INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

- 1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.
- 2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame.
- 3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again-beginning below the first row and continuing on until complete.
- 4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.
- 5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.



300 N ZEEB ROAD, ANN ARBOR, MI 48106 18 BEDFORD ROW, LONDON WC1R 4EJ, ENGLAND KHANNA, RITA

t

٦,

ROLE OF BICARBONATE AND OF MANGANESE IN PHOTOSYSTEM II REACTIONS OF PHOTOSYNTHESIS

University of Illinois at Urbana-Champaign

1

Рн.D. 1980

University Microfilms International 300 N Zeeb Road, Ann Arbor, MI 48106

18 Bedford Row, London WC1R 4EJ, England

ROLE OF BICARBONATE AND OF MANGANESE IN PHOTOSYSTEM II REACTIONS OF PHOTOSYNTHESIS

¢

BY

RITA KHANNA

B.Sc., University of Delhi, 1971 M.Sc., University of Delhi, 1973 Diploma, Indian Institute of Science, 1973

THESIS

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology in the Graduate College of the University of Illinois at Urbana-Champaign, 1980

Urbana, Illinois

.

	THE GRADUATE COLLEGE
	November, 1979
WE HE	REBY RECOMMEND THAT THE THESIS BY
	RITA KHANNA
ENTITLED_	ROLE OF BICARBONATE AND OF MANGANESE IN
	DUOTOSVETEM II DEACTIONS OF DUOTOSVNTUESIS
	PHUIUSISIEM II REACTIONS OF PHUIUSININESIS
ВЕ АССЕРТ	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO
BE ACCEPT THE DEGRE	E OF DOCTOR OF PHILOSOPHY
BE ACCEPT THE DEGRE	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY
BE ACCEPT THE DEGRE	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY Director of Thesis Researce
BE ACCEPT THE DEGRE	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY Director of Thesis Researce Head of Department
BE ACCEPT THE DEGRE	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY Director of Thesis Researc Director of Thesis Researc Head of Department
BE ACCEPT THE DEGRE Committee on	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY Director of Thesis Researce Head of Department Email Examination [†]
BE ACCEPT THE DEGRE Committee on	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY Iowindyce Director of Thesis Researce Head of Department Eternal Examination† Chairman
BE ACCEPT THE DEGRE Committee on	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY Director of Thesis Researce Director of Thesis Researce Head of Department Email Examination† Chairman Email Examination†
BE ACCEPT THE DEGRE Committee on	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY Jowindyce Director of Thesis Researce Head of Department Etent Examination† Chairman Towindyce Chairman
BE ACCEPT THE DEGRE Committee on	ED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FO E OF DOCTOR OF PHILOSOPHY

ACKNOWLEDGMENT

I wish to express my most sincere appreciation to Professor Govindjee for his guidance, help and constant encouragement during the course of my thesis research. I am indebted to him for sharing with me his enthusiasm, insights and broad understanding of the field of photosynthesis. I would also like to thank Professor H. S. Gutowsky for many fruitful discussions, for supporting my research on Photosynthetic Oxygen Evolution, and for the use of NMR equipment.

The NMR measurements were made in collaboration with Dr. S. Rajan who was reponsible for many painstaking and innovative improvements of the NMR equipment. I am grateful to him for having made both the theory and practice of NMR more lucid, and for being such a good companion through the successes and frustrations of research work. I am grateful to Drs. U. Siggel and G. Renger for their valuable help in the measurement of the absorption changes at 334, 265 and 703 nm. I would like to thank Mr. R. Waggner for his help in the measurement of the pH changes, Professor W. Junge for his interest and for the use of his laboratory facilities in these measurements, Dr. K. Pfister for collaborating in herbicide binding experiments, and Dr. K. Steinback for her expert help in the preparation of trypsin treated thylakoid membranes.

I have profited immensely from the large photosynthesis group at the University of Illinois. In particular, I am grateful to Professors Charles Arntzen, Colin Wraight and Tony Crofts for many useful discussions, for the use of their equipment and for their helpful comments on my research.

iii

I have been fortunate in having overlapped with a large number of fellow students at this laboratory, and I am grateful to them for their friendship and consideration. I would especially like to thank Daniel Wong, Paul Jursinic, Tom Wydrzynski and Jim Fenton for helpful discussions.

I owe a special note of appreciation to the Govindjees for their warm friendship and hospitality during my years at Urbana. I am especially grateful to Rajni Govindjee for her kindness and companionship.

I wish to thank Amar for his resolute support and understanding which sustained my efforts throughout these years. Finally, I thank my parents and the rest of my family for their patience and encouragement.

This work was partially supported by National Science Foundation (grant PCM 76-11657) and the U.S. Department of Agriculture (grant 5901-40).

To My Parents

TABLE OF CONTENTS

CH/	APTER			Page
1	GENER	AL INTRO	DUCTION	. 1
	1.1 1.2 1.3 1.4	Photosyr Substrai Bicarbor Kinetic	nthesis te for Oxygen Evolution nate Requirement for Oxygen Evolution Studies of Oxygen Evolution	. 1 . 4 . 6 . 9
		1.4.1 1.4.2 1.4.3 1.4.4 1.4.5 1.4.6	Kinetic Experiments and the Model for Oxygen Evolution Stability of the S States Misses and Double Hits Relaxation Step Deactivation Proton Release	. 9 . 11 . 12 . 14 . 14 . 16
	1.5	Involven	nent of Manganese in Oxygen Evolution	. 17
		1.5.1 1.5.2 1.5.3 1.5.4 1.5.5	Different Pools of Manganese ESR Signals Reactivation of the Oxygen Evolving System Chloroplast Manganese Proteins Measurements of Water Proton Relaxation Related to Oxygen Evolution	 . 18 . 21 . 22 . 25 . 26
	1.6	Purpose	and Scope of the Present Thesis	. 28
2	MATER	IALS AND	METHODS	. 31
	2.1 2.2 2.3 2.4 2.5 2.6 2.7 2.8	Isolatic Bicarbor Trypsin Glutara Isolatic Electror Absorpt Changes	on of Thylakoid Membranes nate Depletion of Thylakoid Membranes Treatment Idehyde Fixation of Thylakoid Membranes on and Purification of the Light Harvesting Complex n Transport Rates ion Changes at 334, 265 and 703 nm ion Changes at 524 and 574 nm for Measuring pH	 31 31 32 33 33 34 36 36
		2.8.1 2.8.2	Measurement of Internal pH by Neutral Red Measurement of External pH by Bromocresol Purple	. 36 . 38
	2.9 2.10 2.11 2.12	Herbicio Meausure ESR Meas Manganes	le Binding Studies ement of Proton Relaxation Rates surements se Determination by Neutron Activation Analysis	• 38 • 39 • 43 • 44

C	HAPTER		F	PAGE
3	ROLE	OF BICAR	BONATE IN PHOTOSYSTEM II REACTIONS OF GREEN PLANTS	46
	3.1 3.2	Introdu Charact	ctionof the Bicarbonate Effect	46 46
		3.2.1 3.2.2	Bicarbonate Concentration Required for the Stimula- tion of the Hill Reaction: Physiological Importance Effect of External pH on the Bayarbonate Stimulation	46
		3.2.3	of the Hill Reaction Effect of Uncouplers on the Bicarbonate Effect	49 51
	3.3	Site of	the Bicarbonate Effect	54
		3.3.1	Biochemical Studies on Partial Reactions of the Electron Transport Chain	55 56
			(Reaction 2) 3.3.1.3 Reduced Diaminodurene to Methyl Viologen	58
		3.3.2	 Effect of Bicarbonate on the Absorption Changes due to Different Redox Components of the Electron Transport Chain 3.3.2.1 Absorption Changes at 334 nm Induced by 20 µs Repetitive Flashes 3.3.2.2 Absorption Changes at 265 nm Induced by 20 µs Repetitive Flashes 3.3.2.3 Absorption Changes at 265 nm and 703 nm Induced by 20 µs Repetitive Flashes 3.3.2.4 Absorption Changes at 265 nm by 85 ms Repetitive Flashes 	59 60 65 68 74
	3.4	Mechani	sm of Bicarbonate Action	82
		3.4.1 3.4.2 3.4.3	Bicarbonate Effect in Trypsin Treated Thylakoid Membranes Herbicide Binding to Bicarbonate Depleted Thylakoid Membranes Measurement of pH Changes in the Inner and Outer Phase of Thylakoids	86 88 93
			3.4.3.1 Measurement of Internal pH 3.4.3.2 Measurement of External pH	94 96
	3.5	Conc lud	ing Remarks	99

•

ţ,

,

~

СН	APTER		PA	GE
4	4 STUDIES ON THE ROLE OF MANGANESE IN OXYGEN EVOLVING SYSTEMS			01
	4.1 4.2	Introdu Effects Membran	ction	01 03
		4.2.1 4.2.2 4.2.3	Effects of Tetraphenylboron 1 Effects of Hydroxylamine 1 Effects of Hydrogen Peroxide 1	03 13 26
	4.3	Studies Membran	on the Different Pools of Manganese in Thylakoid es	28
		4.3.1 4.3.2 4.3.3	Effect of Mg(II) on Cation Depleted Thylakoid Membranes	31 .36 .41
	4.4	Is Ther Paramag	re a Contribution to Water Proton Relaxation from Inetic Species Other than Mn(II)?	45
		4.4.1 4.4.2 4.4.3	Aging of Thylakold Membranes at 35°C	46 48 54
	4.5	Conc lud	ling Remarks	56
5	SUMMA	RY AND C	ONCLUDING REMARKS 1	58
	5.1	Bicarbo	nate Requirement for PS II Reactions	58
		5.1.1 5.1.2 5.1.3	A Few Characteristics of the Bicarbonate Effect 1 Site of the Bicarbonate Effect is Between Q and the PQ Pool	58 59 61
	5.2	Involve	ment of Manganese in Photosynthetic Op Evolution 1	62
	w	5.2.1 5.2.2 5.2.3	Effect of TPB, NH ₂ OH and H ₂ O ₂ on T $\overline{2}^1$ of Thylakold Membranes	63 64 66
LI	TERATU	RE CITED) 1	68
VI	та			79

LIST OF TABLES

1

,

.

,

TABL	Ε	PAGE
3.1	Bicarbonate Effect in the Presence of Uncouplers of Photophos- phorylation: Rates of Electron Transport	. 53
3.2	Effect of Bicarbonate on Various Isolated Electron Transport Systems	. 57
3.3	Absorption Changes at 265 and 703 nm Induced by 20 $_{\mu}s$ Repetitive Flashes for CO2-Depleted and Reconstituted Thylakoids	e 73
3.4	Comparison of Absorption Changes at 265 nm in Fresh, and Frozen and Thawed Thylakoid Membranes	. 75
4.1	Effect of MgCl ₂ on T_2^{-1} and O_2 Evolution of Trypsin Treated Thylakoid Membranes	. 138
4.2	Comparison of T_2^{-1} and Total Mn Content of Isolated LHC and Thylakoid Membranes	. 143
5.1	Conclusions Made in the Thesis	. 167

ix

LIST OF FIGURES

FIGURE	P/	AGE
1.1	Electron flow from H ₂ O to NADP ⁺	2
2.1	Definition of T_1^{1} and T_2^{1} relaxation	40
2.2	Block diagram of the pulsed NMR spectrometer	42
3.1	Effect of bicarbonate on O2 evolution in the presence and absence of formate in the assay medium	48
3.2	Rate of O2 evolution as a function of pH in the presence and absence of bicarbonate	50
3.3	Comparison of the stimulatory effect of HCO_3/CO_2 with the relative concentrations of CO_2 , HCO_3 and CO_3 at different pH values	52
3.4	Time course of the absorption change at 334 nm induced by 20 μs repetitive flashes	62
3.5	Semilogarithmic plots of the curves from Fig. 3.4	63
3.6	Time course of the absorption change at 265 nm induced by 20 $_{\mu}s$ repetitive flashes	67
3.7	Absorption changes at 265 and 703 nm induced by 20 $_{\mu}s$ repetitive flashes, measured simultaneously, for CO2-depleted and reconstituted thylakoid membranes	69
3.8	Semilogarithmic plots of the signals of Fig. 3.7 ($-CO_2$ sample).	70
3.9	Semilogarithmic plots of the signals of Fig. 3.7 (+CO ₂ sample).	72
3.10	Time course of the absorption change at 265 nm induced by 85 ms repetitive flashes for CO_2 -depleted and reconstituted thylakoid membranes	78
3.11	Effect of far red background illumination on absorption changes at 265 nm induced by 85 ms repetitive flashes	81
3.12	Absorption changes at 265 nm induced by 20 μs and 85 ms flashes, measured on the same batch of CO2-depleted thylakoids.	83

FIGURE

3.13	Simplified hypothetical scheme of functional and structural organization of some components of PS II and vectorial electron transport showing protolytic reaction sites in thylakoids	84
3.14	Effect of bicarbonate on oxygen evolution in trypsinated thy- lakoid membranes	87
3.15	Binding of $14C$ -atrazine to control CO_2 -depleted and reconstituted pea thylakoids	90
3.16	Inhibition of DCPIP reduction in control, CO2-depleted and reconstituted thylakoids by atrazine	92
3.17	Kinetics of proton release into internal phase of spinach thylakoids as indicated by absorption change of neutral red at 524 nm	95
3.18	Absorption changes of bromocresol purple at 574 nm induced by a single-turnover flash in control and CO2-depleted spinach thylakoids	97
4.1	Effect of TPB on T_2^{-1} , O_2 evolution and DCPIP reduction in peathylakoids	104
4.2	Room temperature ESR spectra of thylakoid membranes in the presence of different concentrations of TPB	107
4.3	T_2^{-1} of washed pea thylakoid membranes as a function of exogenously added TPB with and without MgCl ₂	109
4.4	Effect of TPB on O2 evolution and DCPIP reduction in washed thylakoid membranes with and without MgCl2	110
4.5	Room temperature ESR spectra of thylakold membranes in the presence of TPB, MgCl2 and TPB plus MgCl2	111
4.6	Effect of NH ₂ OH on T_2^{-1} of thylakoid membranes	115
4.7	Room temperature ESR spectra of thylakoid membranes in the presence of different concentrations of NH2OH	116
4.8	Room temperature ESR spectra of thylakoid membranes in the presence of NH2OH, MgCl2 and NH2OH plus MgCl2	118
4.9	T_2^{-1} as a function of time after the addition of 0.01 to 0.1 mM NH ₂ OH	120
4.10	T_2^{-1} as a function of time after the addition of 1 or 2 mM $\rm NH_2OH$	121

PAGE

•

FIGURE

4.11	T_2^{-1} as a function of time after the addition of 10 or 100 mM NH ₂ OH	122
4.12	Time course of the increase in T_2^{-1} and the release of manganese induced by the addition of 0.5 mM NH ₂ OH to thylakoid membranes	123
4.13	T_2^{-1} as a function of time after the addition of H_2O_2	127
4.14	Room temperature ESR spectra for thylakoid membranes in the presence of $\rm H_2O_2$	129
4.15	Effect of MgCl ₂ on T_2^{-1} , O ₂ evolution and the release of manganese in washed thylakoids	132
4.16	Room temperature ESR spectra of washed thylakoid membranes in the presence of different concentrations of MgCl ₂	134
4.17	Room temperature ESR spectra of control and trypsin treated thylakoid membranes in the absence and presence of MgCl ₂	139
4.18	SDS-polyacrylamide slab gel electrophoresis of polypeptides from control thylakoid membranes, trypsin treated thylakoid membranes and purified light harvesting complex	140
4.19	Effect of time of incubation at 35°C of thylakoid membranes upon T_2^{-1} , O_2 evolution and total Mn content	147
4.20	Effect of heating of thylakold membranes upon T_2^{-1} and ESR signal for Mn(II)	149
4.21	Effect of heating upon T_2^{-1} of glutaraldehyde fixed thylakold membranes	151
4.22	Effect of KCN upon T_2^{-1} of heat treated thylakoid membranes	153
4.23	Effect of pH of the suspension medium on T_2^{-1} and ESR detectable free Mn(II) of thylakoid membranes	155

PAGE

LIST OF ABBREVIATIONS

ADRY: reagents which accelerate the deactivation reactions in the oxygen evolving system \underline{Y}

- β: double hit parameter
- BCP: bromocresol purple
- BSA: bovine serum albumin
- CCCP: carbonyl cyanide m-chlorophenylhydrazone
- Chl: chlorophyll
- cyt f: cytochrome f
- D: alternate electron donor to the reaction center chlorophyll <u>a</u> of photosystem II
- DI: primary donor to the reaction center of photosystem I
- DAD: 2,3,5,6,-Tetramethyl-p-phenylenediamine; also called diaminodurene
- DBMIB: 2,5-Dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; also called dibromothymoquinone
- DCMU: 3-(3,4-Dichlorophenyl)-1,1-dimethylurea; also called diuron
- DCPIP: 2,6-dichlorophenol indophenol
- DPC: diphenylcarbazide
- DQH₂ reduced duroquinone
- EDTA: ethylenediamine tetraacetic acid
- ESR: electron spin resonance
- Ho: external applied magnetic field
- H_i: rf magnetic field, applied orthogonal to H_O
- HEPES: N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
- LHC: light harvesting (chlorophyll a/b protein) complex
- M: charge accumulator involved in oxygen evolution

M:	net magnetization of a spin system
MV:	1,1-dimethy1-4,4-dipyridinium dıchloride; also called methyl viologen
NADP ⁺ :	nicotinamide adenine dinucleotide phosphate
obs:	observed
P680:	photosystem II reaction center
P700:	photosystem I reaction center
PQ:	plastoquinone
PRR:	proton relaxation rate
PS I:	photosystem I
PS II:	photosystem II
Q(X320):	first stable electron acceptor of photosystem II
R(B):	a 2-electron carrier accepting electrons from Q ⁻ (X320 ⁻)
rf:	radio frequency radiation
SiMo:	silicomolybdate
S _n :	intermediate associated with oxygen evolving mechanism; n refers to the state of the intermediate where $n = 0, 1, 2, 3, 4$
t _{1/2} :	halftime
τ_1^{-1} :	longitudinal or spin-lattice relaxation rate
T_2^{-1} :	transverse or spin-spin relaxation rate
t _d :	darktime between flashes
TPB:	tetraphenylboron
Tricine:	N-tris (hydroxymethyl) methyl-2-aminomethanesulfonic acid
Tris:	Tris (hydroxymethyl) aminomethane
Z, Z ₁ :	first electron donor to the photosystem II reaction center
Z2:	second electron donor to the photosystem II reaction center

í

CHAPTER 1

GENERAL INTRODUCTION

1.1 Photosynthesis

Photosynthesis is the process by which green plants convert solar energy into chemical energy according to the following equation:

$$2 H_2 0 + CO_2 \xrightarrow{\text{light}} (CH_2 0) + H_2 0 + O_2$$
 (1.1)

The free energy of light drives a series of oxidation reduction reactions which lead to the transfer of electrons from water to carbon dioxide (CO_2) , ultimately resulting in the oxidation of water to oxygen, and the reduction of CO_2 to carbohydrates (CH_2O) . In eukaryotes light energy is absorbed by chlorophyll and other photosynthetic pigments functionally organized in cellular organelles called chloroplasts. These consist of a lamellar system and a stroma matrix surrounded by a double membrane. Chloroplasts of higher plants have dense regions called grana, that are stacks of sac like membraneous discs (the thylakoids), and less dense regions composed of stroma lamellae, which interconnect the grana (see Arntzen and Briantais, 1975). The membranes of thylakoids contain the photosynthetic pigments and a number of redox components constituting the photosynthetic electron transport chain.

Figure 1.1 shows a scheme for electron transport in photosynthesis; this scheme (see Govindjee, 1979) is based on the original scheme of Hill and Bendall (1960). The transfer of electrons from water to nicotinamide adenine dinucleotide phosphate ($NADP^+$) is driven by two photochemical reactions connected in series. The light energy, absorbed by the accessory pigments and chlorophyll <u>a</u> in both pigment systems, is transferred



Figure 1.1 Electron flow from H_2O to NADP⁺. Excitation of the PS II reaction center (P680) results in the transfer of an electron from P680 to the first "stable" electron acceptor Q; pheophytin has been suggested to be the primary acceptor of PS II. Oxidized P680 is reduced by electrons from H_2O mediated by M, the charge accumulator associated with 0_2 evolution, and electron transfer intermediates Z_2 and Z_1 . Upon excitation of PS I, the reaction center P700 becomes oxidized giving up an electron to the primary electron acceptor which may be a chlorophyll a mole- $P700^+$ is reduced by electrons from Q^- via the intersystem electron cule. transport components: a two electron carrier R(B), the plastoquinone pool (PQ), a Rieske protein, cytochrome f (cyt f) plastocyanin (PC) and an intermediate D. The reduced primary acceptor of PS I then leads to the reduction of NADP⁺ via several other intermediates, A_1 , A_2 , P430 (A and B), ferredoxin and ferredoxin-NADP-reductase (FNR). Suggested halftimes for electron transport reactions are indicated with the arrow for each step (redrawn from Govindjee, 1979).

to specialized chlorophyll a molecules, P680 and P700, the reaction centers for Photosystem II (PS II) and Photosystem I (PS I), respectively. PS II oxidizes water and transfers electrons from water to an intermediate pool of plastoquinone (PQ) between the two photosystems while PS I transfers electrons from plastohydroguinone to $NADP^+$. Upon excitation of PS II, the reaction center chlorophyll molecule P680 becomes oxidized by giving up an electron to Q, the first "stable" electron acceptor of PS II; the existence of an unidentified acceptor component (X_E) between P680 and Q has been suggested (Eckert et al., 1979). The possibility that pheophytin may act as the primary acceptor has gained some support from the work of Klimov et al. (1978). In the subsequent dark reaction, $P680^+$ is reduced by electrons from water oxidation mediated by the charge accumulator M (of the oxygen evolving system) and the electron transfer intermediates Z_2 and Z_1 . Excitation of the PS I reaction center leads to the oxidation of P700 to $P700^+$ and the reduction of the primary electron acceptor which may be a chlorophyll a molecule (Shuvalov et al., 1979). The reduced primary electron acceptor of PS I is oxidized by donating electrons to NADP via intermediates A_1 , A_2 , P430 (A and B) (Sauer et al., 1978), ferredoxin, and ferredoxin-NADP-reductase (FNR). The P700⁺ is reduced by the transfer of electrons from Q^- via several intermediary electron components: a two electron carrier R (or B), the PQ pool, a Rieske Fe-protein (Malkin and Posner, 1978), cytochrome f (cyt f), and an intermediate D (Bouges-Bocquet and Delosme, 1978). The transfer of electrons from water to $NADP^+$ is coupled to a phosphorylating mechanism by which ADP is converted to ATP. NADPH and ATP are then utilized for the fixation of CO_2 into carbohydrates and other organic compounds.

In this study we investigated several reactions on the oxidizing and reducing side of PS II. On the reducing side of PS II, we studied the role of the bicarbonate^{*} ion and characterized some aspects of the mechanism of its action on the electron transport from PS II to PS I, and on the oxidizing side, we studied the involvement of manganese in 0_2 evolution employing the techniques of nuclear magnetic resonance (NMR) and electron spin resonance (ESR). A brief survey of the role of the bicarbonate ion in PS II reactions and studies on 0_2 evolution follows.

The general equation 1.1 of photosynthesis does not distinguish between H_2^0 and CO_2 as the source of O_2 . The establishment of water as the source of molecular oxygen was first suggested by Wurmser (1930) and supported by Van Niel (1931). Based on the metabolic comparison of higher plant and bacterial photosynthesis, Van Niel generalized photosynthesis as follows:

$$CO_2 + 2H_2A \xrightarrow{hv} (CH_2O) + H_2O + 2A$$
 (1.2)

where, H_2A represents some substrate which is oxidized in light. In bacteria H_2A could be H_2S , H_2 or isopropanol, while in higher plants H_2A would be water. The water decomposition hypothesis became more acceptable when Hill and Scarisbrick (1940) showed that isolated chloroplasts could evolve oxygen in the absence of CO_2 fixation, using ferrioxalate as an artificial electron acceptor. The most direct evidence for the concept that O_2 was derived from water came from isotopic labelling experiments done with H_2 ¹⁸O (Ruben <u>et al.</u>, 1941). It was shown

^{*}Except in section 3.2.2, CO2 and bicarbonate have been used interchangeably without implying the identity of the active species.

that oxygen produced by a suspension of <u>Chlorella</u> cells is relatively enriched in ¹⁸0 when ¹⁸0 labelled water is used than when ¹⁸0 labelled CO₂ is used.

The concept of oxygen evolution directly from water has been challenged by Metzner (1966, 1976) who measured the ¹⁸0 content of oxygen evolved in a suspension of <u>Chlorella</u> cells after the addition of labelled bicarbonate. The ¹⁸0 content measured between the 15th and 60th second after the addition of NaHC¹⁸O₃ was more than the ¹⁸0 content expected if O₂ were a product of the decomposition of water. This observed higher content of ¹⁸0 was used by Metzner as a proof against water being the substrate for O₂ evolution. Metzner also criticizes Van Niel's argument on the basis that green plants, in contrast to photosynthetic bacteria, require two photoreactions to transfer electrons to CO₂. The bacterial system is not capable of O₂ evolution and is, therefore, more similar to PS I of green plants rather than O₂ evolving PS II in this respect.

Recently, however, Stemler and Radmer (1975) showed that bicarbonate is not the substrate for photosynthetic oxygen evolution within the first few minutes (3-9 min) of CO_2 addition. In this study isolated broken chloroplast fragments were employed in order to minimize the amount of carbonic anhydrase which speeds up the exchange reaction between H_2O and CO_2 . These chloroplasts were depleted of bicarbonate, thus making O_2 evolution dependent on the addition of bicarbonate ion (see section 1.3). When $HC^{18}O_3^-$ was added, the O_2 evolved was only ${}^{16}O_2$ reflecting the isotopic composition of the unlabelled water rather than the labelled bicarbonate. This suggests that O_2 is indeed evolved from water.

