Regulation of photosynthetic electron flow in isolated chloroplasts by bicarbonate, formate and herbicides

Regulering van fotosynthetisch elektronentransport in geïsoleerde chloroplasten door bicarbonaat, formiaat en herbiciden

Promotor: dr. W. J. Vredenberg
hoogleraar in de plantenfysiologie,
met bijzondere aandacht voor de fysische aspekten

Co-promotor: dr. ir. J. J. S. van Rensen
wetenschappelijk hoofdmedewerker

J. F. H. Snel

Regulation of photosynthetic electron flow in isolated chloroplasts by bicarbonate, formate and herbicides

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C. C. Oosterlee,
in het openbaar te verdedigen
op woensdag 26 juni 1985
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

ISN = 227658-03

Stellingen

1. De procedure die door Badger en Canvin gebruikt is om het in dit proefschrift beschreven bicarbonaateffekt op het elektronentransport in fotosysteem II aan te tonen, is daarvoor inadequaat.

   Badger, M.R., D.T. Canvin. 1981. Oxygen uptake during photosynthesis in C_3 and C_4 plants. In: Photosynthesis IV: Regulation of Carbon Metabolism (G. Akoyunoglou, ed.), pp. 151-161, Balaban International Science Services, Philadelphia, Pa.

2. Op grond van de in dit proefschrift beschreven effekten van formiaat op het fotosynthetisch elektronentransport kan deze verbinding aangeduid worden met de term "inhibitory uncoupler", zoals deze door Moreland voor herbiciden is geïntroduceerd.

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

   Dit proefschrift, hoofdstuk 4.

3. Het onvermeld laten van het belang van de adenylaatkinase aktiviteit bij het bepalen van de flits-geïnduceerde ATP synthese leidt gemakkelijk tot de misvatting dat deze adenylaatkinase aktiviteit niet relevant is voor de betreffende meting.

   Schreiber, U., S. Del Valle-Tascon. 1982. ATP synthesis with single turnover flashes in spinach chloroplasts. FEBS lett. 150: 32-37.

4. Bij de interpretatie van de kinetiek van de licht-geïnduceerde potentiaalverschillen over het thylakoidmembraan in intacte chloroplasten dient ook de invloed van de fosfaatpotentiaal, en daarmee het koolzuurmetabolisme, in beschouwing genomen te worden.

   Bulychev, A.A. 1984. Different kinetics of membrane potential formation in dark-adaptated and pre-illuminated chloroplasts. Biochim. Biophys. Acta 766: 647-652.

5. De veronderstelling van Schuurmans dat de flits-geïnduceerde ladingsscheiding in chloroplasten pas na enkele millisekonden resulteert in een gedelokaliseerde transmembraanpotentiaal is niet houdbaar.

   Schuurmans, J.J. 1984. Dynamic aspects of photosynthetic energy transduction. Dissertatie, Vrije Universiteit, Amsterdam.

   Van Kooten, O., F.A.M. Leermakers, R.L.A. Peters, W.J. Vredenberg. 1984. Indications for the chloroplast as a tri-compartment system: micro-electrode and P515 measurements imply semi-localized chemiosmosis. in: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol. II, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague.

6. De optische en elektrische eigenschappen van de nieuwe typen lichtgevende dioden (LED) maken deze LED's bij uitstek geschikt voor gebruik als moduleerbare lichtbron in het fotobiologisch onderzoek.

   Ruraïnski, H.J., G. Mader. 1980. Light-emitting diodes as a light source in photochemical and photobiological work. in: Methods in Enzymol., Vol. 69, pp. 667-675.

7. De export van, in het westen verboden, herbiciden (o.a. DDT) naar de landen van de derde wereld is een onverteerbare zaak.

8. De grote rol die het toevalselement speelt in het praktisch gedeelte van het huidige rijexamen vraagt om het opnemen van een additionele rijvaardigheidstest in een rit-simulator.

9. De simpele visie van de nederlandse overheid op mogelijke consequenties van de introduktie van het luchtafweersysteem "Patriot" op het machtsevenwicht in Europa staat in schril contrast tot de gecompliceerde aard van dit wapensysteem.

   Eerste situatierapport over de vervanging van het Nike luchtverdedigingssysteem. Tweede Kamer, vergaderjaar 1983-1984, 18000 hoofdst X, nr 17.

10. De mening van Chodorow dat door een grotere betrokkenheid van beide seksen bij de opvoeding van kinderen de maatschappelijke verschillen tussen mannen en vrouwen verkleind kunnen worden, verdient een grotere aandacht in het sociale overheidsbeleid.

    Chodorow, N. (1980). Waarom vrouwen moederen. Feministische Uitgeverij Sara, Amsterdam.

J.F.H. Snel
Regulation of photosynthetic electron flow in isolated chloroplasts by bicarbonate, formate and herbicides
Wageningen, 26 juni 1985

CONTENTS

VOORWOORD

Veel personen hebben op de één of andere manier bijgedragen aan het tot stand komen van dit proefschrift; hen wil ik hier bedanken voor hun medewerking. Een aantal van hen heeft echter een zo duidelijk stempel gedrukt op dit proefschrift, dat ik de bijdragen van deze mensen graag apart wil vermelden.

Allereerst wil ik hier de rol vermelden van mijn co-promotor, Jacques van Rensen, zonder wiens inspanningen dit onderzoek zeker niet tot stand gekomen zou zijn. Beste Jacques, jouw gedegen aanpak, jouw kritische opmerkingen en prettige begeleiding hebben er mede voor gezorgd dat het onderzoek voorspoedig verlopen is. Ook de rol van mijn promotor, Wim Vredenberg, mag hier niet onvermeld blijven. Beste Wim, tijdens het verloop van het onderzoek, maar zeker ook bij het tot stand komen van dit proefschrift, heb jij mij gedwongen om de resultaten en interpretaties daarvan kritisch te bekijken en nauwkeurig te formuleren.

Verder wil ik hier ook de bijdragen van "mijn" doctoraal studenten vermelden. André, jij was de eerste en je hebt je met veel enthousiasme gestort op het werken met "levend" materiaal en jouw onderzoek heeft geleid tot een beter begrip van de rol van bicarbonaat en formiaat. Dirk, jouw onderzoek met intacte chloroplasten toonde, voor het eerst, aan dat het "bicarbonaat-effect" ook in intacte chloroplasten optreedt. Free, jij hebt geprobeerd de interactie(s) tussen bicarbonaat, formiaat en herbiciden verder op te helderen, hetgeen je slechts ten dele gelukt is in de korte tijd die je er aan kon besteden.

Olaf, Rob en ook, gedurende korte tijd, Wim Vermaas, jullie hebben er door jullie enthousiasme en stimulerende discussies een leuke en vruchtbare tijd van gemaakt.

Als laatste wil ik nog de medewerksters van de afdeling Tekstverwerking, met name Marjon van Hunnik, bedanken voor de vlotte en accurate verwerking van dit proefschrift.

LIST OF ABBREVIATIONS, SYMBOLS AND TRIVIAL NAMES

A_{518}	absorbance at 518 nm
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
ATP-ase	ATP-hydrolase/synthase
atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine
azido-atrazine	2-azido-4-(ethylamino)-6-(isopropylamino)-*s*-triazine
BQ	1,4-benzoquinone
BSA	bovine serum albumine
Chl	chlorophyll
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DHAP	dihydroxyacetone phosphate
Diuron	see DCMU
DNOC	dinitro-*o*-cresol
DTE	1,4-dithioerythritol
E_m	midpoint redox potential
EDTA	ethylenediaminetetraacetate
EPR	electron paramagnetic resonance
F6P	fructose-6-phosphate
FBP	fructose-1,6-bisphosphate
FBP-ase	fructose-1,6-bisphosphate phosphatase
FeCy	potassium ferrihexacyanate
FNR	ferredoxin-$NADP^+$ oxidoreductase
G1P	glucose-1-phosphate
ΔG_{ATP}	phosphoryl group transfer potential of ATP formation reaction
GAL3P	glyceraldehyde-3-phosphate
GMCD	gramicidin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i-dinoseb	2,4-dinitro-6-isobutylphenol

K_d	dissociation constant
K_i	inhibitor constant
K_i'	apparent inhibitor constant
K_m	Michaelis constant
K_r	reactivation constant
K_r'	apparent reactivation constant
kD	kilodalton
MES	2-(N-morpholino)-ethanesulfonic acid
MV	methylviologen
NADP	nicotinamide-adeninedinucleotide (oxidized)
NADPH	nicotinamide-adeninedinucleotide (reduced)
n_H	Hill coefficient
OEC	oxygen evolving complex
P_{430}	secondary acceptor of photosystem I
P_{680}	primary electron donor of photosystem II
P_{700}	primary electron donor of photosystem I
P_i	orthophosphate
PGA	3-phosphoglycerate
P-glycolate	2-phosphoglycolate
Pheo	pheophytin
pmf	proton motive force
PQ	plastoquinone
PQH_2	plastoquinol
PSI	photosystem I
PSII	photosystem II
Q_A	primary acceptor plastoquinone of photosystem II
Q_B	secondary acceptor plastoquinone of photosystem II
Q_B-protein	Q_B-binding protein
RPP-pathway	reductive pentose phosphate pathway
RU5P	ribulose-5-phosphate
RUBISCO	ribulose-1,5-bisphosphate carboxylase/ oxygenase
RUBP	ribulose-1,5-bisphosphate
simeton	2-methoxy-4,6-bis(ethylamino)-1,3,5-triazine

tricine	N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl) glycine.
V_{Hill}	Hill reaction rate
V_{max}	maximal Hill reaction rate
Z	secondary donor of photosystem II
↑	Small arrows mark the point of time at which a single turn-over actinic light flash is given.
⇧	Open arrows indicate the point of time at which continuous actinic light is switched on.
⬇	Closed arrows indicate the point of time at which the continuous actinic light is turned off.

CHAPTER 1

GENERAL INTRODUCTION

This Chapter presents a brief survey of some aspects of photosynthesis in plants. Only the aspect that are of relevance for a good comprehension of the effects of bicarbonate, formate and herbicides on plant photosynthesis will be covered. In this Chapter the reader will be referred to recent review articles for more detailed information.

Photosynthesis in plants

In plant photosynthesis carbon dioxide is fixed in the form of carbohydrates. Starch is the major storage product. In higher plants this process takes place in special organelles, the chloroplasts. A chloroplast consists of two compartments: the stroma matrix and the thylakoid lumen. The stroma matrix is bound from the cytoplasm by two closely associated membranes, the chloroplast envelope, and separated from the thylakoid lumen by the thylakoid membrane (1).

In the stroma matrix the reduction of CO_2 to sugar phosphates and the subsequent conversion into starch takes place. These processes, which proceed in the dark, require reducing equivalents in the form of NADPH and chemical energy in the form of ATP. NADPH and ATP are supplied to the stroma by light-driven processes which take place at the thylakoid membrane. These processes serve the reduction of $NADP^+$ and the phosphorylation of ADP. All enzymes required for the reduction of CO_2 are located in the stroma.

The thylakoid membrane contains the apparatus necessary for the conversion of light energy into chemical energy (ATP) and reducing equivalents (NADPH). Light is absorbed by the photosynthetic antenna pigment systems and the associated reaction centers and its energy is used to withdraw electrons from water. The electrons are transferred to $NADP^+$ via an electron transfer chain. Electron transport from water to $NADP^+$ is associated with proton translocation across the thylakoid membrane resulting in a proton electro-chemical potential difference across the thylakoid membrane. This proton potential is the driving force for the phosphorylation of ADP to ATP. Posphorlylation is catalyzed by the H^+-ATP-ase, which is a membrane-spanning protein complex present in the thylakoid membrane.

Photosynthetic carbon metabolism

CO_2-reduction in plants

Chloroplasts are able to reduce CO_2 to sugar phosphates in the Reductive Pentose Phosphate-pathway (RPP-pathway, other names are: Calvin cycle, Benson-Calvin cycle, Photosynthetic Carbon Reduction cycle). The enzymes for the RPP pathway are located in the chloroplast stroma. The RPP pathway can be divided in three stages (2):

a. fixation of CO_2. A single CO_2-acceptor molecule, ribulose-1,5-bisphosphate (RUBP), is carboxylated yielding two molecules of 3-phosphoglycerate (PGA). This reaction is catalyzed by the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO). This enzym also catalyzes the oxygenation of RUBP.
b. reduction of PGA. The next step is the reduction of PGA to triosephosphates, mainly dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAL3P). This process requir-

es ATP and NADPH. The overall reduction of PGA to triose phosphate is highly reversible and subject to control by energy charge and concentration of end products.

c. regeneration of RUBP. Finally triosephosphates are converted to RUBP in a complicated series of reactions. In this conversion five molecules of triosephosphate yield three molecules of ribulose-5-phosphate (RP5P). RU5P is phosphorylated by ATP forming RUBP.

For every three molecules of CO_2 fixed one molecule triosephosphate is formed. This triose phosphate can be used to store carbon in the form of starch, but it can also be used to increase the concentration of intermediates in the RPP-pathway. In this way the RPP-pathway becomes auto-catalytic: by fixation of CO_2 the concentration of acceptor molecules (RUBP) is increased which in turn will increase the rate of fixation of CO_2. The auto-catalytic nature of the RPP-pathway allows a rapid response from a dark-situation, where the concentration of intermediates is low, to a state in the light where CO_2-fixation can proceed rapidly (2).

In several plant species an additional CO_2-fixation mechanism exists, but this mechanism only has as a temporary storage function. Ultimately CO_2 is fixed and reduced in the RPP-pathway. For more details the reader is referred to e.g. ref. 4.

Apart from starch and glucose, triosephosphates appear to be a major end product of photosynthetic carbon reduction in chloroplasts. According to Heber and Heldt (3) the chloroplast might be considered as an organelle that imports phosphate and exports triosephosphate (mainly DHAP). Both import of phosphate and export of triosephosphate are catalyzed by the same (exchange) carrier: the phosphate translocator. This enzyme which is located in the envelope, catalyzes the exchange of 1 triose phosphate for 1 phosphate (see ref. 3 for more details).

CO_2-fixation through the RPP-pathway is regulated by many factors (see e.g. refs. 3, 4 for reviews). One of these fac-

tors is of special interest with respect to the topic of this thesis. This factor is the regulation of carbon flow through the RPP-pathway by pH-dependent modulation of the activity of several key enzymes (3, 4). Salts of weak acids e.g. acetate, formate, bicarbonate and glyoxylate have been shown to affect the pH in the stroma (5, 6). Thus at neutral or moderately alkaline pH values of the suspension medium these weak acids, can inhibit carbon reduction.

Photorespiration

At a high O_2/CO_2 ratio RUBISCO functions as an oxygenase instead of a carboxylase; in fact CO_2 and O_2 compete for the same binding site at the enzyme (7). When RUBP is oxygenated PGA and phosphoglycolate are the reaction products. After dephosphorylation of phosphoglycolate in the stroma, glycolate can be oxidized in the peroxisome. This oxidation is catalyzed by glycolate oxidase and the products are glyoxylate and H_2O_2 (8). Under normal conditions two molecules of glyoxylate are converted to 1 molecule of CO_2 and 1 molecule of glycerate in a series of peroxisomal and mitochondrial reactions (8). Under conditions in which the conversion of glyoxylate to glycerate is blocked, glyoxylate can react with H_2O_2, yielding formate and CO_2 (9). Although the reactions involved in photorespiration have been characterized, the physiological function of photorespiration is still poorly understood (8, 9, 10).

Photosynthetic electron flow in plants

Most components of the photosynthetic electron transport chain in higher plants and eucaryotic algae are located within three major protein complexes that are embedded in the thylakoid membrane (11, 12). Two of these protein complexes contain chlorophyll and are known as the PSI and the PSII protein com-

plexes. The third major protein complex is known as the cytochrome b_6-f complex. These complexes are intrinsic, membrane-spanning, thylakoid membrane protein complexes (11, 12). Electron flow between these major protein complexes is mediated by the smaller electron carriers plastoquinone (actually a hydrogen carrier), plastocyanin and ferredoxin (11). These mobile carriers are located either within the lipid bilayer (plastoquinone) or at its surface (plastocyanin and ferredoxin), see e.g. ref. 11.

For the sake of conciseness this section will deal further only with those aspects of photosynthetic electron flow that are relevant to the topic of this thesis. The reader is referred to ref. 13 for several recent surveys of other interesting aspects of photosynthetic electron flow.

Electron flow in photosystem II

Light absorbed by chlorophylls located in the antenna of PSII is transferred to the reaction center chlorophyll of PSII (P_{680}) in the form of excitons (14). The primary event of electron flow in PSII is the generation of the singlet excited state (P_{680}^*) upon arrival of an excition. P_{680}^* can donate its electron via at least one intermediate acceptor, pheophytin (16), to the first "stable" electron acceptor of PSII, Q_A, creating the state $[P_{680}^+ \cdot \text{Pheo}] \cdot Q_A^-$ within 2 ns after excitation (15, 16). As P_{680} and Q_A are located at opposite sides of the thylakoid membrane, electron transfer from P_{680} to Q_A is accompanied by a transmembrane charge separation (17). Recombination of charges in the state $[P_{680}^+ \cdot \text{Pheo}] \cdot Q_A^-$ is normally prevented by reduction of P_{680}^+ by the secondary donor of PSII, Z. This electron donation to P_{680}^+ occurs within several μs (15), which is much faster than the rate of recombination in the state $[P_{680}^+ \cdot \text{Pheo}] \cdot Q_A^-$ (15).

After donation of an electron to P_{680}^+, Z is left in the state Z^+. Z^+ can be reduced by the oxygen evolving complex

(OEC) which ultimately derives its electrons from H_2O. In the OEC water is decomposed into protons, which are liberated in the thylakoid lumen (17), molecular oxygen and electrons. According to the Kok-scheme, the OEC can be in one of five different oxidation states, generally known as the s-states (18). The overall rate constant for oxygen evolution has been reported to be around 1000 s^{-1} (18). One important feature of the Kok-scheme is that all OEC's are assumed to operate independently from each other, i.e. no charge transfer occurs between OEC's (18).

After the reduction of Pheo by P_{680}^{*}, Q_A is reduced by Pheo within 200 ps (15, 16). Upon reduction Q_A^-, a special plastoquinone that accepts only one electron, stays in the semiquinone anion form (Q_A^-) for 0.2 ms to 1.2 ms, depending on the redox state of the secondary electron acceptor Q_B (19). Q_A^- is normally not protonated (21). Q_B, which is also a special plastoquinone, appears to function as a two-electron gate (19, 20). Only after receiving a second electron from Q_A, Q_B^- is fully reduced to Q_B^{2-} and becomes protonated (21). These protons ultimately come from the stroma of the chloroplast. Q_B appears to be a bound plastoquinone species. In the oxidized and in the fully reduced form Q_B is assumed to equilibrate rapidly with the plastoquinones that reside in the lipid bilayer; the semi-reduced forms of Q_B (Q_B^- and, perhaps, Q_B^{2-}) are firmly bound to a binding site at the PSII protein complex (21). The identity of the Q_B-binding site is not exactly known; probably a 32 kD protein, the Q_B-protein, is involved (12). The overall half-time for plastquinone (PQ) reduction is about 2-3 ms (11). Proton uptake, as measured by alkalinization of the outer aqueous phase, is however much slower (17), an enigma which has not been solved yet. For more details about the reactions involving quinones the reader is referred to e.g. ref. 22. For auxillary electron acceptors and donors of PSII that appear to be present, some under special conditions, see refs. 23 and 18.

Electron flow from PSII to $NADP^+$

Freely diffusing plastoquinol (PQH_2) arises from the dissociation of Q_BH_2, a plastoquinol species bound to the Q_B-protein. PQH_2 can bind to the cytochrome b_6-f complex which is a plastoquinol-plastocyanin oxidoreductase (see ref. 19 for a review). After oxidation of PQH_2 the protons are released in the thylakoid lumen (17). The rate of oxidation of PQH_2 and the concomittent release of protons appear to be pH-dependent; the oxidation of PQH_2 is the major rate-determining step in photosynthetic electron flow with a half-time of about 15 ms (11). There is a pool of about 7 freely diffusing PQ molecules per electron transfer chain (11).

Plastocyanin, a copper containing protein located at the inner surface of the thylakoid membrane, is the immediate donor to P_{700}, the reaction center chlorophyll of PSI (11).

Excitation of P_{700} and subsequent electron transfer to P_{430}, an optically detected acceptor species, results in a transmembrane charge separation (17). P_{430}, the secondary electron acceptor of PSI may be a bound ferredoxin species (11).

Freely diffusing ferredoxin, reduced by PSI, can be oxidized in, at least, three ways (24):

a. Ferredoxin can be oxidized by $NADP^+$ via the enzyme ferredoxin-$NADP^+$ oxidoreductase (FNR). Electron flow from water to $NADP^+$ is named linear electron flow.
b. Ferredoxin can be oxidized by oxygen in a non-enzymatic reaction. Electron flow from water to O_2 is named pseudo-cyclic electron flow.
c. Ferredoxin can be oxidized by components of the cytochrome b_6-f complex, resulting in a cyclic electron flow around PSI without reduction of $NADP^+$ but associated with proton translocation across the thylakoid membrane.

Artificial electron donors and acceptors

A variety of artificial electron acceptors and electron donors have been used, sometimes in combination with inhibitors of electron flow, to characterize various parts of the electron transport chain. In this thesis only the electron acceptors ferricyanide (FeCy), benzoquinone (BQ) and methylviologen (MV) were used. MV and FeCy have been reported to accept electrons mainly from PSI, while BQ can accept electrons from the intersystem electron transport chain. A detailed review on artificial electron donors and acceptors is given in ref. 25.

Energy conversion by thylakoids

Photosynthetic electron transport in thylakoids is coupled to phosphorylation of ADP ("photophosphorylation"). Under steady state conditions the mechanism of coupling of electron flow to photophosphorylation appears to be explained best by the chemiosmotic theory postulated by Mitchell (26). According to this theory the electrochemical proton gradient across the thylakoid membrane, generated by electron flow, is assumed to be the high-energy intermediate that drives the phosphorylation of ADP.

The electrochemical proton gradient, or proton motive force (pmf), contains two components: the transmembrane proton gradient and the electrical potential difference across the thylakoid membrane. The transmembrane potential is generated by the charge separations in PSI and PSII (17) and perhaps also in the cytochrome b_6-f complex (20). The proton gradient is caused by the liberation of protons in the thylakoid lumen during water oxidation in the OEC and the translocation of protons across the thylakoid membrane accompanying the reduction and subsequent oxidation of PQ. The transmembrane potential is dissipated by the movement of ions across the thylakoid membrane (27); during continuous illumination the membrane potential in

thylakoids has been reported to be only a few millivolts (27, see however ref. 17). Much more important for photophosphorylation under steady state conditions is the proton gradient across the thylakoids membrane (17). During continuous illumination the contribution of the proton gradient to the proton motive force may be more than 95% (17). Under non-phosphorylating conditions the proton flux across the thylakoid membrane is small compared to the fluxes of other ions and therefore protons hardly contribute to the dissipation of the membrane potential (17). However when photophosphorylation is taking place, the additional proton flux through the ATP-ase does contribute significantly to the dissipation of the transmembrane potential (17). The dissipation of the electrical field can be greatly stimulated by ionophores and protonophores. The proton gradient can also be dissipated, both by protonophores and permeant buffers.

Role of bicarbonate in photosynthesis

Properties of bicarbonate in aqueous solutions

When bicarbonate is dissolved in an aqueous solution, the following reactions occurs (28):

$$2H^+ + CO_3^{2-} \underset{(1)}{\rightleftharpoons} H^+ + HCO_3^- \underset{(2)}{\rightleftharpoons} H_2CO_3 \underset{(3)}{\rightleftharpoons} H_2O + CO_2$$

$$(4)\ \downarrow\uparrow$$

$$CO_2 + OH^-$$

The reactions (1) and (2), which involve protonation, are very fast and for our purposes only the equilibrium constants need to be considered. Reactions (3) and (4) are much slower; dissociation of H_2CO_3 to H_2O and CO_2 occur with a rate constant of 20 s^{-1} (28). However, when bicarbonate is dissolved, the combined half-times of reactions (3) and (4) (in series) and

reaction (4) yield an overall half-time for the conversion of HCO_3^- to CO_2 of ~ 15 s at pH 7.4. This half-time is pH-dependent; at pH 5 it is only 0.9 s (28). The pK of reaction 1 is 10.35 and the pK of the combined reactions 2, 3 and 4 is 6.35.

A main part of this thesis deals with experiments in which reactions of isolated thylakoids were studied studied in dependence of $[CO_2]$ and $[HCO_3^-]$ in the reaction medium. As in most experiments carried out CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} were in equilibrium with each other, the term bicarbonate will be used in this thesis to indicate properties or effects of a solution to which bicarbonate is added, without implying that the bicarbonate ion is the species responsable for the observed effects.

Bicarbonate effects on processes involved in photosynthesis

Bicarbonate is involved in the proces of photosynthesis in several ways :

1. CO_2 is the substrate for the carboxylation of RUBP, the CO_2-fixing reaction of photosynthesis (7).
2. CO_2 is required to activate RUBISCO, which catalyzes the carboxylation of RUBP (7).
3. Bicarbonate can inhibit the reduction of CO_2 in the RPP pathway by dissipation of the proton gradient across the chloroplast envelope (5, 6).
4. Bicarbonate is a weak uncoupler of photosynthetic electron flow (29).
5. Bicarbonate can stimulate photophosphorylation in broken chloroplasts. This appears to be due to an effect on the conformation of the chloroplast ATP-ase (32).
6. Bicarbonate stimulates electron flow in isolated broken chloroplasts. Although stimulation can be observed in non-treated chloroplasts (31), the most dramatic effects of bicarbonate on electron flow are observed when the chloroplasts have been depleted of CO_2 in a pretreatment (30).

This thesis will mainly deal with the effects of bicarbonate on electron flow as outlined in item 6, although also items 3, 4 and 5 will be briefly discussed. Therefore a more detailed description of the effects of bicarbonate on photosynthetic electron flow is appropriate here.

Bicarbonate effects on photosynthetic electron flow

Basically two different stimulating effects of bicarbonate on photosynthetic electron flow have been described.

1. Bicarbonate effects in isolated non-treated chloroplasts.
   Addition of bicarbonate to non-CO_2-depleted chloroplasts can result in a stimulation of the Hill reaction (31). This stimulation requires relatively high concentrations of bicarbonate and the magnitude of the stimulation is relatively small, usually less than 100%. Stimulation of the Hill reaction is thought to be due to inhibition of a cyclic electron flow around PSII, increasing the efficiency of linear electron flow from PSII to PSI or artificial electron acceptors (31).
2. Bicarbonate effects in CO_2-depleted chloroplasts.
   In contrast with the bicarbonate effects electron flow mentioned above, the effects described here require much lower bicarbonate concentrations and the magnitude of the stimulation of electron flow is much higher; usually the rate in the presence of bicarbonate is more than 5 times higher than in the absence of bicarbonate.

   Since the discovery of the stimulating effect of bicarbonate on the Hill reaction in isolated chloroplasts by Warburg and Krippahl in 1958 (33), considerable efforts have been made to unravel the mechanism of bicarbonate action on photosynthetic electron flow. The results of these efforts can be summarized as follows (see also refs. 36; an alternative interpretation is given by ref. 34).

- CO_2-depletion of isolated broken chloroplasts results in a strong inhibition of electron flow. The CO_2-depletion method is based on dark-incubation of broken chloroplasts in a medium containing high anion concentrations at low pH in the absence of CO_2 (35).
- Dark-incubation of CO_2-depleted chloroplasts with bicarbonate results in binding of bicarbonate to the chloroplasts and in an increased capacity to reduce artificial electron acceptors. Actually two types of bicarbonate-binding sites were described, but only the high-affinity binding sites were correlated with stimulation of electron flow (37).
- In CO_2-depleted chloroplasts electron flow appears to be inhibited between Q_A and PQ (38). Although minor effects on the oxidizing side of PSII have been reported (39), there is evidence that these effects may partly be due to interactions between the reducing and the oxidizing side of PSII (36). In the absence of bicarbonate the oxidation of Q_A^- is slowed down considerably (38), but the major site of inhibition appears to be on the protonation of Q_B^{2-} and/or the exchange Q_B of with the PQ pool (40).
- The actual species involved in the stimulation of electron flow in CO_2-depleted chloroplasts is not known. Both bicarbonate and CO_2 have been suggested to be the active species. A special problem is that the bicarbonate binding site may be located in the vicinity of negative charges at the thylakoid surface (41), causing a change in the local pH and therefore a shift in the equilibruim concentrations of HCO_3^-, CO_2 and CO_3^{2-} near the membrane surface.
- The stimulation of the Hill reaction in CO_2-depleted chloroplasts by bicarbonate appears to obey kinetics that resemble Michaelis Menten kinetics; a double reciprocal plot of the Hill reaction rate vs. the bicarbonate concentration yields straight lines (42). From these plots an apparent K_m of about ~ 1 mM was calculated (42).

