, as is the case in triazine-resistant thylakoids), the S_2 and S_3 decay kinetics in the absence and in the presence of HCO_3^- were measured in the absence of diuron (Fig. 7.2). The major S_2 and S_3 decay phase (80 %) was rather insensitive to HCO_3^-. Since the Q_A^- oxidation by S_2 in the presence of diuron is about a factor of 2 faster in the presence of HCO_3^- than in its absence, the Q_A^- concentration after one flash in the absence of diuron is probably about a factor of 2 higher in the absence of bound HCO_3^- than in its presence. Therefore, HCO_3^- depletion may lead to a small shift of the $Q_A^-.Q_B \rightleftharpoons Q_A.Q_B^-$ equilibrium to the left, but this equilibrium shift cannot account for the large effects of HCO_3^- on electron transport. Thus, the HCO_3^- effect appears to be mainly due to a change of kinetic parameters of electron transport (see, for example, Siggel *et al.* (1977)) rather than of thermodynamic parameters of Q_A and Q_B.

A feature in Fig. 7.2 that is perhaps much more interesting than the $t_{\frac{1}{2}}$ of the major decay phase is the small fast phase of S_2 and S_3 decay present in control thylakoids as well as in CO_2-depleted thylakoids after the addition of HCO_3^-. This phase disappears when no HCO_3^- is bound. As mentioned in Chapter 5, the small fast phase of S_2 and S_3 decay is interpreted as due to a one-electron donation by an unknown component, possibly the quinol-type molecule that gives rise to ESR signal II_s upon oxidation (Velthuys and Visser, 1975), to S_2 and S_3 (Vermaas *et al.*, 1984c). Thus, the one-electron donor is, in some way, isolated from the water splitting system in the absence of HCO_3^-. It remains to be established whether or not it is merely fortuitous that this HCO_3^- effect probably involves quinol oxidation, but it is interesting to note that dinoseb is reported to abolish ESR signal II_s (Rutherford *et al.*, 1984). Dinoseb can possibly act as a quinol analog (Vermaas and Arntzen, 1983) and a close relationship between the sites of action of HCO_3^- and phenolic inhibitors (such as dinoseb) has been postulated (van Rensen and Vermaas, 1981).

In conclusion, it is clear that there is a close interaction between HCO_3^- and inhibitor (herbicide) binding, and that (at least in the presence of formate) HCO_3^- is needed for efficient electron transport through quinones in PS II. However, no real progress has been made in the last few years in answering the question of how $CO_2/HCO_3^-/CO_3^{2-}$ is able to influence electron transport so dramatically.

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Summary

In this thesis experiments are described which are directed towards a further characterization of the interaction of the native bound plastoquinone Q_B, artificial quinones, herbicides and bicarbonate with their binding environment at the acceptor side of Photosystem II in the thylakoid membrane. The most important thylakoid component involved in binding of, *e.g.*, herbicides and quinones appears to be the lysine-free, rapidly turned-over 32,000 M_r protein that is attacked readily by trypsin. This protein, that is involved in creating the herbicide/quinone binding environment is designated in this thesis "ABP-32" (azidoatrazine-binding protein of 32,000 M_r). Chapter 3 describes, however, that a lysine-containing protein complex also appears to modify binding of the herbicides atrazine and bromoxynil. This protein complex might be related to the Photosystem II reaction center (Section 3.3). In many earlier reports, where polypeptide staining with Coomassie Brilliant Blue was used for monitoring the polypeptide content of a preparation, herbicide binding was assigned to the wrong 32,000 M_r protein. The ABP-32 is poorly stainable with Coomassie Brilliant Blue. The other 32,000 M_r protein associated with Photosystem II is probably related to the water splitting process (Section 3.1).