1.3 Bicarbonate Requirement for Oxygen Evolution

In 1960 Warburg and Krippahl discovered that oxygen evolution by isolated chloroplasts, in the presence of an acceptor such as ferricyanide, was dependent on the presence of CO_2 . Warburg and Krippahl (1960) argued that this bicarbonate effect supported the concept of bicarbonate complex as the substrate for oxygen evolution. Willstätter and Stoll (1918) had described the photochemical process as a hydroxylhydrogen exchange reaction in a carbonic acid-chlorophyll complex. This idea of a carbonic acid-chlorophyll complex was adopted by Warburg and Krippahl to explain the requirement for bicarbonate in the Hill reaction. Warburg termed the complex "photolyte" and considered it the direct precursor of O_2 . The carbonic acid-chlorophyll complex is hydrolyzed and decomposed into O_2 , formic acid, and phosphoric acid. A second molecule of oxidant then oxidizes the formic acid to CO_2 . Thus, it was proposed that CO_2 functions as a metabolite and yet catalytically.

Stern and Vennesland (1960, 1962) repeated Warburg's experiments and showed that CO_2 stimulation of ferricyanide reduction by chloroplasts is completely reversible. They also tested chloroplasts from a number of species and found the CO_2 effect to be a general one. This, however, could not be demonstrated for chloroplasts isolated from sugar beet (Abeles <u>et al.</u>, 1961).

Izawa (1962) characterized the phenomenon further and showed that addition of carbonic anhydrase to the reaction medium enhanced blcarbonate depletion and markedly shortened the time required to obtain maximal CO_2 effect. He observed that the bicarbonate effect was more marked in "washed grana" than in whole chloroplasts. He also conducted experiments to determine whether CO_2 influenced the Hill reaction in its photochemical phase or in the dark process. The results indicated that the stimulatory effect of CO_2 upon the Hill reaction observed at high light intensity (30,000 lux) was reduced to nearly a tenth at low light intensity (3,000 lux). These results would imply that CO_2 is involved in a dark rather than a photochemical reaction.

Good (1963) confirmed that the absence of CO_2 was more inhibitory at high light intensities than at low light intensities. He tried to substitute CO_2 by a number of inorganic and organic anions (e.g., phosphate, citrate, glycine, etc.) but came to the conclusion that the stimulatory effect of Hill reaction is specific to bicarbonate. Furthermore, preincubation of chloroplasts with chloride and acetate markedly increased the dependence of O_2 evolution on CO_2 .

Punnett and Iyer (1964) extended the observations of CO_2 effect to phosphorylation accompanying the Hill reaction. Their method differed strikingly from that of the previous workers in that CO_2 depletion of the chloroplasts was not required in order to see stimulation by added bicarbonate. They discovered that phosphorylation was enhanced by CO_2 addition even more than electron flow, thus increasing the P/2e ratio. From their results with phosphorylation as well as the Hill reaction, they postulated that bicarbonate allows the formation of some "high energy intermediate" to drive phosphorylation. These results were confirmed by Batra and Jagendorf (1965). They compared the methods of Punnett and Iyer with those of Warburg and Krippahl, and suggested that the effect of added bicarbonate was probably different in the two systems: the observations of Warburg and Krippahl related to 0_2 evolution and those of Punnett and Iyer related to the phosphorylation mechanism.

Stemler and Govindjee (1973) reinvestigated the bicarbonate problem and attempted to locate the site of its action along the electron transport chain, using heat treated chloroplasts which were unable to evolve oxygen. Diphenyl carbazide (DPC) was used as an electron donor to PS II and the rate of reducton of dichlorophenol indophenol (DCPIP) was measured in the presence and absence of bicarbonate. The rate of electron flow from DPC to DCPIP was insensitive to the bicarbonate ion thereby suggesting that the site of action of bicarbonate is located before the site of donation by DPC. This conclusion had to be modified as it became known later that DPC itself caused an enhancement in DCPIP reduction in the bicarbonate depleted chloroplasts (Wydrzynski and Govindjee, 1975). Thus, the absence of the bicarbonate effect on DPC \Rightarrow DCPIP reaction could not be accepted as evidence for the requirement of bicarbonate on the oxygen evolving side of PS II (see reviews by Govindjee and Khanna, 1978; Govindjee and van Rensen, 1978 for further details).

When work on this thesis was started, the site of the bicarbonate effect was still ambiguous. Above all, the mechanism by which bicarbonate stimulates Hill reaction was a complete mystery. The purpose of this investigation was to locate the site of action of bicarbonate and to arrive at a better understanding of the mechanism by which bicarbonate stimulates 0_2 evolution.

1.4 Kinetic Studies of Oxygen Evolution

1.4.1 Kinetic Experiments and the Model for Oxygen Evolution

Biochemical studies have provided very little information about the chemistry and mechanism of 0_2 evolution except that manganese is somehow involved in this process. The most effective approach for probing the 0_2 evolving system has been to measure 0_2 evolution in sufficiently short single turnover flashes.

Emerson and Arnold (1932a; 1932b) illuminated <u>Chlorella</u> cells by repetitive brief (10 μ s) saturating flashes and measured average 0₂ produced per flash. The amount of 0₂/flash reached a maximum value of ~10₂/2500 chlorophyll molecules when the dark period between the flashes was optimum (>10 ms at 20°C). This result led to the concept of a photosynthetic unit, i.e., several hunderd chlorophyll molecules cooperating in evolving 0₂.

Allen and Franck (1955) found that oxygen yield was increased substantially if anaerobic cells of <u>Scenedesmus</u> were preilluminated by another flash or a weak background light. These observations suggested that during preillumination a precursor for 0_2 evolution is formed. Subsequent experiments (Whittingham and Brown, 1958; Whittingham and Bishop, 1963) confirmed the conclusion that some priming reaction was required before 0_2 evolution occurred.

Joliot (1961, 1965) confirmed these observations under aerobic conditions and showed that after dark adaptation, there was a lag in 0_2 evolution in continuous weak light. He also showed that at least two photoacts were required for 0_2 evolution after a dark period. This led to the idea of charge accumulation. The development of a sensitive, rapid response polarographic technique by Joliot (1967) allowed the precise measurement of small quantities of O_2 by short saturating (1-10 µs) flashes. In these experiments (Joliot <u>et al</u>., 1969; Kok <u>et al</u>., 1970) the O_2 flash yields of the first two flashes are close to zero; maximum O_2 is produced after the third flash; and thereafter the yield varies as a function of flash number showing a damped oscillation of period four, with maxima after the 3rd, 7th and 11th flashes. The oscillations eventually damp out after the fourth or fifth cycle. To account for this unique pattern several models were proposed (Joliot, 1968; Joliot <u>et al</u>., 1969; Kok <u>et al</u>., 1970; Forbush <u>et al</u>., 1971; Mar and Govindjee, 1972). However, the model proposed by Kok <u>et al</u>. (1970) (also see Forbush <u>et al</u>., 1971) accounts well for all the available data.

According to Kok's model the O₂ center (denoted S) can exist in four photoactive states and at least five non-photoactive states. Different steps in the oxidation of S states can be represented as follows:

$$S_{0} \xrightarrow{h\nu} S_{0} \longrightarrow S_{1} \xrightarrow{h\nu} S_{1} \longrightarrow S_{2} \xrightarrow{h\nu} S_{2} \longrightarrow S_{3} \xrightarrow{h\nu} S_{3} \longrightarrow S_{4} (1.3)$$

$$0_{2} + 4H^{+} 2H_{2}0$$

The S states (S_n) differ at least by the number of oxidizing equivalents as represented by the respective subscripts. Each flash induces a single turnover of the system producing one oxidizing equivalent. S_n^{-1} refers to a light activated state which relaxes to the succeeding S_{n+1} state in the dark. Upon the accumulation of four positive charges (in S_4), two water molecules are rapidly decomposed to yield one 0_2 molecule plus four protons, and the S intermediate returns to the S_0 state

(for more recent analysis of the proton yield flash pattern see section 1.4.6). The dark reactions $S_n \rightarrow S_{n+1}$ represent reoxidation of Q⁻ to Q. Some basic features of the model are: (a) all the four photoacts involve one step; (b) the reaction chains with an associated 0_{2} evolving system operate independently of each other; (c) the S_1 state is stable in the dark; (d) the states S_2 and S_3 are unstable and decay to S_1 in darkness, with the lifetime of S_2 and S_3 longer (~15 min) than the dark steps represented by S_n^{-} to S_{n+1}^{-} reaction (~500 -600 μ s); (e) after each flash there is a finite relaxation time before the S states can be activated by another flash ($S_n \rightarrow S_{n+1}$); (f) during each flash a fraction of the centers (α) do not undergo the transition $S_n \ge S_{n+1}$ (called "misses"); (g) if the flashes are of long duration, a small fraction of the centers (B) can undergo two transitions $(S_n \Rightarrow S_{n+2})$ during each flash (called "double hits"); and (h) the $S_4 \Rightarrow S_0$ transition is a dark reaction occuring in ≤ 1 ms, and during this transition 0_2 is released. A detailed description of these features of the model is presented in the following sections (see reviews by Joliot and Kok, 1975; Radmer and Cheniae, 1977; Harriman and Barber, 1978 for further details).

1.4.2 Stability of the S States

The hypothesis that the S_1 state is stable in the dark was introduced (Kok <u>et al.</u>, 1970) to explain the maximal yield of O_2 after the third flash. Since the S_2 and S_3 states are rather short lived, the distribution of S states after a sufficient dark period is: $1S_0:3S_1:$ $0S_2:0S_3$. During the steady state all four S states are equally distributed. Doschek and Kok (1972) gave an alternate explanation for the peak in the 0_2 yield on the third flash. They proposed that all centers start out in S_0 and go through a double transition (advance two steps, $S_0 \Rightarrow S_1 \Rightarrow S_2$) after the first flash (also see Thibault, 1978).

The best evidence for the stability of S_1 is that the S_1/S_0 ratio can be varied by appropriate preillumination (Forbush et al., 1971). The ratio of the S_1/S_0 was determined by measuring the ratio of oxygen yields on the third and fourth flashes (Y_3, Y_4) . If one preflash was given and a long enough dark period was allowed for S_2 and S_3 to deactivate, then most centers were found to be in the S_1 state. Similarly, after three preflashes and dark adaptation, most centers were in the S₀ state. Bouges-Boucquet (1973a) showed that the ratio S_1/S_0 can also be changed chemically by redox reagents $([Fe(CN)_6]^{3-}, DCPIP_{-})$ ascorbate). Addition of 0.1 M $[Fe(CN)_6]^{3-}$ increases the S_1/S_0 ratio indicating a chemical oxidation of $S_0 \Rightarrow S_1$. Likewise, DCPIPascorbate decreases the S_1/S_0 ratio suggesting a chemical reduction of S_1 to S_0 . However, other data suggest that these effects might be more indirect (Kok et al., 1974; Velthuys and Visser, 1975). To account for the high 0_2 yield after the 4th flash in the presence of DCPIPascorbate, Velthuys and Visser proposed a high miss parameter in the first flash.

1.4.3 Misses and Double Hits

In order to account for the damping of the 0_2 oscillations, Forbush <u>et al.</u> (1971) proposed that some reaction centers fail to undergo phototransition in the flash (misses) while some other reaction centers undergo two photoacts and accumulate two positive charges in a single flash (double hits). Misses can result either from the failure of charge separation due to Q being in the reduced state or from annihilation of the charge separation by back reaction. The miss factor α remains constant when the flash is supersaturating. Misses are usually assumed to be uniformally distributed among the four photoacts with an average value of ~10% for isolated chloroplasts (Forbush <u>et al</u>., 1971). According to Velthuys and Visser (1975) misses occur more during the first flash. Delrieu (1974), however, calculated a higher value of misses for the centers in the S₂ and S₃ states in the first cycle.

Kok et al. (1970) assumed that misses are of photochemical origin, therefore implying that if the percent misses increase then the quantum yield at steady state would decrease accordingly. Lavorel (1976) pointed out an apparent contradiction: in <u>Chorella</u> the O2 oscillation damps out faster (high misses) than in isolated chloroplasts, although the quantum yield for oxygen evolution is higher in the former. To explain the positive correlation between the steady state oxygen evolution and the miss factor, Lavorel (1976) proposed a model in which the oxygen evolving enzyme (E) is in free or bound form with the charge separating system (P); the latter is integrated in the thylakoid membrane. E can exist in four possible states (E_0 , E_1 , E_2 , E_3) and can only advance to the next state when bound to P. Thus, when [E] > [P], as might be the case in <u>Chlorella</u>, the yield for 0_2 evolution is high (all photocenters are active in 0_2 evolution) although the number of misses (many of the enzyme units are not activated) appears large. In chloroplasts, on the contrary [E] < [P], the percent misses are low (all enzyme units are activated) but 0_2 yield is low (not all photocenters are active in 0_2

evolution). Since E is mobile with respect to P, the probability of double hits increases as an enzyme can be activated by more than one photocenter during the flash. The extent of double hitting remains small because the lifetime of the active photocenter is short.

Double hits (β) are predicted when flash is of longer duration than the half times of relaxation of the reaction $S_n \Rightarrow S_{n+1}$. Accordingly, the probability of double hits decreases if sufficiently short flashes are used (Weiss and Sauer, 1970; Joliot <u>et al.</u>, 1971). In short flashes, no 0_2 is observed on the second flash indicating that all S_2 states disappear in the dark as predicted from deactivation studies. 1.4.4 Relaxation Step

After light activation each S state recovers by dark relaxation before it can be activated by another quantum. This relaxation is limited by the oxidation of Q⁻ to Q. The half times for the transition $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ were measured to be ~200 µs and ~400 µs at room temperature (Forbush <u>et al</u>., 1971). Later studies yielded somewhat different results for the $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions (Bouges-Bocquet, 1973a). The reason for this discrepancy is not known. The $S_3 \rightarrow S_0$ transition showed a much slower halftime of ~1.2 ms. This transition involves the S_4 state which immediately reacts with water (see equation 1.3).

1.4.5 Deactivation

Since the first burst of 0_2 is seen on the third flash, it is assumed that S_2 and S_3 states deactivate in dark. The process of deactivation involves loss of oxidizing equivalents by the 0_2 system. The kinetics and halftimes of the decay of the S_2 and S_3 states vary

widely depending on the material used. Deactivation is slower in whole cells of algae than in isolated chloroplasts without added acceptor. The variation in the deactivation of the S_3 is probably dependent on the reducing state of the cells (Radmer and Kok, 1973); the S_3 state has a halftime of 5 seconds in Chlorella (Joliot et al., 1971), ~60 s in chloroplasts with added electron acceptor (to keep the intersystem pool oxidized (Forbush et al., 1971; Joliot et al., 1971)), and 0.6 seconds in chloroplasts in the absence of added acceptor (to keep the intersystem pool reduced) (Radmer and Kok, 1973). In chloroplasts the rate of deactivation of S_2 and S_3 are approximately the same, whereas in algae the deactivation of ${\rm S}^{}_3$ is about five times faster than the deactivation of S₂ (Forbush <u>et al.</u>, 1971; Joliot <u>et al.</u>, 1971). Joliot <u>et al</u>. (1971) showed that in algae and chloroplasts deactivation of S_2 follows first order kinetics, whereas ${\rm S}_3$ deactivation does not. On the contrary, Forbush et al. (1971) showed that in chloroplasts all of the decay curves show second order kinetics as if the reductant and the S states are present in approximately equal concentrations.

Forbush <u>et al</u>. (1971) also observed a transient increase in S_2 state in the dark, as though it were simultaneously being produced (by the deactivation of S_3) and consumed. Thus, it was suggested that the deactivation process is a one step mechanism, i.e., $S_3 \Rightarrow S_2 \Rightarrow S_1$. Joliot <u>et al</u>. (1971) have shown that a two step mechanism is also involved, i.e., $S_3 \Rightarrow S_1$ and $S_2 \Rightarrow S_0$.

The deactivation of S₃ is faster after preillumination with PS II light than with PS I light (Lemasson and Barbieri, 1971), suggesting that deactivation proceeds via the back reaction which depends on the extent of reduction of the Q and PQ pool. However, there is no effect of the redox state of the PQ pool on the deactivation of S_2 . Alternatively, deactivation could be due to a direct reduction of the oxidizing equivalents by some electron donor other than water.

Renger (1972a,b,c) reported that several reagents (called ADRY reagents for the acceleration of the deactivation reactions of the water splitting enzyme system, \underline{Y}) markedly increase the deactivation rate. These reagents such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) and anilinothiophenes increase the rate of deactivation by ~100 fold. The mode of action of these agents could be by inducing cyclic electron flow, involving the S intermediate and the reduced acceptors.

1.4.6 Proton Release

According to Kok's model presented above, the conversion of S_4 to S_0 yields one 0_2 molecule and four protons. Using a sensitive pH electrode, Fowler and Kok (1974) observed that protons are released in synchrony with 0_2 . However, they pointed out that there are significant differences, especially on the second flash when the release of protons is much greater than that of 0_2 . This suggests the possibility of some proton release on the $S_2 > S_3$ transition. The pattern of proton release related to 0_2 evolution is also complicated by the proton release and uptake by the plastoquinone pool. More recent data of the proton yield flash pattern by Fowler (1977) and Saphon and Crofts (1977) using dark adapted chloroplasts showed that protons are released approximately as 1, 0, 1, 2 in $S_0 > S_1$, $S_1 > S_2$, $S_2 > S_3$ and $S_3 > S_0$ steps. On the other hand, Junge <u>et al</u>. (1977) obtained proton release as 0, 1, 1, 2 for the above steps; these results were obtained under different experimental conditions (using an ADRY reagent and repetitive flashes). There is now independent evidence in support of the model of Saphon and Crofts (1977) and Fowler (1977). Bowes and Crofts (1978) measured the enhancement of delayed light fluorescence due to the release of protons accompanying transitions of the S-states and concluded that protons are released in all the transitions of the S-states with the exception of $S_1 \Rightarrow S_2$.

1.5 Involvement of Manganese in Oxygen Evolution

In section 1.4 we concluded that 0_2 evolution occurs via a concerted four-electron mechanism in which four oxidizing equivalents are sequentially stored on a "charge accumulator species". Although the chemical nature of the charge accumulating species has not been established, there is indirect evidence that it may involve manganese. The involvement of manganese in photosynthesis was first suggested by Pirson (1937; also see Pirson et al., 1952). He observed that manganese deficiency decreased photosynthetic activity in Ankistrodesmus cells without affecting other physiological activities such as respiration and total chlorophyll content. This phenomenon was later found to be universal in all 0, evolving organisms. The requirement of manganese in photosynthesis was confirmed by Kessler et al. (1957) and shown to be specific for PS II reactions. Moreover, the photoreduction of $NADP^+$ in the presence of DCPIP-ascorbate (a PS I reaction) was not affected in Mn deficient Scenedesmus (Hoch and Martin, 1963) indicating that the requirement of manganese is specific for the 0_2 evolving PS II reactions. Cheniae and Martin (1966) demonstrated that mild temperature shock released Mn

with a concomitant loss of 0_2 evolving activity without affecting PS I reactions. Thus, the role of Mn in photosynthesis was shown to be in the 0_2 evolving system II.

Manganese can be incorporated into Mn-depleted cells so as to restore O_2 evolution. The amount of manganese required for maximum activity is one atom of bound Mn per 50-100 chlorophyll molecules (Cheniae and Martin, 1970).

1.5.1 Different Pools of Manganese

The role of manganese in 0_2 evolving system is further supported by experiments in which chloroplast Mn is released from its binding site by various treatments, such as washing with alkaline Tris, extraction with hydroxylamine (NH₂OH) and gentle heating. Upon progressive Mn depletion the relative 0_2 yield declines almost linearly in relation to the abundance of Mn in the chloroplasts (Cheniae and Martin, 1968, 1970). These treated chloroplasts, however, still show electron flow through PS II in the presence of artificial electron donors. Thus, it seems that the extractable Mn which is involved in the oxygen evolving process is not required for the photooxidation of primary electron donor P680 and for the oxidation of intermediates to which artificial donors donate electrons.

The availability of specific extraction methods for Mn allows us to study the relationship between the amount of bound Mn and 0_2 evolution. In chloroplasts extracted with alkaline Tris a 90% loss of 0_2 evolution is correlated with a loss of about 2/3rd of the functional Mn pool. This 2/3rd Mn pool (loosely bound) is readily extractable but the other 1/3rd of the pool (tightly bound) requires exhaustive treatments

for removal (Cheniae and Martin, 1970). Similar results were obtained when NH₂OH is used to release the bound Mn (Cheniae and Martin, 1970). In all these experiments a linear relationship exists between the loosely bound Mn and O₂ evolving activity. The binding constants for the two fractions of manganese have been estimated to be K_(loosely bound) = $1.2 \times 10^4 \text{ M}^{-1}$ and K_(tightly bound) = $1.9 \times 10^5 \text{ M}^{-1}$ (Takahashi and Asada, 1976).

Blankenship and Sauer (1974) confirmed the two pool hypothesis by measurements of ESR detectable aqueous Mn(II). When manganese is in the bound state it does not show a definite ESR signal. Release of the bound manganese from chloroplasts results in the appearance of a six line signal characteristic of $Mn(II) \cdot 6H_{2}O$ (Lozier <u>et al.</u>, 1971). Blankenship and Sauer (1974) showed that alkaline Tris washing resulted in the appearance of the ESR signal from the aqueous Mn(II). They found that 60% of the chloroplast Mn pool was indeed correlated with the loss of 0_2 evolution. Mn(II) was released into the inside space of the thylakoid vesicle and was not lost from the thyalkoids to the outside suggesting that the Mn associated with 0_2 evolution is located on the inner side of the thylakoid vesicles. Diffusion of the released Mn(II) from the intrathylakoid space was slow with a $t_{1/2} = 2.5$ h. Permeability of the thylakoid membranes can vary considerably depending on the method of preparation and extraction. Recently, however, Siderer et al. (1977) have reported an ESR signal suggested to be due to bound Mn(II) which shows light induced changes attributed to PS II activity.

Bound manganese is not released from chloroplasts with chelating agents such as ethylenediamine tetraacetic acid (EDTA) (Possingham and

Spencer, 1962; Cheniae and Martin, 1966; Homann, 1967) even though this reagent penetrates the membrane. Such results imply that Mn binding sites have a higher affinity for Mn than EDTA. However, upon prolonged storage at 4°C or under conditions common for protein denaturation, the chelating agents become more effective in removing manganese. Cheniae and Martin (1966) showed that this manganese does not exchange readily with externally added Mn(II) although it is slowly released from its binding site by high concentrations of Mg²⁺ (Chen and Wang, 1974).

More evidence for the concept of two Mn pools of the oxygen evolving system came from the studies of development of O_2 evolution capability in algae (Cheniae and Martin, 1973; Tel-Or and Stewart, 1975) and higher plants (Phung-Nhu-Hung <u>et al.</u>, 1974). Under developing conditions when there is no O_2 evolution, only the small tightly bound pool of Mn is present. The development of O_2 evolution activity is correlated with the binding of the larger Mn pool.

The release of the large loosely bound Mn pool does not affect the primary charge separation of the PS II trap $(P680^+Q^-)$ or electron flow between the two photosystems. This implies that the functional site of the largely loosely bound Mn pool is within S states (the intermediate "M", Fig. 1.1) or between the S states and P680.

The function of the smaller tightly bound pool of Mn 1s unknown. The capacity for photooxidation of certain artificial electron donors 1s correlated with the smaller tightly bound pool (Chennae and Martin, 1970) suggesting that it may be associated with the oxidant side of PS II, perhaps Z (Z_1 , Z_2). However, exhaustive treatments of chloroplasts with NH₂OH, 35-50° shock, or pH < 5 results in the release of even the
smaller tightly bound Mn without affecting the photooxidation of NH_2OH (Cheniae and Martin, 1971) and DPC (Kimimura and Katoh, 1972) by PS II. This casts doubts about the function of the tightly bound Mn on reactions linking P680 to the O_2 evolving system. One possible role of this pool of Mn has been suggested to be in the stacking of the lamellae (Possingham <u>et al.</u>, 1964). Alternatively, this pool may be associated with superoxide dismutase (Lumsden and Hall, 1975).

1.5.2 ESR Signals

Using fast time response ESR instrumentation, a flash-induced transient radical with a rise time of ~100 μ s has been observed (Chen and Wang, 1974; Babcock and Sauer, 1975a,b; Blankenship <u>et al.</u>, 1975a; Warden <u>et al.</u>, 1976). In normal chloroplasts this signal (Signal II_{vf}) decays very rapidly with a half time of 400-900 μ s, while in Tris washed or heat treated chloroplasts the decay is ~1000 times slower (Signal II_f).

Signal II_{vf} occurs in an abundance equal to P700 (Warden <u>et al.</u>, 1976). It is most effectively sensitized by red light and is not observed in 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) poisoned chloroplasts (Blankenship <u>et al.</u>, 1975a). The decay of Signal II_{vf} varies with the flash number in the same way as the relaxation times of the S states (Babcock <u>et al.</u>, 1976). The ESR spectra of the two rapidly forming signals (Signal II_f and Signal II_{vf}) are identical suggesting that both species arise from the same endogenous molecule presumably Z_2^+ , oxidized form of an electron donor of PS II.

Any treatment that inactivates O_2 evolution and releases bound manganese results in the conversion of Signal II_{vf} to Signal II_f. The

rise time of Signal II_f appears to be equal to Signal II_{vf} (Blankenship <u>et al.</u>, 1975a), suggesting that the rate constant for electron flow from Z_2 to P680⁺ is not affected by the loss of O_2 evolution activity. Accordingly, the difference (~1000 fold) between the decay of Signal II_{vf} (t_{1/2} ~400-900 µs) and Signal II_f (t_{1/2} ~ 1 s) probably reflects the decreased rate of electron flow from water to Z_2^+ due to the release of Mn.

As described in section 1.5.1, Tris washing of chloroplasts results in the appearance of ESR signals from the free Mn(II); the release of Mn is correlated with the loss of 0_2 evolution. These chloroplasts which have lost their ability to evolve oxygen can be reactivated by the addition of lipophilic reducing agents, e.g., DCPIPH₂ and hydroquinone (Yamashita <u>et al.</u>, 1971, 1972; Yamashita and Tomita, 1974). Upon reactivation, the ESR signal from free Mn(II) disappears, apparently as a result of rebinding of the free Mn to the chloroplast membrane (Blankenship <u>et al.</u>, 1975b) and Signal II_f is converted to Signal II_{vf} (Blankenship <u>et al.</u>, 1975a). These experiments further support the involvement of the Mn pool in the 0_2 evolving system and exclude a requirement of this bound Mn pool for electron flow from Z₂ to P680⁺. 1.5.3 Reactivation of the Oxygen Evolving System

In an earlier section evidence was presented showing that manganese deficiency of green plants preferentially affects the oxygen evolving activity of the photosynthetic apparatus. Studies on the effect of rein-corporation of Mn into chloroplast membranes has provided further evidence for the requirement of Mn in 0_2 evolution. Activation of 0_2 evolution activity in Mn deficient tissues is light dependent (Homann,

1967; Cheniae and Martin, 1971). The photoactivation of O_2 evolution by the reincorporation of Mn has been shown in algae grown in Mn-deficient medium (Gerhardt, 1966; Cheniae and Martin, 1971), in dark grown green and blue green algae (Cheniae and Martin, 1973), and in algae extracted with hydroxylamine (Cheniae and Martin, 1972). This process has also been demonstrated in higher plants grown in Mn-free medium (Anderson and Pyliotis, 1969), devoid of Mn by aging (Margulies, 1972) and grown in the dark (Inoue <u>et al.</u>, 1975).