- The bicarbonate effects on electron flow appear to interfere with the action of herbicides on electron flow (see next section).

Mechanism of herbicide action on electron flow at the acceptor side of photosystem II.

Several classes of herbicides have been shown to inhibit electron flow at the acceptor side of PSII; in this thesis these herbicides will be called PSII herbicides (see refs. 43 and 48 for a classification of herbicides).

The PSII herbicides inhibit the oxidation of Q_A^-. It was suggested by Velthuys and Amesz (44) that binding of e.g. DCMU (diuron) to PSII affects the E_m of the redox couple Q_B/Q_B^- in an allosteric way. The E_m was sugested to be decreased upon binding of DCMU, i.e. the equilibrium of the reaction $Q_A^- \cdot Q_B \rightleftarrows Q_A \cdot Q_B^-$ is shifted to the left. Reversed electron flow from Q_B^- to Q_A was observed when DCMU was added to chloroplasts in the state $Q_A \cdot Q_B^-$ (44).

Several classes of PSII herbicides have been shown to bind to a common binding site at the thylakoid membrane. This was judged from experiments which showed that a bound radio-active labeled herbicide could be displaced from the binding site by another, unlabeled herbicide (45). Using the technique of photo-affinity labeling, it was shown that an atrazine analog, azido-atrazine, was specifically bound to a polypeptide of 32 kD molecular weight (46).

As both PSII herbicides and CO_2-depletion cause inhibiton of electron flow at the acceptor side of PSII, it has been suggested that the modes of action of herbicides and bicarbonate might have a common base. It was shown that in the presence of PSII herbicides the affinity of thylakoid membranes for bicarbonate was decreased by at least a factor 2 (42). Moreover CO_2-depletion of chloroplasts caused a decrase in affinity of the chloroplasts for atrazine and this effect of CO_2-depletion was abolished after readdition of bicarbonate (47). These experi-

ments suggest that the modes of action of bicarbonate and herbicides may have a common base.

Objectives of this thesis

This thesis deals with some aspects of regulation of photosynthetic electron flow in plants. The main objectives of this thesis is to get a deeper insight into the mechanism and physiological significance of the regulation of electron flow by bicarbonate, formate and, to some extent, herbicides.

References

1. Coombs, J., A.D. Greenwood. 1976. *In*: The intact chloroplast. (J. Barber, ed.), pp. 1-51, Elsevier/North-Holland Biomedical Press, Amsterdam.
2. Edwards, G., D.A. Walker. 1983. C_3, C_4: mechanisms, cellular and environmental regulation of photosynthesis. Blackwell Scientific Publications, Oxford.
3. Heber, U., H.W. Heldt. 1981. Ann. Rev. Plant Physiol. 32: 139-168.
4. Bassham, J.A., B.B. Buchanan. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol II, pp. 141-191, Academic Press, New York.
5. Enser, U., H.W. Heber. 1980. Biochim. Biophys. Acta 592: 577-591.
6. Werdan, K., H.W. Heldt, M. Milovancev. 1975. Biochim. Biophys. Acta 396: 276-292.
7. Lorimer, G.H. 1981. Ann. Rev. Plant Physiol. 32: 349-384.

8. Tolbert, N.E. 1981. Ann. Rev. Biochem. 50: 133-157.

9. Ogren, W.L., R. Chollet. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. II, pp. 191-230, Academic Press, New York.

10. Lorimer, G.H., T.J. Andrews. 1981. *In*: The Biochemistry of Plants; Vol. 8 (M.d. Hatch, N.K. Boardman, eds.), pp. 329-374, Academic Press, New York.

11. Haehnel, W. 1984. Ann. Rev. Plant Physiol. 35: 659-693.

12. Kaplan, S., C.J. Arntzen. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. I, pp. 67-151, Academic Press, New York.

13. Govindjee (ed.). 1982. Photosynthesis, Vols. I and II, Academic Press, New York.

14. Pearlstein, R.M. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. I, pp. 293-331, Academic Press, New York.

15. Parson, W.W. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. I, pp. 331-386, Academic Press, New York.

16. Klimov, V.V. 1984. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol. I, M. Nijhoff/Dr. W. Junk Publishers, The Hague.

17. Junge, W., J.B. Jackson. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. I, pp. 589-647, Academic Press, New York.

18. Wydrzynski, T.J. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol I, pp. 470-507, Academic Press, New York.

19. Cramer, W.A., A.R. Crofts. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. I, pp. 389-468, Academic Press, New York.

20. Velthuys, B.R. 1980. Ann. Rev. Plant Physiol. 31: 545-567.

21. Wraight, C.A. 1982. *In*: Function of Quinones in Energy conserving systems (B.L. Trumpower, ed.), pp. 181-197, Academic Press, New York.

22. Trumpower, B.L. (ed.). Function of Quinones in Energy conserving systems, Academic Press, New York.

23. Vermaas, W.F.J., Govindjee. 1981. Photochem. Photobiol. 34: 775-793.

24. Hall, D.O. 1976. *In*: The Intact Chloroplast (J. Barber, ed.), pp. 135-170, Elsevier/North-Holland Biomedical Press, Amsterdam.

25. Trebst, A. 1972. *In*: Methods in Enzymology, Vol XXIV, Photosynthesis and Nitrogen Fixation, Part B (A. San Pietro, ed.), Academic Press, New York.

26. Ort, D.R., B.A. Melandri. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. I, pp. 539-588, Academic Press, New York.

27. Vredenberg, W.J. 1976. *In*: The Intact Chloroplast (J. Barber, ed.), pp. 53-88, Elsevier/North-Holland Biomedical Press, Amsterdam.

28. Walker, N.A., F.A. Smith, I.R. Cathers. 1980. J. Membrane Biol. 57: 5158.

29. Good, N.E. 1962. Arch. Biochem. Biophys. 96: 653-661.

30. Govindjee, J.J.S. van Rensen, 1978. Biochim. Biophys. Acta 505: 183-213.

31. Barr, R., R. Melhem, A.L. Lezotte, F.L. Crane. 1980. J. Bioenerg. Biomembr. 12: 197-203.

32. Nelson, N., N. Nelson, E. Racker. 1972. J. Biol. Chem. 247: 6506-6510.

33. Warburg, O., G. Krippahl. 1958. Z. Naturforsch. 15B: 509-514.

34. Stemler, A. 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. II, pp. 513-540, Academic Press, New York.

35. Stemler, A., Govindjee, 1973. Plant Physiol. 52: 119-123.

36. Vermaas, W.F.J., Govindjee, 1982. *In*: Photosynthesis (Govindjee, ed.), Vol. II, pp. 541-558, Academic Press, New York.

37. Stemler, A. 1977. Biochim. Biophys. Acta 545: 36-45.

38. Govindjee, M.P.J. Pulles, R. Govindjee, H.J. van Gorkom, L.N.M. Duysens. 1976. Biochim. Biophys. Acta 449: 602-605.

39. Stemler, A. 1980. Biochim. Biophys. Acta 593- 103-112.

40. Vermaas, W.F.J., Govindjee, 1982. Biochim. Biophys. Acta 680: 202-209.

41. Vermaas, W.F.J., J.J.S. van Rensen. 1981. Biochim. Biophys. Acta 636: 168-174.

42. Van Rensen, J.J.S., W.F.J. Vermaas. 1981. Physiol. Plant 51: 106-110.

43. Moreland, D.E. 1980. Ann. Rev. Plant Physiol. 31: 597-638.

44. Velthuys, B.R., J. Amesz. 1974. Biochim. Biophys. Acta 333: 85-94.

45. Tischer, W., H. Strotmann. 1977. Biochim. Biophys. Acta 460: 113-125.

46. Gardner, G. 1981. Science 211: 937-940.

47. Khanna, R., K. Pfister, A. Keresztes, J.J.S. van Rensen, Govindjee. 1981. Biochim. Biophys. Acta 634: 105-116.

48. Van Rensen, J.J.S., 1982. Physiol. Plant. 54: 515-521.

CHAPTER 2

KINETICS OF THE REACTIVATION OF THE HILL REACTION IN CO_2-DEPLETED CHLOROPLASTS BY ADDITION OF BICARBONATE IN THE ABSENCE AND IN THE PRESENCE OF HERBICIDES

Introduction

Carbon dioxide (or bicarbonate) is required for photosynthetic electron transport between Photosystem II and Photosystem I in isolated broken chloroplasts. The major effect of bicarbonate on electron transport is located between the reoxidation of the first stable Photosystem II acceptor Q_A and the reduction of the PQ pool. For reviews on this matter, see Govindjee and Van Rensen (3) and Vermaas and Govindjee (15).

Usually broken chloroplasts are depleted of CO_2 in a CO_2-free medium containing formate (pH 5.0). If the Hill reaction of these depleted chloroplasts is measured in a CO_2-free medium at a higher pH with FeCy as an electron acceptor, it is almost completely lost. The Hill reaction can be partially restored by incubating the chloroplasts with bicarbonate in the dark, according to Stemler and Govindjee (11), Stemler (10) and Vermaas and Van Rensen (17). Maximal restoration occurs at pH 6.5 (16). About 60-80% of the original activity remains lost, and this irreversible deactivation is probably located at or before the reoxidation of Q_A^- according to Khanna et al. (5) and Vermaas and Van Rensen (16).

Whether CO_2, bicarbonate or carbonate is the active species remains to be established. Vermaas and Van Rensen (16) found a strong pH-dependence of the stimulation of the Hill reaction by 10 mM bicarbonate, with a sharp pH-optimum near pH 6.5, suggesting the involvement of both CO_2 and bicarbonate. They hypothezised that bicarbonate is the binding species, whereas CO_2 is involved in diffusion from the bulk aqueous phase to a binding site below the membrance surface. Sarojini and Govindjee

(8) also pointed to an active role of CO_2 in diffusion to the binding site.

Stemler (10) and Vermaas and Van Rensen (16, 17) have shown that, in the presence of 100 mM formate, bicarbonate can only reactivate electron transport when incubation takes place in the dark; reactivation does not occur in the light. The restoration by dark incubation with bicarbonate takes some time. So, when the incubation time is varied, the restored initial Hill reaction rate can be used as an indication for the fraction of the binding sites that are occupied by bicarbonate. This procedure enabled us to measure rate constants for the binding of bicarbonate to the thylakoids.

There are many electron transport inhibitors, acting between Q_A and PQ. Van Rensen and Vermaas (13) have shown that e.g. DCMU, simeton and DNOC interfere with bicarbonate action. If these herbicides act by displacing bicarbonate from its binding site, the kinetics of reactivation should be influenced by these herbicides. Recently the idea has emerged that the binding sites for the phenolic herbicides on one hand, and for the phenylureas and the triazines on the other, are close to each other although not identical (Oettmeier et al. (7), Van Rensen (12)). Therefore it was investigated whether the phenylurea DCMU and the dinitrophenol *i*-dinoseb have different effects on the kinetics of the reactivation of the Hill reaction by bicarbonate in CO_2-depleted chloroplasts. If so, this could lead towards a more precise localization of the action of bicarbonate on electron flow.

Materials and Methods

Peas (*Pisum sativum* L., cv Rondo) were grown in a growth chamber at 20°C. Broken chloroplasts were obtained from 10 to 15 days old plants by the following procedure. Freshly harvested leaves (20 g) were washed once with icecold distilled water and ground in a Sorvall omnimixer for 5 s in 50 ml isolation

medium, consisting of 0.4 M sorbitol, 20 mM tricine-NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 2 mM Na-ascorbate and 0.2% (w/v) BSA. The homogenate was squeezed through eight layers of cheese cloth, and the chloroplasts were collected in a centrifuge step: acceleration to full speed (2500 *g*) in 15 s, and after 30 s full speed the rotor was decelerated. The supernatant was decanted and the chloroplasts were broken in 50 ml Na-phospate buffer (50 mM, pH 7.8). The thylakoids were collected again by centrifugation at 1000 *g* for 5 min. and the pellet was resuspended in the isolation medium to a final concentration of 2 mg Chl/ml. The entire procedure was carried out at 2°C. The thylakoids were stored at -80°C. The chlorophyll (*a+b*) concentration was determined according to Bruinsma (1).

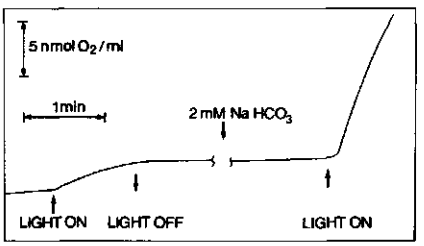
After thawing, the chloroplast fragments were suspended in CO_2-free standard medium, containing 50 mM Na-phosphate, 100 mM NaCl, 100 mM Na-formate and 5 mM $MgCl_2$ (pH 5.0); chloroplast concentration was equivalent to 50 µg Chl/ml. The chloroplast suspension was gently shaken on a Brunswick shaker for 10 min at room temperature, while N_2-gas ($[CO_2] < 1$ ppm) was flushed over the solution. The chloroplasts were pelleted and stored on ice until used. Media and tubes were made CO_2-free by flushing or bubbling with N_2-gas.

The chloroplasts were resuspended in CO_2-free standard medium (pH 6.5). The Hill reaction was measured as oxygen evolution according to Van Rensen et al. (14), with 0.5 mM FeCy as an electron acceptor. Normally the O_2-electrode was equipped with a teflon membrane (Yellow Springs Instruments Co., standard) with a response time of about 30 s (to 90% of final value), but for the kinetic experiments a thinner teflon membrane (YSI Co., high sensitivity) was used, providing a response time of 5 s.

All experiments shown were repeated at least three times.

Results

Figure 2.1 shows a typical reactivation experiment. The depleted chloroplasts were incubated in CO_2-free standard medium (pH 6.5) for 2 min in the dark, and then the Hill reaction was measured during 1 min in the absence of added bicarbonate. Subsequently the chloroplasts were incubated for 5 min in the dark, during which bicarbonate (or herbicides) was added. In this particular experiment bicarbonate was added 2 min before the light was switched on; the Hill reaction was increased from 4.4 to 34.6 µmol O_2/mg Chl·h by the addition of bicarbonate.



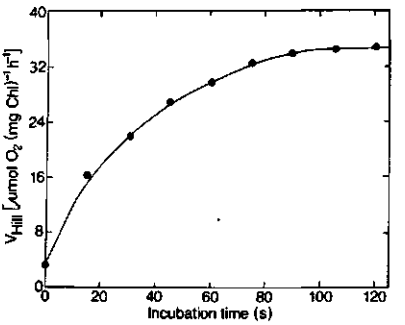


Fig. 2.1 (left): Reactivation of the Hill reaction in CO_2-depleted pea chloroplasts by 2 mM $NaHCO_3$ (measured as O_2-evolution). Reaction medium: 100 mM Na-formate, 100 mM NaCl, 50 mM Na-phosphate (pH 6.5), 5 mM $MgCl_2$ and 0.5 mM FeCy. Chloroplasts were added to a final concentration equivalent to 33 µg Chl/ ml.

Fig. 2.2 (right): Reactivation of the Hill reaction in CO_2-depleted chloroplasts by 2 mM $NaHCO_3$ as a function of the dark incubation time with bicarbonate. The Hill reaction at the start of bicarbonate incubation could not be measured accurately due to the pertubation caused by the injection of bicarbonate. Instead we used the average of the Hill reaction rates in the absence of bicarbonate. Reaction medium as in Fig.2.1.

The kinetics of the reactivation of the Hill reaction were studied by varying the bicarbonate incubation period (Fig. 2.2). The reactivation is almost complete after 120 s, which is in agreement with earlier observations of Stemler (18). The shape of this reactivation curve suggests that the binding of bicarbonate to the thylakoids is a reaction with (pseudo) first order kinetics and a half time of 23 s.

As preliminary experiments indicated that this half-time was influenced by the presence of a herbicide, a reaction scheme is proposed, which can explain these effects. It is assumed that the following reactions occur:

$$A + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\,A \qquad (K_r = \frac{k_2}{k_1}) \qquad \text{reaction 1}$$

$$I + E \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\,I \qquad (K_i = \frac{k_4}{k_3}) \qquad \text{reaction 2}$$

with A = activator (bicarbonate or CO_2)
I = inhibitor (herbicide)
E = bicarbonate/inhibitor binding site at the thylakoids membrane
K_r, K_i = dissociation constants for the activator - resp. inhibitor - binding site complex.

The relaxion of this system upon a change in the concentration of A and/or I can be described by the following differential equations.

$$\frac{d[EA]}{dt} = k_1 \cdot [A] \cdot [E] - k_2 \cdot [EA] \qquad (2.1)$$

$$\frac{d[EI]}{dt} = k_3 \cdot [I] \cdot [E] - k_4 \cdot [EI] \qquad (2.2)$$

This set of differential equations has been solved in Appendix 1. This solution is however rather complex and may be simplified by making the following assumption.
If it is assumed that the inhibitor equilibrates much faster with the binding site than the activator, the solution of eqs. 2.1 and 2.2 can be obtained by substituting $[E]=K_i \cdot \frac{[EI]}{[I]}$.
When [A] and [I] are constant, the solution becomes:

$$[EA]\,(t) = [E_{tot}] \cdot \{\frac{[A']}{K_r+[A']} + C \cdot e^{-(k_1 \cdot [A'] + k_2) \cdot t}\} \qquad (2.3)$$

where $A' = \frac{A}{1+\frac{[I]}{K_i}}$,

t = time after addition of A (or I) to the reaction of mixture,
E_{tot} = total number of binding sites,
C = integration constant.

Starting with a situation where no activator A is present and we add A to a mixture of E, I en EI at time t=0, the following equation describes the association of E and A:

$$[EA](t) = [E_{tot}] \cdot \frac{[A']}{K_r+[A']} \cdot \{1-e^{-(k_1 \cdot [A'] + k_2) \cdot t}\} \qquad (2.4)$$

It is assumed that E is the binding site for bicarbonate at the thylakoids, which allows electron flow between Q_A and PQ only when bicarbonate is attached. If the Hill reaction rate is proportional to the number of binding sites occupied by bicarbonate, the bicarbonate supported Hill reaction may be represented by equation (2.4), with the actual rate (V_{Hill}) substituted for [EA] and the maximal rate (V_{max}) for $[E_{tot}]$:

$$V_{Hill}(t) = V_{max} \cdot \frac{[A']}{K_r+[A']} \cdot \{1-e^{-(k_1 \cdot [A'] + k_2) \cdot t}\} \qquad (2.5)$$

Several implications of equation (2.5) were investigated:

1 The time-independent part of equation (2.5) closely resembles the Michaelis-Menten equation for a one-substrate enzyme catalyzed reaction. This can easily be seen by multiplying numerator and denominator with $1 + \frac{[I]}{K_i}$ yielding:

$$V_{Hill} = V_{max} \frac{[A]}{K_r' + [A]} \qquad (2.6)$$

with $K_r' = K_r \cdot (1 + \frac{[I]}{K_i})$

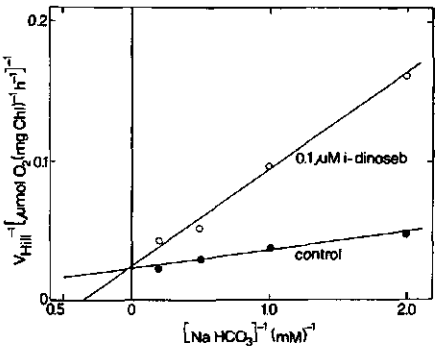


Fig. 2.3: Double reciprocal plot of the Hill reaction of CO_2-depleted chloroplasts as a function of the bicarbonate concentration in the absence and the presence of i-dinoseb. Reaction medium as in Fig. 2.1.

Therefore, we measured the Hill reaction as a function of the bicarbonate concentration. Figure 2.3 shows a double reciprocal plot of a typical reactivation experiment. From these plots the dissociation constant (K_r) of the enzyme-activator complex and the maximal Hill reaction rate (V_{max}) that can be obtained in the presence of bicarbonate can be calculated. It turns out that

$$V_{max} = 39.9 \pm 15 \text{ µmol } O_2/\text{mg Chl} \cdot \text{ h} \qquad (n=6)$$

$$K_r = 1.5 \pm 1 \text{ mM } NaHCO_3 \qquad (n=6)$$

Figure 2.3 also shows the effect of 100 nM *i*-dinoseb on the bicarbonate supported Hill reaction. V_{max} is not significantly influenced by *i*-dinoseb, but the apparent dissociation K_r' is raised more than 4 times. Thus, in terms of the Michaelis-Menten formalism, *i*-dinoseb is a competitive inhibitor of bicarbonate binding, resulting in an inhibited electron transport. From 3 identical experiments the inhibitor constant (K_i) of the inhibitor-enzyme complex was calculated using equation (2.6): $K_i = 31 \pm 6$ nM.

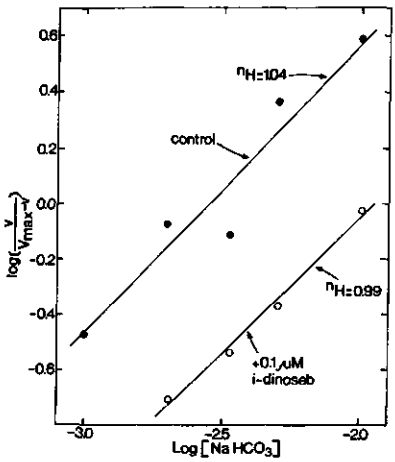


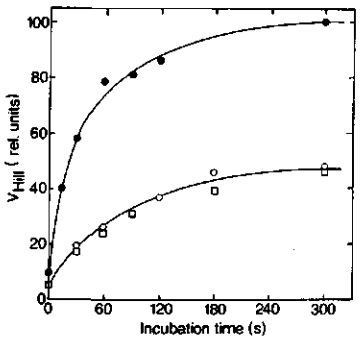
Fig. 2.4: Hill plot of the Hill reaction of CO_2-depleted chloroplasts as a function of the bicarbonate concentration. v=Hill reaction rate at the indicated bicarbonate concentration; Vmax was calculated from a Lineweaver Burk plot of these data. Reaction medium as in Fig. 2.1.

Figure 2.4 shows a Hill plot of the fully reactivated Hill reaction as a function of the bicarbonate concentration. The apparent Hill coefficient (n_H) is about 1, both in the absence and in the presence of *i*-dinoseb, indicating that only one type of activation-site for bicarbonate exists and that there is no cooperativity between the activation-sites.

2. The time-dependent part of equation (2.5) represents a simple exponental decay with a half-time

$$t_{\frac{1}{2}} = \frac{0.693}{k_1 \cdot [A^-] + k_2} \text{ (s)}$$

Figure 2.5 shows the effect of the herbicides *i*-dinoseb and DCMU on the reactivation of the Hill reaction by 2 mM $NaHCO_3$. In the absence of herbicides the half-time of the reactivation is about 27 s, whereas in the presence of 100 nM *i*-dinoseb or 100 nM DCMU this half-time is increased to approx. 57 s. From the half-time $t_{\frac{1}{2}}$ and the dissociation constant K_r, the rate constants k_1 and k_2 can be calculated, $k_1 = 7.3\ M^{-1} \cdot s^{-1}$; $k_2 = 0.011\ s^{-1}$.



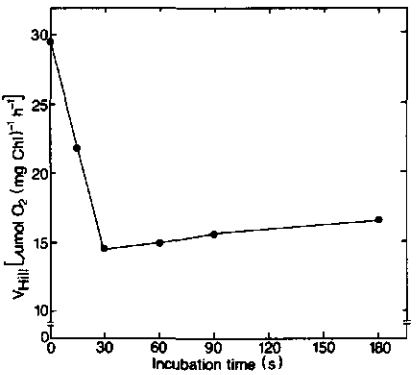


Fig. 2.5 (left): Reactivation of the Hill reaction of CO_2-depleted chloroplasts by 2 mM $NaHCO_3$ in the presence of herbicides,●control;○+100 nM i-dinoseb;□+100 nM DCMU. Reaction medium as in Fig. 2.1.

Fig. 2.6 (right): Hill reaction of CO_2-depleted chloroplasts in the presence of 1 mM $NaHCO_3$ and 100 nM i-dinoseb as a function of the i-dinoseb incubation time in the dark. The bicarbonate incubation time was 300 s. Reaction medium as in Fig. 2.1.

3. An inhibitor, which competes with bicarbonate for the same binding site, is expected to inhibit by displacing bicarbonate from its binding site. Therefore, the kinetics of the inhibition of *i*-dinoseb should be related to the kinetics of the dissociation of the bicarbonate-binding site complex. Figure 2.6 shows the kinetics of the inhibition by 100 nM i-dinoseb in the presence of 1 mM $NaHCO_3$. The half-time ($t_{\frac{1}{2}}$) of the inhibition is less than 15 s, which is much smaller than predicted by the equation (2.5).

$$(t_{\frac{1}{2}} = \frac{0.693}{k_1 \cdot [A'] + k_2} \sim 54 \text{ s}).$$

Discussion

It has now been widely accepted that the major effect of bicarbonate is located between Q_A and PQ. Van Rensen and Vermaas (13) have reported that it is a difficult procedure to deplete thylakoids of CO_2. This is reflected in a rather high variability in our values for V_{max} and K_r. These values are, however, consistent with data given by Khanna et al. (4) and Van Rensen and Vermaas (13), although the latter authors report somewhat lower values for V_{max}.

Van Rensen and Vermaas (13) first demonstrated an interaction between the bicarbonate ion and the herbicides DCMU, simeton and DNOC. They suggested that these herbicides act as (partially) competitive inhibitors of bicarbonate binding to the thylakoid membrane. They did not check, however, whether the thylakoid-herbicide-bicarbonate system could be treated as an enzyme-inhibitor-substrate system.

The reaction scheme as presented can account for the observed kinetics of bicarbonate dependent electron flow. Figures 2.3 and 2.4 show that the reactivation of the Hill reaction can be described by the time-independent part of equation (2.5). So, it seems that the assumption that was made by putting V_{Hill} proportional to the number of binding sites occupied by bicarbonate, is justified. This gives additional support for the conclusion that in CO_2-depleted chloroplasts, the rate-limiting step in linear electron flow is located at the site of action of bicarbonate and not at the oxidation of PQH_2 (cf. 9).

Competitive inhibition of the bicarbonate stimulation of electron flow by DNOC was already reported by Van Rensen and Vermaas (13). The results presented in this Chapter show that *i*-dinoseb has the same effect. It appears that this competitive inhibition is a general feature of the dinitrophenol herbicides. The dissociation constant (K_i = 31 nM) that was calculated for the thylakoid-inhibitor complex, is slightly lower than the binding constant (K_b = 69 nM) given by Oettmeier and

Masson (6). These authors used however "normal" chloroplasts at pH 8.0, while the experiments shown here were performed at pH 6.5 with CO_2-depleted chloroplasts. Oettmeier and Masson (6) have shown that at pH 6.0 three times more *i*-dinoseb is bound than at pH 8.0 and this might explain the difference.

The half-time of the reactivation of the Hill reaction is increased from 27 s in the absence of herbicides to about 58 s in the presence of 100 nM *i*-dinoseb or 100 nM DCMU. It has been shown by Van Rensen and Vermaas (13) that DCMU apparently decreases the affinity of the thylakoids for bicarbonate, with an inhibition constant of about 30 nM, which is almost equal to the value shown here for *i*-dinoseb. Within experimental limits, no difference is found in the effects of DCMU and *i*-dinoseb on the half-time of the reactivation of the Hill reaction by bicarbonate.