Chapter 4 shows that herbicides and quinones appear to displace each other from the binding environment in a seemingly competitive fashion. However, after covalent linkage of a quinone to the binding site herbicide binding still occurs, albeit with a low affinity (Section 4.3). This can be taken as evidence of an allosteric interaction between herbicide and quinone binding: upon binding of one the affinity of the other is decreased. This hypothesis is supported by other data in this thesis, which show differential effects on binding of quinones and different types of herbicides. However, we consider the interaction of two related molecules (for example, two herbicides belonging to related chemical groups) for binding to the binding environment to be truly competitive.

Herbicide/quinone interactions were studied not only under equilibrium conditions, but the binding and release rates of the inhibitor to the site were also estimated and calculated (Chapter 5). Herbicides like diuron, atrazine, bromoxynil and phenisopham exchange slowly with the native quinone, whereas phenol-type inhibitors (for example, dinoseb), *o*-phenanthroline, cyanoacrylates

and synthetic quinones exchange faster ($\geq 0.1\ s^{-1}$ at 50 % inhibition of electron transport). In the case of *o*-phenanthroline a good fit between experimental data and theoretical values calculated from a model of competitive quinone/inhibitor interaction could be obtained. When using a phenol-type inhibitor, fitting of experimental data and theory was less successful in the sense that the results obtained could not be fitted in a scheme where Q_B and Q_BH_2 have a low binding affinity and Q_B^- has a high affinity. It is possible that this is caused by an interaction between Q_B and the phenol-type inhibitor, which is not, to a first approximation, competitive.

During the last decade triazine-resistant biotypes of weeds have developed in fields that were sprayed repeatedly with triazine herbicides (for example, atrazine). All triazine-resistant biotypes characterized thus far differ from the "wild type" by one amino acid in the ABP-32. This minor change leads to a large effect on, for example, the affinity of some herbicides and quinones, and on the semiquinone equilibrium between the first electron-accepting quinone in Photosystem II, Q_A, and Q_B. This equilibrium is shifted to the Q_A side considerably in triazine-resistant plants, thus decreasing photosynthetic efficiency under limiting light intensity (Chapter 6).

Photosynthetic electron transport on the acceptor side of Photosystem II can also be modified by bicarbonate, at least in the presence of formate (Chapter 7). Absence of bicarbonate /CO_2 leads to an inhibition of electron transport whereas readdition of HCO_3^- restores electron flow through Q_B. The binding site of HCO_3^- is functionally close to that of herbicides: herbicide affinity is sensitive to CO_2-depletion and HCO_3^--readdition (Section 7.2). Although the precise role of HCO_3^- in electron transport is not yet known, it is speculated here that HCO_3^- may be involved in protonation of reduced Q_B. Bicarbonate depletion also appears to slow down Q_A^- oxidation by the water splitting system, and to block the reduction of the S_2 and S_3 state of the water splitting system by -probably- a bound quinol (Section 7.3).

In conclusion, this thesis provides many detailed data and analyses, which may add to form a basis for the understanding of the molecular mechanism of ligand binding at the Photosystem II acceptor side and of electron transfer from Q_A to the plastoquinone pool. At this moment, however, the description of electron transport, inhibition and quinone binding at Photosystem II is still rather phenomenological. For a thorough understanding of the underlying molecular processes much more research, especially interdisciplinary, is re-

quired. In this way progress in solving this problem, that contains (bio)physical, (bio)chemical, physiological and genetic components, may best be made.

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Samenvatting

Dit proefschrift beschrijft resultaten - en interpretaties daarvan - van onderzoek aan een klein deel van het fotosyntheseproces. In dit proces, dat in o.a. de groene plant plaatsvindt, worden koolzuur (CO_2) en water (H_2O) onder invloed van licht omgezet in suikers (zetmeel) en zuurstof (O_2). Dit proces zorgt zowel primair voor de voedselvoorziening als voor een groot deel van de energievoorziening (d.m.v. fossiele brandstoffen en hout) op de aarde, en maakt een O_2/CO_2 kringloop mogelijk (de ademhaling gebruikt O_2 en produceert CO_2, terwijl bij de fotosynthese CO_2 wordt gebruikt en O_2 geproduceerd). Dit maakt de fotosynthese tot één der fundamenteelste biologische processen op aarde.