Upon the addition of Mn to Mn-deficient cells, the following reactions are observed: (a) accumulation of Mn by the cells is enhanced by light, this enhancement being presumably linked to photophosphorylation as indicated by the inhibitory effect of uncoupling agents; and (b) an apparent photoinhibition of photoactivation by moderate and high light intensities.

After prolonged incubation (two hours or more) in darkness with $MnCl_2$, the cells accumulate sufficient Mn in the bound state necessary for subsequent photoreactivation of the O_2 evolving centers (Homann, 1967; Cheniae and Martin, 1969, 1973). Following this dark incubation, the photoreactivation process can be studied without interference from the secondary light effect upon Mn influx. Photoreactivation is inhibited by DCMU, an inhibitor of electron flow from PS II to PS I, suggesting that the activation of the Mn(II) complex to form an active O_2 evolving center requires electron flow through PS II. Photophosphorylation is not involved in photoreactivation since the uncoupling agent CCCP has no effect on it.

Data obtained in continuous and flashing light show that activation is a multi (minimally, two) quantum process. The product of the first photoact is a photosensitive state of limited stability which is converted by light into a stable active 0_2 -evolving center. Since photoactivation is inhibited by reductants such as hydroquinone, it has been suggested that the photoactivation process first involves photooxidation of Mn(II) to a higher oxidation state before the binding of Mn to the 0_2 -evolving center occurs. The bound Mn must then be activated by another quantum absorbed in PS II before 0_2 evolution can take place.

In chloroplasts where 0_2 evolution has been inactivated by Tris washing, reactivation can be achieved by treatment with lipophilic reducing agents (such as DCPIPH₂ and hydroquinone) (Yamashita <u>et al</u>., 1971) or by ADRY reagents (Cheniae and Martin, 1978). This reactivation is only achieved for Tris-treated chloroplasts, and is not observed with chloroplasts inactivated by temperature shock, NH₂OH treatment or with cells grown in Mn-free medium. In order to understand the mechanism of the Tris induced inactivation, Cheniae and Martin (1978) studied the kinetics and quantum requirements for the Tris induced inactivation of 0_2 evolution. Effects of DCMU, light flashes, and chemicals known to destabilize the S₂ and S₃ states were also studied. Cheniae and Martin (1978) showed that inactivation is accelerated by weak light absorbed by PS II. They also found that chemicals which destabilize the higher oxidation states (S₂ and S₃) inhibit inactivation and promote reactivation of 0_2 evolution.

1.5.4 Chloroplast Manganese Proteins

The chemical nature of the intermediate involved in O_2 evolution is unknown. Experiments with antibodies (Zilinskas-Braun and Govindjee, 1974), chemical modifiers of protein (Giaquinta <u>et al.</u>, 1974), UV light (Jones and Kok, 1966) and heat treatment (Katoh and San Pietro, 1967) seem to indicate that proteins are involved in oxygen evolution. It has been suggested that Mn in chloroplasts is bound to a protein, perhaps the oxygen evolving enzyme.

Extraction of chloroplasts with organic solvents results in the separation of Mn with the protein phase (Park and Pon, 1963; Cheniae and Martin, 1966; Lagoutte and Duraton, 1975; Henriques and Park, 1976) indicating no association of Mn with chlorophyll. This casts considerable doubt on early speculation suggesting a complex of Mn with a special chlorophyll molecule. Cheniae and Martin (1966) isolated a manganese containing protein but the O_2 evolving system could not be reconstituted by readdition of this protein. Isolation of a protein called the "Oxygen Evolving Factor" (OEF) was reported by Huzisige <u>et al</u>. (1968) and it was suggested to be involved in O_2 evolution. In subsequent experiments, Cheniae and Martin (1970) showed that OEF did not increase rates of O_2 evolution of Mn extracted chloroplasts.

A Mn containing, low molecular weight (1000 daltons) polypeptide has been isolated from the blue green alga <u>Phormidium luridum</u> (Tel Or and Avron, 1974). In contrast to the general belief that the "Oxygen Evolving Enzyme" is unstable to heat, this polypeptide (called the "Hill Factor") is stable even after boiling. When purified "Hill Factor" was added to washed spheroplasts of <u>Phormidium</u> there was a many fold stimulation in $[Fe(CN)_6]^{3-}$ mediated 0_2 evolution. The site of action of this factor was suggested to be close to the 0_2 evolving system. However, it was shown later that the stimulatory effect on 0_2 evolution in washed spheroplasts could be seen simply by adding salts (Binder <u>et al</u>., 1976), thus casting doubts on the role of "Hill Factor" in 0_2 evolution.

Recently, Winget and Spector (1979) have isolated a Mn-containing protein (65,000 molecular weight) from chloroplasts by 2% cholate treatment followed by ammonium sulfate fractionation. When this protein is incorporated into liposomes containing cholate extracted chloroplasts, lacking 0_2 evolution activity, there is a large stimulation of $[Fe(CN)_6]^{3-}$ mediated 0_2 evolution. This protein is not required in the photooxidation of artificial electron donors by PS II, thus suggesting that the site of action of the protein is in close proximity to the 0_2 yielding reactions.

1.5.5 Measurement of Water Proton Relaxation Related to Oxygen Evolution

Nuclear magnetic relaxation (NMR) has been used quite successfully in the study of paramagnetic ions, such as manganese, bound to biological molecules (Mildvan and Cohn, 1970; Dwek, 1973). Using the pulsed NMR technique, Wydrzynski <u>et al</u>. (1975, 1976, 1978) found a close correlation between changes in the relaxation rates of water protons in thylakoid membranes and the known behavior of membrane-bound manganese.

The applicaton of pulsed NMR to studies of bound paramagnetic ions has been reviewed by Mildvan and Cohn (1970). Manganese ion maintains some water ligands even when it becomes bound to macromolecules. Protons of water molecules coordinated to manganese experience a large magnetic dipole of the paramagnetic ion's electron spin such that the relaxation rates of the protons in the solvation sphere are strongly enhanced. Furthermore, all of the water protons experience the enhancement because of the fast proton exchange between the solvation sphere and the solvent.

Wydrzynski et al. (1975, 1978) showed that treatments which affect the amount of bound manganese in chloroplasts have a large effect on the spin-lattice or longitudinal relaxation rate (T_1^{-1}) of water protons. Conditions which are known to remove most of the bound manganese from thylakoid membranes (NH₂OH-EDTA treatment and Tris acetone washing) decrease T_1^{-1} of thylakoids. They also observed that the addition of oxidants caused a decrease in the spin-spin or transverse relaxation rate (T_2^{-1}) and the addition of reductants led to an increase, suggesting that the proton relaxation rates could be used to detect changes in the oxidation state of the manganese. Gribova et al. (1978) confirmed these conclusions from their measurements of water proton relaxation rates in chloroplasts and PS II particles. Furthermore, Wydrzynski et al. (1978) concluded from the frequency and temperature dependence that the proton relaxation rates are characteristic of Mn(II). The frequency dependence of T_1^{-1} and T_2^{-1} for chloroplasts showed that T_1^{-1} has a broad peak in the 10-25 MHz region whereas T_2^{-1} increases with increasing frequency. These results suggest that the electronic spin relaxation rates dominate the measured relaxation rates. The electronic spin relaxation lifetime (τ_{s}) for Mn(II) -- calculated to be $\sim 10^{-9}$ s -- is two orders of magnitude larger than that for Mn(III) and high spin Fe(II) and Fe(III) (Dwek, 1973). On the other hand the electronic relaxation time of Cu(II) is comparable to

Mn(II), but in chloroplasts at least half of the copper is in plastocyanin which has no effect on the relaxation rates because the bound copper is inaccessible to the solvent water (Blumberg and Peisach, 1966). However, the remaining copper in chloroplasts 1s mostly associated with polyphenoloxidase, an enzyme tightly bound to the chloroplast lamellar structure (Tolbert, 1973). The contribution of copper associated with polyphenoloxidase could perhaps account for the background in the proton relaxation rate (PRR) signal when all manganese has been removed.

1.6 Purpose and the Scope of the Present Thesis

The purpose of the work reported in this thesis was two fold: (a) to study the role of bicarbonate and (b) to study the involvement of Mn in PS II reactions. To establish the significance of the bicarbonate requirement for photosynthesis, we undertook to precisely locate the site of action of bicarbonate and discover as much as possible the mechanism by which bicarbonate stimulates the Hill reaction. The bicarbonate reaction was studied on the partial segments of the electron transport chain. It is shown here that the major effect of bicarbonate is on the electron flow from Q to PQ, more specifically between R and PQ. Experiments were also done to understand the mechanism of bicarbonate action. It appears that bicarbonate functions as an allosteric regulator by binding to the protein component covering the Q-R-PQ region, such that a functional connection between Q, R and PQ pool is made to allow electron and proton flow. All experiments on the role of bicarbonate are described in Chapter 3 of this thesis. A portion of the data presented here has been published by the author (Khanna et al., 1976, 1977; Siggel et al., 1977; Govindjee and Khanna, 1978).

Manganese is known to be required for 0_2 evolution and is believed to be part of the 0_2 evolving system (see section 1.5). Recently it has been shown that the relaxation rates of water protons can be used to monitor native bound manganese in chloroplast membranes (see section 1.5.5). The objective of our work was to extend these NMR studies to determine the role of Mn in 0_2 evolution. These experiments are presented in Chapter 4 of this thesis. We have established a good correlation, under several conditions, between water proton relaxation rates and the content of bound Mn(II); contribution from other paramagnetic species appears minimal in normal thylakoids. Treatment of thylakoids with tetraphenylboron (TPB), NH₂OH and H_2O_2 appears to convert higher oxidation states of Mn to the more efficient relaxer species Mn(II) thus causing an enhancement in water proton relaxation rates (Khanna et al., 1979). However, release of bound Mn(II) by the addition of MgCl₂ or incubation with NH_2OH causes a decrease in T_2^{-1} ; this release of Mn is independently detected by ESR measurements. Detailed correlative experiments, using NMR, ESR and 0_2 evolution activity measurements, show that there are at least three pools of Mn in chloroplasts. One pool (to be referred to as very loosely bound) is non-functional in electron transport, whereas the other two (loosely bound and tightly bound) appear to be involved in the photosynthetic reactions. Previously, it has been shown that the loosely bound pool of ~4 Mn/PS II trap is associated with 0_2 evolution but no definite role has been assigned to the smaller tightly bound pool of Mn (2 Mn/PS II trap). We have observed that the purified light harvesting complex (LHC) contains tightly bound manganese that accounts for about one third of the functional Mn, suggesting a structural role for this pool of manganese.

Chapter 2 describes the Materials and Methods used and Chapter 5 provides a general summary of the major conclusions of the research described in this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation of Thylakoid Membranes

Dwarf pea (Pisum sativum var. Progress No. 9) seedlings were grown under 16 h light/8 h dark cycle in vermiculite. Broken chloroplasts (hereafter called thylakoid membranes) were isolated from leaves of 12-18 day old pea plants or fresh market spinach (Spinacia oleracea). Thylakoid membranes were prepared by homogenizing leaves in a Waring blender for approximately 10 s in a buffer. Thylakold membranes used for experiments described in Chapter 3 were homogenized in a medium containing 0.4 M sucrose, 0.01 M NaCl and 0.05 M phosphate buffer (pH 6.8). Unless otherwise stated, the homogenizing medium for thylakoids used for experiments described in Chapter 4 contained 0.4 M sucrose, 0.01 M NaCl and 0.05 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (pH 7.5). The slurry was filtered through four and then 12 layers of cheesecloth and the filtrate centrifuged at 6,000xg for 10 min. The thylakoid pellet was osmotically shocked by resuspending in the isolation medium without sucrose. The thylakoid suspension was centrifuged at 6,000xg for 10 min and the pellet obtained was resuspended in the original isolation buffer or in the buffers described in the legends of figures. Chlorophyll concentration was determined according to the method of Arnon (1949).

2.2 Bicarbonate Depletion of Thylakoid Membranes

Thylakoids were depleted of bicarbonate by a modified method of Stemler and Govindjee (1973). Thylakoid membranes were suspended in a medium containing 50 mM phosphate buffer (pH 5.0), 100 mM sodium formate

and 100 mM NaCl at room temperature. The depletion medium had been previously made free of CO_2 by bubbling with N_2 gas. The chlorophyll concentration was 50 µg/ml. N_2 gas was passed over the suspension while it was shaken gently for about 15 min at room temperature. Then, the suspension was transferred with the help of a syringe into cold screw-capped tubes previously flushed with N_2 gas. After centrifugation, these tubes were stored on ice until use. The supernatant was discarded just before the assay and the thylakoid membranes were resuspended in a buffer containing 50 mM phosphate (pH 6.8), 100 mM sodium formate and 100 mM NaCl. For developing maximum bicarbonate depletion, high anion concentrations (chloride and formate) and low pH conditions are essential.

2.3 Trypsin Treatment

Trypsin treatment of thylakoid membranes, used for experiments described in Chapter 3, was done according to the method of Renger (1976). Thylakoid membranes were depleted of bicarbonate and suspended (40 μ g chlorophyll/ml) in a medium containing 50 mM phosphate (pH 6.8), 100 mM NaCl and 100 mM sodium formate. Trypsin from bovine pancreas, Type XI (8575 units/mg protein) was added to a final concentration of 40 μ g/ml and incubated at room temperature (23°C) for different time periods (1-5 min).

Trypsin treated membranes, used for experiments described in Chapter 4, were obtained according to the method of Steinback <u>et al</u>. (1979). Thylakoid membranes were isolated in Tricine buffer (pH 7.8) and washed in unbuffered 10 mM NaCl. The washed thylakoids were suspended (100 μ g chlorophyll/ml) in 20 mM Tricine (pH 7.8), 100 mM sorbitol, 10 mM NaCl

and 5 mM MgCl₂. Trypsin from bovine pancreas, Type III (12,000 units/mg protein) was added to a final concentration of 0.25 μ g/ml and incubated at room temperature (23°C). Trypsin digestion was stopped after 12 min by the addition of a twenty fold excess of trypsin inhibitor (from soybean, Type I-S, Sigma Chemical Co.). Thylakoid membranes were centrifuged at 10,000xg for 10 min, washed in unbuffered 10 mM NaCl and finally resuspended in 10 mM Tricine (pH 7.0), 10 mM NaCl and 100 mM sorbitol.

Membrane polypeptides of trypsin treated membranes were analyzed using SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970).

2.4 Glutaraldehyde Fixation of Thylakoid Membranes

Thylakoid membranes were washed twice in HEPES homogenization buffer without sucrose (see section 2.1) and then resuspended in the same buffer at a chlorophyll concentration of 150 μ g/ml. Purified glutaraldehyde was diluted to a concentration of 10% and added to the washed thylakoids to a final concentration of 1% (Zilinskas and Govindjee, 1976). The suspension was stirred at 0°C in the dark for 5 min and then centrifuged (6,000xg for 15 min) to obtain a pellet. The pellet was washed twice with the buffer mentioned above and finally resuspended in the original homogenizing buffer.

2.5 Isolation and Purification of the Light Harvesting Complex

Purified LHC was prepared according to the method of Burke <u>et al</u>. (1978). Thylakoids were isolated in Tricine buffer and dispersed in 5 mM EDTA (pH 7.8) containing 100 mM sorbitol. The pH of the suspension was adjusted to 6.0, and the sample was centrifuged at 10,000xg for 10 min to

obtain a pellet. The thylakoid membranes were resuspended in 100 mM sorbitol and then recentrifuged at 10,000xg for 10 min. This washed pellet was stirred with 0.5% Triton X-100 in deionized water for 30 min at 25° C (chlorophyll concentration, 0.5 mg/ml). The suspension was centrifuged at 40,000xg for 30 min to remove membrane fragments and the supernatant was loaded on a linear sucrose gradient (0.1 - 1.0 M) and spun for 15 h using a Beckman SW-27 rotor at 100,000xg. The region of sucrose gradient showing high fluorescence (containing the LHC) was removed and the LHC was precipitated from this fraction by adding MgCl₂ (10 mM) and KCl (100 mM). The sample was stirred briefly and then it was centrifuged for 10 min at 9,000xg over a 500 mM sucrose cushion. The resulting pellet yielded purified LHC. Electrophoretic separation of membrane pigmentprotein complexes was achieved by preincubating samples for 1 h in 100 mM Tris buffer (pH 8.2) containing 1% SDS, followed by SDS (0.1%) polyacrylamide gel electrophoresis as described by Laemmli (1970).

2.6 <u>Electron Transport Rates</u>

The steady state 0_2 evolution rate of thylakoid membranes under continuous light was measured with a Pt/Ag-AgCl₂ Clark electrode, using a Yellow Springs oxygen monitor (Model 53) and an Esterline Angus recorder (Model E11015). The sample chamber (2 ml) was temperature regulated by circulating water from a constant temperature bath through the water jacket surrounding the reaction vessel. Saturating continuous illumination (250 mW/cm²) was provided by an incandescent lamp using a Corning CS 3-71 glass filter and an additional two inch water filter. Thylakoids were suppended at a chlorophyll concentration of 30 µg/ml in the appropriate buffer with 0.5 mm [Fe(CN)₆]³⁻ as an electron acceptor. To measure O_2 evolution under flashing light conditions, using the Clark electrode, a General Radio Model 1538-A xenon strobe lamp was used for excitation. Saturating flashes, with a pulse width of 3 μ s and extended tail up to 10 μ s, were given at a rate of one flash per second and data was recorded with a Midwestern Instruments Model 801 oscillograph.

Electron transport rates from H_2O to $[Fe(CN)_6]^{3-}$, silicomolybdate (SiMo) or oxidized diaminodurene (DAD_{0x}) were measured as rates of O_2 evolution. Electron transport from ascorbate-reduced diaminodurene (DAD_{red}) to methylviologen (MV) was measured as a rate of oxygen uptake. For electron flow from H_2O to Q, 25 µM SiMo and 5 µM DCMU were used. This electron flow stops within 1 min after the addition of SiMo; therefore only the initial rates were monitored (for details see Zillinskas and Govindjee, 1975). For electron flow from H_2O to PQ, 0.5 mM DAD and 0.5 µM dibromothymoquinone (DBMIB) were used; DAD was kept oxidized with 0.5 mM $[Fe(CN)_6]^{3-}$ (for details see Ouitrakul and Izawa, 1973). For electron flow involving only PS I, DAD_{red} (0.5 mM DAD plus 2.0 mM ascorbate) was the electron donor, 50 µM MV the electron acceptor and 1 µM DCMU the electron flow inhibitor at Q (for details see Izawa et al., 1973).

PS II mediated DCPIP reduction was measured at 580 nm using an Hitachi Model 100-60 spectrophotometer. The sample was illuminated from the side through a red Corning CS 2-58 filter. A blue Corning CS 4-96 filter was placed in front of the photomultiplier to protect it from scattered actinic light. Electron transport rates were calculated from direct recordings of the absorbance change using the extinction coefficient of DCPIP as described by Armstrong (1964).

2.7 Absorption Changes at 334, 265 and 703 nm

The absorption changes due to the turnover of the electron acceptor of PS II Q (X320) were detected at 334 nm according to the method described by Renger and Wolff (1976) except that excitation flashes were approximately 20 μ s in duration. The maximum absorption change of X320 is located around 325 nm, but the absorption changes were measured at 334 nm because the signal to noise ratio is higher at the latter wavelength as the UV mercury lamp (Hanovia) used has an emission band there. Furthermore, experiments at 325 nm provided the same conclusions.

The absorption changes at 265 nm (due to PQ) and 703 nm (due to reaction center I P700) were measured with a double beam difference spectrophotometer for simultaneous measurements at two wavelengths with repetitive excitation and signal averaging (Fabri-Tek 1072) as described by Döring <u>et al</u>. (1967). Long (83 ms) flashes were provided by the combination of an actinic lamp and a shutter, and short (20 μ s) light flashes by a xenon flash lamp. The cuvette was flat (thickness, l mm) and was traversed by the monitoring light at an angle of 45°. 2.8 <u>Absorption Changes at 524 and 574 nm for Measuring pH Changes</u>

2.8.1 Measurement of Internal pH by Neutral Red

Thylakoid membranes were prepared in phosphate buffer (pH 6.8) as described in Section 2.1 and suspended, at a chlorophyll concentration of 10 µg/ml, in a medium (20 ml) containing 20 mM KCl, 2 mM MgCl₂, 60 µM benzylviologen, 0.3 µM nonactin and 10 µM neutral red. At 524 nm the absorption changes of neutral red are superimposed on the peak of the intrinsic electrochromic carotenoid changes (Junge and Witt, 1968). Therefore, to observe the changes mainly due to neutral red, the decay of

the electrochromic absorption changes was greatly accelerated by the addition of nonactin (Schmidt and Junge, 1975). The absorption changes were measured at 524nm by a rapid kinetic spectrophotometer as described by Ausländer and Junge (1975). Short $(t_{1/2} = 15 \ \mu s)$ saturating flashes of light were used for excitation. Transient absorption changes were recorded and the signal to noise ratio was improved by averaging over 10 transients induced by repetitive flashes (darktime between flashes, 10 s). The intensity of the monitoring light was low, (300 ergs cm⁻² s⁻¹) and the observed absorption changes were independent of it.

The nitrogenous indicator neutral red has access to both the external and internal space of thylakoids. Addition of the macromolecular broad band buffer bovine serum albumin (BSA) buffers away the dye in the external space such that the absorption changes are then entirely due to the pH changes in the internal space. Absorption changes of neutral red thus observed can be abolished by the addition of a penetrating buffer, such as imidazole.

To observe pH changes in the internal space of thylakoids, the absorption changes were measured at 524 nm in the presence of BSA (1.3 mg/ml). A small background signal observed in the presence of BSA plus imidazole (1 mM) was subtracted from the signal observed in the presence of BSA alone. This subtraction eliminated any contribution from redox-reactions of neutral red in the resulting difference signal. However, the redox contributions to the signal at 524 nm were negligible in our experiments.

2.8.2 Measurement of External pH by Bromocresol Purple

For measurement of external pH the suspension medium (20 ml) contained 20 mM KCl, 2 mM MgCl₂, 60 μ M benzylviologen, 30 μ M bromocresol purple (BCP) and 10 μ g chlorophyll/ml. The absorption changes of BCP were measured at 574 nm by a spectrophotometer as described by Ausländer and Junge (1974). Single turnover flashes ($t_{1/2} = 15 \ \mu$ s) were used for excitation and the signal to noise ratio was improved by averaging 10 transients (darktime between flashes, 10 s). The absorption changes were measured with and without phosphate buffer, and the former signal was subtracted from the latter to obtain the response of BCP to pH changes without the contribution of the intrinsic absorption changes.

2.9 Herbicide Binding Studies

For herbicide binding analysis, thylakoid membranes were isolated in phosphate buffer and depleted of bicarbonate as described in Section 2.2 of this chapter. Binding studies were conducted in the assay medium (50 mM phosphate (pH 6.8), 100 mM formate and 100 mM NaCl) according to the procedure described by Pfister and Arntzen (1979). Suspension medium (1 ml) for binding experiments contained 5-20 μ l of ¹⁴C-labelled atrazine (5.37 Cl/mole) and 50 μ g chlorophyll/ml. Atrazine was dissolved in methanol and the final methanol concentration in the incubation medium was less than 2%. After 2 min incubation under room light at 22°C, the samples were centrifuged for 3 min at 12,000xg in an Eppendorf 5415 centrifuge. Aliquots (0.5 ml) of the clear supernatant were removed and added to 9 ml of Phase Combining System - scintillation fluid (Amersham - Buchler). Radioactivity of the samples was measured by a liquid scintillation counter (Searle Analytic Inc., Model Delta 300). The amount of

bound inhibitor was calculated from the difference between the total inhibitor added to the thylakoids and the amount of free inhibitor found in the supernatant after centrifugation. The amount of bound atrazine increased as the concentration of free atrazine was increased. A quantitative analysis of the binding data was done by plotting [Ch1]/[bound inhibitor] versus 1/[free inhibitor] (double reciprocal plot). The intercept on the ordinate is a measure of the maximum number of available binding sites on a chlorophyll basis. To determine the affinity of the inhibitor for its binding site, the binding constant (K) was calculated from the slope of the double reciprocal plot.

2.10 Measurement of Proton Relaxation Rates

In order to appreciate the measurements of the relaxation rates, a brief background of some basic concepts of NMR measurements is presented here (for details see Dwek, 1973; Farrar and Becker, 1971). When a sample is placed in a strong magnetic field $\overline{H}_{_{O}}$ parallel to the z axis (Fig. 2.1 (a)), the individual nuclear dipoles tend to align with it and precess about the z axis at the Larmor frequency. In a rotating frame of reference (x', y', z', Fig. 2.1 (b)) the individual dipoles appear stationary to an observer rotating along the frame in the x'y'plane. The vector sum of the nuclear dipoles is the net magnetization \overline{M} . Since more dipoles are aligned with \overline{H}_{0} rather than against it, \overline{M} lies parallel with the +z direction (Fig. 2.1 (c)). If a rf pulse, \overline{H}_1 , is applied orthogonally to \overline{H}_0 in the x⁻ direction (Fig. 2.1 (d)), \overline{M} will precess about \overline{H}_1 . If \overline{H}_1 is applied for a long enough time \overline{M} will tip to the x'y'plane (this is called a 90° pulse). After \overline{H}_1 is turned off, \overline{M} tends to return to its original equilibrium position. The



Figure 2.1 Definition of T_1^{-1} and T_2^{-1} relaxation. (a) Precession of magnetic moments of nuclei (I = 1/2) about a fixed magnetic field \overline{H}_0 . The net magnetization is oriented along \overline{H}_0 (the z axis) and has the equilibrium value \overline{M} . (b) In a rotating frame of reference (x', y', z') the individual dipoles appear stationary. (c) Net magnetization (\overline{M}) of the nuclear spins. (d) A 90° pulse (\overline{H}_1) along x' rotates \overline{M} from the equilibrium position to the y' axis. (e) After rf is turned off, \overline{M} relaxes back to equilibrium condition. The growth of \overline{M} along +z' axis is called longitudinal relaxation (T_1^{-1}). (d) Dephasing of nuclear dipoles by spin-spin relaxation (T_2^{-1}) in the x'y' plane (see Farrar and Becker, 1971). rate of growth of \overline{M} along the z axis is defined as T_1^{-1} while the decay of \overline{M} along the y axis is defined as T_2^{-1} (Fig. 2.1 (e)). The decay of \overline{M} along the y axis arises from a dephasing of the individual dipoles in the x'y plane (Fig. 2.1 (f)) and contains contributions from both field inhomogeneities as well as the real T_2^{-1} .