The kinetics of the inhibition of the Hill reaction by *i*-dinoseb cannot be explained by equation (2.5), with the calculated values for k_1, k_2 and K_i. The estimated half-time of the inhibition is less than 15 s, which is almost 4 times lower than predicted by equation (2.5). This discrepancy can be explained by assuming that an additional reaction occurs:

$$X + E \underset{k_6}{\overset{k_5}{\rightleftarrows}} EX \qquad \text{reaction 3}$$

X is another compound that can bind to the bicarbonate/*i*-dinoseb binding site. This compound is most probably formate, which is abundantly present in the reaction medium. A number of investigators, e.g. Good (2), Stemler and Govindjee (11), Vermaas and Van Rensen (16) and Sarojini and Govindjee (8), have already suggested that formate and acetate compete with bicarbonate for the same binding site. It is obvious that if reaction 3 occurs, the calculated values for K_r, k_1 and k_2 are not correct. This means that in the absence of formate the half-time of the reactivation could be less than 15 s. Such an effect of formate on the half-time of the reactivation was re-

ported by Vermaas and Van Rensen (17), but in those experiments the reactivation of the Hill reaction was studied by the addition of bicarbonate in the light to CO_2-depleted chloroplasts in reaction media containing low formate concentrations. Results presented in the next Chapter indicate that formate indeed is a competitive inhibitor of the stimulation of electron flow by bicarbonate. Moreover, it appears that the dissociation of the thylakoid-formate complex may be a rate-limiting step in the binding of bicarbonate.

So far only the case in which bicarbonate, i-dinoseb and formate are competing for the same binding site has been considered. The effect of a competitive inhibitor is an apparent decrease in the affinity of the thylakoids for bicarbonate; k_1 and k_2 are, however, not influenced by the inhibitor. The present data do not allow to distinguish between a competitive inhibitor and an allosteric inhibitor that only alters K_r, while V_{max} is not affected.

Thus it appears that the binding of bicarbonate to its binding site at the thylakoid membrane is retarded in the presence of herbicides and metabolites like formate and acetate. It would be interesting to see if the opposite effect also occurs. If so, the binding sites for herbicides and bicarbonate could be identical (in which case displacement of herbicides by bicarbonate should occur) or merely overlapping (allowing binding of bicarbonate and herbicide at the same time).

References

1. Bruinsma, J. 1963. The quantitative analysis of chlorophylls *a* and *b* in plant extracts. Photochem. Photobiol. 2: 214-249.

2. Good, N.E. 1963. Carbon dioxide and the Hill reaction. Plant Physiol. 38: 298-304.

3. Govindjee, J.J.S. van Rensen. 1978. Bicarbonate effects on the electron flow in isolated broken chloroplasts. Biochim. Biophys. Acta 505: 183-213.

4. Khanna, R., Govindjee T. Wydrzynski. 1977. Site of bicarbonate effect in Hill reaction. Evidence from the use of artificial electron acceptors and donors. Biochim. Biophys. Acta 462: 208-214.

5. Khanna, R., K. Pfister, A. Keresztes, J.J.S. van Rensen, Govindjee 1981. Evidence for a close spatial location of the binding sites for CO_2 and for Photosystem II inhibitors. Biochim. Biophys. Acta 634: 105-116.

6. Oettmeier, W., K. Masson. 1980. Synthesis and thylakoid membrane binding of the radioactively labeled herbicide dinoseb. Pest. Biochem. Physiol. 14: 86-97.

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

8. Sarojini, G., Govindjee. 1981. On the active species in bicarbonate stimulation of Hill reaction in thylakoid membranes.- Biochim. Biophys. Acta 634: 340-343.

9. Siggel, U., R. Khanna, G. Renger, Govindjee. 1977. Investigation of the absorption changes of the plastoquinone system in broken chloroplasts. The effect of bicarbonate depletion. Biochim. Biophys. Acta 462: 196-207.

10. Stemler, A. 1979. A dynamic interaction between the bicarbonate ligand and Photsystem II reaction center complexes in chloroplasts. Biochim. Biophys. Acta 545: 36-45.

11. Stemler, A., Govindjee. 1973. Bicarbonate ion as a critical factor in photosynthetic oxygen evolution. Plant Physiol. 52: 119-123.

12. Van Rensen, J.J.S. 1982. Molecular mechanism of herbicide action near Photosystem II. Physiol. Plant. 54: 515-521.

13. Van Rensen, J.J.S., W.F.J. Vermaas. 1981. Action of bicarbonate and Photosystem II inhibiting herbicides on electron transport in pea grana and in thylakoids of a blue-green alga. Physiol. Plant. 51: 106-110.

CHAPTER 3

REEVALUATION OF THE ROLE OF BICARBONATE AND FORMATE IN THE REGULATION OF PHOTOSYNTHETIC ELECTRON FLOW IN BROKEN CHLOROPLASTS

Introduction

Although it has been recognized for a long time that formate is somehow involved in the regulation of electron flow by bicarbonate, the mode of action of formate is still unknown. Good (2) first demonstrated that formate and acetate increase the dependency of the Hill reaction on bicarbonate. It has been suggested (2, 6, 15, 20) that formate and acetate compete with bicarbonate for binding sites at the thylakoid membrane. Assuming that bicarbonate is required for electron flow between Q_A and PQ, it was argued that displacement of bicarbonate by formate would result in a lowering of the apparent affinity of the binding sites for bicarbonate. This hypothesis was supported by Stemler (12), who described two different types of bicarbonate binding sites and showed that formate can remove bicarbonate from these binding sites. The high-affinity binding sites were shown to be involved in the regulation of electron flow: removal of bicarbonate from these high-affinity binding sites was correlated with an inhibition of electron flow. As bicarbonate was actually removed from the thylakoid membrane by formate in these experiments, it is conceivable that the inhibition of electron flow is not a consequence of the removal of bound bicarbonate, but due to the inhibitory action of formate. Stimulation of electron flow by bicarbonate may then be explained by assuming that formate is displaced by bicarbonate. In the former explanation (6, 15, 20) electron flow should be strongly inhibited in the absence of bicarbonate,

whether or not formate is present. As experimental data on this matter are conflicting (1, 2, 6, 15, 20), this Chapter describes experiments that were carried out to elucidate the mode of action of formate. These experiments are based on the observation that there appears to be a Michaelis-Menten type of relationship between the uncoupled Hill reaction rate and the bicarbonate concentration in the reaction medium (19). As has been pointed out in Chapter 3, this means that the Hill reaction rate can be described by two parameters: the maximal Hill reaction rate (V_{max}) and the apparent reactivation constant (K_r'). The effects of formate on K_r' and V_{max} were investigated to see whether formate is a competitive inhibitor of the bicarbonate stimulation of electron flow. The Hill reaction in CO_2-depleted chloroplasts was measured also in the virtual absence of both formate and bicarbonate to determine the mode of action of formate and bicarbonate.

These experiments require large amounts of CO_2-depleted chloroplasts with low activity in the absence of bicarbonate and a good stimulation of electron flow by bicarbonate. As these requirements were not met by the current CO_2-depletion method, a new CO_2-depletion procedure was developed.

Another reason for examing the role of formate in the regulation of linear electron flow in more details comes from the fact that formate is a metabolite which is involved in e.g. photorespiration (3, 9). If linear electron flow *in vivo* is regulated by formate and bicarbonate, then metabolic pathways affecting formate levels in the chloroplast would influence electron flow.

Materials and Methods

Broken chloroplasts (*Pisum sativum* L.cv Rondo) were isolated as described in Chapter 2. Thylakoids were depleted of CO_2

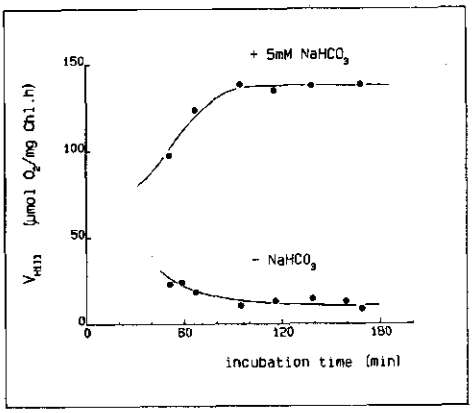
using the following procedure (Snel, Vermaas and Van Rensen, unpublished data). A 0.5 ml thylakoid suspension containing 2 mg Chl/ml was, washed once in 4.5 ml medium consisting of 0.3 M sorbitol, 50 mM sodium phosphate (pH 5.8), 10 mM NaCl and 5 mM $MgCl_2$. The thylakoids were collected by centrifugation (10 min 1000 *g* and resuspended in 4.0 ml depletion medium. This depletion medium contained 0.3 mM sorbitol, 10 mM sodium phosphate (pH 5.8), 10 mM NaCl, 5 mM $MgCl_2$ and 10 mM sodium formate. The final chlorophyll concentration was 250 µg Chl/ml. Depletion was accomplished by incubating the chloroplasts at 25°C in depletion medium for at least 60 min in the dark under a nitrogen atmosphere. The Hill reaction was measured as oxygen evolution with FeCy an electron acceptor at 25°C as described before (18). For the determination of the initial Hill reaction rate we used the first 20 s of the Hill reaction. All media were made CO_2-free by bubbling with pure N_2-gas.

The kinetic constants K_r' and V_{max} were determined graphically from Lineweaver Burk plots that were obtained from measurements (in duplo) of the Hill reaction rates at 6 different bicarbonate concentrations.

Results

CO_2-depletion of chloroplasts

Application of the new procedure for depleting chloroplasts of CO_2 yields large amounts of CO_2-depleted chloroplasts. Figure 3.1 shows the results of a typical CO_2-depletion procedure. At the indicated times a sample of 100 µl of the chloroplast suspension was injected into 1150 µl of CO_2-free reaction medium and the Hill reaction was measured as illustrated in Fig. 3.2. After 1 min dark-equilibration the Hill reaction was recorded during 30 s. Subsequently the light was switched off and $NaHCO_3$ was added to a final concentration of 5 mM. The



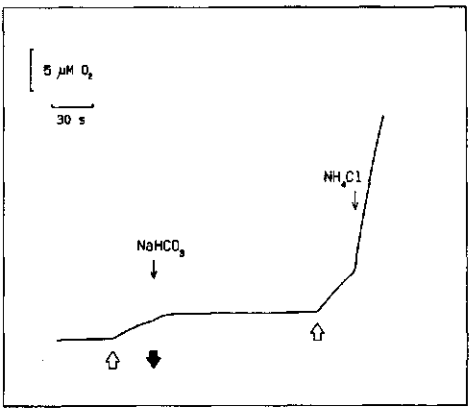


Fig. 3.1 (left): The Hill reaction rate in the absence and presence of bicarbonate as a function of the incubation time in the depletion medium. Reaction mixture: 0.3 M sorbitol, 10 mM NaCl, 5 M $MgCl_2$, 20 mM sodium phosphate (pH 6.5), 20 mM Na-formate, 5 mM NH_4Cl, 0.5 mM FeCy and thylakoids equivalent to 20 µg/ml. Hill reactions were determined as described in Fig. 3.2.

Fig. 3.2 (right): Effect of NH_4Cl upon the reactivation of the Hill reaction of CO_2-depleted chloroplasts by bicarbonate. Reaction medium as in Fig. 3.1 with the exception that NH_4Cl was added at the indicated time.

chloroplasts were incubated in the dark for 2 min and subsequently the Hill reaction (now in the presence of $NaHCO_3$) was measured. Addition of 5 mM NH_4Cl causes a 4-fold stimulation of electron flow. Figure 3.1 shows further that it takes about 90 min, in this particular experiment, to reach a steady-state situation during which the Hill reaction rate in the absence of added bicarbonate is less than 20 µmol O_2/mg Chl·h. In the presence of 5 mM added bicarbonate the uncoupled Hill reaction rate is about 130 µmol O_2/mg Chl·h. These rates approach the uncoupled rates of non-depleted control chloroplasts suspended in the same reaction mixture. The time needed to reach a steady state with minimal activity in the absence of bicarbonate (Fig. 3.1) depends on the composition of the depletion medium; at higher formate concentrations and/or at low pH the steady state can be reached much faster (data not shown). At low formate concentrations (≦ 10 mM) this CO_2-depletion method is not reli-

able when the pH in the depletion medium is above 5.9; 10 mM formate and pH 5.8 were choosen to allow for easy variations in the formate concentration and/or the pH-value of the reaction medium without additions or adjustments to this reaction medium, which might introduce CO_2-contamination.

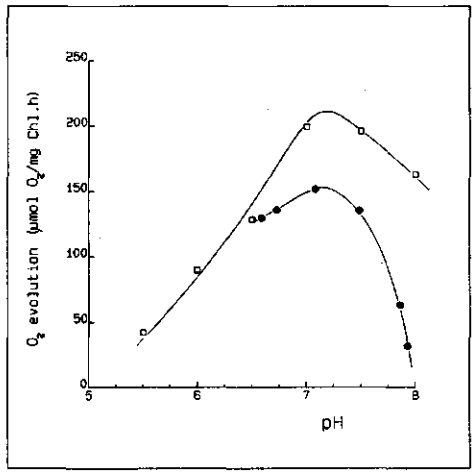


Fig. 3.3: Effect of the pH on the uncoupled Hill reaction in control (□) and in CO_2-depleted chloroplasts that were reactivated by 20 mM $NaHCO_3$ (●). Reaction medium: 0.3 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM buffer, 5 mM NH_4Cl, 0.5 mM FeCy and the chlorophyll concentration was 20 µg Chl/ml. Buffers used were MES, phosphate, HEPES and Tricine. The reaction medium of the reactivated chloroplasts contained additionally 20 mM Na-formate and 20 mM $NaHCO_3$.

Figure 3.3 depicts the effect of the pH of the reaction medium on the Hill reaction rate in reactivated CO_2-depleted chloroplasts. Although the reactivation of the Hill reaction by bicarbonate is almost complete at pH 6.5, this appears to be not the case at higher pH values. Omission of formate and bicarbonate from the reaction medium did not prevent this inhibition at alkaline pH and therefore this inhibition might not be related to the "bicarbonate-effect".

In the following experiments the chloroplasts were injected into the reaction medium containing the appropriate amounts of formate, bicarbonate and NH_4Cl. After 2 min. dark-incubation the Hill reaction was measured. The determination of the Hill reaction in the absence of added bicarbonate was performed on separate samples at the beginning and the end of a set of experiments to check whether the chloroplasts were still "CO_2-depleted". This procedure was adopted to avoid effects of light

on the binding of bicarbonate (cf. ref. 13) and possibly formate (7). In the presence of 10 mM formate the stimulation of the Hill reaction by saturating amounts of bicarbonate was always at least 8-fold, when measured as in Fig. 3.2 in the presence of uncoupler.

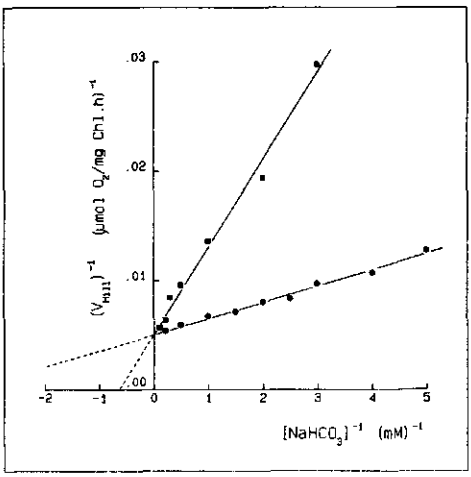


Fig. 3.4: Double reciprocal plot of the Hill reaction rate against the bicarbonate concentration added to the reaction mixture in the presence of 5.8 (●) and 50.8 mM (■) Na-formate. Chloroplasts were previously depleted of CO_2 and incubated in the reaction medium for 2 min. The reaction medium consisted of: 0.3 M sorbitol, 20 mM sodium phosphate (pH 6.5), x mM Na-formate (50.8-x) mM NaCl, 5 mM $MgCl_2$, 0.5 mM FeCy and 5 mM NH_4Cl. The chlorophyll concentration was 20 µg Chl/ml.

Interaction of formate and bicarbonate on the Hill reaction

In order to establish the kind of interaction of formate and bicarbonate on electron flow, we have measured the reactivation of the Hill reaction by bicarbonate in the presence of 5.8 and at 50.8 mM $HCOO^-$. Figure 3.4 shows a double reciprocal plot of the results. It appears that the maximal Hill reaction rate (V_{max}) is not affected by formate, whereas the apparent reactivation constant (K_r') is increased from 0.29 mM bicarbonate in the presence of 5.8 mM formate to 1.6 mM bicarbonate in the presence of 50.8 mM bicarbonate. Formate apparently acts as a competitive inhibitor of the reactivation of the Hill reaction by bicarbonate.

This inhibition can be interpreted in two ways:

1. Electron flow is not possible when no bicarbonate is bound to the binding site at the thylakoid membrane. Formate merely acts by competing with bicarbonate for the same binding site or by lowering the affinity of the binding site for bicarbonate. This view is presented in e.g. refs. 2, 6, 15 20.
2. Electron flow is possible when no bicarbonate is bound to its binding site. Formate inhibits electron flow when bound to this site. Bicarbonate can relieve this inhibition of electron flow by displacing formate from its binding site, see refs. 16, 17.

At high formate and bicarbonate concentrations the two mechanisms yield the same result: at these concentrations nearly all binding sites will be occupied, either by formate or by bicarbonate and in both models electron flow should only occur in those PS II centers that contain a bicarbonate molecule attached to its binding site. Only at bicarbonate and formate concentrations far below their respective dissociation constants the majority of the binding sites will be "free", i.e. these binding sites contain neither bicarbonate nor formate. Under these conditions we should be able to distinguish between the two mechanisms.

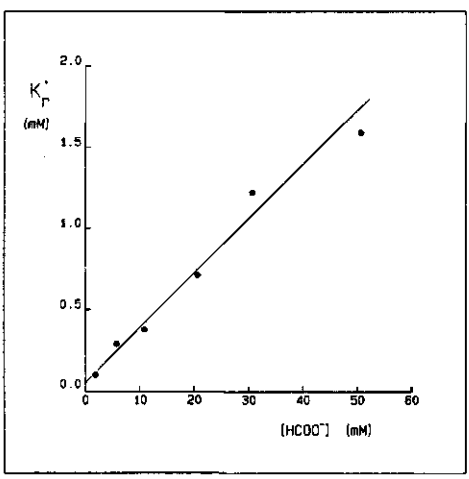
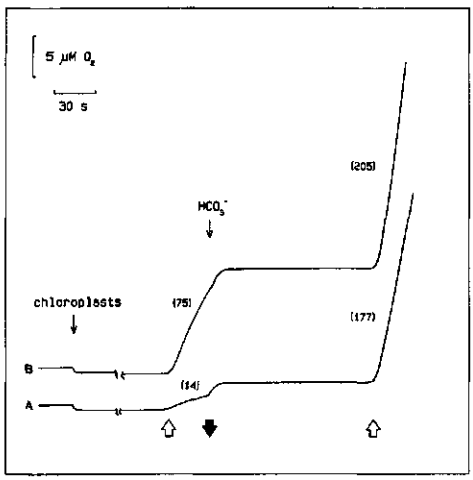


Fig. 3.5: The apparent reactivation constant (K_r') as a function of the formate concentration in the reaction medium. Chloroplasts were previously depleted of CO_2. Reaction medium as in Fig. 3.1; the chloroplasts were incubated for 2 min in reaction medium + bicarbonate at the appropriate bicarbonate and formate concentrations .

The dissociation constants of the bicarbonate- and the formate-binding site complexes were estimated by measuring the reactivation constant K_r and the inhibition constant K_i. Figure 3.5 shows that K_r' is linearly dependent on the formate concentration in the reaction medium. According to eqn. (2.6) extrapolation to $[HCOO^-]$ = 0 mM yields the reactivation constant K_r and K_i can be calculated from the slope of the line. K_r appears to be 78 ± 31 µM bicarbonate and K_i = 2.0 ± 0.7 mM formate at pH 6.5.



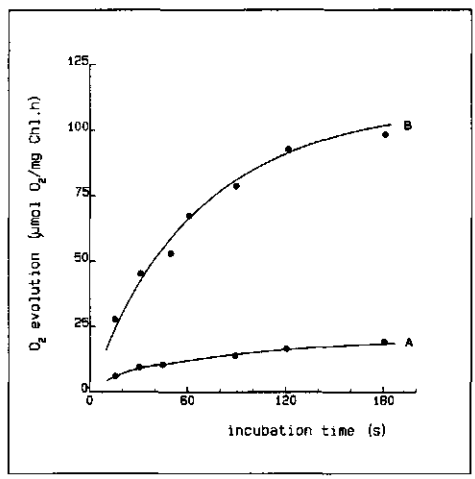
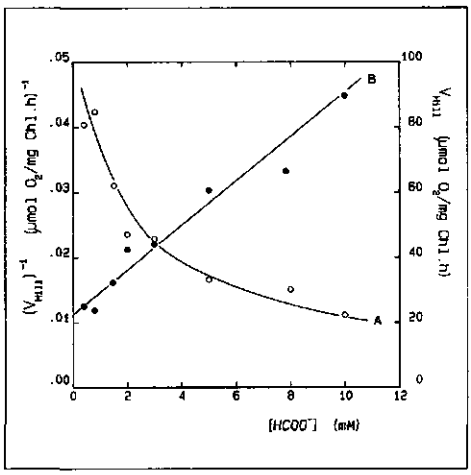


Fig. 3.6 (left): Reactivation of the Hill reaction by bicarbonate in CO_2-depleted chloroplasts in the absence (B) and in the presence (A) of formate. Chloroplasts were transferred from the depletion vessel to the reaction medium in the reaction vessel and incubated for 90 s in the dark. Reaction medium as in Fig. 3.8, but in the experiments shown in trace A 10 mM formate was added.

Fig. 3.7 (right): Time-course of the reactivation of the Hill reaction in CO_2-depleted chloroplasts in the absence of bicarbonate. Reaction media as in Fig. 3.6. A: +10 mM formate, B: -formate.

In the experiment shown in Fig. 3.6 CO_2-depleted chloroplasts were transferred to CO_2-free reaction medium containing either 10 mM formate (A) or no formate (B); due to transfer of formate together with the chloroplasts the final formate concentration in the sample is about 0.8 mM higher. In the pres-

ence of 10 mM formate the Hill reaction rate is about 14 µmol O_2/mg Ch·h after 90 s incubation in the reaction medium; subsequent addition of 10 mM bicarbonate results in a nearly complete reactivation of electron flow (trace A). At low formate concentration a relatively high rate of electron flow is observed. Addition of bicarbonate still stimulates electron flow, but only by a factor of 3 (trace B). It is noteworthy that in the virtual absence of formate and bicarbonate there is still some reversible deactivation of electron flow during illumination (Fig. 3.6B, first illumination period), which is not observed when bicarbonate has been added. Figure 3.7 shows the dependence of the initial Hill reaction rate in the presence of 0.8 or 10.8 mM formate on the dark incubation time in the reaction medium. At low formate concentration electron flow is



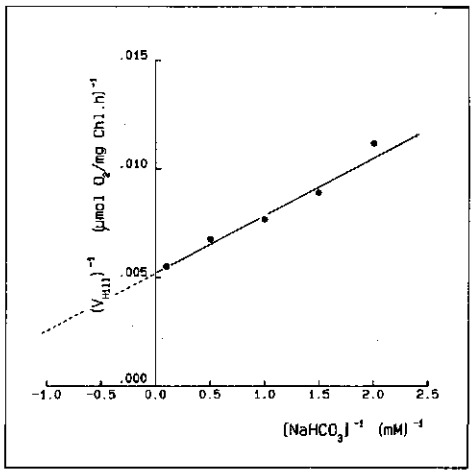


Fig. 3.8 (left): Inhibition of the Hill reaction by formate in the absence of bicarbonate. Reaction medium: 0.3 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM sodium phosphate (pH 6.5), 5 mM NH_4Cl, 0.5 mM FeCy and chloroplasts equivalent to 20 µg Chl/ml. A: Hill reaction rate as a function of the formate concentration (○).
B: reciprocal Hill reaction rate as a function of the formate concentration (Dixon plot) (●).

Fig. 3.9 (right): Double reciprocal plot of the Hill reaction rate as a function of the bicarbonate concentration. Reaction mixture as in Fig. 3.8, except that 10 mM formate was added.

reactivated to a final value of 95 µmol O_2/mg Chl·h with a half-time of approximately 35 s. At 10.8 mM formate the Hill reaction remains much lower and even after 3 min incubation the Hill reaction rate is less than 20 µmol O_2/mg Chl·h. These experiments suggest that in the absence of bicarbonate and formate electron flow can proceed from Q_A to PQ.

Figure 3.8 shows the effect of low formate concentrations on the Hill reaction of CO_2-depleted chloroplasts in CO_2-free reaction medium. The relationship between the Hill reaction rate and the formate concentration appears to be hyperbolic (curve A); a plot of the reciprocal Hill reaction rate against the formate concentration yields a straight line (curve B). The intercept with the y-axis gives the Hill reaction rate in the absence of formate and bicarbonate. The Hill reaction rate is about 88 µmol O_2/mg Chl·h and K_i in this experiment was 3.45 mM formate, which is however not significantly different from the value for K_i given above. Figure 3.9 shows that CO_2-depleted chloroplasts from the same batch exhibit a V_{max} of about 190 µmol O_2/mg Chl·h in the presence of 10 mM formate and saturating amounts of $NaHCO_3$. It is obvious from Fig. 3.8 that electron flow can proceed at $[HCO_3^-] << K_r$ and $[HCOO^-] << K_i$, although V_{max} is not reached. Therefore we conclude that electron flow from Q_A to PQ can proceed when no bicarbonate is bound to the high-affinity bicarbonate binding sites; formate might be regarded as an inhibitor of PS II electron flow.

Discussion

CO_2-depletion of chloroplasts

In contrast to earlier methods (e.g. 15, 21, 22) the modified CO_2-depletion procedure yields thylakoids that can evolve oxygen in excess of 150 µmol O_2/mg Chl·h at pH 6.5 at saturating bicarbonate concentrations. Figure 3.1 shows that these

high rates can only be observed after prolonged incubation of the chloroplasts in depletion medium at pH 5.8. The rather slow development of the dependence of the Hill reaction on bicarbonate probably reflects the binding of formate. As the action of formate is pH dependent, i.e. more pronounced at low pH (10, 14) it could be that the protonated base is involved in the rate-limiting step of the inhibition.

In the CO_2-depleted chloroplasts obtained by the new procedure electron flow in the presence of saturating bicarbonate concentrations can be stimulated about 4-fold by 5 mM NH_4Cl or 5 µM Gramicidin (not shown) when FeCy is the electron acceptor. This strongly suggests that in these chloroplasts FeCy accepts electrons after PQ.

In CO_2-depleted chloroplasts that have been reactivated by incubation with bicarbonate, the pH dependence is different from that in non-treated chloroplasts (Fig. 3.4); at more alkaline pH electron flow is only poorly reactivated. This might be due to an irriversible ageing process during one hour depletion at 25°C causing another step in electron flow to become rate-limiting.

Interaction of formate and bicarbonate on the Hill reaction

Stemler (12) has demonstrated the existence of two types of binding sites for bicarbonate. The high-affinity binding sites, with a pool size of about 1 binding site per 400 Chl molecules, were shown to be involved in the regulation of electron flow. The dissociation constant of this binding site/bicarbonate complex was reported to be 80 µM $NaH^{14}CO_3$ at pH 6.5 (17), which is practically identical to the value of 78 µM $NaHCO_3$ obtained here for K_I at pH 6.5 (see Fig. 3.5). In my opinion this close agreement indicates that the initial Hill reaction rate reflects the state of the regulatory high-affinity binding sites, perhaps at the Q_B-protein, with respect to the binding of formate and bicarbonate. This means that in the experiments illustrated in Figs. 3.5, 3.6B and 3.7B, a large fraction of

the binding sites must have been empty, i.e. not occupied by either formate or bicarbonate. Therefore I suggest that the high-affinity binding sites do not require that a complex is formed with bicarbonate to allow electron flow from Q_A to PQ.

However, the question whether the binding of bicarbonate to the regulatory site at the Q_B-protein is a requirement for electron flow is still a rather hypothetical one. The results do not exclude a possible involvement of a small pool of bicarbonate-binding sites with a very high affinity for bicarbonate. So far however, there are no indications that such a pool exists. Another possibility is that binding of bicarbonate brings the regulatory site in an "active" state after dissociation of the regulatory site-bicarbonate complex. In this way only a few bicarbonate molecules could keep all regulatory sites in the "active" state when no formate is present.