Het fotosyntheseproces kan ruwweg worden opgedeeld in twee delen:

1. de lichtabsorptie en het elektronentransport, die leiden tot produktie van O_2, energierijke verbindingen (ATP) en reducerend vermogen ($NADPH(+H^+)$), en
2. de fixatie van CO_2 tot suikers m.b.v. ATP en $NADPH(+H^+)$.

In de groene plant vinden beide onderdelen van het fotosyntheseproces plaats in een daarop gespecialiseerd celorganel (een door een membraan omgeven compartiment binnen de plantecel): de chloroplast. Het eerste onderdeel (lichtabsorptie en elektronentransport) vindt plaats in een omvangrijk membraansysteem binnen de chloroplast (het thylakoidmembraansysteem) dat zó gevormd is dat het een relatief kleine ruimte binnen de chloroplast geheel omsluit (de intrathylakoidale ruimte). Aan de andere zijde van de thylakoidmembranen, in het zgn. stroma, bevinden zich de enzymen die voor de CO_2-fixatie zorgdragen.

Aangezien dit proefschrift de nadruk op bepaalde delen uit het fotosynthetisch elektronentransport legt, wordt er hier iets nader ingegaan op processen binnen het thylakoidmembraan. Het thylakoidmembraan bestaat uit een dubbellaag van vetmolekulen (waarin andere organische molekulen, zoals chinonen, "opgelost" zijn) met daarin ingebed een rijke verscheidenheid aan eiwitcomplexen. Al die verschillende eiwitcomplexen hebben hun eigen funktie. Voor het mogelijk maken van de omzetting van lichtenergie in chemische energie zijn vooral twee eiwitcomplexen van belang, de reaktiecentrumeiwitten, die elk chlorofylmolekulen bevatten, die op een speciale manier in het eiwit liggen. Deze reaktiecentrumchlorofylmolekulen kunnen, wanneer ze door een lichtkwant in aangeslagen toestand gebracht zijn, een elektron aan een nabijgelegen intermediair in de elektronentransportketen overdragen, die het verder transporteert. De eerste overdrachten

gaan razendsnel (in minder dan een miljardste deel van een sekonde, waarschijnlijk) om terugreakties zo weinig mogelijk kans te geven. Na de eerste elektronenoverdrachten wordt een terugreaktie uit sterisch en thermodynamisch oogpunt zo ongunstig dat dat niet meer snel op kan treden. Zoals gezegd zijn er twee verschillende chlorofyl/eiwitcomplexen, reaktiecentra genaamd, waarin zo'n ladingsscheiding op kan treden. Om de waarschijnlijkheid van een frequente ladingsscheiding bij normale lichtintensiteiten groter te maken, zijn er per reaktiecentrum meestal nog zo'n 200-300 andere pigmentmolekulen (chlorofyl, carotenoiden, etc.) die hun energie naar het reaktiecentrumchlorofyl kunnen overdragen. De twee reaktiecentra staan min of meer "in serie" met elkaar geschakeld: een elektron gaat van het ene reaktiecentrum (dat van "Fotosysteem II") via intermediairen (o.a. zowel eiwitgebonden als "vrij" plastochinon) naar het andere reaktiecentrum, dat van Fotosysteem I. Het geoxideerde Fotosysteem II reaktiecentrum krijgt (indirekt) een elektron van H_2O (netto reaktie: $\frac{1}{4}(2H_2O \rightarrow 4H^+ + O_2 + 4e^-)$), terwijl het Fotosysteem I reaktiecentrum zijn elektron over kan dragen (indirekt) op $NADP^+$. Op deze manier ontstaat er een lineair elektronentransport van H_2O naar $NADP^+$ via twee lichtreakties (zonder licht is netto H_2O oxidatie door $NADP^+$ energetisch niet te verwachten). Het gereduceerd $NADP^+$ wordt, zoals boven vermeld, gebruikt voor o.a. de CO_2-fixatie.