To measure the true T_2^{-1} the CPMG (Carr, Purcell, Meiboom and Gill) pulse sequence is employed (see Carr and Purcell, 1954; Meiboom and Gill, 1958). This method can be described as 90°, τ , 180°, 2 τ , 180°, 2 τ ... pulse sequence. The 90° pulse tips \overline{M} to the +y' direction (Fig. 2.1). \overline{M} starts to dephase because of T_2^{-1} processes and field inhomogeneities. The succeeding 180° pulse inverts the direction of the dipoles to the -y' axis. The dipoles which dephase due to field inhomogeneities tend to refocus back along the y' axis, while the dephasing due to the real T_2^{-1} processes continues to decrease \overline{M} . A series of 180° pulses are used to allow a continuous refocusing of the dipoles to eliminate field inhomogeneity errors. The Meiboom-Gill phase modification of the $\overline{H_1}$ pulse after the initial 90° pulse is used to overcome pulse width errors (see Farrar and Becker, 1971). The slope of a plot of $\ln \frac{M_2(\tau)}{M_0}$ versus τ gives - T_2^{-1} .

The water proton relaxation rates were measured on a constant frequency (26.86 MHz) pulsed NMR spectrometer constructed in Professor H. S. Gutowsky's laboratory. Fig. 2.2 shows the basic design of the spectrophotometer. The NMR sample probe was placed in a sufficiently strong and stable magnetic field. A crystal controlled oscillator was the source for the NMR frequency of 26.86 MHz. The rf signal was gated and phase shifted for the CPMG pulse sequence. A laboratory-built pulse programmer



Figure 2.2 Block diagram of the pulsed NMR spectrometer. The rf transmitter consists of a rf synthesizer, a gating and phase shifter unit and a rf amplifier. The rf receiver consists of a preamplifier, a wide band amplifier and a phase sensitive detector. The sample probe is positioned in the magnetic field. A pulse programmer determines the pulse sequence. An analog to digital converter samples the echo heights and the data is processed by a PDP-8f minicomputer.

i

was used to obtain appropriate 90° and 180° pulse sequence. The gated low-level rf was amplified using an Arenberg Model PG 650 tunable oscillator operated in the gated amplifier mode. A single coil design of the probe was used both for transmission of rf pulses and reception of nuclear induction signals. The transmitter and receiver units were isolated from each other by cross diodes. The induced signal was amplified by a low noise tunable preamplifier (Radio Amateur Model PCL) and a broad band amplifying unit (Kay Electronics Amplifier Model 1024C). A Hewlett-Packard double balanced mixer was incorporated for phase sensitive detection.

 T_2^{-1} was measured by the standard CPMG pulse sequence (90°, τ , 180°, 2 τ , 180°, 2 τ ...). A 90° rf pulse was followed by a 180° pulse after 500 µs and then a series of 2,000 180° pulses spaced 1 ms apart were given. The 90° pulse widths were approximately 2 µs. The T_2^{-1} values were calculated from the decay of spin echo envelopes with time. The echo heights of the envelope were sampled with an analog to digital converter and, the data were analysed by a PDP-8f minicomputer using a least squares analysis program. All measurements were done at room temperature (23°C).

2.11 ESR Measurements

The ESR spectra (first derivative) were measured on a Varian E-9 spectrometer (X Band, 9.5 GHz). The cavity was continuously flushed with dry N_2 gas. Unless otherwise stated, the instrument conditions were: microwave power, 50 mW; modulation frequency, 100 kHz; modulation ampli-tude, 10 G; time constant, 0.3 s; scan rate, 125 G/min. Thylakoid samples (2.0 - 3.0 mg chlorophyll/ml) were placed in a flat cell positioned with clips and all the spectra were recorded at room temperature (23°C).

2.12 Manganese Determination by Neutron Activation Analysis

The basic concept of neutron activation analysis is that the stable nuclei present in a sample become radioactive when bombarded with neutrons in the 1.5 MW TRIGA reactor. In subsequent radioactive decay processes, one or more high energy photons or gamma rays are emitted. A high resolution semiconducter detector interacts with a gamma ray and yields a pulse with the maximum voltage of the pulse being proportional to the gamma ray energy. This pulse is amplified and shaped and then sorted by a pulse height analyzer so that gamma rays of different energies result in different locations. The energy of a gamma ray is characteristic of a particular isotope of a specific element so that a qualitative analysis can be made by observing the spectrum of gamma ray energies emitted by the activated samples. A quantitative analysis can be made by relating the number of gamma rays emitted by the sample relative to a standard containing a known amount of the element.

For the measurement of Mn content of the thylakoid membranes by neutron activation analysis, the samples were sealed in precleaned polyethylene vials. For comparison, a standard was prepared by pipetting microliter quantities of a solution of known Mn concentration on a Whatman #42 filter paper and sealed in another precleaned vial. The samples and standard were then irradiated for 1 hr at a flux of 2 x 10^{12} neutrons cm⁻² s⁻¹ in the TRIGA reactor. After a delay of two to three hours to permit the decay of ³⁸Cl, each sample was counted for 900 s using a Ge(Li) detector with 10% efficiency and 2.10 keV resolution at ⁶⁰Co. The detector is connected to a charge integrating preamplifier (Canberra Model 2001), an amplifier with baseline restoration (Canberra Model 2101)

and a pulse pile-up-rejection system (Canberra Model 1468) which permits the use of high count rates. The gamma ray spectra were accumulated in a 4096 channel analyzer (Canberra Model 8100) and recorded on magnetic tape. The counting data was then reduced to elemental concentration by a fully automatic computer code (developed by J. P. Maney, J. L. Fasching and P. K. Hopke, University of Illinois). The values reported represent the weighted mean values calculated by using two different gamma ray lines emitted by radioactive 56 Mn.

Other necessary details of experimental conditions are described at appropriate places in the text, or in the legends of figures and tables.

CHAPTER 3

ROLE OF BICARBONATE IN PHOTOSYSTEM II REACTIONS OF GREEN PLANTS 3.1 Introduction

Warburg and Kripphal (1960) discovered that bicarbonate (HCO_3^-) is required for O_2 evolution by isolated chloroplasts illuminated in the presence of an oxidant ferricyanide. When 10-20 mM bicarbonate is added to chloroplast membranes previously depeleted of bicarbonate, a large increase (4-10 fold) in O_2 evolution is observed (Stemler and Govindjee, 1973). For a detailed introduction to the bicarbonate phenomenon see Chapter 1, section 1.3. Investigations presented in this chapter clearly show that bicarbonate is required for PS II reactions.

In section 3.2, we present data on the characteristics of the bicarbonate effect: the concentration dependence, the pH dependence and the effect of uncouplers on bicarbonate stimulation of the Hill reaction. The following section (3.3) deals with the question of the site of action of this effect, and in the last section (3.4) we provide experiments to elucidate the possible mechanism of bicarbonate action.

3.2 Characterization of the Bicarbonate Effect

3.2.1 <u>Bicarbonate Concentration Required for the Stimulation of the Hill</u> Reaction: Physiological Importance

The requirement of high concentrations of sodium bicarbonate to observe the maximum bicarbonate effect has not been fully explained (Good <u>et al.</u>, 1966; West and Hill, 1967). <u>In vivo</u> concentration of bicarbonate has been calculated to be 0.5 mM at pH 7.5 (Ogren and Hunt, 1978). The requirement of high concentrations (10-20 mM) of bicarbonate for the stimulation of Hill activity in bicarbonate depleted chloroplasts casts doubt on the physiological importance of the phenomenon. It is quite likely, however, that formate and chloride at high concentrations required for the depletion process, saturate the bicarbonate binding sites. Since the need for bicarbonate is specific (Good, 1963) such competitive action by other anions may effectively reduce the stimulatory effect of exogenously added bicarbonate. To check this point we measured the bicarbonate stimulation of the Hill reaction after washing out the excess formate.

Stimulation of Hill activity (0_2 evolution) measured as a function of bicarbonate concentration is shown in Fig. 3.1. In the presence of 100 mM formate in the assay medium, the amount of bicarbonate required to see the half maximal stimulation is in the range of 5 mM. When formate containing thylakoids were washed once and resuspended in an assay medium from which formate was omitted, the half-maximal stimulation was in the range of 0.5 mM, which is close to the reported <u>in vivo</u> concentration (0.5 mM). Thus it appears that the bicarbonate phenomena studied here may have a physiological significance.

Binding of bicarbonate to chloroplasts has provided more evidence for the physiological significance of the phenomenon. Recently Stemler (1977, 1979) showed that there are two pools of binding for bicarbonate in thylakoid membranes; a smaller high affinity pool at a concentration of one HCO_3^- bound/380-400 chlorophyll molecules and a much larger pool at a concentration approaching that of the bulk chlorophyll. The larger pool may be due to a loose association of bicarbonate on the thylakoid membrane and it does not appear to affect PS II reactions in any way (Stemler, personal communication). Removal of bicarbonate from the



Figure 3.1 Effect of bicarbonate on O_2 evolution in the presence and absence of formate in the assay medium. In the formate containing spinach thylakoids (open circles), the concentration of formate was 100 mM. For samples without formate (filled circles), the formate containing thylakoids were washed once and resuspended in a formate-free medium. The reaction mixture (2 ml) contained 50 mM phosphate buffer (pH 6.8), 100 mM NaCl, 100 mM sodium formate and 0.5 mM [Fe(CN)₆]³⁻. Chlorophyll concentration, 33 µg/ml; light intensity, 250 mW/cm²; maximum rate of electron flow, 250 µequiv./mg Chl per h. Other details as given in Chapter 2 (sections 2.2 and 2.6).

small pool requires low pH (5.0) and a high salt medium, and results in a 99% inhibition of oxygen evolution. This inhibition is reversed by adding bicarbonate. Since removal of bicarbonate from the high affinity pool requires special conditions there is no dependence observed in normal chloroplasts when exogenous bicarbonate is added.

3.2.2 <u>Effect of External pH on the Bicarbonate Stimulation of the Hill</u> Reaction

In order to determine whether dissolved CO_2 or HCO_3^- is the active species in stimulating Hill reaction, the stimulatory effect was studied as a function of pH, although no definite conclusion could be reached. Effect of HCO_3/CO_2 was studied as a function of pH in the 5 to 9 range. If HCO_3^- is the active species, one would expect a larger effect in the 6-8 pH range where its concentration predominates. A suboptimal concentration of 2.0 mM HCO_3/CO_2 (see section 3.2.1) was used to stimulate the Hill reaction (measured as 0_2 evolution). Use of low concentration of HCO_3^-/CO_2 was essential to avoid any pH changes upon the addition of HCO_3^2/CO_2 , especially at pHs outside the pKa of the phosphate buffer used. Fig. 3.2 shows that a much larger stimulation in Hill activity is observed around pH 6-7; a 2-fold effect is seen at pH 6.8 but none at pH 5.0. These studies confirm the measurements of Stemler and Govindjee (1973) at pH 5.8 and 6.8 for low concentrations of bicarbonate. Addition of 20 mM $NaHCO_3$ gave the same fold effect at pH 5.8 and 6.8. However, this effect could have been distorted due to an increase in pH upon the addition of 20 mM bicarbonate at pH 5.8(phosphate is not a good buffer in this pH range).



Figure 3.2 Rate of 0_2 evolution as a function of pH in the presence and absence of bicarbonate. Upper curve (open squares), with subsaturating concentration of NaHCO₃ (2 mM); lower curve (filled circles) without NaHCO₃. Maximum rate of electron flow, 250 µequiv./mg Chl per h. Other conditions as described in the legend of Fig. 3.1.

A plot of the relative concentrations of CO_2 , HCO_3^- and CO_3^- (Fogg, 1968) as a function of pH shows (Fig. 3.3) that the bicarbonate species predominates in the 6-7 pH range where we have the maximum effect. From this data, it would appear that HCO_3^- is the active species in stimulating the Hill reaction. However, the affinity of HCO_3^-/CO_2 to the membrane component(s) may be different at different pH values. It is possible that CO_2 is the active species, but at lower pH the affinity of the active site for CO_2 is lowered even though CO_2 occurs at higher concentrations. Thus, no definite conclusion could be made regarding the active species involved.

3.2.3 Effect of Uncouplers on the Bicarbonate Effect

Punnett and Iyer (1964) while investigating the bicarbonate effect on the Hill reaction discovered that photophosphorylation was enhanced even more than the electron flow, thereby increasing the P/2e ratio. They argued that there were two distinct effects of bicarbonate: (1) related to electron flow and 0_2 evolution and (2) related to the phosphorylation mechanism. The bicarbonate effect on the enhancement of phosphorylation, that did not require prior $C0_2$ -depletion of membranes, was suggested to be due to the increased formation of a "high energy intermediate". Batra and Jagendorf (1965) confirmed these results but found that although bicarbonate increased phosphorylation and the P/2e ratio, the "high energy intermediate" was actually suppressed by bicarbonate.

It was necessary to be sure that we were not dealing with an indirect stimulation of electron flow produced by an enhancement of photophosphorylation by bicarbonate. Thus, the bicarbonate effect was measured in the presence of several uncouplers of photophosphorylation (Table 3.1).



Figure 3.3 Comparison of the stimulatory effect of HCO_3^-/CO_2 with the relative concentrations of CO_2 , HCO_3^- and CO_3^- at different pH. Filled circles, stimulatory effect of CO_2 (calculated from Fig. 3.2); dots, $[CO_2]$; dashes, $[HCO_3^-]$; dots and dashes, $[CO_3^-]$; data for concentration of various species is in sea water at 34% salinity at 18°C (redrawn from Fogg, 1968).

TA	BI.	E	3.	1
	-		•••	-

		Electron (µequiv./mg_chl -HCO3	transport orophyll per hr) +10 mM HCO3	Ratio +HCO3 /-HCO3
(1)	Control	26 ± 3	171 ± 9	6.6
	Plus NH4Cl	24 ± 4	163 ± 7	6.8
(2)	Control	22 ± 3	134 ± 14	6.1
	Plus methylamıne • HCl	23 ± 3	147 ± 18	6.4
(3)	Control	22 ± 4	165 ± 15	7.5
	Plus nigerıcın	21 ± 1	144 ± 5	6.9
(4)	Control	13 ± 1	135 ± 4	10.4
	Plus gramıcıdin D	12 ± 1	125 ± 7	10.4

Bicarbonate Effect in the Presence of Uncouplers of Photophosphorylation: Rates of Electron Transport

The 2 ml reaction mixture contained 50 mM phosphate buffer (pH 6.8), 100 mM NaCl, 100 mM sodium formate, and 0.5 mM $[Fe(CN)_6]^{3-}$. Concentrations of uncouplers used were: 1 mM NH4Cl, 0.3 mM methylamine HCl, 5 μ M nigericin, and 1 μ M gramicidin D. Average of three experiments is shown. Other conditions as described in the legend of Fig. 3.1. The bicarbonate effect ranged from 6 to 10 fold and was independent of the presence of uncouplers. There was no change in the rate of 0_2 evolution when 1 mM NH₄Cl [Table 3.1 (1)], 0.3 mM methylamine HCl [Table 3.1 (2)], 5 µM nigericin [Table 3.1 (3)] or 1 µM gramicidin D [Table 3.1 (4)] was included in the reaction mixture. It appears that our samples were already uncoupled, perhaps due to the high concentration of salts used in the depletion medium. Thus, the bicarbonate effect we have studied in this thesis is not related to its effect on photophosphorylation. 3.3 Site of the Bicarbonate Effect

To identify the site of bicarbonate action along the electron transport chain, Stemler and Govindjee (1973) used thylakoids in which 0_2 evolution was inhibited by heat treatment. In such preparations the rate of electron flow from DPC to DCPIP was found to be insensitive to bicarbonate, thereby suggesting that the site of its action is before the site of electron donation by DPC. This conclusion was later modified by Wydrzynski and Govindjee (1975) when they showed that in the absence of bicarbonate DPC itself sustained a high rate of DCPIP reduction implying an absence of bicarbonate requirement for this reaction. Moreover, Harnishfeger (1974) had shown that, in addition to its function as an electron donor, DPC increases the efficiency of PS II. Thus, the absence of bicarbonate effect, reported by Stemler and Govindjee (1973), on DPC to DCPIP reaction could not be accepted as an evidence for the requirement of bicarbonate on the oxygen evolving side of PS II. In spite of these complications, a 2 fold bicarbonate effect was observed in the DPC \Rightarrow DCPIP reaction when the bicarbonate effect in the H₂O \Rightarrow DCPIP reaction was 10 fold (Wydrzynski and Govindjee, 1975). Furthermore, the

effect of bicarbonate on chlorophyll <u>a</u> fluorescence transients in the presence of artificial electron donors (hydroquinone, $MnCl_2$, NH_2OH and DPC) suggested at least one site of bicarbonate action on the reducing side of PS II.

We have obtained new information regarding the site of bicarbonate effect in photosynthetic electron transport chain through the study of various partial reactions of redox segments of the electron transport chain. In this section experiments are described which show that the major site of the bicarbonate effect is between the primary electron acceptor of PS II and the PQ pool. We adopted two different approaches to identify the site of bicarbonate action. The first approach (see section 3.3.1) was to study the bicarbonate stimulation in partial segments of the electron transport chain, and the second approach (see section 3.3.2) was to study the individual electron transfer reactions by monitoring the absorption changes due to different redox components (Q, R, PQ and P700) of the chain.

3.3.1 <u>Biochemical Studies on Partial Reactions of the Electron Transport</u> Chain

For a better understanding of the experiments, a brief description of the different electron donor and acceptor systems is presented. The electron transport pathway in chloroplast membranes can be divided into several segments by the use of appropriate exogenous electron donoracceptor combinations in conjunction with specific inhibitors of certain intermediate electron carriers as shown below:



where, SiMo accepts electrons from Q⁻ (Zilinskas and Govindjee, 1975; Giaquinta and Dilley, 1975), DCMU is an inhibitor that blocks electron flow from Q⁻ to R, DAD_{ox} accepts electrons somewhere before the PQ pool (Ouitrakul and Izawa, 1973), reduced duroquinone (DQH₂) donates electrons at PQ (Izawa and Pan, 1979), dibromothymoquinone (DBMIB) is an inhibitor of electron flow at PQ (Böhme <u>et al.</u>, 1971), DAD_{red} acts as an artificial electron donor to PS I (Izawa <u>et al.</u>, 1973), and MV accepts electrons from X⁻ (the reduced primary electron acceptor of PS I). Using various partial reactions, indicated by 1, 2 and 3 in the above scheme, the bicarbonate effect was measured on these segments of the electron transport chain.

The bicarbonate effect in various partial reactions is shown in Table 3.2.

3.3.1.1 Water to Silicomolybdate System (Reaction 1)

This abbreviated reaction involves the oxygen evolution apparatus, the photochemistry of PS II and the primary electron acceptor (Q). For electron flow from water to Q, 25 μ M silicomolybdate in the presence of 5 μ M DCMU, was used as the electron acceptor. All measurements were made within 30 seconds of illumination since silicomolybdate mediated electron transport stops 1 min after the addition of DCMU and SiMo (for details
TA	BL	.E	3	2

Effect of Bicarbonate on Various Isolated Electron Transport Systems

System (see Eq. 3.1)	Electron transport (uequiv./mg chlorophyll per h)		
	-HCO3	+10 mM HCO3	
 (1) H₂O to silicomolybdate (2) H₂O to oxidized diaminodurene (3) Bodurand diaminodurene to 	117 ± 16 12 ± 1	108 ± 17 90 ± 2	
(3) Reduced drammodurene to methyl viologen	662 ± 12	673 ± 16	

Spinach thylakoids containing 33 µg Ch1/m1 were illuminated in a continuously stirred reaction mixture (2 ml) containing 50 mM phosphate buffer (pH 6.8), 100 mM NaCl, 100 mM sodium formate and the indicated donor and acceptor system. These systems were: (1) H₂O > SiMo, 5 µM DCMU and 25 µM SiMo; (2) H₂O > DAD_{OX}, 0.5 mM DAD, 0.5 mM [Fe(CN)6]³⁻ and 0.5 µM DBMIB; (3) DAD_{red} > MV, 50 µM MV, 0.5 mM DAD, 2.0 mM sodium ascorbate and 1 µM DCMU. Average of three experiments is shown. Other details as given in Chapter 2 (section 2.6). see Zilinskas and Govindjee, 1975). In CO_2 -depleted thylakoid membranes the H₂O to SiMo reaction was much higher than the H₂O to DAD_{ox} reaction (reaction 2) implying that the former may not require bicarbonate. Furthermore, no significant stimulation of O_2 evolution was observed upon the addition of 10 mM bicarbonate to the depleted samples [Table 3.2 (1)]. This implies that there is no major site of bicarbonate action on the oxidizing side of PS II. The silicotungstate system (Zilinskas and Govindjee, 1975) showed similar results (data not shown).

In addition to the effect mentioned above, absence of $HCO_3^$ causes a reversible inactivation of up to 40% of the PS II reaction center activity (Stemler <u>et al.</u>, 1974; Jursinic <u>et al.</u>, 1976). The results presented in section 3.3.2 show that the inactivation of reaction centers could range from 5 to 40%. The absence of any bicarbonate effect in the $H_2O \Rightarrow$ SiMo reaction at saturating light intensities may be due to the low inactivation of reaction center II in these preparations. Alternatively, silicomolybdate somehow overcomes this inactivation. 3.3.1.2 Water to Oxidized Diaminodurene (Reaction 2)

For electron flow from water to PQ, 0.5 μ M DBMIB was used to block electron flow beyond PQ, (Böhme <u>et al.</u>, 1971) and oxidized DAD was the electron acceptor (0.5 mM DAD and 0.5 mM [Fe(CN)₆]³⁻) (see Ouitrakul and Izawa, 1973). In this reaction, a 7-8 fold enhancement of O₂ evolution was observed when 10 mM sodium bicarbonate was added to samples depleted of bicarbonate [Table 3.2 (2)]. The complete electron transfer chain from H₂O to X showed the same 7-8 fold effect. Since DAD_{OX} accepts electrons before the site of DBMIB inhibition (Ouitrakul and Izawa, 1973), these results suggest that the bicarbonate effect is located between Q and the PQ pool.

3.3.1.3 Reduced Diaminodurene to Methyl Viologen (Reaction 3)

For electron flow involving only PS I, the electron donor was reduced DAD (0.5 mM DAD + 2.0 mM ascorbate) and the electron acceptor was 50 μ M methyl viologen; 1 μ M DCMU was used to block the electron flow from H₂O to PQ (see Izawa <u>et al.</u>, 1973). No significant effect of bicarbonate is observed [Table 3.2 (3)] in electron flow from the site of donation of diaminodurene (PQ or cyt f) to methyl viologen. Thus, the major bicarbonate effect 1s located between Q and PQ.

After this work was completed and published (see Khanna <u>et al</u>., 1976; 1977) Izawa and Pan (personal communication, 1979) have recently studied the bicarbonate effect on the partial reaction 4 (Eq. 3.1), and found that bicarbonate does not affect this reaction.

3.3.2 <u>Effect of Bicarbonate on Absorption Changes Due to Different Redox</u> Components of the Electron Transport Chain

In the second approach to locate the site of bicarbonate action, we directly monitored the absorption changes corresponding to individual electron transfer reactions due to the redox reactions of Q, R, PQ and P700. A brief description of the various absorption changes measured is essential in order to appreciate the results of the experiments described in this section. When the electron acceptor Q of PS II is reduced, it shows a positive absorption band at 320 nm which is due to the formation of a semiquinone (the transition of quinone to semiquinone anion) (Stiehl and Witt, 1969; Renger and Wolff, 1976). The negative absorption changes

measured at 265 nm are due to the transition of plastoquinone to plastohydroquinone (Stiehl and Witt, 1968; Siggel, 1976). Thus, changes in absorption at 265 nm indicate changes in both R and the PQ pool. 3.3.2.1 Absorption Changes at 334 nm Induced by 20 µs Repetitive Flashes

Absorption changes at 320 nm indicate the formation and decay of semiquinone as noted earlier. We studied the transition of quinone to semiquinone anion of the electron acceptor X320 (Q) of PS II and its dark decay by monitoring absorption changes at 334 nm. The maximum of X320 absorption is located around 325 nm, but the absorption changes were measured at 334 nm because at this wavelength the UV mercury lamp has an emission band which allowed a higher signal to noise ratio. Absorption changes at 325 and 334 nm, however, provided the same conclusions.

Using the repetitive flash method, absorption change at 334 nm shows a very fast rise ($\leq 1 \mu s$) and a biphasic dark relaxation dominated by an exponential phase with a halftime of ~500 μs (Figs. 3.4 and 3.5). The fast rise is attributed to the rapid photoreduction of X320 (Q) and the 500 μs decay to the subsequent reoxidation of X320⁻ (Q⁻). A minor portion of the signal (10-15%), with a halftime of 10-20 ms, is due to an uncharacterized signal, probably from the formation of plastohydroquinone, cyt f⁻ or the reduction of oxidized P700. Electron flow from X320⁻ (Q⁻) to the PQ pool takes place via a two electron carrier R (or B) (Bouges-Bocquet, 1973b; Velthuys and Amesz, 1974) as shown below:

$$Q^{-} + R \xrightarrow{K_{1}} Q + R^{-} \qquad (3.2(a))$$

 $Q^{-} + R^{-} \xrightarrow{K_{2}} Q + R^{2-}$ (3.2(b))

The amplitude of the phases corresponding to k_1 and k_2 should be proportional to $(\Delta E_Q - \Delta E_R)$ and $[\Delta E_Q - (\Delta E_R^2 - \Delta E_R)]$, where ΔE_Q , ΔE_R , and ΔE_R^2 - are the extinction coefficient differences for the redox system Q⁻/Q, R⁻/R and R²⁻/R, respectively. In spite of the two reactions (Eqs. 3.2 (a) and (b)) shown, the decay of the signal at 334 nm shows one dominant fast phase. If both Q⁻ and R⁻ are assumed to be plastosemiquinone anions then the differential extinction coefficient for Q and R may roughly be equal, i.e., $\Delta E_Q \sim \Delta E_R$. This would imply that the decay of the signal represents mainly the disappearance of two semiguinones and the formation of one hydroquinone (Eq. 3.2(b)).