I did not succeed in achieving a total restoration of electron flow in the absence of formate and bicarbonate (Figs. 3.5 and 3.8). An explanation for this observation is not known, obviously further experiments are needed to clarify the exact mechanism of formate/bicarbonate regulation of electron flow.

In Chapter 2 the kinetics of the reactivation of the Hill reaction by bicarbonate have been analyzed . There the assumption was made that electron flow from Q_A to PQ can only proceed when bicarbonate is bound to the binding site. According to the present data this assumption might not be correct. Those experiments were done, however, in the presence of 100 mM formate, a condition at which the number of "empty" binding sites must have been negligible. Therefore the only conformation of the regulatory site that allowed electron flow in those experiments was the one in which bicarbonate was bound to the binding site.

Physiological consequences of the formate/bicarbonate interaction on electron flow

The results presented in this chapter clearly show that formate and bicarbonate can regulate linear electron flow in CO_2-

depleted thylakoids. These CO_2-depleted chloroplasts were obtained by incubation with formate at pH 5.8. This incubation may seem a rather unphysiological treatment, but Stemler (14) has already shown that CO_2-depleted chloroplasts can also be obtained by illuminating broken chloroplasts at pH 8 in the presence of high formate concentrations and an artificial electron acceptor.

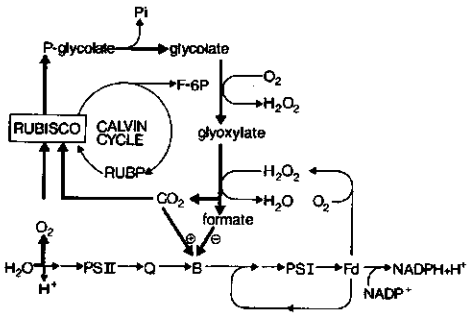


Fig. 3.10: A hypothetical scheme for the interaction between photosynthetic electron flow and photorespiration.

Formate is a metabolite that is involved in several metabolic reactions, among other things breakdown of glyoxylate by H_2O_2 to formate and CO_2 in peroxisomes (3, 9) and chloroplasts (23). Glyoxylate is an intermediate in photorespiration and high rates of glyoxylate decarboxylation have been observed in isolated peroxisomes (3). Figure 3.10 schematically shows how photorespiration and photosynthetic electron flow could interact *in vivo* via the formate and bicarbonate pools. Under highly photorespiratory conditions, e.g. high light intensity and low CO_2 concentration, there is a large carbon flux through the glycolate pathway. If the amount of "free" H_2O_2 (i.e. H_2O_2 not bound to catalase) is high enough, high rates of glyoxylate decarboxylation can be expected in the peroxisomes (3). In order to inhibit photosynthetic electron flow the produced formate would have to enter the chloroplast. Alternatively glyoxylate could enter the chloroplast and react with H_2O_2, generated in a reaction of O_2 with reduced ferredoxin (11), to give

formate and bicarbonate. The bicarbonate formed subsequently will be remetabolized in the Calvin Cycle and the remaining formate will inhibit linear electron flow. So, at a high O_2/ CO_2 ratio there is a direct path from photosynthetically produced oxygen to formate, which inhibits linear electron flow. Such a pathway could be regarded as a negative feedback mechanism that is involved in the regulation of linear electron flow. As inhibition of linear electron flow can stimulate cyclic electron flow (8), regulation of linear electron flow by formate and bicarbonate will result in an altered ratio between linear and cyclic electron flow.

Conclusions

The present data show that formate inhibits linear electron flow in isolated broken pea chloroplasts. Bicarbonate can abolish this inhibition, probably by displacing formate from the thylakoid membrane. The physiological significance of this regulation of electron flow is still far from being understood. However, as photorespiration may be involved as a source of formate, regulation of photosynthetic electron flow by formate and bicarbonate may provide a mechanism by which photorespiration might control photosynthetic efficiency in intact cells or leaves.

References

1. Fischer, K., H. Metzner. 1981. Bicarbonate effects on photosynthetic electron transport. I. Concentration dependence and influence of manganese reincorporation. Photochem. Photobiophys. 2: 133-140.

2. Good, N.E. 1963. Carbon dioxide & the Hill reaction. Plant Physiol. 38: 298-304.

3. Grodzinski, B. 1979. A study of formate production and oxidation in leaf peroxisomes during photorespiration. Plant Physiol. 63: 289-293.

4. Izawa, S. 1962. Stimulatory effect of carbon dioxide upon the Hill reaction as observed with the addition of carbonic anhydrase to reaction mixture. Plant & Cell Physiol. 3: 221-227.

5. Jursinic, P., J. Warden, Govindjee. 1976. A major site of bicarbonate effect in system II reaction. Evidence from ESR signal II_{vf}, fast fluorescence yield changes and delayed light emission. Biochim. Biophys. Acta 440: 322-330.

6. Khanna, R., Govindjee, T. Wydrzynski. 1977. Site of bicarbonate effect in Hill reaction. Evidence from the use of artificial electron acceptors and donors. Biochim. Biophys. Acta 452: 208-214.

7. Lavergne, J. 1982. Mode of action of 3-(3,4-dichlorophenyl)-1, 1-dimethyl urea. Evidence that the inhibitor competes with plastoquinone for binding to a common site on the acceptor side of photosystem II. Biochim. Biophys. Acta 682: 345-353.

8. Mills, J.D., R.E. Slovack, G. Hind. 1978. Cyclic electron transport in isolated intact chloroplasts. Further studies with antimycin. Biochim. Biophys. Acta 504: 298-309.

9. Oliver, D.J. 1981. Mechanism and control of photo-respiratory glycolate metabolism: CO_2 loss from (the C-2) carbon of glycolate. *In*: Proceedings of the 5^{th} International Congress on Photosynthesis (G. Akoyunoglou, ed.), Vol. IV. pp. 501-508, Balaban International Science Services, Philadelphia Pa.

10. Robinson, H.H., J.J. Eaton-Rye, J.J.S. van Rensen, Govindjee. 1984. The effects of bicarbonate depletion and formate incubation on the kinetics of the oxidation-reduc-

tion reactions of the photosystem II quinone acceptor complex. Z. Naturforsch. 39c: 382-385.

11. Steiger, H., Beck E. 1981. Formation of hydrogen peroxide and oxygen dependence of photosynthetic CO_2 assimilation by intact chloroplasts. Plant & Cell Physiol. 22: 561-576.

12. Stemler, A. 1977. The binding of bicarbonate ions to washed chloroplast grana. Biochim. Biophys. Acta 460: 511-522.

13. Stemler, A. 1979. A dynamic interaction between the bicarbonate ligand and photosystem II reaction center complexes in chloroplasts. Biochim. Biophys. Acata 545: 36-45.

14. Stemler, A. 1980. Forms of dissolved carbon dioxide required for photosystem II activity in chloroplasts membranes. Plant Physiol. 65: 1160-1165.

15. Stemler, A., Govindjee. 1973. Bicarbonate ion as a critical factor in photosynthetic oxygen evolution. Plant Physiol. 52: 119-123.

16. Stemler, A., P. Jursinic. 1983. The effects of carbonic anhydrase inhibitors formate, bicarbonate, acetazolamide, and imidazole on photosystem II in maize chloroplasts. Arch. Biochem. Biophys. 221: 227-237.

17. Stemler, A., J. Murphy. 1983. Determination of the binding constant of $H^{14}CO_3^-$ to the photosystem II complex in maize chloroplasts: effect of inhibitors and light. Photochem. Photobiol., 38: 701-707.

18. Van Rensen, J.J.S., W. van der Vet, W.P. van Vliet. 1977. Inhibition and uncoupling of electron transport in isolated chloroplasts by the herbicide 4,6-dinitro-*o*-cresol. Photochem. Photobiol. 25: 579-583.

19. Van Rensen, J.J.S., W.F.J. Vermaas. 1981. Action of bicarbonate and photosystem II inhibiting herbicides on electron transport in pea grana and in thylakoids of a blue green alga. Physiol. Plant 51: 106-110.

20. Vermaas, W.F.J., Govindjee. 1981. Unique role(s) of carbon dioxide and bicarbonate in the photosynthetic electron transport chain. Proc. Indian Natl. Sci. Acad. B47: 581-214.

21. Vermaas, W.F.J., J.J.S. van Rensen. 1981. Mechanism of bicarbonate action on photosynthetic electron transport in broken chloroplasts. Biochim. Biophys. Acta 636: 168-174.

22. Wydrzynski, T., Govindjee. 1975. A new site of bicarbonate effect in photosystem II of photosynthesis: Evidence from chlorophyll fluorescence transients in spinach chloroplasts. Biochim. Biophys. Acta 387: 403-408.

23. Zelitch, I. 1972. The photooxidation of glyoxylate by envelope-free spinach chloroplasts and its relation to photorespiration. Arch. Biochem. Biophys. 150: 698-707.

CHAPTER 4

EFFECTS OF FORMATE AND BICARBONATE ON PHOTOSYNTHETIC ELECTRON FLOW IN THE ABSENCE AND IN THE PRESENCE OF UNCOUPLER

Introduction

Effects of formate on photosynthetic electron flow are known for some time. Isolated thylakoids that are incubated in media containing formate are inhibited in their ability to produce oxygen in the presence of an artificial electron acceptor (5, 10, 12, 19). The inhibitory effect of formate is counteracted by bicarbonate. As several other anions besides formate are also inhibitory and as bicarbonate is the only compound that has been shown to relieve this inhibition (5), it has been suggested that binding of bicarbonate to "active sites" on the thylakoids is a requirement for the Hill reaction in (12). The stimulating-effect of bicarbonate on the Hill reaction the presence of inhibitory salts is generally known as the "bicarbonate-effect" on electron flow.

The stimulation of the Hill reaction by bicarbonate is dependent on the formate concentration (8); formate appears to be a competitive inhibitor of both the binding of bicarbonate to thylakoids (13) and the reactivation of the Hill reaction by bicarbonate in CO_2-depleted chloroplasts (see Chapter 3). It has been suggested that binding of bicarbonate may not be required for electron flow at the acceptor side of PSII (13, see also Chapter 3) but that bicarbonate relieves the inhibitory action of monovalent anions such as formate, acetate and Cl^-.

In Chapter 3 a scheme has been presented in which formate acts as a negative feedback inhibitor of linear electron flow at low bicarbonate concentrations. At higher bicarbonate concentrations the feedback inhibition will be decreased due to a decrease of photorespiratory formate production and a higher

reactivation of electron flow by bicarbonate. As formate is produced during photorespiration, inhibition of electron flow *in vivo* develops during illumination and not in a previous dark period. During illumination of isolated non-depleted chloroplasts the Hill reaction rapidly declines in the presence of formate (5, 9, 11) and bicarbonate is rather ineffective in stimulating electron flow in CO_2-depleted chloroplasts when added in the light in the presence of formate (9, 18).

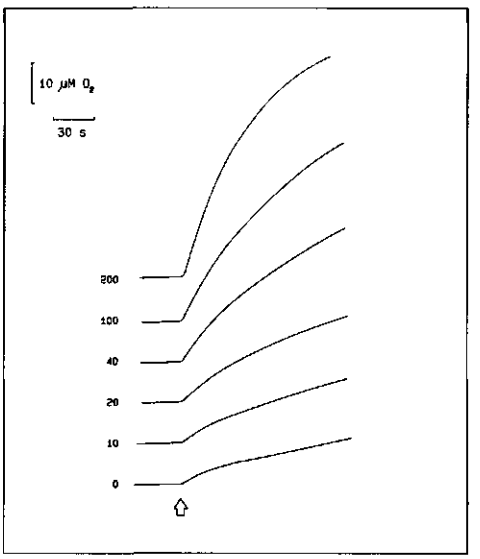
In the model presented in Chapter 3 bicarbonate and formate are suggested to affect the relative yields of NADPH and ATP only by inhibition of linear electron flow. There are however reports that anions may uncouple electron transport from photophosporylation (4). Some of these anions (e.g. citrate, phosphate) are particularly effective while other ions are only weak uncouplers (e.g. bicarbonate). Acetate and butyrate also uncouple, but electron flow rapidly declines during illumination (4). This decline is caused by inhibition of electron flow; acetate and butyrate resemble formate in being able to increase the dependence of the Hill reaction on bicarbonate (5). Although formate was not investigated with respect to uncoupling of electron flow (5, 6), it is to be expected that formate posesses some uncoupling properties as well.

If formate would uncouple at a concentration lower than that required for inhibition of linear electron flow, the negative feedback mechanism might not function *in vivo* since ATP is required to generate RUBP, the substrate for photorespiration. Therefore the inhibitory and uncoupling properties of formate were compared in isolated broken chloroplasts.

Materials and methods

Isolation of pea thylakoids and measurement of oxygen evolution were performed as described in Chapter 2. Isolation of spinach thylakoids was essentially the same with the exception that the isolation medium was different: 0.4 M sorbitol, 10 mM

NaCl, 5 mM $MgCl_2$, 2 mM Na-ascorbate, 20 mM tricine/NaOH (pH 7.8) and 0.2% (w/v) BSA. Reaction medium used with pea chloroplasts (PRM): 100 mM NaCl, 100 mM Na-formate, 50 mM Na-phosphate (pH 6.5), 5 mM NH_4Cl and 1 mM FeCy. For spinach thylakoids the following reaction medium was used (SRM): 0.3 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 50 mM buffer (MES/KOH or HEPES/KOH). Bicarbonate and formate were added to the reaction medium were appropriate.



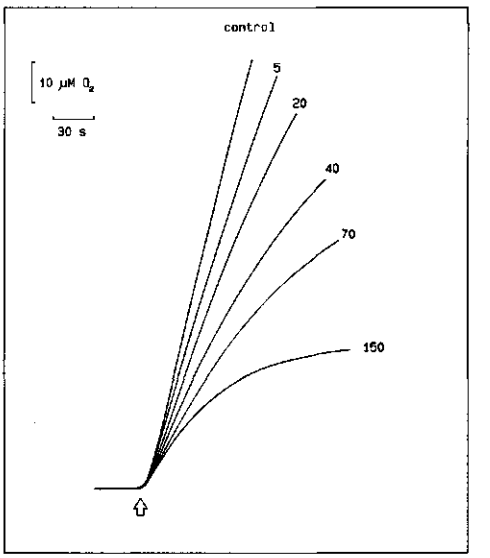


Fig. 4.1 (left): Stimulation of the initial Hill reaction rate by formate in the absence of uncoupler. Reaction medium: SRM (pH 6.9), 1 mM FeCy and spinach chloroplast at a concentration of 25 µg Chl/ml. Numbers along the traces indicate the formate concentration (in mM) present in the reaction medium.

Fig. 4.2 (right): Inhibition of the Hill reaction by formate in the presence of uncoupler. Reaction conditions as described in Fig. 4.1 with the exception that 5 mM NH_4Cl was included in the reaction medium. The numbers along the traces indicate the formate concentration used (mM).

Results

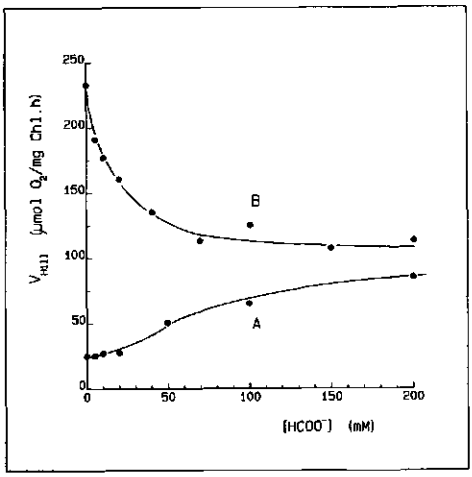
Effects of formate on basal electron flow

Figure 4.1 shows the effect of increasing formate concentrations on basal electron flow in broken chloroplasts. At low formate concentrations (≤ 10 mM) the Hill reaction is hardly affected but at concentrations of 20 mM formate and above a significant stimulation is observed. Especially at high formate concentrations (≥ 100 mM) the stimulation of electron flow is only maximal during the initial phase of illumination. Initially proceeding at a high rate, the Hill reaction progressively slows down. This type of response can also be observed in the presence of high acetate concentrations but not in the presence of citrate (data not shown). The effects of formate on the initial Hill reaction rate (as determined from the initial slope) are indicated in Fig. 4.3A. Half-maximal stimulation of electron flow occurs at ~ 70 mM formate.

Effects of formate on uncoupled electron flow.

In the presence of an uncoupler formate has no stimulatory effect on the initial Hill reaction rate (Fig. 4.3B); uncoupled electron flow is only inhibited by formate. The inhibition is not constant but increases during the illumination period (Figs. 4.2 and 4.4). At a high formate concentration the decrease of the Hill reaction rate appears to be mono-exponential (data not shown). The half-time of the deactivation of the Hill reaction is dependent on several parameters, e.g. formate concentration (Fig. 4.2), pH (Fig. 4.4), surface potential (15), light intensity (9) and biotype (14). In Fig. 4.3B the inititial Hill reaction rate in the presence of NH_4Cl is plotted as a function of the formate concentration. Half-maximal inhibition is observed at approx. 20 mM formate. Thus it appears that at pH 6.9 formate inhibits uncoupled electron flow

at concentrations at which hardly a stimulatory (uncoupling) effect on basal electron flow is observed.



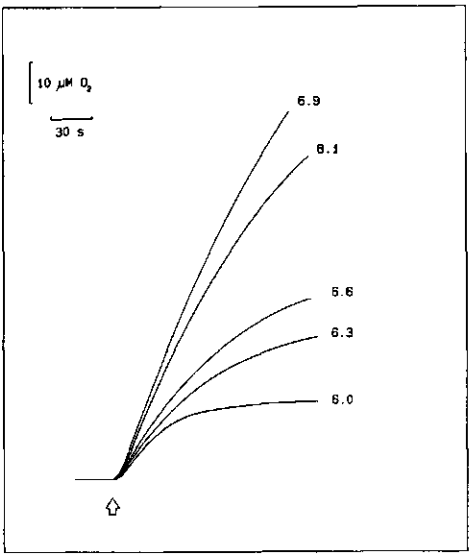


Fig. 4.3 (left): Effects of formate on the initial Hill reaction rate in spinach thylakoids in the absence (A) and the presence (B) of uncoupler (5 mM NH_4Cl). Reaction conditions as described in Figs. 4.1 resp. 4.2.

Fig. 4.4 (right): Effect of the ambient pH on the Hill reaction in the presence of 20 mM formate. Reaction conditions as described in Fig. 4.2 with the exception that HEPES/KOH was used at pH 8.1. The numbers along the traces indicate the pH of the reaction medium.

Effects of bicarbonate on the inhibition of electron flow by formate during illumination.

After dark-incubation of CO_2-depleted thylakoids with 10 mM bicarbonate the (initial) Hill reaction rate is almost completely reactivated, even in the presence of high formate concentrations (8, 18). Figure 4.5A shows that in the absence of bicarbonate the Hill reaction rate rapidly declines and that a subsequent incubation in the dark in the absence of bicarbonate does not result in a reactivation of the Hill reaction; only dark-incubation with bicarbonate results in a reactiva-

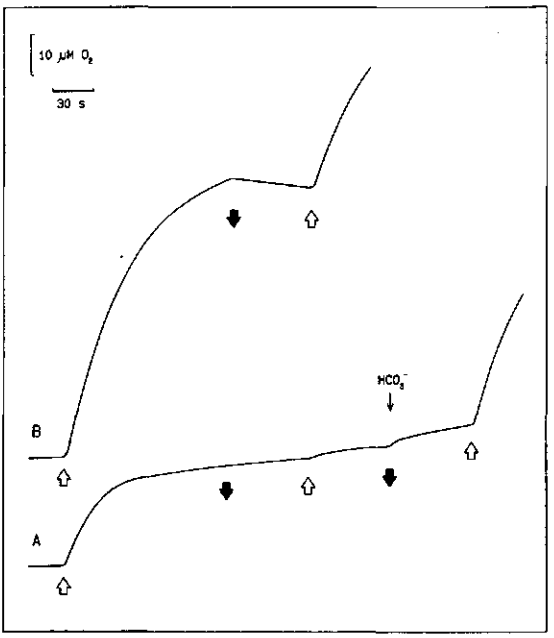


Fig. 4.5: Inhibition of the Hill reaction by formate during prolonged illumination in the absence (A) and the presence (B) of 10 mM bicarbonate. Reaction medium: PRM (pH 6.5), 1 mM FeCy, 5 mM NH_4Cl and pea thylakoids equivalent to 25 µg Chl/ ml.

tion of the Hill reaction. In Fig. 4.5B a similar experiment was carried out in the presence of 10 mM bicarbonate. It appears that also in the presence of bicarbonate the Hill reaction becomes inhibited during illumination. The inhibition is however less severe and only a dark-incubation suffices to reactivate the Hill reaction. This observation is in agreement with observations by Good (5) and Stemler (9) and demonstrates once more that reactivation of electron flow by bicarbonate is only optimal after a dark-incubation period (see also Chapter 2). Figure 4.6 shows that the bicarbonate concentration required for half-maximal reactivation of the initial rate of the Hill reaction is about 1 mM.

The same bicarbonate concentrations that reactivate the initial Hill reaction rate in CO_2-depleted chloroplasts after a dark-incubation period (see e.g. Fig. 4.6), are much less effective in preventing the inhibitory action of formate during

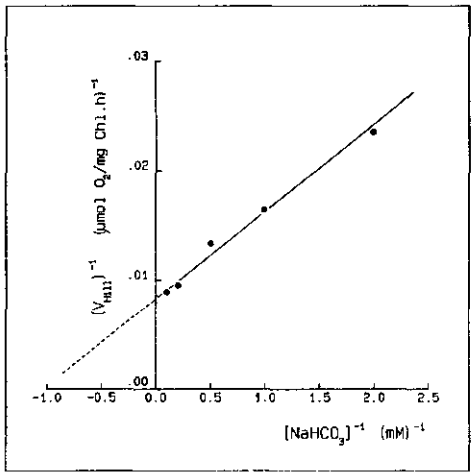


Fig. 4.6: Reactivation of the Hill reaction by bicarbonate. Pea thylakoids were illuminated for 3 min. in the presence of formate and subsequently reactivated by incubation in the dark for 2 min. with various bicarbonate concentrations as described in Fig. 4.5A. The initial Hill reaction rates were determined from the first 20s of the Hill reaction and plotted versus the bicarbonate concentration in a double-reciprocal plot. Reaction conditions as described in Fig. 4.5A.

illumination (see e.g. Fig. 4.5B). Inclusion of 5 mM bicarbonate in the reaction medium, a concentration which usually reactivates the initial rate of electron flow for about 60-80%, cannot prevent formate (100 mM) from inhibiting electron flow considerably in non-depleted chloroplasts (Fig. 4.7C); even in the presence of 20 mM bicarbonate inhibition can be observed (Fig. 4.7C). After some time the Hill reaction rate reaches a steady state. Figure 4.7 shows that the Hill reaction rate in steady state is dependent on the bicarbonate concentration. As in the presence of 20 mM bicarbonate and 100 mM formate the Hill reaction rate is only ~ 13% of the initial rate in the control without formate (compare Figs. 7A en B), it can be concluded that more than 20 mM bicarbonate is required to counteracts the inhibitory effect of 100 mM formate. This indicates that in the light either formate is a more effective inhibitor of electron flow than in the dark or alternatively that bicarbonate is less effective in stimulating of electron flow during prolonged illumination.

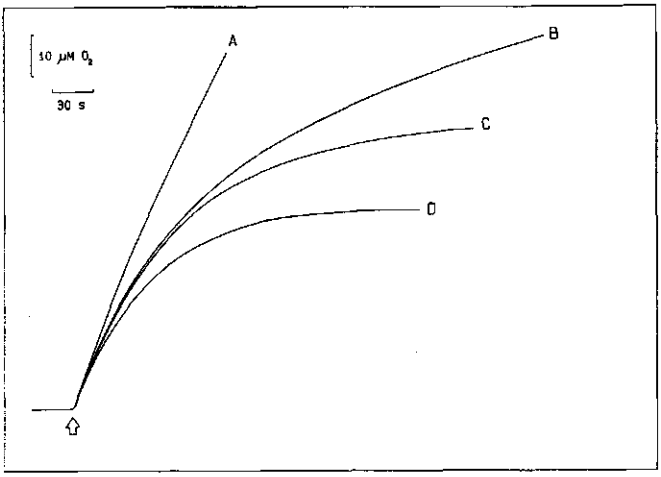


Fig. 4.7: The kinetics of the Hill reaction in the presence of formate and bicarbonate. A: control, -formate, -bicarbonate; B: + 100 mM formate, + 20 mM bicarbonate; C: + 100 mM formate, + 5 mM bicarbonate; D: + 100 mM formate, + 1 mM bicarbonate. Reaction medium: 0.3 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM MES/NaOH (pH 6.5), 1 mM FeCy and pea chloroplasts equivalent to 25 µg Chl/ ml.

Discussion

Uncoupling of electron flow by formate

Formate is shown to posess uncoupling properties (Figs. 4.1 and 4.3). The half-maximal stimulation of basal electron flow is observed at approximately 70 mM formate at pH 6.9. This value may be slightly underestimated since at high formate concentrations the concomittant inhibitory effect will interfere, even in the first 20s of the Hill reaction (see Fig. 4.3B). This means that at a high formate concentration electron flow would have proceeded faster in the absence of concurrent inhibition. The result of this inhibition is that the maximal stimulation of the Hill reaction rate is observed at a lower formate concentration. Thus the true half-maximal stimulation of basal electron flow due to the uncoupling action of formate

may occur at formate concentrations higher than 70 mM under the conditions used.

The fact that formate uncouples electron flow in thylakoids indicates that formate does not enter the thylakoid lumen as formic acid. If only the protonated species would be diffusing into the thylakoid lumen, this would lead to an acidification in the lumen. As acidification of the thylakoid lumen slows down the reoxidation of PQH_2 (6), inhibition electron flow by formate would be expected. This is not the case (see Figs. 4.1 and 4.3) and therefore formate must enter the chloroplasts either as a neutral complex or as an anion. Preliminary measurements of the flash-induced change in the membrane potential by means of the P515-absorbance changes, show that at 25°C Na-formate enhances the dissipation of the transmembrane potential (J. Snel, unpublished data), indicating that formate moves across the membrane as anion.

Inhibition of electron flow by formate

The experiments shown in Figs. 4.2 and 4.3 demonstrate that the initial Hill reaction rate is inhibited by formate. The initial rate is inhibited for 50% by approx. 80 mM formate (Fig. 4.3). The Hill reaction rate decreases however during illumination in the presence of formate (Fig. 4.2) and after 2 min illumination the inhibition is much higher than 50% at 80 mM formate (not shown). Several carbonyl compounds (e.g. bicarbonate, dimethyl carbonate) have been shown to stimulate linear electron flow from PSII to PSI (1). It was suggested that these compounds act by inhibiting a cyclic electron flow around PSII (1). A stimulation of electron flow by formate was never observed in the presence of NH_4Cl. This indicates that formate probably does not inhibit a cyclic electron flow around PSII (cf. ref. 2). Obviously formate either does not bind to the bicarbonate-binding site involved in inhibition of cyclic electron flow around PSII, or it may bind but then with a much

lower affinity than the affinity for the binding site that is involved in inhibition of linear electron flow. Results of Barr et al. (1) and Bublaugh and Govindjee (2) indicate that the bicarbonate-binding site involved in inhibition of cyclic electron flow has a low affinity for bicarbonate.

The action of formate is markedly pH dependent. The kinetics of the decline of the Hill reaction rate (Fig. 4.4) suggest that either formic acid or a protein moiety with a pK_a of approx. 6.5~6.8 is involved in the mechanism of formate action in the light. As the limited time-resolution of the oxygen electrode prevents a precise determination of the kinetics of the formate action at low pH, a discrimination between these two mechanisms has not been possible so far.

Interaction of formate and bicarbonate on electron flow during prolonged illumination.

Figures 4.5 and 4.6 show that after illumination in the presence of formate and an electron acceptor chloroplasts appear to be in a state that is very similar to the state obtained after CO_2-depletion in the dark at low pH. The apparent reactivation constant K_r' obtained by illumination of chloroplasts in the presence of formate appears to be approx. 1 mM (Fig. 4.6), which is identical to the value obtained for K_r' in CO_2-depleted chloroplasts (8, 16; see also Chapter 2) and the dissociation constant K_d for bicarbonate in the presence of formate (13), both in the presence of 100 mM formate. The main difference between the two CO_2-depletion methods is that electron flow in the presence of saturating bicarbonate concentrations is much lower in chloroplasts depleted of CO_2 as described in e.g. Chapter 2.