Dit proefschrift houdt zich vooral bezig met een stukje van het elektronentransport tussen de reaktiecentra van Fotosysteem II en I. De eerste elektronenacceptor van Fotosysteem II die in gereduceerde vorm redelijk stabiel is (d.i. een levensduur van meer dan een miljoenste deel van een sekonde heeft onder fysiologische omstandigheden) is een plastochinonmolekuul, Q_A genaamd. Dit molekuul is waarschijnlijk min of meer ingebed in het eiwitcomplex dat ook het Fotosysteem II reaktiecentrumchlorofyl bevat. Gereduceerd Q_A wordt geoxideerd door een ander plastochinon, Q_B, dat in geheel geoxideerde en gereduceerde vorm betrekkelijk vrij kan uitwisselen met de plastochinonen in de lipidenfase (d.w.z. "opgelost" tussen de vetmolekulen). Het Q_B is gebonden op een specifieke plaats aan een eiwitcomplex dichtbij Q_A, zodat Q_B het gereduceerde Q_A kan oxideren. Zodra Q_B volledig is gereduceerd (Q_B kan twee elektronen opnemen; Q_A maar één tegelijk) en geprotoneerd, kan het van de bindingsplaats aan het eiwitcomplex loskomen, met het plastochinon in de lipidenfase mengen, en een ander plastochinonmolekuul kan de plaats van Q_B weer innemen. Het gereduceerd plastochinon wordt, indirekt, geoxideerd door geoxideerde Fotosysteem I reaktiecentra. Het concept van het hierboven geschetste "mobiele" Q_B is nog vrij nieuw (1981).

Resultaten in dit proefschrift ondersteunen dit model.

Hoofdstuk 4 van dit proefschrift beschrijft dat binding van Q_B verhinderd kan worden door binding van bepaalde onkruidbestrijdingsmiddelen (herbiciden) aan hetzelfde eiwitcomplex. Omgekeerd voorkomen ook chinonen herbicidebinding, terwijl binding van herbiciden ook binding van andere herbiciden (soms uit een andere chemische familie) aan hetzelfde complex verhindert. Uit een nadere analyse blijkt dat de herbicide/herbicide en herbicide/chinon interactie competitief lijkt, d.w.z. dat maar één molekuul, hetzij chinon, hetzij herbicide, op een gegeven moment aan het eiwitcomplex gebonden kan zijn. Als men echter een covalente binding tussen het chinon en het eiwitcomplex tot stand brengt, dan blijkt er toch nog herbicidebinding op te kunnen treden, zij het met verlaagde affiniteit. Hieruit is in dit proefschrift voorlopig geconcludeerd dat de bindingsomgeving van herbiciden en chinonen niet helemaal identiek is, en dat -onder bepaalde omstandigheden- toch binding van een chinon en een herbicidemolekuul gelijktijdig op kan treden. Dit wijst op een zgn. allosterische interaktie tussen chinonen en bepaalde herbiciden.

Hoofdstuk 3 van dit proefschrift vermeldt resultaten van onderzoek naar herbicidebinding, waarbij het Q_B- en herbicide-bindend eiwitcomplex door eiwitsplitsende enzymen wordt aangetast. Verschillende typen van herbiciden (behorend tot verschillende chemische groepen) reageren hier niet hetzelfde op, hetgeen aan kan duiden dat de verschillende herbicidegroepen inderdaad niet-identieke bindingsomgevingen in het eiwitcomplex hebben (hoewel ze allen Q_B binding verhinderen). Hoewel tot nu toe werd aangenomen dat uitsluitend een specifiek eiwit, dat geen lysine bevat, bij binding van een bepaalde herbicidegroep, de triazinen, was betrokken, blijkt dat behandeling met een lysine-specifiek protease de binding van atrazine (een triazine) modificeert. Hieruit wordt geconcludeerd dat -direkt of indirekt- ook (een) ander(e) eiwit(ten) bij de triazinebinding betrokken is (zijn), en dat een funktionerende herbicide/chinon bindingsomgeving uit een complex van meerdere eiwitten bestaat, waarvan het al eerder genoemde lysine-vrije eiwit overigens een zeer belangrijk deël uitmaakt.