Bicarbonate depletion (Fig. 3.4 and 3.5) has two effects on the absorption change at 334 nm:

- the amplitude is reduced by 35-40% which is in agreement with the reduction of the absorption change of P680 (cited by Jursinic <u>et al.</u>, 1976) and reflects inhibition of the reaction center II; and,
- (2) the decay becomes clearly biphasic showing two exponential phases of about equal magnitude with halftimes of $500 \pm 100 \ \mu s$ and 7 ± 3 ms (average of 5 experiments). Both effects of bicarbonate depletion are reversible as addition of 10-20 mM bicarbonate (+ $C0_2$) restores all of the values to that of the control.

The reduction (40%) in the amplitude of the absorption change at 334 nm could be either due to the inactivation of the reaction centers or



Figure 3.4 Time course of the absorption change at 334 nm induced by 20 μ s repetitive flashes. The absorption changes (indicating mainly Q⁻ and R⁻) are shown for untreated spinach thylakoid membranes (frozen and thawed, control), after bicarbonate depletion (-CO₂) and after readdition of 20 mM bicarbonate (+CO₂). The amplitudes $\Delta I/I$ are 4.2 x 10⁻⁴, 2.7 x 10⁻⁴ and 4.5 x 10⁻⁴, respectively. The reaction mixture contained 20 mM phosphate buffer (pH 6.8), 100 mM NaCl, 100 mM sodium formate, 0.5 mM [Fe(CN)₆]³⁻, 0.02 mM gramicidin and 100 μ g Chl/ml. 512 flashes were averaged; darktime (t_d) between flashes, 250 ms; electrical bandwidth, 10 kHz.



Figure 3.5 Semilogarithmic plots of the curves from Fig. 3.4. The relative amplitudes and halftimes $(t_{1/2})$ of the two exponential phases are indicated.

due to the inability of the centers to undergo charge separation in repetitive flashes if they were not able to relax during the short (250 ms) dark period between flashes.

The biphasic relaxation in the thylakoid suspensions reflects heterogeneity of the system introduced by the depletion procedure - about 30% of the total signal seems to have been unaffected by the procedure of depletion, another 30% exhibits slow kinetics, and the rest (40%) appears totally inactivated. It is not clear whether the heterogeneity lies within the same thylakoid membrane or in different populations of membrane fragments (cf. section 3.4.2). It is also not possible to say which of the rate constants, k_2 or both k_1 and k_2 , are affected by bicarbonate depletion. However, a change in both of the rate constants is suggested by the fact that the electron transfer is between Q and R species in both cases and by the possibility that the change may be due to structural reorganization.

Jursinic <u>et al</u>. (1976) measured the decline in Chl <u>a</u> fluorescence yield as a monitor of Q⁻ decay and reported a $t_{1/2}$ of 2.6 ms in bicarbonate depleted chloroplasts. Our data on X320 shows a $t_{1/2}$ of 7 ± 3 ms. This difference in the two values could be due to differences in samples or due to the fact that fluorescence decay monitors the Q⁻R to QR⁻ reaction (Eq. 3.2(a)), whereas the absorption change monitors mainly the Q⁻R⁻ to QR²⁻ reaction (Eq. 3.2(b)). However, our data is consistent with the observed decrease in the relaxation of S⁻_n to S_{n+1} (Stemler <u>et al</u>., 1974) from a halftime of approximately 500 µs to ~10 ms upon bicarbonate depletion. Relaxation of S⁻_n to S_{n+1} is mainly due to the reoxidation of Q⁻ to Q.

3.3.2.2 Absorption Changes at 265 nm Induced by 20 µs Repetitive Flashes

Absorption changes at 265 nm are indicative of changes in Q, R and the PQ pool. A portion of the electron flow in the intersystem electron carriers (ignoring Rieske iron center) can be represented as:

P680
$$\longrightarrow$$
 Q $\frac{t_{1/2}}{(1)}$ R $\frac{t_{1/2}}{(2)}$ PQ $\frac{t_{1/2}}{(3)}$ cyt f (3.3)

Dark relaxation of the light induced absorption change at 265 nm is composed of a minor (20% amplitude) fast phase $(t_{1/2} = 500 \ \mu s)$ and a major slow phase $(t_{1/2} = 20 \ ms)$ for osmotically shocked chloroplasts (Stiehl and Witt, 1969). The 500 μs phase represents reaction (1) and the 20 ms phase represents reaction (3) (the oxidation of plastohydroquinone) in Eq. 3.3.

In a short flash (< < 0.6 ms), one electron is transferred from water via P680 to R in ~500 μ s. The electron of the reduced plastohydroquinone (PQH₂) is accepted by P700⁺ via several intermediates in 20 ms. This reoxidation of PQH₂ is the rate limiting step of photosynthesis. Thus, in short (20 μ s) flashes, separated by 1.3 s dark period, the absorption change is primarily due to the formation of R²⁻ but the relaxation in the subsequent dark period is governed by reaction (3). The transfer of a pair of electrons from R²⁻ to PQ would not have caused significant absorption changes as both are quinones (Pulles <u>et al.</u>, 1977).

The control thylakoids of this study behaved somewhat differently than the "normal" chloroplasts. As shown in Fig. 3.6, the relaxation kinetics of the control thylakoids can be separated into a slow and a fast phase: the fast phase is larger (33% versus 20%) and slower $(t_{1/2} - 10 \text{ ms versus } 500 \text{ }\mu\text{s})$ than in normal chloroplasts; and the slow phase is also slower ($t_{1/2} = 50-75$ ms) than normal (20 ms). The halftime of the fast phase is not resolved due to the slow response time of the instrument used. The halftime of the slow phase in the control is 75 ms instead of 50 ms in the reconstituted (+CO₂) sample. This increase in $t_{1/2}$ is probably due to aging, since for the control the data are shown for the second illumination (from 513^{rd} to 1024^{th} flashes) whereas in the case of the reconstituted sample the data are shown for the first illumination (from 1^{st} to 512^{nd} flashes).

The major effect (in 70% of the signal) of bicarbonate depletion $(-CO_2)$ is the retardation of the slow phase from 50 ms to about 170 ms halftime (Fig. 3.6). The signal amplitude is reduced by ~20% in the depleted sample. This can be due to a reversible inactivation of reaction center II. Upon the addition of 10 mM bicarbonate, the halftime of the slow phase is restored to 50 ms and the signal amplitude ($\Delta I/I$) is comparable to that of the control (2.7 x 10⁻⁴ versus 2.6 x 10⁻⁴). The absorption change due to Q, with a halftime of 500 µs, in reconstituted and control thylakoid membranes has not been resolved due to the electrical bandwidth (1.7 kHz) chosen for these measurements. In CO₂-depleted thylakoid membranes, it is slowed down to ~7 ms (Fig. 3.5) and remains unobserved in Fig. 3.6 because it is masked by the presence of a 10 ms phase already present in the control. The only indication of its existence is that the 10 ms phase becomes larger (from ~30% to 44%)in CO₂-depleted sample.

The 170 ms phase in CO_2 -depleted thylakoid membranes could correspond to either (a) the oxidation of PQH₂, if the relaxation of the signal is governed by reaction (3) in Eq. 3.3 or, the reduction of PQ if the relaxation is determined by reaction (2) in Eq. 3.3 (accompanied by a



Figure 3.6 Time course of the absorption change at 265 nm induced by 20 μ s repetitive flashes. The absorption changes (indicating mainly R²⁻ or PQ²⁻) are shown for untreated (control), CO₂-depleted (-CO₂) and reconstituted (+CO₂) thylakoid membranes (freshly prepared). The amplitudes (Δ I/I) are 2.7 x 10⁻⁴, 2.2 x 10⁻⁴ and 2.6 x 10⁻⁴, respectively. The relative magnitudes and halftimes (t_{1/2}) of the two exponential phases are shown in the column on the right. Number of flashes, 512; darktime (t_d) between flashes, 1.3 s; electrical band-width, 1.7 KHz. Intensity of the monitoring light, 150 ergs cm⁻² s⁻¹ ($\Delta\lambda = 5$ nm). Data for second illumination, i.e., from 513 to 1024 flashes are shown for the control (increased halftime because of aging). Other conditions as described in the legend of Fig. 3.4

minor or no absorption change) with the absorption change being brought about by the consecutive faster oxidation of the PQH_2 (reaction (3) in Eq. 3.3). In order to check this, absorption changes at 265 nm were measured with long flashes (see section 3.3.2.4). Furthermore, the present data does not indicate whether the ~200 ms time represents a reaction within the electron transfer chain. Therefore, absorption changes of the PQ system and P700 were measured simultaneously in the same sample.

3.3.2.3 Absorption Changes at 265 nm and 703 nm Induced by 20 μ s

Repetitive Flashes

Absorption changes of the plastoquinone system and P700 were measured simultaneously in the same sample to check that the 170-230 ms halftime does not correspond to an artificial pathway of the electrons directly to ferricyanide.

Both the P700 and PQ signals consist of a slow and a fast phase in the bicarbonate depleted $(-CO_2)$ and reconstituted $(+CO_2)$ samples (Figs. 3.7, 3.8 and 3.9). If the two phases of the PQ signal are due to consecutive reactions, then the P700 signal should relax with the larger time constant, whereas the smaller time constant should lead to a lag in the reduction of oxidized P700. But this lag can be observed only if the intersystem pool (i.e., PQ, cytf, PC) is completely oxidized by giving far red light prior to the flash. In the experiments shown in Fig. 3.7, there was no far red preillumination hence no lag was expected. The appearance of the two phases in the P700 signal indicates heterogeneity in our preparations. The 170-230 ms phase appears with almost equal amplitude (60-70%) in the absorption changes of both PQ and P700 in the bicarbonate depleted samples (Fig. 3.8). This means that oxidized P700



Figure 3.7 Absorption changes at 265 and 703 nm induced by 20 μ s repetitive flashes, measured simultaneously, for CO₂-depleted and reconstituted thylakoid membranes. The amplitudes are 2.85 x 10⁻⁴ and 3.05 x 10⁻⁴ for the PQ system at 265 nm and 9.1 x 10⁻⁴ and 10 x 10⁻⁴ for P700 at 703 nm. Intensity of the monitoring light at 703 nm, 70 ergs cm⁻² s⁻¹ ($\Delta\lambda = 5$ nm). Thylakoids were frozen and then thawed before use. Other conditions as described in the legend of Fig. 3.6.



Figure 3.8 Semilogarithmic plots of the signals of Fig. 3.7 $(-CO_2 \text{ sam-ple})$. The relative magnitudes and halftimes $(t_{1/2})$ of the two exponential phases are indicated for both the PQ and the P700 signals.

is reduced with this halftime, thereby showing that the 170-230 ms time does not correspond to an artificial pathway. The appearance of a small (30% amplitude) 200 ms phase in the reconstituted samples (Fig. 3.9) has been discussed in the preceeding section.

A quantitative analysis of the signals (Table 3.3) shows that bicarbonate depletion $(-CO_2)$ causes the following effects:

- (1) The predominant phase (~70% signal) which has a halftime of 25-30 ms for both P700 and the plastoquinone system in the reconstituted thylakoids (+ CO_2) is replaced by a predominant (~70%) phase having a $t_{1/2}$ of 210-230 ms in the CO_2 -depleted thylakoids (Table 3.3). The fast phase of the P700 signal with a halftime of 25-35 ms represents the transfer of electrons along the normal pathway.
- (2) The 30 ms fast phase constitutes 70% of the total signal in the reconstituted thylakoids. Upon depletion, about 50-60% of this fast phase is converted into the slow ($t_{1/2}$ ~200 ms) phase. This implies 50-60% depletion in these samples.
- (3) Bicarbonate depletion reduces the amplitude of signals for absorption changes at 265 and 703 nm. The data in Fig. 3.7 shows that the amplitude ($\Delta I/I$) for the depleted and the reconstituted samples are 2.85 x 10⁻⁴ and 3.05 x 10⁻⁴ for the PQ system, and 9.1 x 10⁻⁴ and 10 x 10⁻⁴ for P700, respectively. This decrease in amplitude is consistent with a 10-20% inactivation of the reaction center II.
- (4) In reconstituted thylakoids, the absorption change due to Q ($t_{1/2} = 500 \ \mu s$) is not resolved due to the slow response time



Figure 3.9 Semilogarithmic plots of the signals of Fig. 3.7 $(+CO_2 \text{ sam-ple})$. The relative amplitudes and halftimes $(t_{1/2})$ of the two exponential phases for both the PQ and the P700 signal are indicated.

T 0	m 1	÷	2	-
- Δ	К!	-	٠.	•
10	ᅛᄂ	L.	J	•••

Absorption Changes at 265 and 703 nm Induced by 20 μs Repetitive Flashes for CO2-Depleted and Reconstituted Thylakoids

	والمحيري والبرجي كالتبادي النكار بالراكر تعاري	والمراجع والمتحاد والمتحر والمراجع	الورياني محمد ومراجعا مراجع ومعاديا والمراجع	
	Absorption Change at 265 nm Halftime (Amplitude)		Absorption Change at 703 nm Halftime (Amplitude)	
	Fast	Slow	Fast	Slow
+C02	30 ms (69%)	180 ms (31%)	25 ms (70%)	180 ms (30%)
-C02	20 ms (37%)	210 ms (63%)	35 ms (28%)	230 ms (72%)

Relative amplitudes and halftimes of the two exponential phases are indicated for absorption changes at 265 and 703 nm (data of Figs. 3.8 and 3.9).

of the instrument used. The absorption change due to Q becomes significant in the depleted thylakoids because of the increased halftime of 7 ms for 60% of the signal. This new phase has not been separated, but it must have been included in the fast phase of 20 ms halftime, which is therefore faster than the 30 ms phase in the reconstituted case. Since absorption changes due to Q are not included in the P700 signal, the fast phase of the P700 signal is somewhat smaller (28%) as compared to the absorption changes at 265 nm (37%).

Frozen and thawed thylakoid preparations were used for experiments described in Fig. 3.7. Freezing the thylakoids caused some changes in the relaxation kinetics of absorption changes (compare Fig. 3.6 with Fig. 3.7). Data of Figs. 3.6 and 3.7 are tabulated to show these differences (see Table 3.4). In fresh reconstituted thylakoids, the fast and slow phases show a halftime of 8 ms and 50 ms which upon bicarbonate depletion increases to 12 ms and 170 ms repsectively. In frozen thylakoids, the fast phase shows an increased halftime ($t_{1/2} = 20-30$ ms). The frozen thylakoids show a small (30% amplitude) 200 ms phase even in the reconstituted samples. The extent of this phase was doubled by the absence of bicarbonate. The presence of a small proportion of the slow phase in frozen and thawed thylakoids suggests that freezing might have caused some damage to the sample. However, this does not affect our major conclusions.

3.3.2.4 Absorption Changes at 265 nm by 85 ms Repetitive Flashes

The dark relaxation of the absorption change at 265 nm by short flashes (Fig. 3.6) showed that it is slowed down from an approximately 50 ms phase to a \sim 200 ms phase. In the control or reconstituted case, the 50-75 ms phase corresponds to the oxidation of plastohydroguinone

TA	BL	_E	3.	.4
		-	· •	

	(a) Fresh		(b) Frozen and	rozen and Rethawed	
	Halftıme (Amplitude)		Halftime (Ar	ftime (Amplitude)	
	Fast	Slow	Fast	Slow	
+C02	8 ms	50 ms	30 ms	180 ms	
	(30%)	(70%)	(69%)	(31%)	
-C02	12 ms	170 ms	20 ms	210 ms	
	(44%)	(56%)	(37%)	(63%)	

Comparison of Absorption Changes at 265 nm in Fresh, and Frozen and Thawed Thylakoid Membranes

The relative amplitudes and halftimes of the two exponential phases (fast and slow) are shown for (a) fresh and (b) frozen and thawed thylakoids (data of Figs. 3.6 and 3.7). (PQH_2) (reaction (3) in Eq. 3.3). In the CO_2 -depleted thylakoids the ~200 ms phase could either correspond to the oxidation of plastohydroquinone (reaction (3) in Eq. 3.3) or to the formation of PQ^{2-} from R^{2-} (reaction (2) in Eq. 3.3), accompanied by a minor or no absorption change, and the absorption change would then correspond to the consecu- tive fast oxidation of the plastohydroquinone. In order to distinguish between these two possibilities the absorption change was measured in long (85 ms) flashes (dark time between flashes, 5 s). In control thylakoids the electron transfer reactions in an 85 ms flash and the subsequent dark reactions can be represented as follows:

$$Q R PQ \xrightarrow{flash}{85 \text{ ms}} Q^{-} R^{2-} PQ^{2-} \qquad (3.4(a))$$

$$1/2 \text{ PQ}^2 \xrightarrow{\text{dark}} 1/2 \text{ PQ}$$
 (3.4(b))

$$R^{2-} \xrightarrow{\text{dark}} R \qquad (3.4(c))$$

$$Q^{-} \xrightarrow{\text{dark}} Q \qquad (3.4(d))$$

In long flashes (85 ms) of saturating intensity, Q, R and PQ should be mostly reduced (Eq. 3.4(a)). In the dark period following the flash, PQ^{2-} will be oxidized (Eq. 3.4(b)), whereas the electrons from R^{2-} and Q⁻ will be transferred to PQ (Eq. 3.4(c)) and R (Eq. 3.4(d)) respectively, when available in the oxidized form. Once R⁻ is formed it will not be reoxidized before the next flash ($t_d = 5$ s) because the reoxidation of R⁻ takes a few minutes (Velthuys, 1976). The absorption change at 265 nm will be only due to the couples R/R^{2-} and PQ/PQ^{2-} . In control thylakoids, relaxation will correspond to the reoxidation of PQ^{2-} but if the relaxation of R^{2-} is slowed down, then this will govern the rate of dark relaxation.

Fig. 3.10 shows the time course of absorption changes at 265 nm in long (85 ms) repetitive flashes for depleted $(-CO_2)$ and reconstituted $(+CO_2)$ samples. In these experiments continuous far red background light (720 nm) was used to keep the intersystem pool and P700 oxidized. Under these conditions, there is more PQ available for reduction. Bicarbonate depletion causes a 40% reduction in the amplitude of the absorption change. The dark relaxation of the signal in the CO_2 -depleted sample has a $t_{1/2}$ of 100 ms as compared to 26 ms for the reconstituted thylakoids.

If the 265 nm signal in long flashes is composed of absorption changes of the transition PQ/PQ^{2-} and R/R^{2-} , then the absorption changes in control thylakoids can be explained by the following scheme:

 $Q \longrightarrow R \xrightarrow{t_{1/2} \le 1 \text{ ms}} PQ \xrightarrow{t_{1/2} = 25 \text{ ms}} \longrightarrow P700 (3.5(a))$

The reduction of the PQ pool is much faster than the corresponding oxidation. Accordingly, a high degree of reduction will result in a significant (large) absorption change. The dark relaxation will be determined by the slow oxidation of PQH₂. The 25 ms halftime for dark relaxation here should be due to the oxidation time of PQH₂. The depletion procedure slows down dark relaxation to 100 ms. This slowing down could be either due to the slow reduction or slow reoxidation of the PQ pool. The two possibilities are described below:



Figure 3.10 Time course of the absorption change at 265 nm induced by 85 ms repetitive flashes for CO_2 -depleted and reconstituted thylakoid membranes. The amplitudes ($\Delta I/I$) are 6.5 x 10⁻⁴ and 9 x 10⁻⁴, for $-CO_2$ and $+CO_2$ samples, respectively. The halftimes ($t_{1/2}$) of dark relaxa- tion are shown. Number of flashes, 64; darktime (t_d), 5 s; electrical bandwidth, 600 Hz; intensity of 720 nm background light ($\Delta \lambda = 15 \text{ nm}$), 400 ergs cm⁻² s⁻¹. Sample frozen and then thawed before use. Other con- ditions as described in the legend of Fig. 3.4.

(1) The reduction of the PQ pool is unaltered but the corresponding oxidation is slowed down:

$$Q \longrightarrow R \xrightarrow{t_{1/2} \le 1 \text{ ms}} PQ \xrightarrow{t_{1/2} = 100 \text{ ms}} \longrightarrow P700 (3.5(b))$$

PQ is being reduced at a normal fast rate but its' reoxidation is slowed down; this would lead to an accumulation of PQH_2 . If this is the case then in CO_2 -depleted thylakoids the amplitude of the signal should have increased but it did not.

(2) The reduction of the PQ pool is slow compared to its oxidation. Accordingly, the reaction scheme can be represented as:

$$Q \longrightarrow R \xrightarrow{t_{1/2} = 100 \text{ ms}} PQ \xrightarrow{t_{1/2} = 25 \text{ ms}} \longrightarrow P700 (3.5(c))$$

The amplitude of the absorption change will be small and will mainly be due to R^{2-} . During the dark relaxation, R^{2-} will slowly be substituted by PQ^{2-} with subsequent fast oxidation to PQ (i.e., 25 ms), so that the process will be governed by the slow reduction of the PQ pool. The signal from thylakoids depleted of bicarbonate (Fig. 3.10, top) can be qualitatively explained by this assumption because there is a decrease in the amplitude of the signal in the depleted thylakoids. Thus, from the reduction in the amplitude of the absorption change in long flashes, it is concluded that the slow (100 ms) recovery in the dark represents the slowing down of electron flow from R to PQ (as shown in Eq. 3.5(c)). Furthermore, chlorophyll <u>a</u> fluorescence yield measurements following a series of single flashes clearly indicate that bicarbonate depletion blocks electron flow from R^{2-} to PQ (Govindjee <u>et al.</u>, 1976). The absence of bicarbonate effect on the electron flow from PQ to P700 is confirmed by the absence of this effect on DQH_2 (that donates electrons specifically to PQ) to MV reaction noted earlier.

A quantitative evaluation of the present data in terms of the size of the PQ pool is not possible because of difficulties unforeseen at the time these experiments were done. The decay of the signal is incomplete in both the CO_2 -depleted and reconstituted samples. The recovery depends on the intensity of far red background illumination (Stiehl and Witt, 1969). A supplementary far red background light (720 nm) keeps the intermediates between the two light reactions in the oxidized state. A comparison of absorption changes in the presence and absence of far red background light is shown in Fig. 3.11. Although control and reconstituted $(+CO_2)$ samples are shown, comparison is still valid because in all cases the controls and $+CO_2$ were indistinguishable. The amplitude of the absorption change is more $(9 \times 10^{-4} \text{ versus } 6.8 \times 10^{-4})$ in the presence of far red background light. The increase in the amplitude is only 32% because of the low intensity (400 ergs $cm^{-2} sec^{-1}$) of far red background light used in this experiment. Stiehl and Witt (1969) had used far red background light of much higher intensity (3,000 ergs cm^{-2} sec⁻¹) to see maximum absorption change. In our experiments the duration of the long flashes and the dark time between the repetitive flashes were not optimum to see a maximal change in the magnitude of the absorption.

In the absence of bicarbonate, the halftime of the dark relaxation in long flashes should be equal to that of the slow phase in the short flash experiment. But the halftime (100 ms), observed in Fig. 3.11, is shorter by a factor of two for reasons not understood. However, the dark

80



Figure 3.11 Effect of far red background illumination on absorption changes at 265 nm induced by 85 ms repetitive flashes. The upper trace is for control (without far red background illumination) and the lower one is for reconstituted (+CO₂) thylakoids (with 400 ergs cm⁻² s⁻¹ 720 nm background illumination). The amplitudes ($\Delta I/I$) are 6.8 x 10⁻⁴ and 9 x 10⁻⁴, respectively. The halftimes ($t_{1/2}$) for dark relaxation are shown. Other conditions as described in the legend of Fig. 3.10.

81

18....

relaxation time suggested to be due to the reduction of PQ in bicarbonate depleted thylakoids is in fair agreement with the 150 ms obtained from indirect measurements (Govindjee <u>et al.</u>, 1976). To exclude possible effects of variable depletion on the amplitudes and halftimes, the absorption changes at 265 nm, induced by short and long flashes, were measured on the same batch of depleted thylakoids (Fig. 3.12). The results do not differ markedly from those discussed above where different depletions were used. In CO_2 -depleted thylakoids, a major phase of 230 ms in the short flash regime corresponds to an 85 ms phase in the long flash regime. This difference requires further investigation, but it does not affect our major conclusions.

3.4 Mechanism of Bicarbonate Action

Having established the major site of inhibition by bicarbonate depletion, we next attempted to study the mechanism of its action. For an understanding of the mechanism of the bicarbonate effect, it is important to realize that the spatial arrangement and properties of the intersystem electron carriers (especially Q, R and PQ) can be altered by certain specific treatments.

In chloroplast membranes, electrons are driven from water to NADP⁺ by two light reactions in a zig-zag pattern (Trebst, 1974; Witt, 1975; Tiemann <u>et al.</u>, 1979). According to this scheme, the reaction center complexes are oriented in the thylakoid membrane with the primary electron donors near the inner side and the corresponding primary electron acceptors on the outer side (Fig. 3.13). When light-induced vectorial charge separation takes place, electrons are transferred from the inside to the outside, whereas reduced plastoquinone drives the electrons from



Figure 3.12 Absorption changes at 265 nm induced by 20 μ s and 85 ms flashes, measured on the same batch of CO₂-depleted thylakoids. The amplitudes are 2.85 x 10⁻⁴ for the short flash and 5.8 x 10⁻⁴ for the long flash. The number of flashes were 512 and 64; darktime between flashes, 1.3 and 3.2 s, respectively. No far red background light was given. Other conditions as described in the legends of Figs. 3.6 and 3.10.