It has been reported that bicarbonate is not able to stimulate electron flow when added in the light in the presence of high formate concentrations (9, 18). Figure 4.7 shows that in the presence of 100 mM formate the presence of bicarbonate has

a beneficial effect on the rate of electron flow during prolonged illumination. In the presence of 20 mM bicarbonate and 100 mM formate the Hill reaction rate in the steady state is about 13% of the initial rate in control chloroplasts (compare Figs. 4.7A and B). This means that in the presence of 100 mM formate more than 20 mM bicarbonate is required to maintain electron flow in steady state at a half-maximal rate. Assuming that the effects of formate and electron flow are reversible, which is indicated in e.g. Fig. 4.5B, the apparent reactivation constant K_r' can be calculated from the Michaelis Menten equation (rewritten):

$$K_r' = [A] \cdot \frac{1 - \frac{v}{v_{max}}}{\frac{v}{v_{max}}} \simeq 20 \cdot \frac{1-0.13}{0.13} = 134 \text{ mM bicarbonate}$$

(see Chapter 2 for a description of parameters)

This value of 134 mM may be an overestimation since the Hill reaction in the control chloroplasts (Fig. 4.7A) also seems to decline slighthly. But nevertheless it is clear that K_r' in steady state in the light is more than a magnitude higher than in the dark. Stemler and Murphy (13) have reported effects of light on the binding of bicarbonate. In the absence of formate only a small effect was found but in the presence of formate (20 mM) a 3-fold increase in the dissociation constant K_d was measured. This light-effect on K_d is much lower than that on K_r' as estimated above, but in the binding experiments the light-intensity was rather low (5 W/m^2) and no electron acceptor was present (13). Illumination with saturating light in the presence of an electron acceptor and high formate concentrations are conditions that promote the deactivation of electron flow by formate (9; J. Snel, unpublished data). Therefore it is to be expected that under such conditions the effect of light on the binding of bicarbonate is much larger than the factor 3 reported by Stemler and Murphy (13). In the absence of formate only a small effect of light on the binding of bicarbonate was de-

tected (13). Thus the major effect of light must be on the binding of formate, i.e. formate binds much tighter to its binding site in the light. Using the data from Fig. 4.5 in ref. 13, I have calculated the K_d of formate to be about 5 mM in the dark. In Chapter 3 K_i has been determined to be 2-3 mM formate in the dark. Thus K_d and K_i in the light might be smaller than 0.5 resp. 0.2 mM formate.

The results of these calculations are consistent with the observation by Vermaas and Van Rensen (18) that formate concentrations as low as 0.1 mM have a significant effect on the stimulation of electron flow by bicarbonate in the light. From these data K_r was estimated to be about 1 μM HCO_3^- (18); this estimation was based on the assumption that K_i was about 100 mM formate in the dark. The evidence available now (13; see also Chapter 3 and this Chapter) strongly suggests that K_i is more than an order of magnitude lower. Using the new values, the same calculation yields a K_r of ~ 20-150 μM bicarbonate, which is more in agreement with experimental data (13, 3; see also Chapter 3).

Conclusions

The data presented in this chapter indicate that formate inhibits electron flow. It is estimated that in the light formate can inhibit linear electron flow at sub-mM concentrations at pH 6.5. Formate can also stimulate basal electron flow, probably by dissipating the proton gradient across the thylakoid membrane. This effect occurs at formate concentrations above 10 mM. Therefore it is concluded that formate can inhibit electron flow at concentrations that hardly uncouple electron flow from photophosporylation. Bicarbonate can counteract the inhibitory action of formate but not the uncoupling effects of formate as bicarbonate itself also has uncoupling properties (4).

References

1. Barr, R., R. Melhem, A.L. Lezotte, F.L. Crane. 1980. Stimulation of electron transport from photosystem II to photosystem I in spinach chloroplasts. J. Bioenerg. Biomembr. 12: 197-203.

2. Blubaugh, D.J., Govindjee. 1984. Comparison of bicarbonate effects on the variable chlorophyll a fluorescence of CO_2-depleted and non-CO_2-depleted thylakoids in the presence of diuron. Z. Naturforsch. 39c: 378-381.

3. Fischer, K., H. Metzner. 1981. Bicarbonate effects on photosynthetic electron transport. I. Concentration dependence and influence of manganese incorporation. Photochem. Photobiophys. 2: 133-140.

4. Good, N.E. 1962. Uncoupling of the Hill reaction from photophosphorylation by anions. Arch. Biochem. Biophys. 96: 653-661.

5. Good, N.E. 1963. Carbon dioxide & the Hill reaction. Plant Physiol. 38: 298-304.

6. Good, N.E., S. Izawa, G. Hind. 1966. Uncoupling and energy transfer inhibition in photophosphorylation. *In*: Current Topics in Bioenergetics (D.R. Sanadi, ed.), Vol. I, pp. 59-76, Academic Press, New York.

7. Huber, H.L., B. Rumberg. 1977. Determination of potentials and fixed charges and their light-induced changes on the inner surface of the thylakoid membrane. *In*: Photosynthesis I. Photophysical Processes - Membrane Energization (G.Akoyunoglou ed.), pp. 419-429, Balaban International Science Services, Philadelphia, Pa.

8. Khanna, R., Govindjee, T. Wydrzynski. 1977. Site of bicarbonate effect in Hill reaction. Evidence from the use of artifial electron acceptors and donors. Biochim. Biophys. Acta 462: 208-214.

9. Stemler, A. 1979. A dynamic interaction between the bicarbonate ligand and photosystem II reaction center complexes in chloroplasts. Biochim. Biophys. Acta 545: 36-45.

10. Stemler, A. 1980. Inhibition of photosystem II by formate. Possible evidence for a direct role of bicarbonate in photosynthetic oxygen evolution. Biochim. Biophys. Acta 593: 103-112.

11. Stemler, A. 1980. Forms of dissolved carbon dioxide required for photosystem II activity in chloroplasts membranes. Plant Physiol. 65: 1160-1165.

12. Stemler, A., Govindjee. 1973. Bicarbonate ion as a critical factor in photosynthetic oxygen evolution. Plant Physiol. 52: 119-123.

13. Stemler, A., J. Murphy. 1983. Determination of the binding constant of $H^{14}CO_3^-$ to the photosystem II complex in maize chloroplasts: Effects of inhibitors and light. Photochem. Photobiol. 38: 701-707.

14. Van Rensen, J.J.S. 1984. Interaction of photosystem II herbicides with bicarbonate and formate in their effects on photosynthetic electron flow. Z. Naturforsch. 39c: 374-377.

15. Van Rensen, J.J.S, J.H. Hobé, R.M. Werner, J.F.H. Snel. 1984. Reactivation of electron transport in CO_2-depleted chloroplasts by bicarbonate is influenced by thylakoid surface potential. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol. I, M. Nijhoff/Dr. W. Junk Publishers, The Hague.

16. Van Rensen, J.J.S., W.F.J. Vermaas. 1981. Action of bicarbonate and photosystem II inhibiting herbicides on electron transport in pea grana and in thylakoids of a blue-green alga. Physiol. Plant. 51: 106-110.

17. Vermaas, W.F.J., Govindjee. 1982. Bicarbonate or carbondioxide as a requirement for efficient electron transport

on the acceptor side of photosystem II. *In*: Photosynthesis (Govindjee, ed.), Vol. II pp. 541-558, Academic Press, New York.

18. Vermaas, W.F.J., J.J.S. van Rensen, 1981. Mechanism of bicarbonate action on photosynthetic electron transport in broken chloroplasts. Biochim. Biophys. Acta 636: 168-174.

19. Wydrzynski, T., Govindjee. 1975. A new site of bicarbonate effect in photosystem II of photosynthesis: Evidence from chlorophyll fluorescence transients in spinach chloroplasts. Biochim. Biophys. Acta 387: 403-408.

CHAPTER 5

STIMULATION OF THE FLASH-INDUCED TRANSMEMBRANE POTENTIAL IN CO_2-DEPLETED CHLOROPLASTS BY ADDITION OF BICARBONATE

Introduction

In Chapter 3 a model has been proposed that describes a possible regulation of electron flow by formate and bicarbonate *in vivo*. In Chapter 4 some of the effects of formate and bicarbonate on electron flow in isolated broken chloroplasts have been characterized in more detail. Demonstration of formate and bicarbonate effects on electron flow in functionally intact systems (e.g. intact chloroplasts, algae or leaves) *in situ* requires a non-invasive technique. Two optical techniques useful for measuring parameters of electron flow *in situ* are: chlorophyll a fluorescence and electrochromic absorption changes. Both have been used to study bicarbonate effects on electron flow in isolated broken chloroplasts.

P_{515}-absorbance changes

This method has been applied so far in only two cases to measure the effects of bicarbonate on electron flow in chloroplasts. Jursinic and Stemler (7) have measured the effect of bicarbonate on the flash-induced P_{515} response in CO_2-depleted broken chloroplasts and Garab et al. (3) have reported effects of CO_2 on the flash-induced P_{515} response in both chloroplasts and leaves. Transmembrane charge separations in PSI and PSII result in the formation of a transmembrane potential. The resulting electrical field is sensed by special pigments, mainly carotenoids and chlorophylls (5). The flash-induced electrical field causes a red shift of the absorption bands; light-induced absorbance changes at 515 nm appear to be linearly correlated with the transmembrane potential (1, 5, 6). The flash-induc-

ed absorbance change at 515 nm (P_{515}-response) in thylakoids is characterized by complex kinetics. At least 5 different kinetic components can be observed in broken and intact chloroplasts (10). After a fast rise, which takes less than 20 ns (e.g. 5, 6), a slower rise in the P_{515} response is often observed. This slow rise has a risetime varying from several ms (1, 5, 6) to several tens of ms (14). After the slow rise the P_{515} response decays to its dark value with triphasic kinetics with life-times of ~ 100 ms, 1000 ms and > 1 s (10, 14). The different components have been named phase a (initial fast rise), phase b (slow rise[1]) and the decay phases c', c and d (9). Although the interpretation of several components, notably phases b, c and d, is still a matter of discussion (see e.g. reviews by Cramer and Crofts (1), Junge and Jackson (6) and Vredenberg et al. (14)), the assumption that phase a is a linear indicator of the transmembrane potential has been widely accepted.

Materials and Methods

Thylakoids were isolated from pea leaves as described in Chapter 2 and stored at -80°C. Before use the thylakoids were thawed and stored on ice in the dark. Flash-induced absorbance changes were measured using a modified Aminco-Chance spectrofotometer in the double beam mode. By removal of the optical chopper, DC-operation was possible. The measuring beams were positioned next to each other giving a measuring area of 1.75 x 1 cm^2; the optical pathlength was 1 cm and the intensity of the measuring light was 7 $\mu W/cm^2$ at 518 nm. The optical dispersion was 14 nm. Measuring light was provided by a tungsten lamp

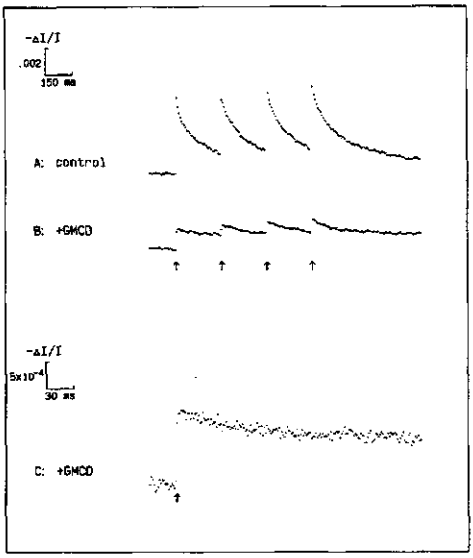
[1] In this chapter phase b will be used to describe the slow rise of the P_{515} response without substraction of any other component.

connected to a stabilized power supply (Oltronics B32-20R). Saturating actinic flashes were generated by two Xenon flash tubes (General Electric FT-230) connected to a 8 μF capacitor giving a flash duration of 8 μs at half-intensity; a small tail of less than 1% of the total energy was however extending to about 150 μs. The light reached the sample via a Schott RG-630/ RG-645 filter combination and a glass-fiber lightguide. The photomultiplier (power supply: Oltronics A2,5k-10HR) was shielded from the actinic light by 9 mm BG-39 filters (Schott). The photomultiplier signal was amplified by a Tektronix 5A22 differential amplifier after compensating the dark signal with an offset voltage. Further processing of the signal and generation of the triggers for the flashes was carried out by a minicomputer (Minc-11/23, Digital Equipment Corporation) equipped with the following modules: MNCAG (preamplifier), MNCAD (AD converter), MNCAA (DA converter), 2 x MNCKW (real-time clock) and MNCDO (triggering). A fast MNCAD processing routine with a maximal sampling-frequency of 6 kHz was written by G.H. van Eck, Computer Centre, Agricultural University, Wageningen. The electrical bandwith of the amplifier was adjusted to the sampling-frequency. Unless indicated otherwise the measurements were carried out at 3°C and signals were averaged at a repetition rate of 0.1 Hz.

Thylakoids were depleted of CO_2 by pre-illumination of the sample with continuous red light for 1 or 2 min. in formate containing reaction medium at 0°C in the measuring cuvette. Illumination was provided by a laboratory-built lamphouse equipped with a 250 W tungsten lamp. Red light was obtained by including a 3 mm RG-630 filter in the light-path; the incident light intensity was approx. 150 W/m^2.

Results

P_{515} response in control chloroplasts



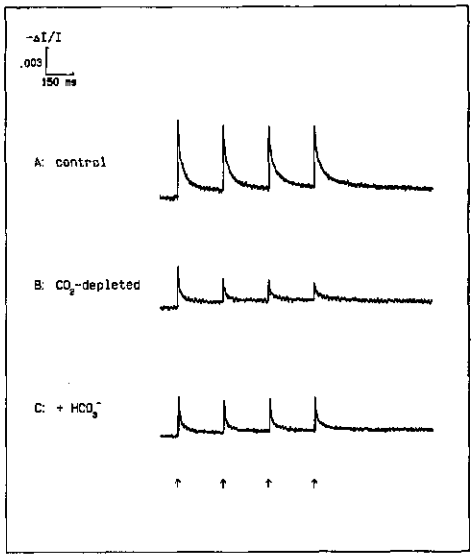


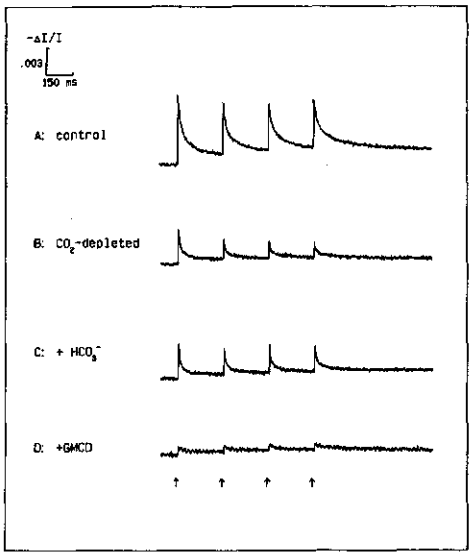
Figure 5.1 (left): Flash-induced absorbance changes (ΔI/I) at 518 nm in pea thylakoids. Reaction medium: 0.3 M sorbitol, 10 mM NaCl, 5 mM Mg Cl_2, 20 mM Na-formate, 25 mM HEPES/KOH (pH 7.2). Further additions are: 0.25 mM BQ and the chlorophyll concentration was 25 µg Chl/ml. A: control; B: same sample after addition of 1 µM gramicidine; C: same sample measured with higher time resolution. In traces A and B 16 signals were averaged at a repetition rate of 0.1 Hz and in trace C 36. The arrows indicate the time at which the actinic flashes were given. In trace C the electrical bandwith (- 3dB) was increased from 100 Hz to 1 kHz.

Figure 5.2 (right): Effects of CO_2-depletion and addition of bicarbonate on the flash-induced ΔA518 at pH 6.3. The reaction medium was essentially the same as in Fig. 5.1 but 25 mM MES/KOH (pH 6.3) was used as a buffer. Further additions are: 5 mM NH_4Cl, 1 mM FeCy and [Chl] = 40 µg Chl/ml. A: chloroplasts not depleted of CO_2; B: sample A pre-illuminated for 2 min at 0°C; C: sample B after 2 min dark-incubation with 10 mM $NaHCO_3$. For these experiments 16 measurements were averaged at a repetition rate of 0.1 Hz; the electrical bandwith was 1 kHz.

Figure 5.1A shows the flash-induced P_{515} response in pea thylakoids that have not been depleted of CO_2. The signal clearly contains the components a and c'. In the presence of gramicidin (Fig. 5.1B) a component remains visible with an unresolved risetime of less than 7.5 ms and a biphasic decay with an overall half-time of ~ 460 ms. The slowest component is probably identical to phase d in intact chloroplasts (7, 9, 13) as it is also observed at $\lambda > 535$ nm and as the half-life of this slower component is larger than 1 s. In previous experiments with broken chloroplasts phase d has been reported to be negligably small (8, 10) but this appears to be only the case in media of relatively high ionic strength in the absence of an electron acceptor (data not shown). In the presence of MV, FeCy or BQ the amplitude of the gramicidin insensitive signal was about 8-25% of the total signal, depending on experimental conditions. Figure 1C shows the gramicidin insensitive signal at higher time resolution; the rise time is less than 1.5 ms. The half-life of the fast decay component is ~ 50 ms.

P_{515} response in CO_2-depleted chloroplasts

Figure 5.2 shows the effects of CO_2-depletion on the P_{515} response in broken chloroplasts before and after readdition of bicarbonate. After 2 min preillumination in the presence of 20 mM formate the P_{515} response is drastically altered (B). The magnitude of phase a is diminished by ~ 50% in the first flash compared to the control (A) and even to a greater extent in subsequent flashes. This is partly due to a combination of a faster decay of the signal and the use of an electrical bandwith of 1 kHz; at 3 kHz the magnitude of phase a was ~ 10% higher (data not shown). After incubation of the sample with 10 mM bicarbonate a stimulation of the P_{515} response is observed in the second and following flashes. Qualitatively the same results were obtained at pH 6.1 (data not shown) and pH 5.7 (Fig. 5.3). At pH 5.7 phase d is clearly present in the signal (Fig. 5.3).



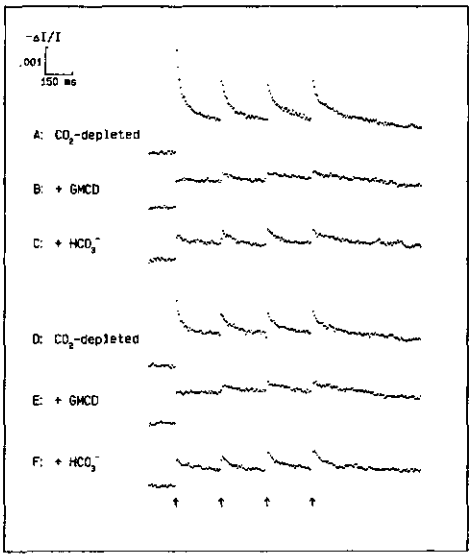


Figure 5.3 (left): Effect of CO_2-depletion and addition of bicarbonate and gramicidin on ΔA518 at pH 5.7. Reaction medium as in Fig. 5.2 with omission of NH_4Cl. A: non-depleted chloroplasts; B: sample A after 1 min illumination at 0°C; C: sample B after 2 min dark-incubation with 10 mM $NaHCO_3$ D: sample C after addition of 2.5 μM gramicidin. Other conditions as in Fig. 5.2.

Figure 5.4 (right): Effect of bicarbonate on the gramicidin insensitive absorbance changes at 518 nm (A, B and C) and 535 nm (D, E and F). Reaction medium as in Fig. 5.3. The chlorophyll concentration was 25 μg Chl/ml and 0.5 mM BQ was used as an electron acceptor. A(D): chloroplasts illuminated for 2 min at 0°C; B(E): Sample A(D) after addition of 1 μM gramicidine; C: Sample B(E) after about 3 min dark-incubation with 10 mM $NaHCO_3$. The electrical bandwith was 100 Hz in these experiments; 16 measurements were averaged at a repetition rate of 0.1 Hz.

The effects of CO_2-depletion and subsequent readdition of bicarbonate on P_{515} response are dependent on the formate concentration, the ambient pH and the preillumination time. Figure 5.4A shows the P_{515} response in thylakoids depleted of CO_2 by 2 min preillumination at pH 5.7. In the presence of 2 μM gramicidin a signal remains (B) which is influenced by the addition of bicarbonate (C).

The same effects were observed at 535 nm, although the gramicidin sensitive part of the response is of course much smaller at this wavelength (Figs. 5.4A, E en F). This means that the gramicidin insensitive signal has a spectrum that bears more resemblence to the spectrum of light scattering (14) than to the spectrum of the P_{515} response (1, 5, 6). The decay of the gramicidin insensitive signal at pH 5.7 in the presence of formate and bicarbonate contains 2 components with half-lives of ~ 50 ms and > 1s. Figure 5.4 clearly shows that the fast component of the gramicidin insensitive signal ($t_{½}$ ~ 50-60 ms) in CO_2-depleted chloroplasts appears to be suppressed in the second and subsequent flashes in the absence of bicarbonate. It seems that this fast component is saturated by the first flash and reverses with a decay which is slower in the absence than in the presence of bicarbonate. This causes an apparent inhibition in the second and subsequent flashes in the absence of bicarbonate. After incubation with bicarbonate the kinetics of the gramicidin insensitive signal appear to be the same as in non-depleted chloroplasts (see e.g. Fig. 5.1B).

Discussion

P_{515} response in CO_2-depleted chloroplasts

The hypothesis that in CO_2-depleted chloroplasts electron flow is inhibited between Q_B and PQ implicates that in CO_2-depleted dark-adapted chloroplasts PSII can make 3 turnovers (4). This has been observed in several cases (2, 4). Since both PSI and PSII contribute to the transmembrane potential, the fast rise of the P_{515} response is composed of the more or less equal contributions of PSI and PSII (5). If the flow of electrons out of PSII is inhibited and the intersystem electron transport components are in a fully oxidized state, PSI can make only 1 turnover, leaving the primary donor P_{700} in the oxidized state: P^+_{700}. In case of a more reduced electron transport chain the

contribution of PSI to the P_{515} response is less clear. Therefore the contribution of PSI to the P_{515} response in Figs. 5.2B and 5.3B is rather uncertain, especially in the second and subsequent flashes. In Figs. 5.2B and 5.3B the amplitude after the second flash is already inhibited with respect to the first flash indicating that either P^+_{700} is not fully reduced in the dark time between the flashes (250 ms) and/or a considerable amount of PSII centers are in one of the states $[P^+_{680} \cdot Q^-_A]$, $[P_{680} \cdot Q^-_A]$ or $[P^+_{680} \cdot Q_A]$. Since the reduction of P^+_{680} by the water splitting system is not strongly inhibited by CO_2-depletion (12) and since the equilibrium $Q^-_A \cdot Q_B \rightleftarrows Q_A \cdot Q^-_B$ may be shifted slightly to the left in the in the absence of bicarbonate (12), the reduced amplitude caused by the second flash is probably a consequence of the presence of the state ($[P_{680} \cdot Q^-_A]$ and perhaps an incomplete reduction of P^+_{700}. From Figs. 5.4B and E it is clear that the reduced amplitude in the second and following flashes is partly caused by suppression of the gramicidin insensitive signal. The half-time of electron flow from PSII to PSI is slowed down to 100-200 ms in CO_2-depleted chloroplasts, as judged from P^+_{700} reduction kinetics (11). The progressive inhibition of the P_{515} response in CO_2-depleted chloroplasts in successive flashes given at a state of 4 Hz also points to an overall half-time of electron flow of 200-250 ms (Figs. 5.2B and 5.3B, see also ref. 7).

In the presence of bicarbonate the amplitude of the fast rise of the P_{515} response remains approximately constant at a flash rate of 4 Hz (Figs. 5.2C and 5.3C), although the amplitude of the 1st flash is always somewhat higher than that in subsequent flashes. But even in the presence of bicarbonate the amplitude is only ~ 50% of the value in non-depleted chloroplasts (Figs. 5.2 and 5.3). This is in agreement with results of Jursinic and Stemler (7). In their experiments only the contribution of PSII to the P_{515} response was measured; the P_{515} response was irreversibly reduced by ~ 50% after CO_2-depletion of the thylakoids. There are basically two explanations; either the electrical field is diminished or the P_{515} pigment pool is

less sensitive to field changes. This reduced sensitivity may be caused by a reduced sensitivity of all the pigments or alternatively a number of P_{515} pigments have been inactivated. The magnitude of the flash-induced transmembrane potential is determined by the number of "open" reaction centers and by the membrane capacitance (4, 5). Several factors might influence the number of reaction centers that participate in the generation of a transmembrane charge separation, e.g. absorption cross section, conversion of PSII centers to a non-electrogenic form and destruction of reaction centers by photoinhibition. The electrical field is affected by changes in physical parameters of the membrane such as the dielectric constant and the thickness of dielectric core. The response of the P_{515} pigment to an applied electrical field is not linear but pseudolinear (5, 6). A lowering of the local static electrical field by e.g. a lowering of a surface potential difference might reduce the magnitude of the P_{515} response. The ionic composition of the reaction medium appears to be important with respect to the amplitude of the P_{515} response; a stimulation of this amplitude in low-salt conditions has been observed (R.L.A. Peters, O. van Kooten, unpublished results). This might indicate that CO_2-depletion lowers the surface potential difference across the thylakoid membrane. Another explanation may be found by the report that a low pH in the thylakoid lumen appears to cause oxidation of some carotenoids (6). It is however not clear whether these carotenoids are involved in the P_{515} response.

Gramicidin insensitive absorbance changes

The absorbance changes observed in the presence of gramicidin have the same amplitude and kinetics at 518 nm and 535 nm (see Fig. 5.4). This suggests that these absorbance changes may be identical to the absorbance changes that have been referred to as reaction III (14). The gramicidin insensitive sig-

nal observed here however contains 2 kinetic components in contrast with reaction III. Both components are characterized by a (unresolved) risetime of < 1.5 ms. A fast component decays with a half-time of ~ 50-60 ms while the slow decay component has a $t_{\frac{1}{2}}$ > 1 s. According to published data (14) the fast component apparently is absent or alternatively the decay is slower in intact chloroplasts and leaves. The fast component appears to be inhibited by bicarbonate depletion (Figs. 5.4B and E) and can be reactivated by incubation with bicarbonate (Figs. 5.4C and F). Vredenberg et al. (14) have shown that reaction III can only be observed in the first two flashes in the presence of DCMU; moreover reaction III shows an oscillatory pattern; at least one component has a periodicity of 2 (14, U. Schreiber, unpublished data). These observations indicate that reaction III can probably be associated with PSII activity (23). The gramicidin insensitive signal observed here probably has the same spectrum as reaction III and is moreover affected by formate and bicarbonate. Therefore these absorbance changes probably are also associated with PSII. Comparison of the kinetics of the fast component of the gramicidin insensitive signal ($t_{\frac{1}{2}}$ ~ 50-60 ms) with published data for other reactions reveals that the fast component might be correlated with proton uptake at the acceptor side of PSII, which occurs with a half-time of approx. 60 ms (9). Obviously further experiments are required to see whether this fast component indeed can be correlated with proton uptake at the acceptor side of PSII.

Conclusions

CO_2-depletion of thylakoids appears to diminish the flash-induced absorbance changes at 518 nm, the P_{515} response, using multiple flashes given at a high rate, e.g. 4 Hz. Part of this diminution is reversible, i.e. can be relieved by bicarbonate addition. However ~ 50% of the P_{515} response is irreversibly diminished, i.e. cannot be restored by addition of bicarbonate.

CO_2-depletion also affects gramicidin insensitive absorbance changes in the 500-550 nm region. In the absence of bicarbonate these flash-induced changes are partly inhibited. This part is restored by addition of bicarbonate. These flash-induced absorbance changes show a spectrum that differs from the spectrum of the flash-induced P_{515}-response.