Door het intensieve gebruik van triazine herbiciden in de landbouw zijn er in de laatste jaren diverse triazine-resistente onkruiden tevoorschijn gekomen. Deze biotypes kenmerken zich, naast een 100-1000 x lagere gevoeligheid voor triazine herbiciden, door een veranderde gevoeligheid voor andere herbiciden. Dit is, tenminste in de tot nu toe onderzochte gevallen, veroorzaakt door een wijziging van één aminozuur in het lysine-vrije eiwit. In hoofdstuk 6 staat be-

schreven dat ook het redoxevenwicht tussen de semichinonvormen van Q_A en Q_B in deze triazine-resistente planten drastisch is gewijzigd, terwijl de affiniteit van Q_B voor zijn bindingsplaats niet veel is veranderd.

Hoewel meestal chinon/herbicide interacties alleen onder evenwichtsomstandigheden worden bestudeerd, is er in dit proefschrift, uitgaande van een -bij benadering- competitieve chinon/inhibitor interactie, een methode ontwikkeld om de snelheid van reacties van de inhibitor met de bindingsplaats te meten als functie van de redoxtoestand van de gebonden chinonen. Het blijkt dat de Q_A^--oxidatie-remmer *o*-phenanthroline veel slechter bindt als Q_B^- verdrongen moet worden dan wanneer Q_B gebonden was, hetgeen op grond van een al eerder gepostuleerde relatief zeer hoge affiniteit van Q_B^- voor het complex goed te begrijpen is. De snelheid waarmee *o*-phenanthroline de bindingsplaats verlaat blijkt overigens vrijwel onafhankelijk van de redoxtoestand van Q_A te zijn. Resultaten verkregen met de inhibitor *i*-dinoseb kunnen echter niet zonder meer met het opgestelde model begrepen worden (hoofdstuk 5). Voorts blijkt er tussen de verschillende groepen van stoffen die de Q_A^- oxidatie remmen grote verschillen te bestaan wat betreft de snelheid van binding. Sommige inhibitoren binden en verlaten hun bindingsplaats veel sneller dan andere (hoofdstuk 5).

Het tot zover besproken deel van het proefschrift heeft zich voornamelijk beziggehouden met de bindingsinteractie van Q_B en remstoffen van de Q_A^- oxidatie. Deze kennis zou o.a. gebruikt kunnen worden bij de ontwikkeling van zowel "safeners" (stoffen die de plant tegen onkruidbestrijdingsmiddelen kunnen beschermen) als andere typen van herbiciden (bijv. bepaalde chinonanalogen die hetzij nog minder schadelijk zijn voor mens en dier hetzij door de bodemflora beter kan worden afgebroken dan veel van de tegenwoordig gebruikte herbiciden).

Op dezelfde plaats als waar sommige herbiciden een remmende functie hebben, blijkt bicarbonaat (dat in chemisch evenwicht staat met CO_2) een stimulerende funktie te hebben (in ieder geval in de aanwezigheid van organische anionen zoals formiaat). Deze stimulering door bicarbonaat wordt het "bicarbonaateffekt" genoemd. Hoewel het duidelijk is dat bicarbonaat ook herbicidebinding modificeert, en daarom verwacht wordt aan of bij het lysine-vrije thylakoid eiwit te binden, is nog niet vast komen te staan hoe het bicarbonaat het elektronentransport via de chinonen reguleert. In hoofdstuk 7 is een aantal suggesties gedaan over mogelijke werkingsmechanismen. Dit bicarbonaateffekt kan *in vivo* een terugkoppelingsmechanisme vervullen, waarbij bij CO_2-gebrek ook het lineair elektronentransport wordt geblokkeerd waardoor een ongewenste NADPH(+ H^+) ophoping of

vorming van radicalen zoals O_2^- kan worden voorkomen. Het zij overigens opgemerkt dat afwezigheid van bicarbonaat niet alleen tot een remming van elektronentransport bij Q_B leidt, maar waarschijnlijk ook een chinoloxidatie aan de watersplitsende kant van Fotosysteem II remt.