Figure 3.13 Simplified hypothetical scheme of functional and structural organization of some components of PS II and vectorial electron transport showing protolytic reaction sites in thylakoids. The water splitting enzyme system and P680 are located near the inner phase of the thylakoid The protein component covering X320(Q)-R-PQ region functions membrane. as an allosteric regulator of the electron flow from X320⁻ to the PQ pool. There are two proton uptake sites on the outer side of the membrane: one associated with the reduction of PQ and the second (not shown) due to the reduction of the electron acceptor used. Two sites of proton release on the inner side of the membrane are associated with water oxidation and the oxidation of PQ. The suggested site of CO_{2} action is shown; the rest of the scheme is based on that of Renger et al. (1976). For more details see text.

the outside to the inside -- from the acceptor side of PS II to the donor side of PS I accompanied by a net proton flux via protonation/deprotonation reactions. The water splitting enzyme, involved in the decomposition of water into 0_2 and protons, is supposed to be located near the inner side (Fowler and Kok, 1974; Zilinskas and Govindjee, 1975; Wydrzynski et al., 1978). On the other hand, the acceptor side of PS I, responsible for $NADP^+$ reduction coupled with proton uptake, is located on the outer side of the membrane (Trebst, 1974). Any structural modification of the thylakoid membrane could alter the functional pattern of electron flow. Treatment with the proteolytic enzyme trypsin can alter the structural organization of photosynthetic electron transport (Renger, 1976). Since trypsin is a large water soluble, impermeable molecule, it can attack protein components of the electron transport chain which are located near the outer surface, i.e., mainly the reducing side of the photosystem. A structural model of PS II has been described by Renger, according to which the primary electron acceptor X320 is located near the outer surface of thylakoid membrane and is covered by a protein component. The protein structure is an essential element for electron flow between X320 and the PQ pool via a two electron acceptor molecule R. The protein component is supposed to contain the binding site for DCMU type inhibitors (Pfister et al., 1979). Modification of this structural protein makes X320 accessible to external redox agents in such a way that $H_{0}0$ to $[Fe(CN)_{R}]^{3-}$ electron flow becomes DCMU insensitive (Renger, 1976). Accordingly, trypsin treatment seems to alter the binding site of DCMU like inhibitors.

3.4.1 Bicarbonate Effect in Trypsin Treated Thylakoid Membranes

Since bicarbonate depletion affects the electron flow between Q and PQ, it is possible that bicarbonate might be acting via the protein component. Fig. 3.14 demonstrates bicarbonate effect on ferricyanide Hill reaction in trypsin treated thylakoid membranes. Bicarbonate depletion was carried out as usual (see section 2.2) and after depletion thylakoid membranes were subjected to trypsin treatment (as described in section 2.3) for varying time periods. As the time of trypsin treatment was increased from 1 min to 5 min the stimulation of electron flow by bicarbonate was reduced (Fig. 3.14). Without trypsin treatment 10 mM bicarbonate showed a 2 fold stimulation. After a 1 min trypsin treatment bicarbonate stimulated O_2 evolution by only 1.6 fold. Trypsin treatment for 5 min, however, completely eliminated any effect of added bicarbonate.

Renger (1976) has shown that after trypsin treatment, $[Fe(CN)_6]^{3-}$ accepts electrons from Q. If this is indeed the case then in trypsinated membranes, the rate of electron flow from water to $[Fe(CN)_6]^{3-}$ in the absence of CO_2 should be as high as in the presence of CO_2 (except for a decrease due to the inactivation of reaction center II). However, in CO_2^{-} depleted sample (Fig. 3.14) the rate of electron flow is decreased suggesting that in our samples, $[Fe(CN)_6]^{3-}$ is accepting electrons from PS I.

The data of Fig. 3.14 show that as a function of time of trypsin treatment there is a decrease in the rate of electron flow both in CO_2 -depleted and reconstituted samples. A small inhibitory effect may be caused by a slow thermal degredation of water splitting enzyme system, occurring independently of the presence of trypsin. The decrease in electron flow by trypsin treatment is different for the two cases, i.e.,



Figure 3.14 Effect of bicarbonate on oxygen evolution in trypsinated thylakoid membranes. Pea thylakoids were depleted of bicarbonate and treated with trypsin (40 μ g/ml) at a chlorophyll concentration of 40 μ g/ml for different time periods (1-5 min). Upper curve (filled circles), with 10 mM NaHCO₃; lower curve (open circles), without NaHCO₃. [Fe(CN)₆]³⁻, 0.5 mM; other conditions as described in the legend of Fig. 3.1.

33% and 68% decrease for CO2-depleted and reconstituted samples respectively. This can be interpreted to imply that trypsin treatment prevents the stimulation of electron flow by bicarbonate in the depleted thylakoids. As a working hypothesis, we may speculate that bicarbonate functions as a regulator of the electron flow from X320 (Q) to R and finally from R to the PQ pool. Bicarbonate binding to the protein component, covering the Q-R-PQ region, may place it in a proper orientation thereby making the functional connection between X320, R and the PQ pool. Trypsin treatment may alter the bicarbonate binding site in such a way that bicarbonate can no longer bind to it. Since trypsin treatment also alters the binding site for DCMU, a very close spatial relationship between the bicarbonate binding site and the site of action of DCMU is suggested. This conclusion is consistent with the binding studies done by Stemler (1977). He observed that the removal of bound bicarbonate by SiMo is inhibited if DCMU is added prior to washing with SiMo, suggesting a close spatial relationship between the binding sites of bicarbonate and DCMU.

3.4.2 <u>Herbicide Binding to Bicarbonate Depleted Thylakoid Membranes</u>

If indeed the binding sites for bicarbonate and herbicide are on the same protein component, as suggested by the experiments described above, then the absence of bicarbonate could alter the binding properties of herbicides. Recently, Pfister and Arntzen (1979) concluded that the site of action of PS II herbicide atrazine is on the reducing side of PS II, more specifically on a protein associated with R (or B). In their binding studies they showed that 14 C-labelled DCMU was displaced by

unlabelled atrazine, thereby supporting the idea (Tischer and Strotmann, 1977) that these herbicides bind to a common site.

We studied the binding of atrazine in normal, bicarbonate depleted $(-CO_2)$ and reconstituted $(+CO_2)$ thylakoids. Binding may be described by the following equation (Tischer and Strotmann, 1977):

$$b = \frac{X_{I} + f}{K + f} \text{ or } \frac{1}{b} = (\frac{K}{X_{I}})\frac{1}{f} + \frac{1}{X_{I}}$$
 (3.6)

where b denotes the concentration of bound inhibitor/mg chlorophyll; f is the concentration of free inhibitor in the solution; X_I is the concentration of inhibitor binding sites; and K is the binding constant. The inverse of the intercept $(1/X_I)$ on the ordinate of a double reciprocal plot (1/b versus 1/f) is a measure of the number of binding sites on a chlorophyll basis. The binding constant (K) is calculated from the slope (K/X_I) of the curve as X_I is already known from the intercept. The binding of ¹⁴C-labelled atrazine as a function of inhibitor concentration, plotted as [Ch1]/[bound inhibitor] versus 1/[free inhibitor] (double reciprocal plots) is shown in Fig. 3.15. The ordinate is mg Ch1/nmoles of bound herbicide, and the abscissa is expressed in μM^{-1} .

Binding of 14 C-labelled herbicide to thylakoid membranes was analysed in the herbicide concentration range of 0.053 nmoles to 0.26 nmoles with the chlorophyll concentration of 50 µg. In the double reciprocal plots (Fig. 3.15), curves for control, bicarbonate depleted (-CO₂) and reconstituted (+CO₂) samples show approximately the same intercept on the y-axis, indicating that the number of binding sites is not affected by the depletion procedure. Binding of 14 C-atrazine for the three (control, bicarbonate depleted and reconstituted) cases is



Figure 3.15 Binding of 14 C-atrazine to control, CO_2 -depleted and reconstituted pea thylakoids. Double reciprocal ([Ch1]/[bound atrazine] versus 1/[free atrazine]) plots are shown. Thylakoid membranes were incubated with various concentrations of 14 C-labelled atrazine. The amount of bound inhibitor was calculated from the difference between the total radioactivity added to the thylakoid suspension and the amount of free inhibitor found in the supernatant after centrifugation. For other details see text and Chapter 2 (section 2.9).

2 nmoles/mg Chl, i.e., 1 binding site per 500 chlorophyll molecues. This ratio agrees well with earlier reported values (Pfister <u>et al.</u>, 1979).

From the double reciprocal plots, the K values for control, bicarbonate depleted and reconstituted samples were calculated as 3.4×10^{-8} , 1.2×10^{-7} and 4.9×10^{-8} M, respectively. This implies that in the bicarbonate depleted sample the affinity of atrazine to its binding site is reduced. It appears that the absence of bicarbonate somehow changes the "conformation" of a protein component acting on the reducing side of PS II, and, this change may lead to a change in the affinity of the herbicide to the protein. Upon the addition of bicarbonate to $CO_2^$ depleted thylakoids, binding affinity for atrazine is restored and becomes comparable with that of the control.

Upon bicarbonate depletion a fraction of the thylakoid membranes remains unaffected, i.e., a fraction still shows normal electron flow (see section 3.3.2). From herbicide binding studies it appears that bicarbonate depletion changes the affinity of the herbicide to its binding site, as noted above. We were interested in finding out if all thylakoid membranes are partially affected or if only a fraction of the membranes is completely inactivated. This was achieved by measuring the concentration dependence of the inhibitory effect of atrazine on the rate of PS II electron transport (Fig. 3.16). From the data shown in Fig. 3.16, I_{50} values (a concentration of herbicide giving half-maximal inhibition of electron transport) were calculated to be ~0.2 μ M for all the three cases: control, $-CO_2$ and $+CO_2$; the I_{50} value of ~0.2 μ M is not too far from the value of 0.5 μ M found by Pfister and Arntzen (1979) for



Figure 3.16 Inhibition of DCPIP reduction in control, CO_2 -depleted and reconstituted thylakoids by atrazine. Thylakoids were suspended, at a concentration of 5 µg Chl/ml, in a medium containing 50 mM phosphate (pH 6.8), 100 mM NaCl, 100 mM sodium formate, 0.05 mM DCPIP, 1 mM NH₄Cl, and 1 µM gramicidin. For other details see Chapter 2 (section 2.6).
their control chloroplasts. If bicarbonate depletion affects the affinity of atrazine for the total population of thylakoids we expect to see a different I_{50} for the depleted $(-CO_2)$ thylakoids. However, the I_{50} value of thylakoids did not change upon depletion suggesting that the membranes which do not undergo depletion have the same affinity for atrazine as the control or reconstituted thylakoids. Therefore, the difference in binding affinities (as calculated from herbicide binding experiments, Fig. 3.15) must be due to a change in the affinity of only those thylakoid membranes which were inactivated completely by depletion of CO_2 . This suggests that the CO_2 effect is an all or none phenomenon and it lends support to the physiological significance of the CO_2 effect (cf. section 3.2.1).

3.4.3 Measurement of pH Changes in the Inner and Outer Phase of

Thylakoids

During illumination of thylakoids there is an uptake of protons from the external phase and a release of protons into the interior space (Witt, 1975). There are four protolytic reaction sites in thylakoid membranes (see Fig. 3.13): two proton uptake sites on the outer side of the membranes (Schliephake <u>et al</u>., 1968) and two sites of proton release on the inner side (Junge and Ausländer, 1973). One of the sites on the outer surface is due to the reduction of the electron acceptor used. The second site of proton uptake is associated with the reduction of plastoquinone (PQ + 2H⁺ + 2e⁻ > PQH₂) which acts as a shuttle such that its oxidation releases protons into the inner side of the membrane. The other site of proton release into the inner phase is attributed to the oxidation of water (1/2 H₂O > 1/4 O₂ + H⁺ + e⁻). The sites of proton binding from the outer phase and proton release in the inner phase have been studied with pH indicating dyes by flash spectrophotometry (Junge <u>et al.</u>, 1979).

Thylakoid membranes depleted of bicarbonate show an increased $t_{1/2}$ for electron flow from R to PQ, i.e., formation of plastohydroquinone is slowed down (see section 3.3). This suggests that the absence of bicarbonate could affect proton uptake and release at the plastoquinone level. It seemed, therefore, of interest to study these protolytic changes in bicarbonate depleted thylakoid membranes.

3.4.3.1 Measurement of Internal pH

Neutral red can be used as an indicator specific for pH changes in the internal phase by selectively buffering away pH changes in the outer phase by bovine serum albumin (BSA) which is a non penetrating buffer. Neutral red is a very sensitive pH indicator with a response time of at least 0.3 ms (Ausländer and Junge, 1975). Neutral red distributes itself to the external as well as internal space of thylakoids but the addition of BSA neutralizes the dye in the external medium. Thus, the absorption change of neutral red, measured at 524 nm, in the presence of BSA represents an acidification of the internal space. A small background absorption change persists even after the addition of imidazole which permeates across the membrane and neutralizes the dye in the internal phase too. Therefore, to obtain a true absorption change of neutral red due to a pH change, the signal in the presence of BSA plus imidazole is substracted from the signal with BSA alone as was done for data shown in Fig. 3.17. In control thylakoids, absorption change as indicated by neutral red shows biphasic kinetics for the release of protons into the internal



Figure 3.17 Kinetics of proton release into the internal phase of spinach thylakoids as indicated by absorption change of neutral red at 524 nm. The signals shown in the figure represent a difference between two transient signals (signal in the presence of BSA and neutral red minus signal in the presence of BSA, neutral red and imidazole). Thylakoids were suspended, at a concentration of 10 µg Chl/ml, in a medium containing 20 mM KCl, 2 mM MgCl₂, 0.5 mM [Fe(CN)₆]³⁻, 0.3 µM non-actin, 10 µM neutral red and 1.3 mg/ml BSA. Saturating single-turnover flashes ($t_{1/2} = 15$ µs) were used; darktime between flashes, 10 s. Signal averaged over 10 flashes. For other details see Chapter 2 (section 2.8).

phase. The more rapid phase is attributable to the protons released from the water oxidizing system, and the slower phase to the protons released from the oxidation of plastohydroquinone (Ausländer and Junge, 1975). In bicarbonate depleted thylakoids, only the fast phase due to the water oxidizing system is seen which is consistent with the previous conclusion that bicarbonate depletion does not significantly affect the oxidizing side of PS II (see section 3.3.1). The amplitude of the rapid phase is slightly smaller indicating a partial inactivation of the centers. In the present experiments, measurement on the bicarbonate buffered away the pH changes. The results with bicarbonate depleted thylakoids indicate, as noted above, that the protons released in the internal space are only from water oxidation, and there is no release of protons from the plastoquinone pool.

3.4.3.2 Measurement of External pH

pH changes in the outer aqueous phase of the thylakoid membrane were measured with the pH indicating dye bromocresol purple. Bromocresol purple is a non permeating pH indicator dye (Ausländer and Junge, 1975). The absorption changes of bromocresol purple are proportional to the changes in proton concentration in the outer phase of thylakoids. The pH indicating absorption change of bromocresol purple at 574 nm is shown in Fig. 3.18. The absorption changes shown have been corrected for the contribution of the intrinsic absorption changes of thylakoid membranes by subtracting the background signal obtained in the presence of the buffer. In the control thylakoid trace, there is an initial alkalization due to proton uptake by the plastoquinone pool. This is followed by a



Figure 3.18 Absorption changes of bromocresol purple at 574 nm induced by a single-turnover flash in control and CO_2 -depleted spinach thylakoids. Reaction mixture (15 ml) contained 20 mM KCl, 2 mM MgCl₂, 20 μ M bromocresol purple and spinach thylakoids at a concentration of 10 μ g Chl/ml. Signal averaged over 10 flashes ($t_{1/2} = 15 \ \mu$ s); darktime between flashes, 10 s. The ordinate is plotted in arbitrary units. For other details see Chapter 2 (section 2.8).

slow acidification due to the release of internal protons (from plastohydroguinone oxidation and water oxidation) to the exterior of the thylakoids. In the bicarbonate depleted thylakoids, there is no-proton uptake by the PQ pool at the outer side of the membrane. The final irreversible acidification level in bicarbonate depleted thylakoids is approximately the same as in the control. However, the total protons released in the bicarbonate depleted samples is about half that of the control. This implies that there is one less proton released (per electron transferred) into the inside of the bicarbonate depleted thylakoids. The acidification in bicarbonate depleted thylakoids indicates protons released during water oxidation. The kinetics of the acidification are the same in both the control and the bicarbonate depleted thylakoids; this implies that permeability of thylakoid membranes for protons is not affected by bicarbonate depletion. This is in agreement with the freeze fracture electron microscopic studies (done in collaboration with Dr. A. Keresztes) which showed that membrane structure does not change markedly by the depletion procedure.

These data on the flash induced H^+ uptake and release in the thylakond vesicles indicate that there is no proton translocation at the plastoquinone pool. Absorption changes at 265 nm show (see section 3.3.2) that in bicarbonate depleted thylakoids, plastoquinone is still being reduced, but at a much slower rate. Accordingly, we had expected to see an uptake and a release of proton with slower kinetics. Since plastoquinone does receive electrons and forms PQ^{2-} we may speculate that in bicarbonate depleted thylakoids, electron flow via the PQ pool takes place without the formation of PQH_2 . However, the experimental

conditions of pH measurements (unbuffered) are, by necessity, entirely different from that of the PQ measurements (buffered). These results raise several questions that need further investigation.

3.5 Concluding Remarks

The results presented in this chapter show that bicarbonate phenomenon studied here may have a physiological importance since it is observed even at low concentrations (close to the <u>in vivo</u> concentrations) of added bicarbonate (section 3.2.1). Maximum bicarbonate effect is seen in the pH range of 6 to 7 (section 3.2.2); and it appears that the bicarbonate stimulation of the Hill reaction is not related to the effect of bicarbonate on photophosphorylation (section 3.2.3).

Using artificial electron donors and acceptors, it is shown that the major bicarbonate effect is located somewhere between the electron acceptor Q (X320) and the PQ pool (section 3.3.1). Thylakoids in the presence of DCMU, which blocks electron flow from Q⁻ to the PQ pool, and silicomolybdate, which accepts electrons from Q⁻, show no siginificant bicarbonate stimulation of electron flow. However, a 6-7 fold stimulation is clearly observed on electron flow from H₂O to oxidized DAD in the presence of DBMIB which acts as an inhibitor of electron flow beyond the PQ pool. In the same thylakoid preparation, no measurable effect of bicarbonate is observed in a PS I reaction as monitored by electron flow from reduced DAD to MV in the presence of DCMU. The effect of bicarbonate on individual electron transfer reactions involving Q, R, PQ and P700 was also examined by monitoring their absorption changes (section 3.3.2). In bicarbonate depleted thylakoids, formation of R²⁻ is retarded by a factor of 10-15 (from a $t_{1/2}$ of ~ 500 µs to ~ 7 ± 3 ms) and the

reoxidation of R^{2-} by the PQ pool becomes the new bottleneck reaction $(t_{1/2} = 100 \text{ to } 200 \text{ ms})$; the bottleneck reaction of normal thylakoids is the reoxidation of PQH₂ $(t_{1/2} = 25-50 \text{ ms})$.

Based on experiments presented in section 3.3 of this Chapter, it is suggested that bicarbonate stimulation of electron flow between Q and the PQ pool my be brought about by an interaction of bicarbonate with a protein component associated with the reducing side of PS II. Trypsin treatment of thylakoids, which selectively alters this protein component, prevents bicarbonate stimulation of electron transport (section 3.4.1). Moreover, bicarbonate depletion alters the binding affinity of the herbicide atrazine to the membrane suggesting a requirement of bicarbonate for the proper conformation of the protein (section 3.4.2). Finally, the absence of proton translocation at the PQ level gives further support to the involvement of bicarbonate via the protein shield covering the Q-R-PQ region.

CHAPTER 4

STUDIES ON THE ROLE OF MANGANESE IN THE OXYGEN EVOLVING SYSTEM 4.1 Introduction

Very little is known about the mechanism of 0_2 evolution in green plants. Several experiments suggest that some form of membrane bound manganese is involved in this process (see reviews by Radmer and Cheniae, 1977; Harriman and Barber, 1979). The kinetic characterization of 0_2 production has provided considerable information about the reactions leading to oxygen evolution involving charge accumulation. For the generation of one molecule of oxygen, four oxidizing equivalents have to be accumulated by four successive photoacts (Joliot and Kok, 1975). Since the manganese ion can take on a number of relatively stable oxidation states, it is a good candidate for charge accumulation (Cheniae, 1970; Olson, 1970; Early, 1973; Renger, 1970, 1977). However, experimental evidence to suggest the involvement of different oxidation states of Mn in 0_2 evolution was provided only recently (Wydrzynski, 1977; Govindjee <u>et al.</u>, 1977; Wydrzynski <u>et al.</u>, 1978; Wydrzynski and Sauer, 1979).

No definite ESR signal has been observed from manganese bound to chloroplasts (with the possible exception of one report by Siderer <u>et</u> <u>al</u>., 1977). As a result, attempts to determine the relationship between bound manganese and O_2 evolution have relied mainly on the correlation between the amount of bound Mn and O_2 evolution activity. Recently, however, NMR studies have been used to monitor the amount of bound Mn (Wydrzynski <u>et al</u>., 1975, 1978). The manganese ion usually maintains water ligands even when it is bound to macromolecules. Protons of water

molecules, coordinated to manganese, experience the large magnetic dipole of the unpaired electrons of manganese. The resulting electron-nuclear dipole-dipole interaction leads to an efficient relaxation of the water protons. Thus, the proton relaxation rates of water may be used to monitor any changes of bound Mn in thylakoid membranes. For a detailed introduction to the role of manganese in 0_2 evolution see Chapter 1 (sections 1.4 and 1.5).

In this chapter parallel measurements of NMR and ESR are described to correlate 0_2 evolution activity with the amount of bound and free manganese. Experiments indicate that treatment of thylakoids with reductants TPB, NH_2OH and H_2O_2 , convert higher oxidation states of Mn to the more efficient relaxer species Mn(II) (section 4.2). Titration of thy lakoids with TPB indicates the presence of several distinct pools of Mn or other paramagnetic species. The effect of NH₂OH appears two fold: conversion of higher oxidation states of Mn to Mn(II) and release of bound Mn. The release of bound Mn can be also brought about by the addition of low (10-20 mM) concentrations of $MgCl_2$ (section 4.3). The pool of Mn released by this treatment is not related to 0_2 evolution as determined by parallel measurements of 0_2 evolution activity. Measurement of T_2^{-1} and Mn content of the purified LHC show the presence of a tightly bound Mn that accounts for about one third of the total functional Mn. In the last part of this chapter (section 4.4) a good correlation is shown for T_2^{-1} , Mn content and O_2 evolution activity. However, treatments like heating and exposure to extreme pH lead to contributions from paramagnetic species other than Mn(II), and/or from changes in the accessibility of Mn to water protons.

4.2 Effects of Reductants on the Manganese of Thylakoid Membranes

4.2.1 Effects of Tetrephenylboron

In thylakoid membranes, bound manganese is believed to exist in different oxidation states (Wydrzynski <u>et al.</u>, 1975, 1978). Addition of redox reagents can shift the oxidation states of bound manganese. However, several redox reagents upon oxidation give rise to free radical intermediates which can interfere with the proton relaxation rates. The reductant TPB does not form free radical intermediates (Geske, 1959, 1962), thus it was used to study the reduction of manganese in thylakoid membranes. Most of the published work on reduction by TPB deals with its mechanism in organic solvents (Geske, 1959, 1962). Under these conditions biphenyl- and diphenylboronium ions have been shown to be the reaction products of a two electron process. TPB is known to interact strongly with the components of the 0_2 evolving center in thylakoids (Homann, 1972; Erixon and Renger, 1974), although preliminary studies have suggested that TPB acts as a 1-electron donor in thylakoids (Erixon and Renger, 1974).

Effect of TPB on T_2^{-1} , rate of O_2 evolution and rate of DCPIP reduction of dark-adapted thylakoid membranes is shown in Fig. 4.1. The plot of T_2^{-1} (filled squares) as a function of added TPB shows four distinct plateaus which might be related to the successive reduction of different pools of ions titratable with TPB. This result is similar to the preliminary results obtained for longitudinal relaxation rates T_1^{-1} (Wydrzynski <u>et al</u>., 1976). In the experiments shown in Fig. 4.1, T_2^{-1} increases with plateaus appearing in the following ranges: 1 mM, 5 mM, 10 mM and 40 mM TPB. There is no increase in T_2^{-1} up to



Fig. 4.1 Effect of TPB on T_2^{-1} , O_2 evolution and DCPIP reduction in pea thylakoids. The difference between DCPIP reduction and O_2 evolution (crosses) indicates electron donation by TPB. For T_2^{-1} measurements the suspension medium contained 50 mM HEPES (pH 7.5), 400 mM sucrose and 10 mM NaCl; chlorophyll concentration, 2 mg/ml. T_2^{-1} was measured at 27 MHz using the CPMG method. For O_2 evolution 0.5 mM [Fe(CN)₆]³⁻, 1 mM NH₄Cl and 0.1 µM gramicidin were included in the reaction mixture; chlorophyll concentration, 20 µg/ml. For DCPIP reduction 0.03 mM DCPIP was added; chlorophyll concentration, 10 µg/ml. Obs. stands for observed. Pea thylakoids were used for all experiments described in Chapter 4.

2 mM TPB added. The rate of 0_2 evolution in the presence of TPB indicates the amount of electrons donated by H_20 , whereas the total electron flow from water and TPB is measured by the amount of DCPIP undergoing reduction. For the measurements of 0_2 evolution and DCPIP reduction, thylakoid membranes were incubated for 30 min with different concentrations of TPB. An aliquot of the incubated sample was diluted 200 fold before measurements were made. There is an inhibition of 0_2 evolution in the presence of TPB (Fig. 4.1, open circles); this inhibition is 100% at 2 mM TPB. This is expected if TPB acts as a competitive electron donor and donates electrons in preference to water (Homann, 1972). For DCPIP reduction only the initial rates are shown (Fig. 4.1, filled circles) because there is a progressive inhibition of electron transport during the illumination period. This inhibitory effect of TPB on DCPIP reduction is probably due to the formation of oxidation products of TPB during illumination as suggested by Homann (1972). A comparison of the rates of 0_2 evolution and DCPIP reduction shows that incubation with 1 mM TPB causes 80% inhibition of 0_2 evolution whereas electron flow from both TPB and H_2O to DCPIP is slowed down by only 26%. A plot of the difference between DCPIP reduction and 0_2 evolution (Fig. 4.1, crosses) represents the net electron flow from TPB to DCPIP. As noted above, incubation of thyalkoid membranes with 2 mM TPB almost completely eliminates 0_2 evolution.