References

1. Cramer, W.A., A.R. Crofts. 1982. Electron and proton transport. *In*: Photosynthesis (Govindjee, ed.), vol. I, pp. 387-467, Academic Press Inc., New York.
2. Farineau, J., P. Mathis. 1983. Effect of bicarbonate on electron transfer between quinones in photosystem II. *In*: The Oxygen-evolving system of Plant Photosynthesis (Y. Inoue, ed.), Academic Press, New York.
3. Garab, G., A.A. Sanchez Burgos, L. Zimànyi, A. Faludi-Dàniell. 1983. Effect of CO_2 on the energization of thylakoids in leaves of higher plants. FEBS lett. 154: 323-327.
4. Govindjee, M.P.J. Pulles, R. Govindjee, H.J. van Gorkom, L.M.N. Duysens. 1976. Inhibition of the reoxidation of the secondary electron acceptor of photosystem II by bicarbonate depletion. Biochim. Biophys. Acta 449: 602-605.
5. Junge, W. 1977. Membrane potentials in photosynthesis. Ann. Rev. Plant Physiol. 28: 503-536.
6. Junge, W., J.B. Jackson. 1982. The development of electrochemical potential gradients across photosynthetic membranes. *In*: Photosynthesis (Govindjee, ed.), Vol. I, pp. 589-646, Academic Press Inc., New York.
7. Jursinic, P. A. Stemler. 1982. A seconds range component of the reoxidation of the photosystem II acceptor, Q. Effects of bicarbonate depletion in chloroplasts. Biochim. Biophys. Acta 681: 491-428.

8. Peters, R.L.A., M. Bossen, O. van Kooten, W.J. Vredenberg. 1983. On the correlation between the activity of ATP-hydrolase and the kinetics of the flash-induced P_{515} electrochromic bandshift in spinach chloroplasts. J. Bioenerg. Biomembr. 15: 335-346.

9. Polle, A., W. Junge. 1984. Diffusion barriers for protons at the external surface of thylakoids. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol. II, M. Nijhoff/Dr. W. Junk Publishers, The Hague.

10. Schapendonk, A.H.C.M., W.J. Vredenberg, W.J.M. Tonk. 1979. Studies on the kinetics of the 515 nm absorbance changes in chloroplasts. Evidence for the induction of a fast and a slow P_{515} response upon saturation light flashes. FEBS lett. 100: 325-330.

11. Siggel, U., R. Khanna, G. Renger, Govindjee. 1977. Investigation of the absorption changes of the plastoquinone system in broken chloroplasts. The effect of bicarbonate depletion. Biochim. Biophys. Acta 462: 196-207.

12. Vermaas, W.F.J. 1984. The interaction of quinones, herbicides and bicarbonate with their binding environment at the acceptor side of photosystem II in photosynthesis. Dissertation, Agricultural University, Wageningen, The Netherlands.

13. Vermaas, W.F.J., Govindjee. 1982. Bicarbonate effects on chlorophyll fluorescence transients in the presence and the absence of diuron. Biochim. Biophys. Acta 680: 202-209.

14. Vredenberg, W.J., O. van Kooten, R.L.A. Peters. 1984. Electrical events and P_{515} response in thylakoid membranes. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol II, M. Nijhoff/dr. W. Junk, Publishers, The Hague.

CHAPTER 6

EFFECTS OF BICARBONATE AND FORMATE ON PHOTOSYNTHETIC ELECTRON FLOW IN ISOLATED INTACT CHLOROPLASTS

Introduction

Several proposals have been made with respect to a possible role of bicarbonate and formate in the regulation of photosynthetic electron flow *in vivo*. Radmer and Ollinger (24) have suggested that regulation of linear electron flow by bicarbonate will also affect cyclic electron flow, because linear and cyclic electron flow share a part of the electron transport chain (16). This means that the NADPH/ATP ratio will be lower at sub-saturating bicarbonate concentrations. Vermaas and Van Rensen (36) suggested that inhibition of linear electron flow at low bicarbonate concentrations might prevent overreduction of the electron transport chain and the build-up of a surplus of reducing equivalents (e.g. NADPH, NADH) which might disturb cell metabolism. In Chapter 3 evidence is given that bicarbonate may not be required for electron flow from Q_A to PQ but that bicarbonate only counteracts the inhibitory action of formate. As formate can be generated during photorespiration, a model was proposed that links regulation of electron flow to photorespiration via the bicarbonate and formate pools in the chloroplasts.

The models mentioned above were however entirely based on experiments carried out with isolated broken chloroplasts. Demonstration of the existence of a bicarbonate-effect on electron flow *in vivo* is a complicated matter (see the discussion section). Therefore demonstration of a bicarbonate-effect on electron flow in isolated intact chloroplasts seemed to be the most logical step.

There are basically two methods to induce a "bicarbonate-effect" in broken chloroplasts. These two methods are (i) prolonged dark-incubation of the chloroplasts with formate at low

pH (35, 41 and Chapter 3) and (ii) illumination of chloroplasts for several minutes in the presence of formate and an artificial electron acceptor (34, see also Chapter 4). Both methods have their advantages and their drawbacks when applied to intact chloroplasts as will be shown below.

A special problem in demonstrating a "bicarbonate-effect" in isolated intact chloroplasts is that usually CO_2 (or bicarbonate) is the ultimate electron acceptor. The use of CO_2 as electron acceptor would make measurement of electron flow in the absence of bicarbonate nearly impossible. Fortunately PGA is readily taken up by intact chloroplasts (11) and PGA can be reduced to GAL3P at the expense of 1 NADPH and 1 ATP (15). In contrast to CO_2 reduction, PGA reduction by intact chloroplasts is nearly independent of the pH of the stroma (40), which implies that acidification of the stroma by anions of weak acids (5) will not interfere with PGA reduction.

In the experiments described in this Chapter both methods of CO_2-depletion have been applied to obtain CO_2-depleted intact chloroplasts. Dark-incubation with formate at low pH appears to be a method that combines a fair degree of CO_2-depletion and a good reactivation of electron flow by bicarbonate.

Material and methods

Spinach plants (*Spinacia oleracea*) were grown in a greenhouse under high-pressure mercury lamps (Philips MGR 102-400) at a light-intensity of approximately 80 W/m^2. Several cultivars were used: Kir, Bergola and Amsterdams breedblad. During the daily illumination period of 8h the temperature of the leaf and the soil surface was kept between 18-20°C. The relative humidity was minimal 70%.

Intact chloroplasts were isolated from freshly harvested leaves of 4-5 weeks old plants. The method was based on the procedure described by Enser and Heber (5). The leaves (ap-

prox. 20 g) were deribbed, cut in small pieces and homogenized in 50 ml isolation medium in a Sorvall Omnimixer in 2-3 bursts of 3 s. After filtration of the brei through two layers of perlon net (pore diameter 40μm) the filtrate was centrifuged for 60 s at 1500 *g* in two centrifuge tubes in a MSE Chill Spin centrifuge. The pellet was gently suspended in 25 ml resuspension medium and centrifuged for another 60 s at 1500 *g*. The supernatant was discarded and the loose pellet (broken chloroplasts) was removed by gently washing the pellet with 0.5-1 ml resuspension medium. The remaining pellet was resuspended in a small volume of resuspension medium and kept on ice. The entire procedure was carried out at 0-4°C. Isolation medium: 0.3 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4, 2 mM ascorbate, 4 mM cystein and 50 mM MES/KOH (pH 6.2). Resuspension medium: 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.2 mM KH_2PO_4, 1 mg BSA/ml and 50 mM HEPES/KOH (pH 6.7).

The percentage intact chloroplasts was usually between 80 and 95%, as determined by the FeCy method (12). In the experiments shown, the percentage intact chloroplasts was minimal 75%. The chlorophyll concentration was determined as described in Chapter 2.

Intact chloroplasts were depleted of CO_2 by incubation with Na-formate at low pH. Formate was added to a concentrated suspension of chloroplasts in resuspension medium (0.5-2 mg Chl/ml) and the pH was adjusted to the desired value with HCl. The incubation took place on ice in darkness; see the results of this Chapter for more details.

Oxygen evolution was measured with a Clark electrode at 20°C as described in Chapter 2. Standard reaction medium: 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.2 mM KH_2PO_4 and 50 mM buffer (MES or HEPES, adjusted with KOH). For measurements with broken chloroplasts phosphate was omitted and 10 mM NaCl was added. Broken chloroplasts were obtained by suspending intact chloroplasts in 2 mM $MgCl_2$ and 5 mM MES/Tris (pH 7); after 1 min incubation an equal volume of double strength reaction medium was added.

P_{515} measurements were carried out as described in Chapter 5. Reaction medium: 0.3 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM buffer (MES or HEPES, adjusted with KOH) and Na-formate when appropriate.

Results

Effects of formate on PGA reduction

CO_2-depletion of broken chloroplasts by illumination in the presence of an electron acceptor and formate can properly be achieved by using a high light-intensity; a low pH and a high formate concentration speed up the depletion process (see Chapter 4 for more details). On the other hand a high pH and a low formate concentration are prerequisites for optimal carbon reduction in intact chloroplasts (5). These two requirements are

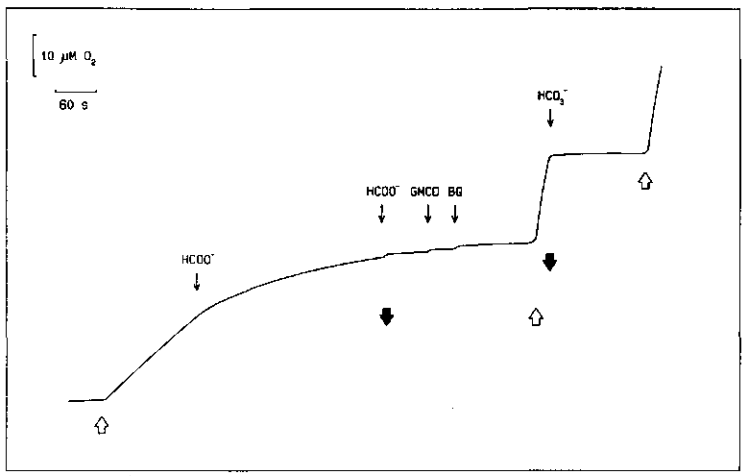
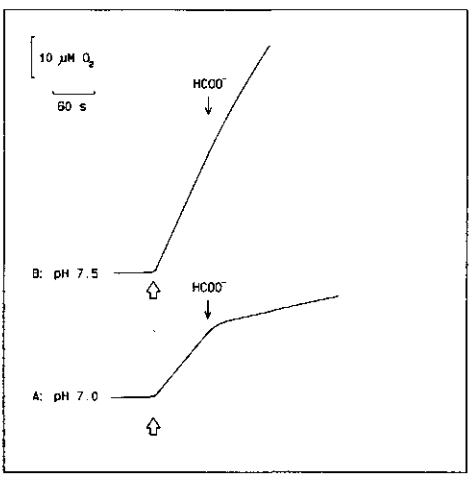


Fig. 6.1: Effect of formate (12.5 mM) on PGA dependent O_2 evolution in isolated intact spinach chloroplasts. The chloroplasts were suspended in standard reaction medium (19 µg Chl/ml) to which 3 mM PGA was added; the pH was 7. Gramicidin (GMCD) and 1,4-benzoquinone (BQ) were added to a final concentration of 2 µM resp. 0.4 mM. At the end of the illumination period the formate concentration was increased to 50 mM.

conflicting and therefore a compromise was chosen by using pH 7 and 10-50 mM formate. Figure 6.1 shows that PGA dependent oxygen evolution at pH 7 is significantly inhibited by 12.5 mM formate, with maximal inhibition attained after about 3 min. Subsequent addition of an uncoupler and an electron acceptor provides a means of assaying electron flow without interference of carbon metabolism. Figure 6.1 shows that electron flow in the presence of BQ and gramicidin is much faster than in the presence of PGA alone. Moreover bicarbonate does not stimulate electron flow in the presence of BQ and gramicidin. So it seems that the inhibition of PGA reduction by formate is not caused by inhibition of electron flow.

Experiments similar to the one described in Figure 6.1 have been carried out at higher formate concentrations and/or longer illumination times. The result of these experiments was that PGA reduction generally was inhibited to a greater extent. Also electron flow was more inhibited in the absence of bicarbon-



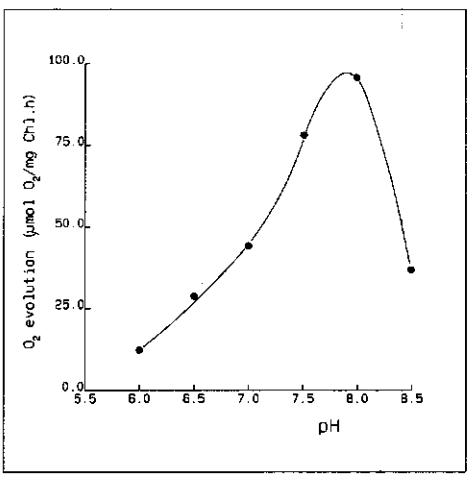


Fig. 6.2 (left): Effect of ambient pH on the inhibition of PGA dependent O_2 evolution in intact chloroplasts by formate. A: standard reaction medium at pH 7.0. B: standard reaction medium at pH 7.5. The PGA concentration was 3 mM; where indicated 20 mM Na-formate was added.

Fig. 6.3 (right): Effect of ambient pH on PGA dependent O_2 evolution in intact chloroplasts. Standard reaction medium with MES as a buffer at pH 6.0 and pH 6.5; HEPES was used at the other pH values. The PGA concentration was 3 mM.

ate, but addition of bicarbonate mostly did not result in a significant stimulation of electron flow.

Figure 6.2 shows that 20 mM formate strongly inhibits PGA reduction at pH 7 while only a slight inhibition is observed at pH 7.5. These observations do suggest that formate might affect PGA reduction in a way similar to the inhibition of CO_2 reduction by formate (5). This, however, implies that there must exist a pH dependent step in the reduction of PGA by intact chloroplasts. Therefore the effect of the ambient pH on PGA reduction by intact chloroplasts was measured. Figure 6.3 shows the pH dependence of PGA reduction. Optimal PGA reduction is observed at pH 7.9, a value pH which is almost identical to the optimum pH for CO_2 dependent oxygen evolution (40).

The results shown in this section indicate that inhibition of PGA reduction by intact chloroplasts in the presence of formate is probably not due to inhibition of electron flow but due to an effect on carbon metabolism; perhaps acidification of the stroma induced by formate could be responsible for the observed inhibition.

CO_2-depletion by dark-incubation with formate at low pH

Prolonged treatment of isolated intact chloroplasts at low pH (< 6) was found to suppress PGA dependent O_2 evolution completely; increasing the pH of the reaction medium did not restore PGA dependent oxygen evolution (D. Naber and J. Snel, unpublished results). Therefore an artificial electron acceptor like e.g. BQ had to be used for our purposes. The use of an artificial electron acceptor has a clear disadvantage: both intact and broken chloroplasts can reduce the electron acceptor. Therefore only bicarbonate and formate effects larger than 5-25% (the percentage broken chloroplasts) can be ascribed with certainty to the intact chloroplasts.

Table 6.1 shows the results of an experiment in which intact chloroplasts were dark-incubated with 100 mM formate at 4 dif-

INCUBATION		ELECTRON FLOW (μmol O_2/mg Chl·h)		
time (min.)	pH	$-HCO_3^-$	$+HCO_3^-$	$+HCO_3^-/-HCO_3^-$
15	6.4	140	169	1.2
46	6.4	53	158	3.0
90	6.4	24	88[1]	3.7
15	6.1	110	180	1.6
45	6.1	46	176	3.8
90	6.1	26	110	4.2
15	5.5	44	174	4.0
47	5.5	24	143	6.0
90	5.5	18	79	4.4
15	5.2	30	153	5.1
45	5.2	24	143	6.0
90	5.2	-	-	-

[1] This value may be an underestimation; the sample was twice illuminated for 30 s in the absence of bicarbonate.

Table 6.1. Effect of bicarbonate on the Hill reaction in isolated intact chloroplasts after dark-incubation with formate at the indicated pH. A suspension of intact chloroplasts was supplemented with 100 mM formate and the pH was adjusted with HCl to the desired value. Subsequently the chloroplasts were incubated on ice in the dark. At the indicated times a small sample of CO_2-depleted chloroplasts was drawn and injected into the reaction vessel containing the standard reaction medium (pH 6.8) supplemented with 10 mM NH_4Cl, 2 mM BQ and 25 mM Na-formate. Bicarbonate was added to a final concentration of 20 mM. The control activity of broken chloroplasts in the presence of FeCy (0.5 mM) and NH_4Cl (10 mM) was 193 μmol/mg Chl.h.

ferent pH values. At various times samples were drawn and oxygen evolution was measured in the absence and in the presence of bicarbonate using the protocol described in Fig. 6.4. The results clearly demonstrate that at all pH values tested a bicarbonate-effect on electron flow is induced during the incubation period; however at pH 5.2 this induction is much faster than at pH 6.4, but nevertheless a clear inhibition of electron flow in the absence of bicarbonate is observed after 90 min incubation at pH 6.4. The initial rate of electron flow in the presence of bicarbonate is high as long as the incubation time is not exceeding 45 min. Figure 6.4 shows that although the initial rate of electron flow is high in the presence of formate and bicarbonate, electron flow is declining during prolonged illumination. A similar phenomenon has also been observed in broken chloroplasts (see Chapter 4 for more details).

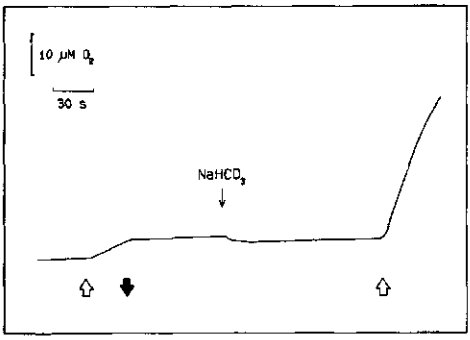


Fig. 6.4: Effect of bicarbonate on O_2 evolution in CO_2-depletion intact chloroplasts in the presence of a Hill oxidant. The chloroplasts were depleted of CO_2 by dark-incubation with 100 mM formate at pH 5.8 as described in materials and methods. The standard reaction medium additionally contained 25 mM Na-formate, 10 mM NH_4Cl and 2 mM BQ.

Effects of bicarbonate and formate on the flahs-induced P_{515} response

The flash-induced P_{515} response of CO_2-depleted intact chloroplasts is shown in Fig. 6.5B. Six flashes were given and the amplitude of the absorbance changes at 518 nm after the first flash is about equal to the amplitude of the fast rise (phase

a) of the P_{515} response in control chloroplasts (Fig. 6.5A). In the following flashes the magnitude of phase a gradually decreases to a value of ~30% of the amplitude in the first flash. The flash-induced ΔA_{518} measured after dark-incubation of the same sample with bicarbonate is shown in Fig. 6.5C. The gradual decrease of phase a, which is apparent in CO_2-depleted chloroplasts, is almost absent in the presence of bicarbonate. The magnitude of phase a after the 6th flash is about 70% of that after the first flash. These results indicate that in the absence of bicarbonate only part of the reaction centers are in an "open" state (i.e. can perform a stable charge-separation) within the dark-period of 250 ms between the flashes. It should be kept in mind that both PS I and PS II contribute to the generation of a transmembrane potential (37) and that total inhibition of e.g. PS II results in only 50% reduction of the amplitude of the P_{515} response (provided PS I is "open").

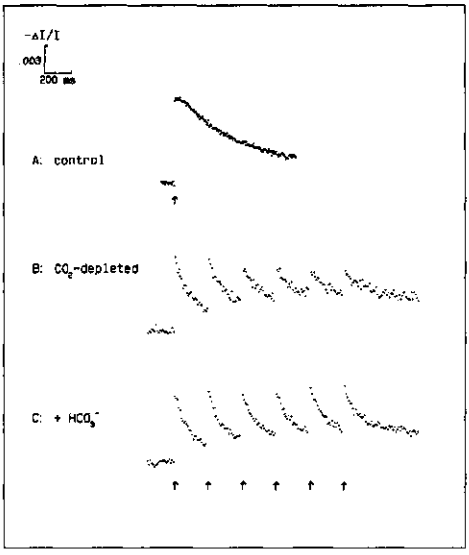


Fig. 6.5: Effect of bicarbonate on the flash-induced P515 response in CO_2-depleted chloroplasts at pH 6.4. A: control (non-depleted); B: CO_2-depleted chloroplasts; C: CO_2-depleted chloroplasts in the presence of 10 mM $NaHCO_3$. The chloroplasts were depleted of CO_2 as described in Fig. 6.4, the formate concentration was 20 mM and the chlorophyll concentration was 27 µg Chl/ml. The number of averages was: A:11, B:3, C:7; the flash-trains were given at a rate of 0.05 Hz.

Another aspect of the P_{515} response in CO_2-depleted chloroplasts is that the kinetics of the dark-relaxation are drastically altered in comparison with the response in control chlo-

roplasts. The slow (phase b) rise apparent in control chloroplasts is absent in CO_2-depleted chloroplasts and phase b is not restored in the presence of bicarbonate. In both cases the half-time of the decay of ΔA_{518} is about 120 ms, which is slightly higher than usually found for the decay of reaction I (38). The decay of reaction 1 is known however to be dependent on the ionic composition of the reaction medium (21,

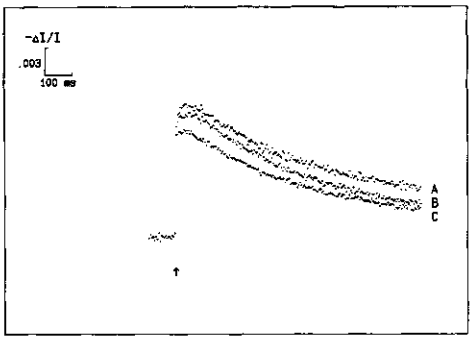


Fig. 6.6: Effect of formate on the flash-induced P515 response in intact chloroplasts (not depleted of CO_2). A: control; B: +50 mM Na-formate; C: sample B after 90 min dark-incubation on ice. The chlorophyll concentration was 33 µg Chl/ml. The number of average was: A:6, B:8, C:13.

26). Under the conditions used here, addition of sodium formate was found not to alter the rate of decay of ΔA_{518} significantly but formate does appear to inhibit the slow rise slightly (Fig. 6.6). Only after prolonged incubation the amplitude of the fast rise (phase a) appears to be decreased as well (Fig. 6.6C). On the other hand the ambient pH has dramatic effects on the kinetics of the flash-induced ΔA_{518}. Figure 6.7 shows that especially at low pH and after long dark-incubation periods the rate of decay is greatly increased and phase b is no longer observable. These results indicate that the absence of the slow rise observed in CO_2-depleted intact chloroplasts is more likely to be due to by the incubation at low pH than by an effect of formate on the kinetics of ΔA_{518}.

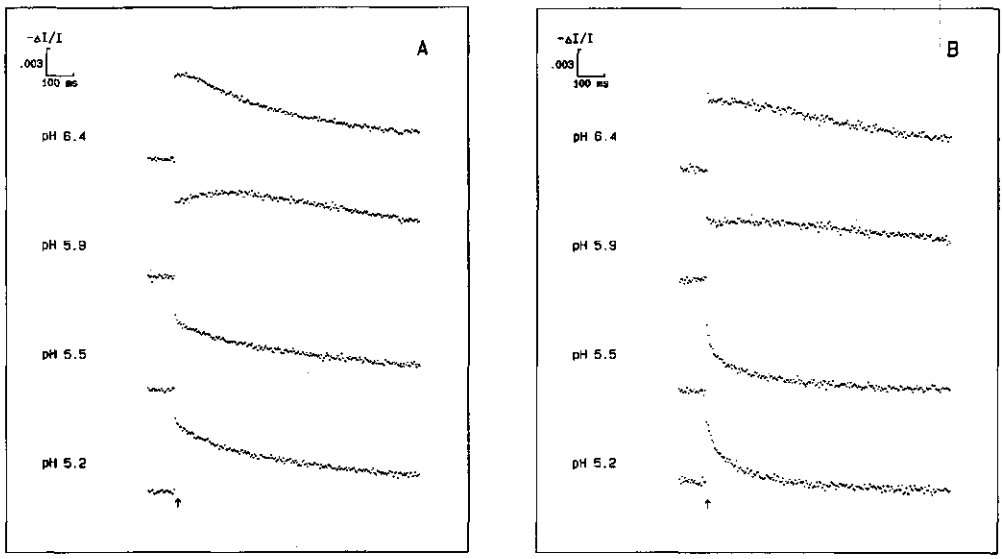


Fig. 6.7: Effect of ambient pH on the flash-induced P515 response in intact chloroplasts. A: measurements started after approx. 20 min incubation; B: measurement started after 60 min incubation. Incubation in reaction medium at the indicated pH value was carried out in the dark on ice. The chlorophyll concentration was 27 µg Chl/ml. The number of averages was 9; the repetition rate 0.1 Hz.

Discussion

Effects of formate on PGA reduction

Figure 6.1 clearly demonstrates that formate inhibits PGA dependent O_2 evolution at another step than photosynthetic electron flow. This inhibition of PGA reduction is more pronounced at low pH than at high pH, a phenomenon that resembles the inhibition of CO_2-dependent O_2 evolution by anions of weak acids (5, 40). PGA dependent O_2 evolution appeared to be dependent on the ambient pH (Fig. 6.3). The maximal rate of PGA dependent O_2 evolution was observed at pH 7.9. This pH dependen-

ce might explain the inhibition of PGA dependent oxygen evolution by formate at pH 7 as formate appears to inhibit the light-induced alkalinization of the stroma (5).

There is however strong evidence that reduction of PGA to GAL3P in vitro (18) and in isolated intact chloroplasts (40) is not dependent on the ambient pH at pH values between 6.7 and 9.2. The experimental conditions in our experiments were however slightly different from the conditions used by Werdan et al. (40) with respect to the P_i concentration; in those experiments 3 mM P_i was used. At a high ratio of PGA/P_i starch formation is stimulated (13) by activation of ADP glucose pyrophosphorylase (23). As starch synthesis requires phosphorylation of G1P (23), a lowered energy charge might result in inhibition of PGA reduction (15, 18). The shape of the curve in Fig. 6.3 might then be explained by the fact that photophosphorylation under continuous illumination is pH dependent. The optimum pH for photophosphorylation has been reported to be pH 8.4 (1).

If photophosphorylation is the rate-limiting step in PGA-dependent O_2 evolution under the conditions used, it is difficult to see how inhibition of FBP-ase activity by formate (5) could inhibit PGA reduction. Inhibition of FBP-ase activity should increase the energy charge, wich in turn would stimulate PGA-kinase activity (18). There are however two effects of formate that could inhibit PGA reduction. The first effect is that formate can uncouple electron flow (see Chapter 4) which will lower the energy charge. The second effect is the dissipation of the ΔpH across the envelope (5). PGA transport across the chloroplast envelope is mediated by the phosphate translocator (7, 11). The kinetic parameters of this enzyme are dependent on the ambient pH and on the magnitude and direction of a transmembrane protongradient (7, 11). In the presence of the light-induced proton gradient across the envelope (inside alkaline), PGA uptake will be faster (17). As formate is known to dissipate the proton gradient, PGA reduction to DHAP may be inhibited by substrate depletion. The reduction of PGA to DHAP

is known to be highly reversible (18). Obviously more experiments are required to elucidate the mechanism of the inhibition of PGA reduction by formate.

The irreversible inhibition of electron flow in isolated intact chloroplasts that have been preilluminated with saturating light in the presence of formate indicates that photoinhibition may have occurred. In chloroplasts or leaves that are illuminated with strong actinic light in the absence of an electron acceptor or in the presence of DCMU photobleaching occurs and electron flow is impaired (17). As formate also inhibits electron flow at the acceptor side of PSII, the effect of formate with respect to photoinhibition might be similar to the effect of DCMU. Inhibition of CO_2 and PGA reduction by formate might result in depletion of electron acceptors which will enhance photoinhibition (17).

CO_2-depletion of intact chloroplasts by dark-incubation with formate at low pH

The data given in Table 6.1 clearly demonstrate that a "bicarbonate-effect" on electron flow can be induced in isolated intact chloroplasts by dark-incubation with formate at low pH. This method has two serious drawbacks. The first is that PGA-dependent O_2-evolution is inhibited after CO_2-depletion and this inhibition is not relieved in the presence of bicarbonate. The second drawback is that after more than 45-90 min incubation (depending on pH) electron flow is inhibited in such a way that it cannot be fully reactivated by bicarbonate (see Table 6.1). The results of Table 6.1 also provide evidence that the irreversible inhibition of PGA reduction is probably not caused by inhibition of electron flow. This depletion method was optimized however to demonstrate a bicarbonate-effect on electron flow. The method can probably be improved, especially with respect to carbon metabolism.