Hoewel in dit proefschrift veel gegevens en analyses beschreven zijn die een basis kunnen vormen voor een goed begrip van het moleculair mechanisme van ligandbinding aan het eiwitcomplex bij Q_A en van elektronentransport van Q_A naar het niet-gebonden plastochinon, is de beschrijving van de binding van Q_B en inhibitoren nog vrij fenomenologisch. Een belangrijke bijdrage tot een verdere oplossing van dit probleem, dat (bio)fysische, (bio)chemische, fysiologische en genetische komponenten bevat, kan geleverd worden door nader interdisciplinair onderzoek.

Nawoord

(De Engelse versie van dit nawoord kan gevonden worden onder "Acknowledgements" op pag. 117).

Er zijn zoveel mensen die me op de één of andere manier geholpen hebben met het schrijven van dit proefschrift of met het kunnen uitvoeren van de hier beschreven experimenten, dat het vrijwel onmogelijk is om een ieder persoonlijk te bedanken zonder iemand te vergeten. Daarom zou ik graag zonder namen te noemen iedereen die op de één of andere manier heeft meegewerkt aan het tot stand komen van dit proefschrift erg hartelijk willen danken voor haar of zijn inzet. Ik denk dat allen hun best hebben gedaan, ieder op zijn terrein: sommigen kweekten planten, bouwden instrumenten, deden proeven, of maakten illustraties; anderen hebben me chemicaliën gestuurd, gegevens bediskussieerd, me met ideeën geïnspireerd, manuscripten getypt, of thee gezet. Ik waardeer het zeer.

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Curriculum vitae

Omdat het promotiereglement het voorschrijft, volgt hier in het kort mijn levensloop. Ik ben op 3 juni 1959 in Rhoon geboren, begon in 1970 de middelbare school aan de Christelijke Scholengemeenschap "Johannes Calvijn" in Rotterdam, en deed in 1976 eindexamen Gymnasium β. Daarna startte ik mijn studie Biologie aan de Landbouwhogeschool in Wageningen, haalde in 1979 het kandidaatsdiploma Biologie (niveau cel/subcellulair), en koos voor de ingenieursstudie de hoofdvakken Fysiologie der Planten (bij Dr. J.J.S. van Rensen en Prof. W.J. Vredenberg) en Moleculaire Fysica (bij Dr. G.H. van Brakel, Prof. T.J. Schaafsma en Dr. G.F.W. Searle). Van najaar 1980 tot voorjaar 1982 deed ik onderzoek aan aspekten uit het fotosynthetisch elektronentransport in de laboratoria van Prof. Govindjee (Departments of Physiology and Biophysics, and Botany; University of Illinois, Urbana/Champaign, Illinois, USA) en Prof. C.J. Arntzen (MSU/DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan, USA). Een deel van dit promotieonderzoek is ook op deze laboratoria verricht. Na het officieel afstuderen in voorjaar 1982 was ik voor 3 maanden werkzaam bij de vakgroep Plantenfysiologisch Onderzoek van de Landbouwhogeschool, daarna vertrok ik voor 10 maanden naar het laboratorium van Prof. G. Renger (Max-Volmer-Institut für biophysikalische und physikalische Chemie, Technische Universität, Berlin, Duitsland) om het onderzoek over plastochinon/inhibitor interacties voort te zetten. Sinds augustus 1983 ben ik weer tijdelijk werkzaam bij de vakgroep Plantenfysiologisch Onderzoek (LH-Wageningen).