We interpret the data of Fig. 4.1 as follows: 0_2 evolution decreases as the concentration of TPB increases because TPB donates electrons in competition with H₂0. There is no increase in T₂⁻¹ up to 2 mM TPB and the increase in T₂⁻¹ starts after 0_2 evolution has completely stopped, i.e., water is no longer acting as a donor and all electrons are coming from TPB. One possibility is that at low concentrations (≤ 2 mM) TPB donates electrons at a site other than the 0_2 evolving site, thus, causing no change in the relaxation rates. At higher concentrations (> 2 mM) TPB perhaps donates electrons to the 0_2 evolving and other Mn-containing systems such that T_2^{-1} starts to increase indicating a reduction of the Mn of the thylakoid membranes. We cannot rule out the possibility that a significant part of the TPB induced increase in T_2^{-1} is not related to the 0_2 system.

The decline in the net electron flow from TPB (as indicated by crosses in Fig. 4.1) could be due to (a) an inhibition caused by the oxidation products of TPB as suggested by Homann (1972) or (b) a reduction of other components of the electron transport chain thereby preventing electron flow to DCPIP.

In order to see if addition of TPB to thyalkoid membranes releases Mn(II), ESR measurements were carried out for samples treated with TPB concentrations up to 50 mM. Fig. 4.2 shows ESR spectra (plotted as the first derivative of absorption) of thylakoid membranes in the presence of TPB. The sharp signal in the middle of the control curve at g = 2 is the well known Signal II, associated with PS II (Weaver, 1968). When Mn(II) is released from its bound state, a 6-line pattern resulting from the hyperfine interaction of the unpaired electrons with the 5/2 spin of the 55Mn nucleus (Garrett and Morgan, 1966) is observed. Addition of TPB showed only a slight change in the amplitude of the 6-line spectrum, thereby suggesting that TPB does not release any significant amount of bound Mn at least not in the form of Mn(II). In a control experiment it



Fig. 4.2 Room temperature ESR spectra of thylakoid membranes in the presence of different concentrations of TPB. ESR spectra (here and else-where in this chapter) are shown as the first derivative of absorption. The suspension medium contained 50 mM HEPES (pH 7.5), 400 mM sucrose and 10 mM NaCl; chlorophyll concentration, 2 mg/ml. Instrumental conditions: microwave power, 50 mW; modulation amplitude, 10 G; time constant, 0.3 s; scan rate, 150 G/min; receiver gain, 1.25 x 10^4 .

was shown that TPB (0.5-50 mM range) has no effect on ESR signal of $MnCl_2$ (10 μ M) in solution. Signal II associated with PS II shows a decrease as the concentration of TPB is increased; thus us consustent with other data showing that unhibition of the 0_2 evolving system leads to a decrease in this signal (Chen and Wang, 1974).

When thylakoids are washed once in 10 mM NaCl (as described in the legend of Fig. 4.3) the TPB titration curve still shows plateaus but these do not appear as distinct as for the unwashed membranes (Fig. 4.3, open circles). The percent increase in T_2^{-1} is approximately the same (60%) as for the control (Fig. 4.1). If 20 mM MgCl₂ is added to these washed membranes, and then the titration is carried out, there is only ~25% increase in T_2^{-1} with increasing TPB concentration (Fig. 4.3, filled circles). The Mg induced decrease in T_2^{-1} in the absence of TPB is due to the release of Mn from the very loosely bound pool of manganese, as monitored by the appearance of ESR signal of free Mn(II) (see below). Both 0_2 evolution and DCPIP reduction rates, measured as a function of TPB concentration, are unaffected in the washed samples by the addition of 20 mM MgCl₂ (Fig. 4.4). This supports the conclusion that addition of 20 mM MgCl₂ does not release functional manganese associated with the 0_2 evolving system.

Release of Mn(II) was measured using the ESR method in thylakoid samples in the presence of MgCl₂ and/or TPB. Fig. 4.5 shows that when 20 mM MgCl₂ is added to thylakoid membranes there is some release of Mn(II); this release of Mn(II) could explain the observed decrease in T_2^{-1} (shown in Fig. 4.3). Addition of 25 mM TPB also shows only a slight release of Mn(II). Surprisingly, when TPB is added to thylakoid



Fig. 4.3 T_2^{-1} of washed pea thylakoid membranes as a function of exogenously added TPB with and without MgCl₂. T_2^{-1} was measured at 27 MHz and at 25°C using the CPMG method. Thylakoid membranes were washed once in unbuffered 10 mM NaCl and resuspended in 50 mM HEPES (pH 7.5), 400 mM sucrose and 10 mM NaCl; chlorophyll concentration, 1.7 mg/ml.



Fig. 4.4 Effect of TPB on 0_2 evolution and DCPIP reduction in washed thy lakoid membranes with and without MgCl₂. Circles, 0_2 evolution; squares, DCPIP reduction; open symbols, without, and closed symbols, with 20 mM MgCl₂. Other conditions as described in the legend of Fig. 4.1.



Fig. 4.5 Room temperature ESR spectra of thylakoid membranes in the presence of TPB, $MgCl_2$ and TPB plus $MgCl_2$. Thylakoid membranes were washed once in unbuffered 10 mM NaCl and resuspended in 50 mM HEPES (pH 7.5), 400 mM sucrose and 10 mM NaCl; chlorophyll concentration, 2 mg/ml. Instrumental conditions: microwave power, 50 mW; modulation amplitude, 10 G; time constant, 1.3 s; scan rate, 250 G/min; receiver gain, 1.25 X 10^4 .

membranes already containing 20 mM MgCl₂, the amount of free Mn(II) detected by ESR is more than the additive signals of the two treatments (25 mM TPB and 20 mM MgCl₂) separately. This could be due to the enhanced release of Mn(II) by the divalent cation Mg, since in the presence of TPB more Mn(II) may be available for displacement by Mg. This increase in the release of bound Mn(II), leading to a lower concentration of bound Mn(II) in thylakoids, may be responsible for a lowered TPB-induced increase in T_2^{-1} (Fig. 4.3). In a control experiment it was shown that addition of 20 mM MgCl₂ causes a small (15-20%) increase in the ESR signal of 10 μ M MnCl₂ solution but this does not affect our conclusion as Mg-induced increase in thylakoids is much larger (Fig. 4.5). Addition of 25 mM TPB to MnCl₂ solution containing 20 mM MgCl₂ caused no further increase in the ESR signal.

It has been suggested by Erixon and Renger (1974) that TPB exerts its effect on PS II primarily because of its ability to react with oxidants in the water splitting reaction complex. However, the oxidant has not yet been identified. Each oxygen evolving center has 4-6 Mn atoms associated with it (see review by Radmer and Cheniae, 1977). We suggest that addition of TPB to thylakoids causes two separate effects depending upon the concentration used. At low concentrations (≤ 2 mM) TPB does not reduce Mn as there is no increase in T_2^{-1} ; at these concentrations TPB probably donates electrons at a site distinct from the O_2 evolving system. The addition of Anigher (> 2 mM) concentration of TPB reduces the oxidized form of Mn to the more efficient water proton relaxer species Mn(II) thereby causing an enhancement in T_2^{-1} . This implies an interaction of TPB with the Mn of thylakoids. This ability of TPB has also been found by direct Mn X-ray fluorescence edge measurement studies (T. Wydrzynski, personal communication). Several plateaus seen in the TPB titration curve may be indicative of several fractions of Mn or other paramagnetic species not related to 0_2 evolution.

Thylakoid membranes washed with 10 mM NaCl appear as unstacked membrane structures (Arntzen, 1978). In the unstacked thylakoids the distinct plateaus in the TPB titration curve are much less obvious probably because the accessibility of TPB to the titrable paramagnetic ions is altered. The decreased TPB effect in the presence of MgCl₂ (Fig. 4.3) is due to an increase in the release of Mn(II) by MgCl₂ (Fig. 4.5) when more Mn is in the Mn(II) state due to reduction caused by TPB. However, addition of MgCl₂ shows no effect on the rates of O_2 evolution and DCPIP reduction (Fig. 4.4). This is probably because in the low concentration range (< 2 mM), where O_2 evolution activity is measured, TPB is unable to reduce Mn. The T_2^{-1} measurements (Fig. 4.1) show very clearly that TPB starts influencing the conversion of higher oxidation states of Mn to Mn(II) only when the concentration of TPB is above 2 mM. 4.2.2 Effects of Hydroxylamine

Hydroxylamine is known to serve as an electron donor to PS II (Vaklinova <u>et al.</u>, 1966; also see Mohanty <u>et al.</u>, 1971 for other references). At high concentrations it specifically inhibits the reactions on the oxidant side of PS II that lead to 0_2 evolution (Radmer and Cheniae, 1977). Chloroplasts treated with micromolar concentrations (1 NH₂OH/20 Chl molecules) of NH₂OH can evolve 0_2 but the 0_2 flash yield dependence is altered (Bouges, 1971); the maximal yield is at the 5th flash rather than at the 3rd flash. At higher concentrations (6 NH_2OH/Chl molecule), the O_2 evolving capacity is destroyed but NH_2OH can still act as an electron donor to PS II (Cheniae and Martin, 1971). Recently Radmer (1979) has elucidated the mechanism of electron donation by NH_2OH using mass spectrophotometric measurements in chloroplasts illuminated with saturating light flashes. The proposed mechanism involves the removal of one electron from each molecule of NH_2OH for electron flow via PS II to form the oxidized product ($NHOH \cdot$): $2NH_2OH$ $\frac{PS II}{}$ $NHOH \cdot + H^+ + e^-$. Subsequently, two molecules of $NHOH \cdot$ react to form N_2 : $2NHOH \cdot \rightarrow N_2 + 2H_2O$. In this section experiments are described on parallel measurements of T_2^{-1} and ESR detectable Mn(II) as released by NH_2OH .

Fig. 4.6 shows a plot of T_2^{-1} as a function of increasing NH₂OH concentration. Initially, as the concentration of NH₂OH is increased up to 1.0 mM, T_2^{-1} is enhanced (~40%), but at higher concentrations there is a decrease in T_2^{-1} (filled circles). A similar trend is seen for thylakoids washed in 10 mM NaCl. This indicates that the washing of thylakoids with 10 mM NaCl does not remove any Mn that undergoes reduction by NH₂OH. Addition of 20 mM MgCl₂ to the washed thylakoids shows a decreased effect (~25%) of NH₂OH on the enhancement of T_2^{-1} (open squares). This decreased enhancement in T_2^{-1} by NH₂OH could be due to the release of Mn(II) by MgCl₂ (see Fig. 4.5). The Mg induced decrease in T_2^{-1} in the absence of NH₂OH is due to the release of Mn from the very loosely bound pool of Mn (see section 4.3).

Measurements of ESR detectable Mn(II) are shown in Fig. 4.7. The appearance of the 6 line spectrum is typical for free Mn(II) in an



Fig. 4.6 Effect of NH_2OH on T_2^{-1} of thylakoid membranes. Chlorophyll concentration, 2.5 mg/ml. Time of incubation, 30 min. Other condi-tions as described in the legend of Fig. 4.3.

5 . . .



Fig. 4.7 Room temperature ESR spectra of thylakoid membranes in the presence of different concentrations of NH_2OH . The suspension medium contained 50 mM HEPES (pH 7.5), 400 mM sucrose, and 10 mM NaCl; chloro-phyll concentration 2 mg/ml. Instrumental conditions: microwave power, 50 mW; modulation amplitude, 10 G; time constant, 0.3 s; scan rate, 250 G/min; receiver gain, 8 x 10^3 .

aqueous environment. The release of Mn(II) becomes evident even at low concentrations (0.5 mM NH₂OH). The amount of Mn(II) released from the bound state increases as the concentration of added NH₂OH is increased. We interpret the effect of NH₂OH on T_2^{-1} as due to two opposing effects: (i) reduction of the oxidized form of bound Mn to bound Mn(II) which would increase T_2^{-1} and (ii) release of bound Mn to the aqueous phase so that there is a decrease in T_2^{-1} .

The ESR detectable Mn(II) signal was also measured for thylakoids treated with MgCl₂ (20 mM) and/or NH₂OH (10 mM) (Fig. 4.8). Addition of 10 mM NH₂OH releases Mn(II); this released Mn(II) is from the Mn of the 0_2 evolving system because at this concentration of NH₂OH, 0_2 evolution activity is lost. The release of Mn(II) by the addition of 20 mM MgCl₂ is not associated with 0_2 evolution activity (see Fig. 4.4 and section 4.3) since the 0_2 evolution rate is not affected as measured by the Clark electrode. Thus, the release of Mn(II) by $MgCl_2$ is from the very loosely bound pool of Mn which is not related to 0_2 evolution. If $MgCl_2$ (20 mM) and NH_2OH (10 mM) are added together the amount of Mn(II) released is more than the additive effect of the two seen separately. Addition of MgCl₂ (20 mM) and NH₂OH (10 mM) to MnCl₂ (10 μ M) solution does not show such an increase in the ESR signal. It is likely that in thylakoids the reduction of bound Mn to Mn(II), in the presence of NH_2OH , allows more Mn(II) to be released by $MgCl_2$. Alternatively, the presence of $MgCl_2$ may facilitate the release of Mn(II) by NH₂OH.

In order to separate the two effects (reduction of the higher oxidation states of Mn to Mn(II) and the release of Mn(II)) of NH₂OH, we studied the dependence of T_2^{-1} upon the time that elapsed after



Fig. 4.8 Room temperature ESR spectra of thylakoid membranes in the presence of NH_2OH , $MgCl_2$ and NH_2OH plus $MgCl_2$. Chlorophyll concentration, 2 mg/ml. Other conditions as described in the legend of Fig. 4.5.

adding different concentrations of NH2OH. The results of these experiments are shown in Figs. 4.9, 4.10 and 4.11 for seven concentrations of NH₂OH ranging from 0.01 mM to 100 mM. At 0.01 mM NH₂OH (1 NH₂OH to 200 Ch1; Fig. 4.9, open circles), there is no effect on T_2^{-1} even up to 30 min, but as the concentration of NH_2OH is increased to 0.05 mM (1 $NH_{2}OH$ to 40 Ch1; Fig. 4.9, open squares) and 0.1 mM (1 $NH_{2}OH$ to 20 Ch1; Fig. 4.9, open triangles), T_2^{-1} begins to increase as a function of time. As the concentration is increased further (1 NH_2OH to 2 Chl, 1 mM; and 1 NH₂OH to 1 Ch1, 2 mM) T_2^{-1} increases to a peak value at about 5 min and then declines (Fig. 4.10, open circles and open squares, respectively). At still higher concentrations (5 NH₂OH to 1 Chl, 10 mM) the decline begins at < 2 min (Fig. 4.11, open circles) and finally at 100 mM NH_2OH (50 NH_2OH to 1 Chl) only a steady state lower level value is seen (Fig. 4.11, open squares). This steady state level, however, is still higher than the value obtained for thylakoids without any added NH_2OH . This implies that NH_2OH releases only a fraction of the total Mn and the remaining bound Mn is in the Mn(II) state. This remaining pool of Mn could be from the tightly bound pool of Mn to which no role has been assigned yet.

The time course of the release of Mn(II) upon the addition of 0.5 mM NH_2OH was followed by measuring the area under the second peak of the six line ESR spectrum. The release of Mn(II) becomes evident as soon as the first measurement is made at 3 min and there is a subsequent increase in the signal amplitude as the time progresses (Fig. 4.12). The two opposing effects of NH_2OH are clear, both taking place simultaneously; but initially the increase in T_2^{-1} due to the formation of Mn(II) is



Fig. 4.9 T_2^{-1} as a function of time after the addition of 0.01 to 0.1 mM NH₂OH. The suspension medium contained 50 mM HEPES (pH 7.5), 400 mM sucrose and 10 mM NaCl; chlorophyll concentration, 2 mg/ml. Other conditions as described in the legend of Fig. 4.3.



Fig. 4.10 T_2^{-1} as a function of time after the addition of 1 or 2 mM NH₂OH. Other conditions as described in the legend of Fig. 4.9.



Fig. 4.11 T_2^{-1} as a function of time after the addition of 10 or 100 mM NH₂OH. Other conditions as described in the legend of Fig. 4.9.



Fig. 4.12 Time course of the increase in T_2^{-1} and the release of manganese induced by the addition of 0.5 mM NH₂OH to thylakoid membranes. The data for the free manganese (labelled ESR) was obtained by measuring the area under the second peak of the six line ESR spectrum. The suspension medium contained 50 mM HEPES (pH 7.5), 400 mM sucrose and 10 mM NaCl; chlorophyll concentration, 2 mg/ml. Instrumental conditions for ESR experiment as described in the legend of Fig. 4.2.

much more than the decrease in T_2^{-1} as a result of release of bound Mn. The decrease in T_2^{-1} becomes visible only after a longer time period (Figs. 4.10 and 4.12), or at higher concentrations of NH₂OH (Fig. 4.6).

Measurements of 0_2 yield as a function of flash number (Bouges, 1971) showed that when thylakoid membranes are incubated with low concentration (0.5 mM) NH₂OH, the 0_2 flash yield dependence is altered. In the normal case 0_2 evolution flash pattern shows the first peak in 0_2 evolution at the 3rd flash while in the NH₂OH treated sample 0_2 evolution is delayed until the fifth flash and, thereafter, it oscillates with a period of four. As the concentration of NH₂OH is increased further the yield of 0_2 evolution is affected and at high concentrations (>> 1 mM) the 0_2 evolving capacity is destroyed. Even at low concentrations of NH₂OH the yield of 0_2 evolution is less than that of the control sample suggesting that the two processes (reduction and release of Mn) are taking place simultaneously.

The inhibition of 0_2 evolution by NH₂OH is a result of several processes: (i) the reduction of an unidentified oxidizing equivalent (the S state) necessary for 0_2 evolution (Bouges, 1971; Bouges-Bocquet, 1973a); (ii) the release of bound Mn leading to the inactivation of the 0_2 evolving system (Cheniae and Martin, 1970, 1971); and (iii) the inhibition of electron transfer between Z and P680⁺, i.e., in the ZP680⁺ to Z⁺P680 reaction (Den Haan <u>et al.</u>, 1976), where Z is the electron donor to the reaction center P680 (see a review by Govindjee and Jursinic, 1979, for further discussion). Bouges (1971) suggested that in the presence of low concentrations of NH_2OH the maximum yield of O_2 is delayed probably because of the binding of two molecules of NH_2OH or due to the presence of two electrons (donated by NH_2OH) in the PS II complex. Our results show that NH_2OH acts by increasing the concentration of Mn(II), as seen by an enhancement in T_2^{-1} . This suggests that NH_2OH donates electrons to the S states and causes the reduction of higher oxidation states of Mn to Mn(II).

At higher concentrations of NH_2OH there is a progressive loss of O_2 evolution activity which (as mentioned earlier) is correlated with the release of bound Mn (Cheniae and Martin, 1969). We note that the release of Mn(II), as monitored by ESR spectroscopy, becomes apparent even at very low concentrations and increases further at higher concentrations. The amount of Mn(II) released at low concentrations is small and may be from the very loosely bound pool of Mn that is not related to O_2 evolution.

The release of Mn is not complete even at very high (100 mM) concentrations of NH₂OH used suggesting that there is a tightly bound pool of Mn. It has been shown that even when 0_2 evolution activity is completely inhibited, NH₂OH can still donate electrons to PS II perhaps via Z_2 (see Fig. 1.1), the secondary electron donor to P680. It has been suggested that the tightly bound Mn may be associated with Z_2 . In this Chapter (section 4.3.3), however, we present data on the Mn content of the LHC and suggest that the Mn pool associated with the LHC might be related to the tightly bound fraction of Mn.

In summary, experiments described in this subsection show that (i) NH_2OH reduces the oxidizing equivalents of the S state; (ii) NH_2OH releases membrane bound Mn even at low concentrations but this Mn may be from the very loosely bound Mn; at higher concentrations of NH_2OH , however, the release is from the loosely bound pool of Mn involved in O_2 evolution; (iii) release of Mn(II) by MgCl₂ is facilitated by the presence of NH_2OH ; and (iv) even at the maximum concentration of NH_2OH (100 mM) used, some Mn is still in the bound state as indicated by the T_2^{-1} value in Fig. 4.11.

4.2.3 Effects of Hydrogen Peroxide

Velthuys and Kok (1978) have shown that H_2O_2 can reduce S states beyond S_0 state to a state designated as S_{-1} . Electron donation by H_2O_2 , a two electron process, reduces S_1 to S_{-1} and S_2 to S_0 in the dark. On the other hand, two electron oxidation by H_2O_2 regenerates S_2 and S_1 . At high pH the formation of the more reduced state S_{-1} is favored. To check whether the reduction of the O_2 evolving system by H_2O_2 involves the formation of Mn(II), we studied its effect on T_2^{-1} in thylakoid membranes.

In order to perform the above experiment, a catalase inhibitor, such as sodium azide, was added to the sample. Otherwise the H_2O_2 is consumed by the endogenous catalase in a few minutes after equilibration with H_2O_2 .

Fig. 4.13 illustrates T_2^{-1} of thylakoid membranes treated with 0.1% and 1.0% (v/v) H_2O_2 at high pH (8.8). There is an enhancement in T_2^{-1} as a function of time after the addition of H_2O_2 . In



. · . -#+

Fig. 4.13 T_2^{-1} as a function of time after the addition of H_2O_2 . The suspension medium contained 50 mM HEPES (pH 8.8), 400 mM sucrose, 10 mM NaCl, and 0.5 mM sodium azide; chlorophyll concentration, 2 mg/ml. Other conditions as described in the legend of Fig. 4.3.

the presence of $0.1\% H_2 O_2 T_2^{-1}$ increases by ~30%. At a higher concentration of $H_2 O_2$ (1.0%) the increase in T_2^{-1} is ~69%. No increase in T_2^{-1} is observed if catalase inhibitor sodium azide is absent because $H_2 O_2$ is consumed by endogenous catalase.

Measurements of ESR detectable Mn(II) were carried out for thylakoid membranes treated with H_2O_2 . Fig. 4.14 shows the ESR spectra for control and H_2O_2 treated thylakoids. Addition of 0.1% and 1.0% H_2O_2 does not release any appreciable amount of Mn(II).

According to Velthuys and Kok (1978) H_2O_2 puts the S states in the more reduced state S_{-1} which is quite stable in the dark. At high pH (8.8), H_2O_2 accelerates the S_1 to S_{-1} reaction and decreases the rate of the reverse reaction (S_{-1} to S_1), thereby increasing the ratio S_{-1}/S_1 . Since S_{-1} is a more reduced state it would imply that H_2O_2 is increasing T_2^{-1} by reducing oxidized Mn to Mn(II).

4.3 Studies on the Different Pools of Manganese in Thylakoid Membranes

There are several pools of manganese in thylakoid membranes: (1) a tightly bound pool; (2) a loosely bound pool which is related to 0_2 evolution; and (3) a very loosely bound pool which is not related to oxygen evolution. There are several reported values for the amount of Mn present in chloroplasts (Kok and Cheniae, 1966), with extreme values of 1 g atom/14 Chl and 1 g atom/600 Chl. However, 1 Mn atom 'bound' per 50-100 Chl is obtained for the pool of Mn within PS II (Cheniae and Martin, 1970).

The oxygen evolving capacity of chloroplasts can be lost by various treatments, such as, alkaline Tris extraction (Yamashita and Butler, 1968), hydroxylamine extraction (Cheniae and Martin, 1970) or temperature


Figure 4.14 Room temperature ESR spectra of thylakoid membranes in the presence of H_2O_2 . Instrumental conditions: microwave power, 50 mW; modulation amplitude, 10 G; time constant, 0.3 s; scan rate, 250 G/min; receiver gain, 5 x 10^3 . Other conditions as described in the legend of Fig. 4.13.

shock (Katoh and San Pietro, 1967; Homann, 1968). These treated chloroplasts can still maintain electron flow via PS II in the presence of artificial electron donors. Chloroplasts treated with alkaline Tris show a 90% loss of O_2 evolution activity with a parallel loss of the loosely bound Mn pool. The loss of oxygen evolution capacity shows a linear relationship with the Mn content of two thirds of the functional Mn pool associated with PS II. The remaining Mn pool which constitutes one third of the total Mn is tightly bound and cannot be released readily, and its function is unknown.

Incubation of chloroplasts with chelating agents such as EDTA does not release Mn associated with 0_2 evolution (Possingham and Spencer, 1962; Kok and Cheniae, 1966; Homann, 1967) probably because of the high affinity of Mn for its native binding site(s). Also, this Mn does not exchange easily with other divalent cations (Kok and Cheniae, 1966), but is slowly released by incubation with very high (~200 mM) concentrations of MgCl₂ (Chen and Wang, 1974; Wydrzynski <u>et al</u>., 1978). Besides these two pools of Mn (loosely bound and tightly bound) there is a third pool of Mn, which we have so far referred to as the very loosely bound Mn.

In this section we present ESR, NMR and 0_2 evolution measurements confirming the presence of the very loosely bound Mn. Parallel measurements of 0_2 evolution activity show that this Mn pool can be released by the addition of low concentrations of MgCl₂ without having any effect on the steady state 0_2 evolution and 0_2 per flash, measured with a Clark type concentration electrode (section 4.3.1). This result is in contradiction with the reported activation of PS II reaction centers by Mg as described by a stimulation of the flash yield of 0_2

evolution, measured with a Pt/AgCl₂ rate electrode (Bose and Arntzen, 1978). To check if the Mg induced decrease in T_2^{-1} is due to the release of bound Mn and not caused by Mg induced structural changes, we measured the effect of Mg on T_2^{-1} in modified thylakoid membranes which do not exhibit such structural changes (section 4.3.2). In the last part of this section (4.3.3) we present data on the T_2^{-1} of the isolated light-harvesting pigment-protein complex (LHC), since it has been suggested to be involved in cation induced regulation of excitation energy distribution between the two photosystems (Burke <u>et al.</u>, 1978; Wong <u>et al.</u>, 1979). Determination of the Mn content of the isolated LHC shows a significant amount of Mn present which is not readily extracted during the isolation procedure. It is considered possible that this is the tightly bound pool of Mn.

4.3.1 Effect of Mg(II) on Cation Depleted Thylakoid Membranes

Fig. 4.15 (open circles) shows the effect of increasing concentra tions of added MgCl₂ on T_2^{-1} of thylakoid membranes depleted of divalent cations by washing once with 10 mM NaCl. There is a significant (~25%) decrease in T_2^{-1} as MgCl₂ concentration is increased to 20 mM. The concentration of MgCl₂ required to produce half of the maximum change in T_2^{-1} is ~4 mM. A similar decrease in T_2^{-1} is seen in unwashed thylakoids upon addition of MgCl₂, the only difference being that the amount of MgCl₂ required to attain the half-maximal change is much higher than 4 mM (data not shown). The experiments shown in Fig. 4.15 were done at pH 6.5. At pH 7.0 and 7.5 the decrease in T_2^{-1} is definitely observed but it is of a smaller magnitude (10-15%).