Effects of bicarbonate and formate on the P_{515} response

In contrast with the experiments shown in Chapter 5 the amplitude of phase a (the fast rise) of the flash-induced P_{515} response after the first flash is about equal in control and CO_2-depleted chloroplasts (Fig. 6.5). This is in agreement with results of Q_A^- decay experiments which show that Q_A^- is reoxidized within 20 s (6, 14, 25), the time between the flash-trains (Fig. 5). Prolonged dark-incubation of intact chloroplasts at low pH was found to decrease the amplitude of phase a, both in the absence (Fig. 6.7) and in the presence of formate (data not shown).

In the absence of bicarbonate the magnitude of phase a decreases in subsequent flashes. Figure 6.5 shows that in the presence of bicarbonate the magnitude of phase a after the 6th flash is a factor 2 higher than in the absence of bicarbonate. This means that it takes about 250 ms to regenerate 50% of the reaction centers in an "open" state, i.e. reaction centers in which the primary donor is reduced and the acceptor is oxidized. This half-time of about 250 ms is in the same order of magnitude as the half-times for the reduction of PQ and P_{700} in CO_2-depleted broken chloroplasts (33). From the oxygen evolution measurement shown in Figure 6.4 a half-time of about 165 ms was calculated for the turnover of PQ in the absence of bicarbonate. In the presence of bicarbonate the half-time of PQ-turnover must be much smaller than 250 ms. From Fig. 6.4 a half-time of about 30 ms is estimated, a value which is in agreement with data given by Siggel et al. (33) for broken chloroplasts.

In CO_2-depleted intact chloroplasts phase b is absent in the P_{515} response (Fig. 6.5). This observation is in agreement with experiments of Garab et al. (8), but in those experiments phase b reappeared after addition of CO_2. The question arises whether in those "CO_2-depleted" leaves or chloroplasts electron flow really was inhibited at the acceptor side of PS II. The method of CO_2-depletion used in (8) is quite different from

the methods used in this thesis. The depletion times used by Garab and co-workers are very short (5-30 min) when compared with the incubation times shown in Table 6.1, especially if one considers that no formate or acetate was used by Garab et al. (8). Moreover phase b shows some characteristics that make the use of phase b as an indicator of bicarbonate effects on electron flow rather hazardous. These characteristics are:

1. phase b is present in thylakoid vesicles that contain no PS II, which indicates that phase b might be related to cyclic electron flow (19, 20).
2. phase b is dependent on the ambient redox potential (9, 10). At redox potentials higher than 150 mV phase b is very small (10) and phase b appears with an E_m of approx. +200 mV, at pH 7.5, a value which is pH dependent (10).
3. phase b is dependent on the pH of the reaction medium; at low pH phase b is absent (39, see Fig. 6.7).
4. phase b is dependent on the state of energization of the thylakoid membrane. In actively ATP-hydrolyzing thylakoid membrane preparations phase b is absent (30, 31, 32).

With respect to the effect of pre-energization of the thylakoid membrane it is noteworthy to mention that, in the presence of DTE and ATP, the chloroplast ATP-ase can be activated by only a few saturating light-flashes and the ATP-hydrolysis continues for several minutes (30; R.L.A. Peters, unpublished data). According to Garab et al. (8) the effect of CO_2-depletion could only be observed "after a few exciting flashes" and the disappearance of phase b might have been caused by energization of the membrane by ATP-hydrolysis. Reversed electron flow from PQH_2 to Q_A caused by ATP-hydrolysis can partially reduce Q_A to Q_A^- (2, 29) and acidification of the thylakoid lumen lowers Chl a fluorescence yield (3, 4). This means that ATP-hydrolysis, induced by the CO_2-depletion procedure, may have caused the inhibition of phase b and the complex kinetics of Chl a fluorescence induction observed by Garab et al. (8). Addition of CO_2 to these leaves might perhaps lower the ΔG_{ATP} by acting as a substrate in carbon metabolism, thereby slowing

down ATP-hydrolysis. This alternative interpretation is rather speculative and needs further investigations. Nervertheless this alternative interpretation shows that the interpretation of Garab et al. (8) needs further evidence as well.

The absence of an accelerating effect of formate on the decay of the P_{515} response (Fig. 6.6) indicates that, at 3°C, the formate anion does not readily move across either the thylakoid membrane or the chloroplast envelope. Experiments with broken chloroplasts show that formate does accelerate the decay at 25°C, which is in agreement with the fact that formate uncouples electron flow at 25°C (see Chapter 3), but at 3°C formate has no significant effect on the P_{515} response (data not shown).

The effect of the ambient pH on the P_{515} response (Fig. 6.7) is in agreement with data given by Schapendonk (26) for broken chloroplasts. Between pH 5 and pH 6 phase b appears to be strongly dependent on the ambient pH; at the moment it is not known whether the disappearance of phase b is reversible or whether phase b is irreversibly suppressed by incubation at low pH.

Conclusions

It is shown in this Chapter that dark-incubation of isolated intact chloroplasts, that are able to evolve O_2 in the presence of PGA, with formate at low pH renders these chloroplasts "CO_2-depleted". These CO_2-depleted chloroplasts have lost the ability to use PGA as electron acceptor and electron flow to an artificial electron acceptor is inhibited. The inhibition of electron flow in CO_2-depleted chloroplasts is relieved after dark-incubation with bicarbonate. PGA-dependent O_2 evolution however is not restored in the presence of bicarbonate.

Inhibition of electron flow in CO_2-depleted intact chloroplasts was also inferred from the flash-induced P_{515} response As the P_{515} response can be measured in intact leaves of whole plants, measurement of the P_{515} response can be a useful tool in the search for a "bicarbonate-effect" on electron flow *in vivo*.

References

1. Avron, M. 1972. The relation of light induced reactions of isolated chloroplasts to proton concentrations. *In*: Proceedings of the IInd International Congress on Photosyntheisis Research (G. Fort et al., eds.), Vol. 2: p.p. 861-871, Dr. W. Junk Publishers, The Hague.
2. Avron, M., U. Schreiber. 1979. Properties of ATP-induced chlorophyll luminescence in chloroplasts. Biochim. Biophys. Acta 546: 448-454.
3. Briantais, J.M., C. Vernotte, M. Picaud, G.H. Krause. 1979. A quantitative study of the slow decline of chlorophyll *a* fluorescence in isolated chloroplasts. Biochim. Biophys. Acta 548: 128-138.
4. Briantais, J.M., C. Vernotte, M. Picaud, G.H. Krause. 1980. Chlorophyll fluorescence as a probe for the determination of the photo-induced proton gradient in isolated chloroplasts. Biochim. Biophys. Acta 591: 198-202.
5. Enser, U., U. Heber. 1980. Metabolic regulation by pH gradients. Inhibition of photosynthesis by indirect proton transfer across the chloroplast envelope. Biochim. Biophys. Acta 592: 577-591.
6. Farineau, J., P. Mathis. 1983. Effect of bicarbonate on electron transfer between plastoquinones in photosystem

II. *In*: The Oxygen-evolving system of Plant Photosynthesis (Y. Inoue, ed.), Academic Press, New York.

7. Flügge, U.I., H.W. Heldt. 1984. Influence of a proton gradient on the activity of the reconstituded chloroplast phosphate translocator. *In*: Advances in Photosynthesis Research, (C. Sybesma, ed.), Vol. II, M. Nijhoff/dr. W. Junk Publishers, The Hague.

8. Garab, G., A.A. Sanchez Burgos, L. Zimànyi, A. Fàludi-Daniel. 1983. Effect of CO_2 on the energization of thylakoids in leaves of higher plants. FEBS Lett. 154: 323-327.

9. Giorgi, L., N. Packham, J. Barber. 1984. Redox titrations of the fast and slow phase of the 515 nm electrochromic absorption change in chloroplasts. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol. II, M. Nijhoff/dr. W. Junk Publishers, The Hague.

10. Girvin, M., W.A. Cramer. 1984. Redox titration of the slow electrochromic phase in chloroplasts. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol. II, M. Nijhoff/dr. W. Junk Publishers, The Hague.

11. Heber, U., H.W. Heldt. 1981. The chloroplast envelope: structure, function and role in leaf metabolism. Annu. Rev. Plant. Physiol. 32: 139-169.

12. Heber, U., K.A. Santarius. 1970. Direct and indirect transfer of ATP and ADP across the chloroplast envelope. Z. Naturforsch. 25b: 718-827.

13. Heldt, H.W., C.J. Chon, D. Maronde, A. Herold, Z.S. Stankovic, D.A. Walker, A. Kraminer, M.R. Kirk, U. Heber. 1977. Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. Plant Physiol. 59: 1146-1155.

14. Jursinic, P., A. Stemler. 1982. A seconds range component of the reoxidation of the primary photosystem II acceptor, Q. Biochim. Biophys. Acta 681: 419-428.

15. Latzko, E., G.J. Kelly. 1979. Enzymes of the reductive pentose phospate cycle. *In*: Encyclopedia of Plant Physiology, Photosynthesis II (A. Pirson, ed.), New Series Vol. 6, p.p. 239-250, Springer-Verlag, Berlin.

16. Mills, J.D., R.E. Slovacek, G. Hind. 1978. Cyclic electron transport in isolated intact chloroplasts. Further studies with antimycin. Biochim. Biophys. Acta 682: 345-353.

17. Osmond, C.B. 1981. Photorespiration and photoinhibition. Some implications for the energetics of photosynthesis. Biochim. Biophys. Acta 639: 77-98.

18. Pacold, I., L.E. Anderson. 1975. Chloroplast and cytoplasmatic enzymes. VI Pea leaf 3-phosphoglycerate kinases. Plant Physiol. 55: 168-171.

19. Peters, F.A.L.J., R.H.M. van der Pal, R.L.A. Peters, W.J. Vredenberg, R. Kraayenhof. 1984. Studies on well-coupled photosystem I enriched subchloroplast vesicles. Discrimination of flash-induced fast and slow electric potential components. Biochim. Biophys. Acta 766: 169-178.

20. Peters, F.A.L.J., G.A.B. Smit, R. Kraayenhof. 1984. On the origin of the slow electric potential induced by ferredoxin-mediated cyclic electron transfer in photosystem-I-enriched subchloroplast vesicles. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol II, M. Nijhoff/ dr. W. Junk Publishers, The Hague.

21. Peters, R.L.A., O. van Kooten, W.J. Vredenberg. 1984. The kinetics of the flash-induced P515 electrochromic bandshift in dependence of the energetic state of the thylakoid membrane and the ion concentration in the outer aqueous phase. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol. II, M. Nijhoff/Dr. W. Junk Publishers, The Hague.

22. Peters, R.L.A., M. Bossen, O. van Kooten, W.J. Vredenberg. 1983. On the correlation between the activity of ATP-hydrolase and the kinetics of the flash-induced P515 electrochromic bandshift in spinach chloroplasts. J. Bioenerg.

Biomembr. 15: 335-346.

23. Preiss, J., C. Levi. 1979. Metabolism of primary products of photosynthesis. Metabolism of starch in leaves. *In*: Encyclopedia of Plant Physiology, Photosynthesis II (M. Gibbs, E. Latzko, eds.), New Series Vol. 6, pp. 282-312, Springer Verlag, Berlin.

24. Radmer, R., O. Ollinger. 1980. Isotopic composition of photosynthetic O_2 flash yields in the presence of $H_2{}^{18}O$ and $HC^{18}O_3^-$. FEBS lett. 110: 57-61.

25. Robinson, H.H., J.J. Eaton-Rye, J.J.S. van Rensen, Govindjee. 1984. The effects bicarbonate depletion and formate incubation on the kinetics of oxidation-reduction reactions of the photosystem II quinone acceptor complex. Z. Naturforsch. 39c: 382-385.

26. Schapendonk, A.H.C.M. 1980. Electrical events associated with primary photosynthetic reactions in chloroplast membranes. Agric. Res. Rep. 905, ISBN 90 220 0756 1 (doctoral thesis).

27. Schapendonk, A.H.C.M., W.J. Vredenberg. 1979. Activation of the reaction II component of P515 in chloroplasts by pigment system I. FEBS lett. 106: 257-261.

28. Schapendonk, A.H.C.M., W.J. Vredenberg, W.J.M. Tonk. 1979. Studies on the kinetics of the 515 nm asorbance changes in chloroplasts. Evidence for the induction of a fast and a slow P515 response upon saturating light flashes. FEBS lett. 100: 325-330.

29. Schreiber, U., M. Avron, 1979. Properties of ATP-driven reversed electron flow in chloroplasts. Biochim. Biophys. Acta 546: 436-447.

30. Schreiber, U., S. Del Valle-Tascon. 1982. ATP-synthesis with single turnover flashes in spinach chloroplasts. In situ monitoring with the firefly luciferase method. FEBS lett. 150: 32-37.

31. Schreiber, U., K.G. Rienits. 1982. Complementarity of ATP-induced and light-induced absorbance changes around 515 nm. Biochim. Biophys. Acta 682: 115-123.

32. Schuurmans, J.J., A.L.H. Peters, F.J. Leeuwerik, J. Kraayenhof. 1981. On the association of electrical events with the synthesis and hydrolysis of ATP in photosynthetic membranes. *In*: Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (F. Palmieri et al., eds.), pp. 359-369, Elseviers/North-Holland Biochemical Press, Amsterdam.

33. Siggel, U., R. Khanna, G. Renger, Govindjee. 1977. Investigation of the absorption changes of the plastoquinone system in broken chloroplasts. The effect of bicarbonate-depletion. Biochim. Biophys. Acta 462: 196-207.

34. Stemler, A. 1979. A dynamic interaction between the bicarbonate ligand and photosystem II reaction center complexes in chloroplasts. Biochim. Biophys. Acta 545: 36-45.

35. Stemler, A., Govindjee. 1973. Bicarbonate ion as a critical factor in photosynthetic oxygen evolution. Plant Physiol. 52: 119-123.

36. Vermaas, W.F.J., J.J.S. van Rensen. 1981. Bicarbonate action on photosynthetic electron transport and its possible physiological role. *In*: Photosynthesis II. Electron transport and photophosphorylation (G. Akoyunoglou, ed.) pp. 157-165, Balaban Internation Science Service, Philadelphia, Pa.

37. Vredenberg, W.J. 1981. P515: a monitor of photosynthetic energization in chloroplast membranes. Physiol. Plant. 53: 598-602.

38. Vredenberg, W.J., O. van Kooten, R.L.A. Peters 1984. Electrical events and P515 response in thylakoid membranes. *In*: Advances in Photosynthesis Research (C. Sybesma, ed.), Vol. II, M. Nijhoff/Dr. W. Junk Publishers.

39. Vredenberg, W.J., A.H.C.M. Schapendonk. 1981. Reaction kinetics of P515 in chloroplasts: transmembrane (Reaction I) and inner membrane (Reaction II) electric fields. *In*: Photosynthetisis I. Photophysical Processes - Membrane Energization (G. Akoyunoglou, ed.), pp. 489-498, Balaban International Science Services, Philadelphia, Pa.

40. Werdan K., H.W. Heldt, M. Milovancev. 1975. The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2-fixation in the light and dark. Biochim. Biophys. Acta 396: 276-292.

41. Wydrzynski, T., Govindjee. 1975. A new site of bicarbonate effect in photosystem II of photosynthesis: evidence from chlorophyl fluorescence transients in spinach chloroplasts. Biochim. Biophys. Acta 387: 403-408.

CHAPTER 7

GENERAL DISCUSSION

This thesis presents results of experiments, designed to obtain more insight into the regulation of photosynthetic electron flow in isolated chloroplasts by bicarbonate and formate. Moreover the influence of herbicides on the regulation of electron flow by bicarbonate and formate was investigated.

An allosteric relation between the binding of the herbicides i-dinoseb and the binding of bicarbonate/formate

The results presented in Chapter 2 could be explained in two ways. i) Bicarbonate and *i*-dinoseb compete for a common site at the acceptor side of PSII which in their absence regulatory is occupied by another competitive inhibitor. It was suggested that formate might be this competitive inhibitor. ii) Bicarbonate and *i*-dinoseb do not bind to a common site, but i-dinoseb affects the reactivation of the Hill reaction by bicarbonate in an allosteric manner. The results of Chapter 3 show that formate indeed acts as a competitive inhibitor of the stimulation of the Hill reaction by bicarbonate. In the presence of two competitive inhibitors the reactivation of the Hill reaction by bicarbonate can be described by equation 7.1.

$$V_{Hill} = V_{max} \cdot \frac{A}{K_r\left(1 + \frac{I_1}{K_1} + \frac{I_2}{K_2}\right) + A} \qquad (7.1)$$

with I_1, I_2: concentration of formate resp. i-dinoseb
K_1, K_2: dissociation constant of the formate- resp. i-dinoseb-binding site complex.
V_{Hill}, V_{max}, K_r, A: as defined in Chapter 2.

Recalculation of the data presented in Fig. 2.3 yields a value for K_2 of 1.6 nM *i*-dinoseb (I_1 = 100 mM formate, K_1 = 5 mM formate). This low value is inconsistent with measured binding constants, which have been found in the range between 30 nM and 105 nM (5, 10). Therefore it is probable that i-dinoseb and formate/bicarbonate do not compete for a common binding site at the acceptor side of PSII. This leaves an allosteric interaction between *i*-dinoseb binding and bicarbonate/formate binding site as the most likely mechanism. The binding sites of bicarbonate/ formate and *i*-dinoseb are both located in the vicinity of Q_A, since both *i*-dinoseb and formate affect the EPR signal at g = 1.82. This EPR signal is attributed to a Q_A^- Fe^{2+} complex (11). Interaction of other herbicides with bicarbonate/ formate at the acceptor side of PSII have been discussed in detail elsewhere (10).

Quantitative parameters for the interaction of bicarbonate and formate with photosynthetic electron flow

The results of the experiments described in Chapter 3 indicate that formate and bicarbonate compete for a common regulatory site, probably located at the PSII protein complex in the vicinity of Q_A/Q_B. This site, that can bind formate and bicarbonate, is involved in electron flow at the acceptor side of PSII. The data presented in Chapter 3 can be explained by assuming that i) electron flow from Q_A to P_Q is inhibited when formate is bound to this site, ii) electron flow can proceed when bicarbonate is bound to this site and iii) electron flow is allowed when neither formate nor bicarbonate is bound to this site. These assumptions imply that the model for the reactivation of the Hill reaction by bicarbonate in the presence of a competitive inhibitor, as proposed in Chapter 2, should be adapted. According to assumptions i, ii and iii, the Hill reaction rate is linearly dependent on [EA] + [A] (see Chapter 2 and Appendix 1 for a description of the parameters). The Hill reaction rate in the presence of bicarbonate and formate can

be described by the following equation:

$$V_{Hill} = V_{max} \{\frac{EA}{E_{tot}} + a \cdot \frac{E}{E_{tot}}\} \qquad (7.2)$$

The scaling factor, a, has been introduced in eqn. 7.2 to indicate that the rate of electron transport when the binding site is in the state E is only a fraction (a) of the rate when it is in state EA. Equation 7.2 can be reduced to:

$$V_{Hill} = V_{max} \cdot \frac{a \cdot K_r + A}{K_r \cdot (1 + \frac{I}{K_i}) + A} \qquad (7.3)$$

Under the experimental conditions used in Chapter 3 the values of the parameters in eqn. 7.3 were the following:

V_{max} = 150 - 200 µmol O_2/mg Chl.h

a = 0.4 - 0.5

K_r = 50 - 110 µM bicarbonate

K_i = 1.3 - 2.7 mM formate

These values give a satisfactory description of the initial Hill reaction rate as a function of the bicarbonate and formate concentration. In Chapter 4 evidence is given that K_i may be much lower during prolonged illumination. In that case K_i in the light may be as low as 0.2 mM formate at pH 6.5. At higher pH formate may be less inhibitory (see e.g. Fig. 6.4 in Chapter 4).

Effects of formate and bicarbonate on carbon metabolism

The CO_2-depletion method applied in Chapter 6 yields intact chloroplasts both electron flow and the reduction of exogenous

PGA are inhibited. In contrast to electron flow in the presence of BQ, PGA dependent oxygen evolution was found not to be stimulated by addition of bicarbonate. This irreversible inhibition of PGA reduction may have been caused by the high formate concentration or by the low pH of the depletion medium. The CO_2-depletion procedure however was developed primarily to demonstrate the existence of a large and reproducible bicarbonate effect on electron flow. *In vivo* only small effects of bicarbonate and formate are expected. Therefore, when only small effects on electron flow are required, a milder CO_2-depletion procedure is sufficient to evoke these effects.

Formate and other anions of weak acids have been reported to inhibit CO_2-dependent oxygen evolution in isolated intact chloroplasts (1, 12) and protoplasts (3). CO_2-dependent oxygen evolution in intact chloroplasts is inhibited for 50% by 5 mM formate at pH 6.9; the degree of inhibition is pH dependent and inhibition is only observed at sub-optimal pH (1, 10). The inhibition of CO_2 dependent oxygen evolution by formate has been ascribed to a dissipation of the proton gradient across the envelope in the presence of formate and no effect of formate on the proton gradient across the thylakoid membrane could be detected (1). The results presented in Chapter 4 also indicate that formate does not significantly uncouple electron flow at a concentration of 5 mM. Formate apparently also inhibits FBP-ase at relatively high concentrations (1). This inhibition occurs however at much higher formate concentrations than those required for inhibition of electron flow (see Chapter 4) or inhibition of CO_2-dependent O_2-evolution.

In Chapter 4 the inhibitor constant K_i for inhibition of linear electron flow by formate has been estimated to be in the sub-mM range in the light. It seems therefore that formate affects electron flow at lower concentrations than carbon metabolism.

Interaction of photosynthetic electron flow and photorespiration in higher plants

Figure 7.1 shows a scheme in which the link between photorespiration photosynthetic electron flow via the formate and the bicarbonate pools in the cytoplasm is vizualized. This scheme is an extension of the scheme presented in Chapter 3 and is based on the observations that i) formate and bicarbonate can regulate electron flow at the acceptor side of PSII and ii) formate can be produced during photorespiration.

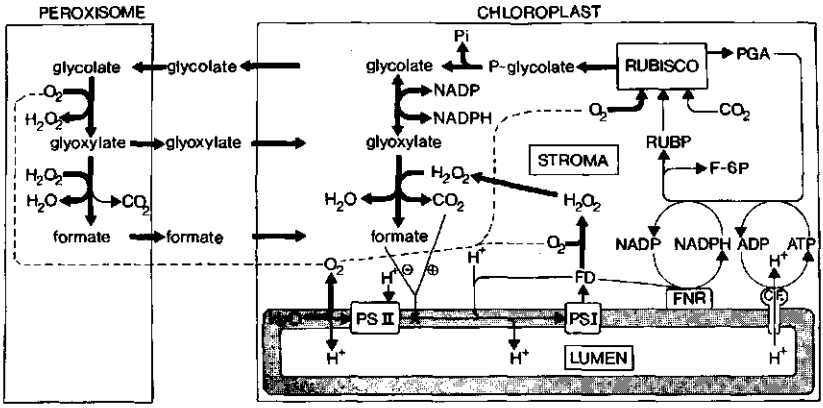


Figure 7.1. Hypothetical scheme of reactions that are involved in the coupling of photosynthetic electron flow to photorespiration in higher plants. Heavy arrows indicate reactions that are proposed to be included in the negative feedback loop from oxygen to formate.

At a high O_2/CO_2 ratio photorespiration proceeds at high rates (4, 6). Photorespiration starts with the oxygenation of RUBP, a reaction which yields PGA and P-glycolate (4, 6, 9). In subsequent reactions (not shown) P-glycolate is converted back to PGA; this series of reactions is also known as the C_2-cycle (4). Glyoxylate is an intermediate in the C_2-cycle and is generated by oxidation of glycolate in the peroxisome (4, 6, 9). Glyoxylate can either be decarboxylated in a reaction with H_2O_2 (2, 6, 7, 8, 9) or converted to glycine by a trans-

amination reaction (4, 6, 8, 9, 13). Although high rates of glyoxylate decarboxylation have been observed in isolated broken peroxisomes (2), recent data suggest that *in vivo* glyoxylate decarboxylation does not significantly contribute to photorespiratory CO_2 evolution (8). It appeared that only in the absence of amino donors, i.e. when transamination of glyoxylate was inhibited, glyoxylate decarboxylation could be observed (8). This means that regulation of electron flow by formate produced during photorespiration is probably limited to conditions in which transamination of glyoxylate is to slow to compensate the synthesis of glyoxylate, i.e. glyoxylate can accumulate and react with H_2O_2 yielding formate and CO_2.

References

1. Enser, U., U. Heber. 1980. Metabolic regulation by pH gradients. Inhibition of photosynthesis by direct proton transfer across the chloroplast envelope. Biochim. Biophys. Acta 592: 577-591.
2. Grodzinski, B. 1979. A study of formate production and oxidation in leaf peroxisomes during photorespiration. Biochim. Biophys. Acta 63: 289-293.
3. Kaiser, G., U. Heber. 1983. Photosynthesis of leaf cell protoplasts and permeability of the plasmalemma to some solutes. Planta 157: 462-470.
4. Lorimer, G.H., T.J. Andrews. 1981. *In*: The Biochemstry of Plants, Vol. 8 (M.D. Hatch, N.K. Boardman, eds.), pp. 329-374, Academic Press, New York.
5. Oettmeier, W., K. Masson. 1980. Synthesis and thylakoid membrane binding of the radioactively labeled herbicide dinoseb. Pest. Biochem. Physiol. 14: 86-97.

6. Ogren, W.L. 1984. Photorespiration: Pathways, Regulation and modification. Ann. Rev. Plant Physiol. 35: 415-442.

7. Oliver, D.J. 1981. Mechanism and control of photorespiratory glycolate metabolism: CO_2 loss from the (C-2) carbon of glycolate. *In*: Proceedings of the 5th International Congress on Photosynthesis (G. Akounoglou, ed.), Vol. IV, pp. 501-508, Balaban International Science Services, Philadelphia, Pa.

8. Somerville, C.R., W.L. Ogren. 1981. Photorespiration-deficient mutants of Arabidopsis thaliana lacking mitochondrial serine transhydroxymethylase activity. Plant Physiol. 67: 666-671.

9. Tolbert, N.E. 1981. Metabolic pathways in peroxisomes and glyoxysomes. Ann. Rev. Biochem. 50: 133-157.

10. Vermaas, W.F.J. 1984. The interaction of quinones, herbicides and bicarbonate with their binding environment at the acceptor side of photosystem II in photosynthesis. Dissertation, Agricultural University, Wageningen, The Netherlands.

11. Vermaas, W.F.J., A.W. Rutherford. 1984. EPR-measurements on the effects of bicarbonate and triazine resistance on the acceptor side of photosystem II. FEBS lett. 175: 243-248.

12. Werdan, K., H.W. Heldt, M. Milovancev. 1975. The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2-fixation in the light and dark. Biochim. Biophys. Acta 396: 276-292.

13. Yu C., Z. Liang, A.H.C. Huang. 1984. Glyoxylate transamination in intact leaf peroxisomes. Plant Physiol. 75: 7-12.

APPENDIX 1

KINETICS OF THE BINDING OF TWO LIGANDS TO A COMMON BINDING SITE AT A MACROMOLECULE

The binding of two ligands A and I to a macromolecule E can be described by the following reactions:

$$A + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \qquad [1]$$

and

$$I + E \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI \qquad [2]$$

The equilibrium constants for these reactions can be expressed as follows:

$$K_a = \frac{k_1}{k_2} = \frac{[E]\cdot[A]}{[EA]} \qquad \text{for reaction 1}$$

and

$$K_i = \frac{k_3}{k_4} = \frac{[E]\cdot[I]}{[EI]} \qquad \text{for reaction 2}$$

Strictly speaking these equilibrium constants are the dissociation constants and are usually expressed in mM.

In the following considerations it is assumed that ligands A and I compete for the common binding site E; A and I only bind to the "free" binding site, i.e. binding to the states EA or EI does not occur. Under these conditions the rate of formation of the two binary complexes can be described by the following differential equations:

$$\frac{d[EA]}{dt} = k_1 \cdot [E] \cdot [A] - k_2 \cdot [EA] \qquad [3]$$

and

$$\frac{d[EI]}{dt} = k_3 \cdot [E] \cdot [I] - k_4 \cdot [EI] \qquad [4]$$

It is assumed that the number of binding sites remains constant:

$$[E]+[EI]+[EA] = \text{constant} = E_{tot} \qquad [5]$$

(For the sake of simplicity the brackets will be omitted in the following calculations.)

The amount of free binding sites (E) can be eliminated from equations [3] and [4] with the aid of eqn. [5]:

$$\frac{d\ EA}{dt} = k_1 \cdot A \cdot E_{tot} - k_1 \cdot A \cdot EI - (k_1 \cdot A + k_2) \cdot EA \qquad [6]$$

and

$$\frac{d\ EI}{dt} = k_3 \cdot I \cdot E_{tot} - k_3 \cdot I \cdot EA - (k_3 \cdot I + k_4) \cdot EI \qquad [7]$$

Generally this set of differential equations is hard to solve. If however A and I are constants, i.e. when $A >> E_{tot}$ and $I >> E_{tot}$, equations [6] and [7] form a set of linear independent differential equations for which an analytical solution exists.

Equations [6] and [7] can be simplified by differentatiation. First define $x \equiv \frac{d\ EA}{dt}$ and $y \equiv \frac{d\ EI}{dt}$ and after differentiation the following result is obtained:

$$\frac{dx}{dt} = -k_1 \cdot A \cdot y - (k_1 \cdot A + k_2) \cdot x \qquad [8]$$

$$\frac{dy}{dt} = -k_3 \cdot I \cdot x - (k_3 \cdot I - k_4) \cdot y \qquad [9]$$

Next it is assumed that $x = X \cdot e^{zt}$ and $y = Y \cdot e^{zt}$ with X and Y being constants. Further simplification is obtained by substituting $a = k_1 \cdot A + k_2$; $b = k_3 \cdot I + k_4$; $c = k_1 \cdot A$ and $d = k_3 \cdot I$.

Substitution in equations [8] and [9] yields:

$$z \cdot X \cdot e^{zt} = -c \cdot Y \cdot e^{zt} - a \cdot X \cdot e^{zt} \qquad [10]$$

and

$$z \cdot Y \cdot e^{zt} = -d \cdot X \cdot e^{xt} - b \cdot Y \cdot e^{zt} \qquad [11]$$

This set of equations has only a solution for $t > 0$ when the coefficients of X and Y have the following relation:

$$\frac{a+z}{d} = \frac{c}{b+z} \qquad [12]$$

or written in a different form:

$$z^2 + (a+b) \cdot z + a \cdot b - c \cdot d = 0 \qquad [13]$$

yielding

$$z_{1,2} = \frac{-(a+b) \pm \sqrt{(a-b)^2 - 4(a \cdot b - c \cdot d)}}{2} \qquad [14]$$

Now X and Y can be calculated from equation [12] for the two distinct values of z:

a) $z = z_1$: let $X = \lambda$, then $Y = -\lambda \cdot \frac{a+z_1}{c}$ [15]

for the second value of z, z_2, the calculation is identical:

b) $z = z_2$: putting $X = \mu$, yields $Y = -\mu \cdot \frac{a+z_2}{c}$ [16]

The general solution is composed of the two partial solutions:

$$x = \lambda \cdot e^{z_1 \cdot t} + \mu \cdot e^{z_2 \cdot t} \qquad [17]$$

and

$$y = -\lambda \cdot \frac{a+z_1}{c} \cdot e^{z_1 \cdot t} - \mu \cdot \frac{a+z_2}{c} \cdot e^{z_2 \cdot t} \qquad [18]$$

Reminding that $x = \frac{d\ EA}{dt}$ and $y = \frac{d\ EI}{dt}$, equations [17] and [18] can be written in their final form:

$$\frac{d\ EA}{dt} = \lambda \cdot e^{z_1 \cdot t} + \mu \cdot e^{z_2 \cdot t} \qquad [19]$$

and

$$\frac{d\ EI}{dt} = -\lambda \cdot \frac{a+z_1}{c} \cdot e^{z_1 \cdot t} - \mu \cdot \frac{a+z_2}{c} \cdot e^{z_2 \cdot t} \qquad [20]$$

These differential equations can be simply integrated simply, because λ, a, z_1, z_2 and c are time-independent. Therefore the general solution becomes:

$$[EA]\ (t) = C' + \frac{\lambda}{z_1} \cdot e^{z_1 \cdot t} + \frac{\mu}{z_2} \cdot e^{z_2 \cdot t} \qquad [21]$$

and

$$[EI]\ (t) = C'' - \lambda \cdot \frac{a+z_1}{c} \cdot e^{z_1 \cdot t} - \mu \cdot \frac{a+z_2}{c} \cdot e^{z_2 \cdot t} \qquad [22]$$

with C' and C" as integration constants.

Equations [21] and [22] describe the time-dependent change in the concentration of the binary complexes EA and EI upon a change in one of the parameters, e.g. [A] or [I]. The values for the constants C', C", λ and μ are determined by the boundary conditions.

For a few cases it may be illustrative to calculate the exact solution.

Example 1

At time t=0 an amount of ligand A is added to a solution containing macromolecules E, competitive inhibitor I and their binary complex EI. This case simulates the experiments described in Figs. 2 and 5 in Chapter 2.

The boundary conditions in this case are:

a) at $t < 0$: $[EI] = E_{tot} \cdot \frac{I}{K_i + I}$

$[I] = I$

$[A] = 0$

b) at $t = 0$: $[A] = A$

$[EA] = 0$

$[EI] = E_{tot} \cdot \frac{I}{K_i + I}$

and c) at $t < \infty$: $[EA] = E_{tot} \cdot \frac{A'}{K_r + A'}$, with $A' = \frac{A}{1 + \frac{I}{K_i}}$

and $[EI] = E_{tot} \cdot \frac{I'}{K_i + I'}$, with $I' = \frac{I}{1 + \frac{A}{K_r}}$

From these boundary conditions the values for C', λ and μ can be calculated:

$$\lambda = \frac{z_1}{z_1 - z_2} \cdot E_{tot} \cdot \{k_1 \cdot A' + z_2 \cdot \frac{A'}{K_r + A'}\} \qquad [23]$$

$$\mu = -\frac{z_2}{z_1 - z_2} \cdot E_{tot} \cdot \{k_1 \cdot A + z_1 \cdot \frac{A'}{K_r + A'}\} \qquad [24]$$

and

$$C' = E_{tot} \cdot \frac{A'}{K_r + A'} \qquad [25]$$

Substitution of these values in equation [21] yields:

$$EA(t) = E_{tot} \cdot \{\frac{A'}{K_r + A'} + \frac{1}{z_1 - z_2} \cdot (k_1 \cdot A' + z_2 \cdot \frac{A'}{K_r + A'}) \cdot e^{z_1 \cdot t} -$$

$$- \frac{1}{z_1 - z_2} \cdot (k_1 \cdot A' + z_1 \cdot \frac{A'}{K_r + A'}) \cdot e^{z_2 \cdot t}\} \qquad [26]$$

with

$$z_1 = -\frac{\{k_1 \cdot A + k_2 + k_3 \cdot I + k_4\} + \sqrt{(k_1 \cdot A + k_2 - k_3 \cdot I - k_4)^2 + 4k_1 \cdot A \cdot k_3 \cdot I}}{2}$$

and

$$z_2 = -\frac{\{k_1 \cdot A + k_2 + k_3 \cdot I + k_4\} - \sqrt{(k_1 \cdot A + k_2 - k_3 \cdot I - k_4)^2 + 4k_1 \cdot A \cdot k_3 \cdot I}}{2}$$

Equation [26] describes the formation of the EA complex as a function of the incubation time t after addition of ligand A to a mixture of E and I. Equation [26] can be split up into three parts: a time-independent part and two time-dependent parts with relaxation constants z_1 and z_2:

$$EA(t) = C_1 - C_2 \cdot e^{z_1 t} - C_3 \cdot e^{z_2 \cdot t} \qquad [27]$$

The constants C_1, C_2, C_3, z_1 and z_2 have been calculated for a number of parameter sets. The results are shown in Table A1. Line 3 shows that, at [I] = K_i and [A] = K_r, the condition that $k_3 = 10 \cdot k_1$ and $k_4 = 10 \cdot k_2$ suffices to make C_3 much smaller than C_2. This means that equation [27] reduces to the following form:

$$EA(t) \simeq C_1 \cdot (1 - e^{z \cdot t}) \qquad [28]$$

with

$$C_1 = E_{tot} \cdot \frac{A'}{K_r + A'} \quad \text{and} \quad z = z_1$$

Line 3 of Table A1 further shows that $Z_1 = -0.0146\ s^{-1}$, which is only slightly different from the value for an infinitely fast inhibitor: $-0.0150\ s^{-1}$. Table A1 further shows that increasing k_3 and k_4 further reduces the error made by omitting the second term from equation [39].

Example 2

At time t=0 an amount of competitive inhibitor I is added to a solution containing macromolecules E, ligand A and their binary complex EA. This example simulates the experiment described in Fig. 6 of Chapter 2.

The boundary conditions are:

a) at $t < 0$: $[EA] = [E_{tot}] \cdot \frac{[A]}{K_r + [A]}$

$[A] = A$

$[EI] = 0$

$[I] = 0$

b) at $t=0$ $[A] = A$

$[I] = I$

$\frac{d[EI]}{dt} = k_3 \cdot I \cdot \{E_{tot} - EA\}$

and c) at $t \to \infty$: $[EA] = E_{tot} \cdot \frac{A'}{K_r + A'}$ $\quad (A' = \frac{A}{1+\frac{I}{K_i}})$

$$[EI] = E_{tot} \cdot \frac{I'}{K_i + I'} \quad (I' = \frac{I}{1+\frac{A}{K_r}})$$

Calculation of the constants as in example 1 yields:

$$EA\ (t) = E_{tot} \cdot [\{\frac{A'}{K_r+A'} - \frac{1}{z_1-z_2} \cdot \frac{c}{a+z_1} \cdot (k_3 \cdot I' + z_2 \cdot \frac{I'}{K_i+I'})\} \cdot$$

$$\cdot e^{z_1 \cdot t} + \frac{1}{z_1-z_2} \cdot \frac{c}{a+z_1} \cdot \{k_3 \cdot I' + z_1 \cdot \frac{I'}{K_i+I'}\} \cdot e^{z_2 t}] \qquad [29]$$

The constants used in equation [29] have been described earlier in this appendix. Comparing equations [26] and [29] it appears that the time-indepent parts are identical, which is of course not surprising for reversible reactions. When inhibitor I equilibrates much faster with E than A equilibrates with E, equation [29] can be simplified by omitting the third term. Although I binds rapidly to E, full equilibrium is only reached after ligand A is in equilibrium with the macromolecule E, i.e. dissociation of the EA-complex is the rate-determining step in attaining equilibrium.

Table A1. Effect of the magnitude of the rate constants k_3 and k on the kinetics of the binding of ligand A to the macromolecule E in the presence of a competitive ligand I. The parameters have been described in example 1 and the values of C_1, C_2, C_3, Z_1 and Z_2 were calculated according to equation [26]. The following values were used for the parameters: [A] = 1 mM, k_1 = 10 M^{-1}, s^{-1}, k_2 = 0.01 s^{-1}, [I] = 1 mM.

k_3 ($M^{-1} \cdot s^{-1}$)	k_4 (s^{-1})	C_1	C_2	C_3	Z_1 (s^{-1})	Z_2 (s^{-1})
1	0.001	0.3333	-0.0968	-0.2366	-0.00146	-0.02054
10	0.010	0.3333	-0.2500	-0.0833	-0.01000	-0.03000
100	0.100	0.3333	-0.3326	-0.0007	-0.01461	-0.20540
1000	1.000	0.3333	-0.3333	-0.0000	-0.01496	-2.00500

SUMMARY

This thesis describes some efforts that were made to gain a better understanding of the processes involved in the regulation of photosynthetic electron flow by bicarbonate, formate and herbicides in chloroplasts. In the past decade a large amount of research has been devoted to get insight into the mechanism of herbicide action on electron flow at the acceptor side of photosystem II. This thesis will deal mainly with studies on the regulation of electron flow at the acceptor side of photosystem II by bicarbonate and formate. Some details of the mechanism of this regulation as well as its integration in the overall process of photosynthesis were investigated.

In Chapter 2 experiments are described that were aimed to provide a more quantitative description of the mutual interaction between herbicides, bicarbonate and their binding environment at the acceptor side of photosystem II. This interaction was studied by measuring the effects of bicarbonate and herbicides on electron flow in CO_2-depleted chloroplasts. The kinetics of the reactivation of the inhibited Hill reaction in CO_2-depleted chloroplasts by dark-incubation with bicarbonate suggest that the binding of bicarbonate involves a reaction with (pseudo) first order kinetics. It is shown that in the presence of the herbicides *i*-dinoseb and DCMU the reactivation of the Hill reaction in CO_2-depleted chloroplasts by bicarbonate is retarded. It is shown that any competitive inhibitor of the binding of bicarbonate can be expected to effectively retard the binding of bicarbonate (a theoretical treatment is given in Appendix 1). Although *i*-dinoseb appeared to be an apparently competitive inhibitor of the stimulation of the Hill reaction by bicarbonate under equilibrium conditions, *i*-dinoseb and bicarbonate probably do not compete for a common binding site. This was inferred from the kinetics of the binding of *i*-dinoseb. These kinetics were shown to be to fast to be ex-

plained by simple competition. Therefore it is concluded that the binding of i-dinoseb affects the binding of bicarbonate in an allosteric way.

In Chapter 3 a new procedure for CO_2-depletion of chloroplasts is presented. This method yields CO_2-depleted chloroplasts in which the Hill reaction can be almost completely reactivated by bicarbonate at pH 6.5. It is further shown that formate is a competitive inhibitor of the reactivation of the Hill reaction by bicarbonate. The true reactivation constant K_r was calculated to be 78 μM bicarbonate at pH 6.5. Under the same conditions the inhibitor constant K_i was calculated to be 2 mM formate. Experiments performed at bicarbonate and formate concentrations lower than K_r resp. K_i show that under these conditions electron flow proceeds at high rates. These observations suggest that, contrary to hitherto presented concepts, binding of bicarbonate to the regulatory site at the acceptor side of PSII is not a requirement for electron flow at the acceptor side of PS II. The results presented in Chapter 3 further suggest that formate is a potent inhibitor of electron flow at the acceptor side of PSII. The inhibitory action of formate is counteracted in a competitive way by bicarbonate.

It is proposed in Chapter 3 that photorespiration may affect photosynthetic electron flow. Under certain conditions formate is produced during photorespiration. As photorespiratory formate production is dependent on the oxygen concentration, a negative feedback loop from photosynthetically produced oxygen to formate may exist under these conditions. This negative feedback loop can be powerful mechanism for the regulation of electron flow *in vivo*.

In Chapter 4 the effects of formate and bicarbonate on the Hill reaction are further studied and characterized, especially during prolonged illumination. Formate is shown to stimulate the Hill reaction in non-depleted chloroplasts. It is therefore proposed that formate acts as an uncoupling agent. In the presence of an uncoupler formate was found not to stimulate elec-

tron flow. Instead only inhibiton of electron flow by formate was observed. The extent of this inhibition was not constant in time but appeared to increase during prolonged illumination. The inhibition of electron flow by formate could be partially prevented by including bicarbonate in the reaction medium, but in the light bicarbonate appeared to be a less efficient counteracting agent than in the dark. It is estimated that the inhibitor constant K_i of formate may be more than a magnitude lower in the light than in the dark, implying that formate affects steady state electron flow at sub mM concentrations.

In Chapter 5 an alternative method is presented which can effectively be used to study the effects of formate and bicarbonate on electron flow in intact chloroplasts. This method is based on measurement of the magnitude of the flash-induced transmembrane potential using the P_{515} response as a linear indicator of the transmembrane potential difference. In CO_2-depleted chloroplasts the overall rate-limiting step of electron flow was found to posess a half-time of about 200-250 ms in the absence of bicarbonate. This half-time was much smaller in the presence of bicarbonate. Moreover gramicidin insensitive absorbance changes in the 500-550 nm region were found to be affected by CO_2-depletion and readdition of bicarbonate. These absorbance changes are speculated to be associated with protolytic reactions at the acceptor side of PSII.

Chapter 6 describes effects of formate and bicarbonate on electron flow in isolated intact chloroplasts. Formate was found to inhibit PGA dependent oxygen evolution, but this inhibition could not be correlated with inhibition of electron flow at the acceptor side of PSII. Inhibition of electron flow was observed when isolated intact chloroplasts were incubated with formate at low pH. This inhibition of electron flow was abolished by addition of bicarbonate. The treatment with formate at low pH however appeared to be detrimental to carbon metabolism; PGA and CO_2-dependent oxygen evolution were found to be irreversibly inhibited.

A summarizing discussion is given in Chapter 7, in which the physiological significance of regulation of electron flow by formate and bicarbonate is discussed in relation to carbon metabolism.

SAMENVATTING

Dit proefschrift beschrijft een aantal experimenten die er op gericht zijn om tot een beter begrip te komen van de processen die betrokken zijn bij de regulering van het fotosynthetisch elektronentransport in chloroplasten door bicarbonaat, formiaat en herbiciden. In de laatste 10 jaar is er al veel onderzoek verricht naar het werkingsmechanisme van herbiciden die het elektronentransport remmen aan de acceptor zijde van fotosysteem II. Het in dit proefschrift beschreven onderzoek zal voornamelijk betrekking hebben op de regulering van het fotosynthetisch elektronentransport door bicarbonaat en formiaat. Het onderzoek omvatte zowel sommige aspecten van het mechanisme van deze regulering als de integratie ervan in het fotosynthese proces.

In hoofdstuk 2 worden experimenten beschreven die zouden moeten leiden tot een meer kwantitatieve beschrijving van de effecten van de interacties van herbiciden en en bicarbonaat met hun bindingsomgeving aan de acceptorzijde van fotosysteem II en de effecten daarvan op het elektronentransport. In geisoleerde chloroplasten waaraan CO_2 onttrokken is, is het elektronentransport sterk geremd. Door toevoeging van bicarbonaat (of CO_2) wordt het elektronentransport gereactiveerd. De kinetiek van deze reactivatie doet vermoeden dat de binding van bicarbonaat aan chloroplasten verloopt volgens een reactie met een (pseudo) eerste orde kinetiek. In de aanwezigheid van het herbicide *i*-dinoseb blijkt de affiniteit van de chloroplasten voor bicarbonaat verlaagd te zijn; er is meer bicarbonaat nodig om tot een maximale reactivering van het elektronentransport te komen. Op grond van theoretische overwegingen wordt aangetoond dat een competitieve remmer van de binding van bicarbonaat deze binding kan vertragen (zie ook Appendix 1), hetgeen experimenteel ook voor *i*-dinoseb wordt aangetoond. Desondanks moet echter aangenomen worden dat bicarbonaat en *i*-dinoseb niet aangrijpen op dezelfde bindings-

plaats. Deze conclusie komt voort uit het feit dat de remming van het elektronentransport in de aanwezigheid van bicarbonaat door *i*-dinoseb sneller optreedt dan verwacht mag worden van een competitieve remmer. De mogelijkheid wordt geopperd dat de interactie tussen de bindingsplaatsen van *i*-dinoseb en bicarbonaat allosterisch van aard is.

In hoofdstuk 3 wordt een nieuwe methode beschreven om CO_2 aan geïsoleerde chloroplasten te onttrekken. Deze methode leverde chloroplasten op waarin het elektronentransport beter gereactiveerd kan worden door bicarbonaat dan bij vorige methoden het geval was. In dit hoofdstuk wordt de rol van formiaat in de regulering van het elektronentransport nader bestudeerd. Formiaat blijkt een competitieve remmer te zijn van de reactivering van het elektronentransport in CO_2-vrij gemaakte chloroplasten door bicarbonaat. De reactiveringsconstante (K_r) blijkt 78 µM bicarbonaat te bedragen bij een pH van 6.5; onder dezelfde omstandigheden werd een waarde van 2 mM gevonden voor de remmingsconstante van formiaat. Uit experimenten uitgevoerd met chloroplasten waaraan CO_2 onttrokken is, blijkt dat het elektronentransport goed verloopt wanneer de bicarbonaat en formiaat concentraties veel lager zijn dan K_r resp. K_i. Deze waarneming doet vermoeden dat, in tegenstelling tot hetgeen tot dan toe aangenomen werd, binding van bicarbonaat aan chloroplasten niet vereist is voor het doen verlopen van elektronentransport aan de acceptorzijde van fotosysteem II. Dit houdt in dat de remming van het elektronentransport in afwezigheid van bicarbonaat veroorzaakt moet zijn door formiaat. Deze, soms sterke, remming van formiaat kan door bicarbonaat op een competitieve wijze ongedaan gemaakt worden.

In hoofdstuk 3 wordt verder nog een mechanisme voorgesteld waarbij fotorespiratie het fotosynthetisch elektronentransport beïnvloedt. Onder bepaalde omstandigheden kan in de fotorespiratie formiaat gevormd worden. Er wordt beschreven hoe de fotorespiratie een negatieve terugkoppeling op het elektronentransport teweeg kan brengen. Een dergelijk mechanisme zou kun-

nen dienen om door middel van fotorespiratie het elektronentransport te reguleren.

In hoofdstuk 4 worden de effecten van formiaat en bicarbonaat op het elektronentransport in onbehandelde chloroplasten nader bestudeerd, met name tijdens langere belichting. Formiaat blijkt in onbehandelde chloroplasten het elektronentransport te kunnen stimuleren. Deze stimulering wordt toegeschreven aan een ontkoppelende werking van formiaat. In volledig ontkoppelde chloroplasten had formiaat alleen een remmende werking op het elektronentransport. De mate van de remming nam toe tijdens de belichting. De aanwezigheid van bicarbonaat bleek de remming van het elektronentransport door formiaat slechts gedeeltelijk te kunnen tegengaan. Volgens een ruwe schatting is de remmende werking van formiaat in het licht meer dan een orde van grootte sterker dan in het donker. Dit betekent dat formiaat het elektronentransport al kan beïnvloeden bij een concentratie van minder dan 1 mM.

In hoofdstuk 5 wordt een methode beschreven waarmee op een andere wijze de effecten van bicarbonaat en formiaat op het fotosynthetisch elektronentransport bestudeerd kunnen worden. Deze methode is gebaseerd op het meten van flits-geïnduceerde potentiaalveranderingen over het thylakoid membraan met behulp van absorptieveranderingen van het pigmentcomplex P_{515}. In chloroplasten waaraan CO_2 onttrokken was kon ook op deze wijze aangetoond worden dat het elektronentransport geremd was. Na toevoeging van bicarbonaat bleek het elektronentransport versneld te verlopen. Het onttrekken van CO_2 aan chloroplasten had ook invloed op absorptieveranderingen in het gebied tussen 500 en 550 nm. Deze absorptieveranderingen, welke hoogstwaarschijnlijk niet gerelateerd zijn aan de flits-geïnduceerde membraanpotentiaalveranderingen, worden verondersteld te zijn gecorreleerd met protonopname aan de acceptorzijde van fotosysteem II.

Hoofdstuk 6 beschrijft effecten van formiaat en bicarbonaat

op het elektronentransport in geïsoleerde intacte chloroplasten. Er werd gevonden dat formiaat een remmende werking heeft op de zuurstofproduktie in aanwezigheid van een natuurlijke electronacceptor, PGA. Deze remming bleek niet veroorzaakt te worden door remming van het elektronentransport aan de acceptorzijde van fotosysteem II. Door incubatie van intacte chloroplasten met formiaat bij lage pH kon het elektronentransport wel geremd worden. Na toevoeging van bicarbonaat bleek deze remming te zijn opgeheven, d.w.z. het "bicarbonaat-effect" blijkt ook in intacte chloroplasten op te kunnen treden. De incubatie met formiaat bij lage pH had echter wel een schadelijk effect op het koolstof metabolisme.

In hoofdstuk 7 tenslotte worden enkele resultaten nog eens besproken en wordt gespeculeerd over de betekenis van de regulering van het fotosynthetisch elektronentransport door bicarbonaat en formiaat.

PUBLICATIONS

Several topics of this thesis are covered in the following papers:

Snel, J.F.H., J.J.S. van Rensen, 1982. Influence of photosynthetic inhibitors on the reactivation of the Hill reaction in CO_2-depleted chloroplasts by bicarbonate. Plant Physiology 69: 114.

Snel, J.F.H., J.J.S. van Rensen, 1983. Kinetics of the reactivation of the Hill reaction in CO_2-depleted chloroplasts by addition of bicarbonate in the absence and in the presence of herbicides. Physiologia Plantarum 57: 422-427.

Van Rensen, J.J.S., J.H. Hobé, R.M. Werner, J.F.H. Snel, 1983. Reactivation of electron transport in CO_2-depleted chloroplasts by bicarbonate is influenced by thylakoid surface potential. *In*: Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague.

Snel, J.F.H., A. Groote-Schaarsberg, J.J.S. van Rensen, 1983. Mechanism and physiological role of bicarbonate action on electron flow; a possible link between photorespiration and photosynthetic electron flow? *In*: Advances in Photosynthesis Research (Sybesma, E., ed.), Vol. I, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague.

Snel, J.F.H., J.J.S. van Rensen, 1984. Reevaluation of the role of bicarbonate and formate in the regulation of photosynthetic electron flow in broken chloroplasts. Plant Physiology 75: 146-150.

Snel, J.F.H., D. Naber, J.J.S. van Rensen, 1984. Formate as an inhibitor of photosynthetic electron flow. Zeitschrift für Naturforschung 39c: 386-388.

Van Rensen, J.J.S., F. de Koning, J.F.H. Snel, (in press). Effects of bicarbonate, formate and herbicides on photosynthetic electron transport in isolated broken chloroplasts. Proceedings of the 3rd EBEC conference, ICSU Press, Miami.

Snel, J.F.H., D. Naber, J.J.S. van Rensen, (in press). Regulation of photosynthetic electron flow by formate and bicarbonate in isolated intact chloroplasts. Proceedings of the 3rd EBEC conference, ICSU Press, Miami.

Van Rensen, J.J.S., J.F.H. Snel, 1985. Regulation of photosynthetic electron transport by bicarbonate, formate and herbicides in isolated broken and intact chloroplasts. Photosynthesis Research (in press).

CURRICULUM VITAE

Ik ben geboren op 31 juli 1952 te Laag-Soeren in de gemeente Rheden (Gld). De middelbare school heb ik gevolgd in Zutphen op het Baudartius College, alwaar ik het diploma HBS-B behaalde in 1970. Daarna heb ik 2 jaar gestudeerd aan de onderafdeling Vliegtuigbouwkunde van de Technische Hogeschool in Delft. In 1974 ben ik aan de Rijksuniversiteit te Groningen begonnen aan de studie Biologie. Het kandidaatsexamen B3, d.w.z. met de hoofdvakken Biologie en Natuurkunde, heb ik behaald in 1978. In mijn doctoraalfase heb ik eerst bij de vakgroep Plantenfysiologie onderzoek gedaan naar het gebruik van bicarbonaat als koolstofbron door waterplanten; de begeleiding door Dr. Hidde Prins en Prof. Riender Helder heeft mij erg gestimuleerd. Vervolgens heb ik bij de vakgroep Fysische chemie onder leiding van Dr. Klaas Nicolay en Prof. Rob Kaptein onderzoek gedaan naar mogelijke toepassing van ^{31}P-NMR op het gebied van de energieconversie in eukaryote fotosynthetische organismen en organellen. Daar heb ik mijn eerste chloroplasten geisoleerd. In 1981 heb ik het doctoraalexamen Biologie behaald met als specialisatie Plantenfysiologie en als bijvak Fysische chemie. Van 1981 tot 1984 ben ik als wetenschappelijk assistent in dienst geweest bij de vakgroep Plantenfysiologisch Onderzoek van de Landbouwhogeschool te Wageningen, alwaar ik onder leiding van Dr.ir. Jacques van Rensen en Prof. Wim Vredenberg het onderzoek gedaan heb dat in dit proefschrift beschreven is. Vanaf december 1984 ben ik als universitair docent in tijdelijke dienst bij de vakgroep Plantenfysiologisch onderzoek.