Figure 4.15 Effect of MgCl₂ on T_2^{-1} , 0_2 evolution and the release of manganese in washed thylakoids. Thylakoid membranes were washed once in unbuffered 10 mM NaCl and resuspended in 10 mM Tricine (pH 6.5), 400 mM sorbitol and 10 mM NaCl. T_2^{-1} was measured at 27 MHz and at 25°C for thylakoid suspensions at a chlorophyll concentration of 3 mg/ml. For 0_2 evolution 0.5 mM [Fe(CN)₆]³⁻ and 1 mM NH₄Cl were included in the reaction mixture; chlorophyll concentration, 20 µg/ml. The data for the free Mn(II) was obtained by measuring the area under the second peak of the six line ESR spectra shown in Fig. 4.16.

The rate of steady state 0_2 evolution (Fig. 4.15, filled squares) in washed membranes does not change when 10 or 20 mM MgCl₂ is added (pH 6.5) (for data on flash yields at pH 7.5, see later). Furthermore, Fig. 4.15 (open squares) shows that the amplitude of the ESR signal, ascribed to free Mn(II), increases with increasing concentrations of MgCl₂. This ESR detectable free Mn(II) signal was obtained from Fig. 4.16. As already noted above, the amplitude of the 6-line spectrum, indicative of free Mn(II), increases as the concentration of added MgCl₂ is raised to 20 mM. Thus, the decrease in bound Mn, as monitored by T_2^{-1} (shown in Fig. 4.15, open circles), is accompanied by an increase in free Mn(II) (as monitored by ESR). This is in agreement with the results described in section 4.2.2 of this chapter. Addition of low concentrations of MgCl₂ increased the ESR signal of 10 μ M MnCl₂ solution and it saturated at ~1 mM MgCl₂; this does not affect our conclusion since the major effect of $MgCl_2$ on Mn(II) signal of thylakoids becomes apparent at MgCl₂ concentrations > 1 mM (Fig. 4.15). Moreover, the effect of Mg on $MnCl_2$ solution is much smaller (~15%) as compared to its effect on thylakoid membranes(80-90%).

 0_2 flash yield was measured in order to see if the release of Mn(II) caused any change in 0_2 evolving activity. Bose and Arntzen (1978) reported that low salt thyalkoids show a decrease in the steady state flash yield of 0_2 evolution (pH 7.8) under short saturating repetitive flashes; addition of 10 mM MgCl₂ resulted in approximately a two fold increase in the steady state flash yield of low-salt chloroplasts. This suggested that approximately half of the PS II centers are photochemically inactive in a low-salt medium. We measured 0_2 yield



Figure 4.16 Room temperature ESR spectra of washed thylakoid membranes in the presence of different concentrations of $MgCl_2$. Chlorophyll concentration, 3 mg/ml. Instrumental conditions: microwave power, 50 mW; modulation amplitude, 10 G; time constant, 0.3 s; scan rate, 250 G/min; receiver gain, 5 x 10^3 . Other conditions as described in the legend of Fig. 4.15. per flash using a Clark type electrode and obtained a value of 10_2 molecule per 2,600 ± 250 chlorophyll molecules, both in the presence and absence of 10 mM MgCl₂ (pH 7.5). These numbers are in fair agreement with the photosynthetic unit size of 2,500 chlorophyll molecules/ 0_2 in <u>Chlorella</u> (Emerson and Arnold, 1932a,b). If salt depleted thylakoids are photochemically inactive, as suggested by Bose and Arntzen (1978), we should have obtained a larger value for the photosynthetic unit size. Also, independent measurements of Govindjee <u>et al</u>. (1979) on charge separation at reaction center II (as measured by 515 and 320 nm absorption changes) and on 0_2 yield/flash support our conclusions.

Removal of Mn from thylakoids by Mg exchange (by incubation with 100-200 mM MgCl₂ for 30 to 90 min at 0° to 2°C) is known to decrease oxygen evolution by the Hill reaction and increase the steady state amplitude of ESR Signal II (Chen and Wang, 1974). The rate of 0_2 evolution was found to be directly proportional to the Mn content of the extracted sample, whereas the steady state amplitude of Signal II was inversely proportional to the Mn content of the extracted sample. Wydrzynski (1977) showed that both the proton relaxation and 0_2 evolution rates decreased with a decrease in Mn content as thylakoids were incubated with increasing ratios of [Mg]/[Ch1] for 2 hrs at 4°C. In this procedure (Chen and Wang, 1974; Wydrzynski et al., 1978), however, thylakoids were centrifuged and resuspended in a fresh medium so as to remove free manganese. In our measurements described here, low concentrations of MgCl₂ were added to salt-depleted thylakoids and the released Mn(II) was not removed. In contrast to the correlation between loosely bound manganese with O2 evolution (Wydrzynskı, 1977), our data show that

the release of Mn(II) is not correlated with the rate of 0_2 evolution, implying that this released Mn must be from the very loosely bound Mn pool. It is quite likely that this pool of Mn is associated with superoxide dismutase. Lumsden and Hall (1975) reported a cyanide-insensitive superoxide dismutase in chloroplasts; however, it is not established whether this superoxide is a Mn-enzyme or Fe-enzyme (both are cyanide insensitive).

4.3.2 Effect of Mg(II) on Trypsin Treated Thylakold Membranes

Cations bring about major structural and functional changes in thylakoid membranes. Addition of low concentrations of divalent cations to low-salt thylakoids containing unstacked lamellae results in the formation of stacked regions (grana) (Izawa and Good, 1966; Arntzen, 1978). This structural change is usually accompanied by an increase in quantum yield of PS II and a decrease in guantum yield of PS I under low light conditions (see reviews by Barber, 1976; Williams, 1977). There is evidence to suggest that LHC of chloroplasts is necessary for cation mediated grana formation and energy redistribution between the two pigment systems (Arntzen, 1978). The proteolytic enzyme trypsin has been shown to modify the LHC at the external surface of the membrane such that these trypsin treated membranes do not exhibit cation induced structural changes (Steinback et al., 1979). Such structural changes could lead to a change in the environment of the loosely bound Mn, thereby affecting the relaxation of water protons in the hydration sphere of the Mn complex. In order to verify that the decline in T_2^{-1} upon MgCl₂ addition (Fig. 4.15) is due to the release of Mn(II) (Fig. 4.16), and not due to some environmental and structural changes, we performed experiments to see if the Mg induced decrease in T_2^{-1} persists in trypsin treated thylakoid membranes.

The effect of MgCl₂ on trypsin treated thylakoids is summarized in Table 4.1. T_2^{-1} decreases when 20 mM MgCl₂ is added to the treated membranes. The decrease in T_2^{-1} for trypsin treated membranes is of the same order of magnitude as for control membranes. The control was carried through the same incubation but without trypsin. The release of Mn(II) is indicated by a corresponding increase in the six line component of the ESR spectrum for aqueous Mn(II) (Fig. 4.17). Furthermore, addition of MgCl₂ does not change the rates of O_2 evolution in trypsin treated membranes after ~12 min of trypsin treatment (Table 4.1). Thus, the Mg effect persists in thylakoids that do not undergo structural changes; this confirms the earlier conclusion that low concentrations of MgCl₂ releases bound Mn from the very loosely bound pool of Mn that is not related to O_2 evolution.

Polypeptides from trypsin treated thylakoid membranes were separated by SDS-polyacrylamide gel electrophoresis (data shown in Fig. 4.18). The polypeptide patterns of control thylakoid membranes and the LHC preparation are also shown for comparison. The data show that trypsin treatment causes a change in the relative mobility of the polypeptides associated with the LHC. Steinback <u>et al</u>. (1979) have concluded that this change in the pattern is caused by a decrease in the molecular weight of the major 25,000 polypeptide of the LHC and an alteration of the 29,000 minor polypeptides of thylakoid membrane indicating that trypsin treatment under the conditions employed specifically modifies a portion of the

	T2 ¹ (obs), s-1 [MgCl2], mM		O2 Evolution μ equiv. O2/mg chlorophyll per h [MgCl2], mM	
	0	20	0 20	
Control	1.97	1.70	418 ± 8 415 ± 7	
Trypsin Treated	1.96	1.73	408 ± 9 414 ± 8	

Tryspin treatment was done according to the procedure of Steinback <u>et</u> <u>al.</u>, 1979. (see Chapter 2, section 2.3, for details). Chlorophyll concentration for the T_2^{-1} experiment was 1.8 mg/ml. For details on T_2 and O_2 evolution measurements see the legend of Fig. 4.1.

Effect of MgCl₂ on T_2^{-1} and O₂ Evolution of Trypsin Treated Thylakoid Membranes



Figure 4.17 Room temperature ESR spectra of control and trypsin treated thylakoid membranes in the absence and the presence of $MgCl_2$. Details of trypsin treatment are described in Chapter 2 (section 2.3). Chloro-phyll concentration, 2.4 mg/ml. Instrumental conditions: microwave power, 50 mW; modulation amplitude, 10 G; time constant, 0.1 s; scan rate, 250 G/min; receiver gain, 10^4 .



Figure 4.18 SDS-polyacrylamide slab gel electrophoresis of polypeptides from control thylakoid membranes, trypsin treated thylakoid membranes and purified light harvesting complex. Molecular weight standards used (S): bovine serum albumin (68,000). ovalbumen (45,000), carbonic anhydrase (29,000), chymotrypsinogen (25,500), cytochrome c (12,500). C, control; T, trypsin-treated; and LHC, light harvesting complex. Protein bands were stained with coomassie brilliant blue R-250. For details on methodology see Steinback <u>et al</u>. (1979).

LHC. Those modified membranes do not show cation-induced grana stacking and cation regulation of energy redistribution between the two photosystems. However, the effect of Mg on T_2^{-1} persists even after trypsin treatment (Table 4.1), suggesting that Mg induced decline in T_2^{-1} cannot be related to structural changes of the kind discussed above.

4.3.3 Manganese Content and Proton Relaxation Rate of the Light

Harvesting Complex

The LHC is a pigment protein complex and has been isolated to a great purity (Burke et al., 1978). It contains three polypeptides (two major polypeptides of 23,000 and 25,000 daltons and one minor polypeptide of 29,000 daltons) and chlorophyll a and b in a ratio of 1:1. The LHC can undergo self association in the presence of cations and thus bring about cation mediated grana formation and regulation of energy distribution between PS I and II in vivo (Arntzen, 1978; also see Wong and Govindjee, 1979). In order to further assess the role of structural effects on changes in the observed T_2^{-1} , we measured T_2^{-1} of isolated LHC. The effect of Mg on the T_2^{-1} of purified LHC could not be measured as the isolated LHC preparation contained high concentrations of MgCl₂ (Burke et al., 1978). Purified LHC preparation was obtained from chloroplast fragments by mild detergent fractionation and aggregation by the addition of cations (MgCl₂ and KCl). The LHC preparation was checked for its purity by electrophoretic separation on polyacrylamide slab-gel (Fig. 4.18). There are three polypeptides associated with the LHC preparation: two major polypeptides of 23,000 and 25,000 and one minor polypeptide of 29,000 daltons. The LHC preparation thus obtained has no PS II and PS I activity (Burke et al., 1978).

Table 4.2 shows the T_2^{-1} and total Mn content (as determined by neutron deactivation method) for thylakoid membranes and purified LHC. The T_2^{-1} and total Mn content of the LHC are unexpectedly high when expressed on a mg Chl basis. However, qualitative correlation between T_2^{-1} and Mn content is confirmed. Since chlorophyll is present in other chlorophyll-complexes, it seems more appropriate to express Mn content on mg protein basis. For isolated LHC the average Chl/protein ratio has been determined to be 13.4 molecules of Ch1/1 molecule of LHC (average molecular weight 23,000) (Burke et al., 1978). The total Mn content of the LHC (0.4 μ g Mn/mg Ch1, Table 4.2) corresponds to 1 Mn for 11 molecules of the LHC (153 Chl molecules). In thylakoid membranes a Chl/protein ratio of 1:3 (on weight basis) has been estimated (C. J. Arntzen, personal communication). Based on the fact that 60% of the total Chl is associated with the LHC, we can calculate the ratio of total thylakoid chlorophyll to LHC protein to be 1:1 (on weight basis), i.e., 25.6 thylakoid Chl molecules/LHC molecule present. The total Mn content in thylakoid membranes (0.8 μ g Mn/mg Chl, Table 4.2) amounts to 3.8 Mn for ~11 molecules of LHC (292 thylakoid Chl). Thus, the amount of Mn associated with isolated LHC (1 Mn/11 molecules of LHC) is approximately one-third of the total Mn (3.8 Mn/11 molecules of LHC present).

In order to verify that the high content of Mn in the LHC is not due to a nonspecific association of free Mn during the isolation procedure, we dialysed the isolated LHC with EDTA. The T_2^{-1} of dialysed LHC is lower (0.3 s⁻¹ for 0.6 mg Ch1/ml) as compared to the undialysed sample (0.66 s⁻¹ for 0.3 mg Ch1/ml) but analysis of the total Mn content shows no loss of Mn during dialysis (data not shown). The decrease in T_2^{-1}

TABLE 4	•	2
---------	---	---

Comparison of T_2^{-1} and Total Mn Content of Isolated LHC and Thylakoid Membranes

Sample	T2 ¹ (corr.) s-1 (0.3 mg Ch1/m1)	Total Mn Content µg Mn/mg Chl
Thylakoid Membranes	0.74	0.8 ± 0.04
LHC	0.66	0.4 ± 0.03

Isolation of the LHC was done according to the procedure of Burke <u>et</u> <u>al.</u> (1978). Mn content was determined by neutron activation analysis. Corr. stands for corrected. For details on T_2^{-1} measurements see the legend of Fig. 4.1.

٩

,

is probably because EDTA replaces the water ligands on the bound Mn. The one third Mn pool which still remains linked to the LHC could perhaps correspond to the tightly bound pool of Mn in chloroplasts (for the concept of two thirds Mn in loosely bound pool and one third Mn in tightly bound pool, see Cheniae and Martin, 1970).

In view of our results it is important to note that Lagoutte and Duraton (1975) have observed a 25 k dalton band following SDS polyacrylamide gel electrophoresis. This protein contains tightly bound Mn (1 Mn/eight 25,000 molecular weight peptide chains). In similar studies Henriques and Park (1976), however, found that the 25,000 molecular weight protein is deficient in Mn. More recently Foyer and Hall (1979) have reported the presence of Mn (~1 Mn/twenty three 25,000 molecular weight polypeptides) in the isolated LHC. There is no cogent explanation for these apparent discrepancies.

No definite role has been assigned for the tightly bound pool of Mn. It is likely that this Mn has a structural role. Possingham <u>et al</u>. (1964) studied the effect of Mn deficiency on the mesophyll cells of spinach leaves and showed that Mn deficiency has a large effect on the structure of chloroplasts. Mn deficiency leads to a progressive disorganization of the lamellar system. A loss of structure could arise directly if Mn acts as an essential structural link in the membranes, or indirectly if Mn participates in some reaction which is required for the production of molecules essential for the formation of these membranes. It is not possible to distinguish between these alternatives, but it appears that Mn of the LHC may have an important structural role in chloroplasts.

4.4 <u>Is There a Contribution to Water Proton Relaxation from Paramagnetic</u> Species Other than Mn(II)?

NMR provides a unique method for monitoring Mn bound to thylakoid membranes as indicated by data presented in sections 4.2 and 4.3 of this Chapter (also see Wydrzynski, 1977). On the basis of experiments described so far we can conclude that: (a) Mn in its bound state is a more efficient relaxer of water protons; (b) Mn(II) is more efficient than Mn(III) in enhancing T_2^{-1} ; and (c) conversion of Mn(III) to Mn(II) increases T_2^{-1} .

In this section, we describe experiments which suggest that water proton relaxation rate in thylakoids is not only dependent on the concentration of bound Mn but also on its accessibility to water. So far we have explained all our data data in terms of the relative contribution of Mn(II). However, the total paramagnetic effect on proton relaxation depends not only on the concentration of [Mn(II)], but also on the number of bound nuclei in the first coordination sphere. If the number of water ligands, and hence the number of bound protons changes, this would represent the loss of a site which communicates the paramagnetic effect to the bulk water. Any changes in the accessibility of Mn would also alter the sites available for water protons. Furthermore, experiments described here could suggest that contributions from other paramagnetic ions may arise, but only when the membrane structure is altered by specific treatments such as heating of thylakoid membranes (section 4.4.2) and suspending thylakoids at very high and low pH (section 4.4.3). However, aging of thylakoids at 35°C (section 4.4.1) does not involve any dramatic changes in the membrane structure so as to increase the contribution of

other paramagnetic ions. In the aging experiments reported below, there is a perfect correlation between the amount of Mn, 0_2 evolution activity and T_2^{-1} . Thus, the aging experiments confirm the earlier conclusion that water proton relaxation rates can monitor the bound Mn related to 0_2 evolution.

4.4.1 Aging of Thylakoid Membranes at 35°C

We varied the chloroplast Mn pool by aging at 35°C and studied its effect upon 0_2 evolution activity and T_2^{-1} . Thylakoids were aged in a buffer (pH 8.3) preequilibrated at 35°C and at specific times aliquots were removed and extracted in EDTA-buffer. Aging of thylakoids at 35°C resulted in a time-dependent loss of 0_2 evolution, T_2^{-1} and the Mn content as shown in Fig. 4.19. After 3 min at 35°C the Mn content, as measured by neutron activation analysis (Chapter 2, section 2.12), was decreased to about 30% (from 0.69 to 0.22 μ g Mn/mg Ch1, see Fig. 4.19, open circles). The steady state saturation rates of 0_2 evolution decreased in parallel as a function of the time of aging; 10 min of incubation at 35°C was enough to inhibit almost all 0_2 evolution (Fig. 4.19, open squares). Similarly, aging causes a parallel decrease in T_2^{-1} (Fig. 4.19, filled circles). This experiment clearly establishes a relationship between T_2^{-1} , the Mn content and 0_2 evolution in thylakoids. This supports the correlation between T_2^{-1} , Mn and 0_2 evolution observed by Wydrzynski (1977) in thylakoids washed after incubation with high concentrations of MgCl₂.

The effects of aging on O₂ evolution and the Mn content of thylakoids are in agreement with the results of Cheniae and Martin (1970). As pointed out by Cheniae and Martin (1970), there is no clear demarcation



Figure 4.19 Effect of time of incubation at 35° C of thylakoid membranes upon T_2^{-1} , O_2 evolution and Mn content. Incubation medium contained 25 mM Tris chloride (pH 8.3), 5 mM MgSO₄, 10 mM (NH₄)₂SO₄ at a chlorophyll concentration of 200 µg/ml. At specified times, aliquots were removed and extracted in EDTA (1 mM) buffer (pH 7.4) at 4°C. Thylakoids were washed and resuspended in 50 mM Tris chloride (pH 7.4), 400 mM sucrose and 10 mM NaCl at a chlorophyll concentration of 2.5 mg/ml. Mn content was determined by neutron activation analysis. For control it was 0.7 µg Mn/mg Chl. For details on T_2^{-1} and O_2 evolution measurements see the legend of Fig. 4.1.

between the two pools when the Mn content is varied by aging. Moreover, the amount of Mn remaining after 15-30 min of aging constitutes about 10% of the total Mn content, implying that aging may also release part of the very tightly bound Mn (~ one third of the total Mn content).

4.4.2 Effect of Heating on Thylakold Membranes

In the heating experiments described here, thylakoid membranes were incubated for 5 min in a water bath maintained at a specific temperature. This treatment is expected to release Mn from its bound state (Katoh and San Pietro, 1967; Homann, 1968). In contrast to the aging experiments described in section 4.4.1, the released Mn was not extracted with EDTA-extraction buffer but was directly measured by ESR.

Fig. 4.20 (open circles) shows that as the temperature of heating is increased, there is an increase in the amount of ESR detectable Mn(II). Based on the experiments described in sections 4.3.2 and 4.3.3, it is concluded that free Mn is less efficient in the relaxation of water proton nuclei. If Mn is indeed released by heat treatment, then there should have been a parallel decrease in T_2^{-1} . However, measurements of T_2^{-1} showed an increase in T_2^{-1} as the temperature of incubation is increased (30-50°C) (Fig. 4.20, filled circles). In order to explain the increase in T_2^{-1} when Mn is being released, we propose that the enhancement of proton relaxation rates may be due to one or more of the following contributions: (a) heat treatment does not release all the bound Mn present in the thylakoids and the accessibility of the remaining tightly bound Mn is altered in such a way that it can make more ligands with water molecules; and/or (b) thylakoid membranes subjected to nonphysiological temperature may undergo structural changes thereby exposing



Figure 4.20 Effect of heating of thylakoid membranes upon T_2^{-1} and ESR signal for Mn(II). For heat treatment, thylakoids in HEPES buffer (see legend of Fig. 4.1) were incubated for 5 min at specified temperatures. Samples were stored on ice and measurements were made at room temperature (25°C). Chlorophyll concentration, 2.5 mg/ml. For details on T_2^{-1} and ESR measurements see the legend of Fig. 4.12.

the inaccessible copper of plastocyanin and other copper containing enzymes like polyphenol oxidase (Tolbert, 1973) to water molecules.

In order to test these possibilities experiments were done with thylakoid membranes in which conformational (structural) changes were minimized by fixation of membranes. Fixation of thylakoids with glutaraldehyde prevents macroconformational changes (see e.g., Zilinskas and Govindjee, 1976). Fig. 4.21 shows T_2^{-1} for unfixed and glutaraldehyde fixed membranes subjected to various temperatures. A comparison of T_2^{-1} for the control and fixed thylakoids at 50°C shows a marked decrease in T_2^{-1} for glutaraldehyde fixed membranes. This suggests that in the fixed membranes the exposure of paramagnetic species, which enhances T_2^{-1} upon heating at 40-50°C, is partly prevented. Hardt and Kok (1976, 1977) showed that treatment of chloroplasts with glutaraldehyde causes an inhibition in the electron transport chain between the two photosystems. Measurements of 0_2 flash yields, pH changes and chlorophyll <u>a</u> fluorescence induction show that the 0_2 evolving apparatus, PS II and its electron acceptor pool are not affected. The reduction of P700 but not its oxidation is severely inhibited. Cyt f can be reduced by PS II but its oxidation by PS I is slow. These observations led to the conclusion that inhibition by glutaraldehyde is at the plastocyanin level and thereby it induces a break between P700 and cyt f. Further support for this conclusion came from the observed sensitivity of isolated plastocyanin to glutaraldehyde. Thus, the decreased effect of heating at 50°C in fixed membranes could be either due to the direct effect of glutaraldehyde on



Figure 4.21 Effect of heating upon T_2^{-1} of glutaraldehyde fixed thylakoid membranes. Thyalkoids were fixed in glutaraldehyde according to the procedure of Zilinskas and Govindjee (1976) (see Chapter 2, section 2.4, for details). Chlorophyll concentration; 1.8 mg/ml. This result was confirmed three times. Details of heat treatment and T_2^{-1} measurements as described in the legend of Fig. 4.20.

the copper-containing plastocyanin or due to the prevention of exposure of the hidden paramagnetic centers to water molecules. At present, it is not possible to distinguish between these two cases.

To test the possible identity of the paramagnetic species involved, we used KCN which interacts with copper. Ouitrakul and Izawa (1973) have demonstrated that KCN reacts guite rapidly with plastocyanin causing an inhibition of electron transport. They showed that KCN blocks PS I-dependent electron transport from reduced DAD to MV without inhibiting electron transport from water to oxidized p-phenylenediamine. Fig. 4.22 shows T_2^{-1} measurements of heat treated membranes when 60 mM KCN is added to thylakoids. In this experiment the heat treatment was carried out as usual and KCN was added afterwards. In the presence of KCN the temperature induced increase in T_2^{-1} disappears. This may suggest that the contribution to T_2^{-1} in heat treated thylakoids is due to the copper-containing plastocyanin. However, this conclusion is subject to the assumption that KCN specifically interacts with Cu(II). Takahashi and Asada (1976, 1977) have shown that NaCN-treatment of thylakoids for 90 min removes a significant amount of Cu (~50%) but has little effect (only 10% reduction) on the Mn content. We observed that the addition of 60 mM KCN to heat treated thylakoids completely eliminated the ESR signal due to Mn(II) (data not shown) suggesting an interaction of KCN with the free Mn(II). Since the incubation of KCN (for 90 min) does not decrease the T_2^{-1} value any further than the control value (see Fig. 4.22), it implies that the remaining bound Mn is not interacting with KCN. Thus, we may conclude that in heat treated $(40^{\circ} \text{ to } 50^{\circ}\text{C} \text{ range})$ thylakoids the



Figure 4.22 Effect of KCN upon T_2^{-1} of heat treated thylakoid membranes. Thylakoids were heat treated as described in the legend of Fig. 4.20 and then incubated with 60 mM KCN for 90 min at 4°C. Chlorophyll concentration, 2 mg/ml. For details on T_2^{-1} measurements see the legend of Fig. 4.1.

major contribution to T_2^{-1} is from Cu but there is also some contribution from Mn (probably the tightly bound Mn pool or the non-specifically bound Mn). However, in normal thylakoids, the major contribution is from bound Mn (section 4.4.1).

4.4.3 NMR and ESR Measurements of Thylakoids at Different pHs

We studied water proton relaxation rates of thylakoid membranes in the pH range of 4 to 9. Fig. 4.23 shows T_2^{-1} and ESR detectable Mn(II) as a function of pH of the suspension medium. There is no significant change in the observed T_2^{-1} between pH 4 and 7 (Fig. 4.23, filled circles). As the pH is increased from 7 to 9, T_2^{-1} increases by about 40%. Free Mn(II), as calculated by measuring the area under the second peak of the six line ESR spectrum, shows no change in the 7 to 9 pH range (Fig. 4.23, open circles). The increase in T_2^{-1} may be due to an increase in the accessibility of the bound Mn to water protons. As discussed earlier, water proton relaxation is dependent not only on the concentration of the bound Mn but also upon the number of protons in the first coordination sphere. At pH < 7, however, the amount of free Mn(II) as indicated by the six line ESR spectrum, increases as the pH is lowered to 4. In a control experiment 10 μ M MnCl₂ solution showed a 30-40% increase in the ESR signal as the pH was lowered from 7 to 4; this, however, is negligible as compared to the effect of pH on ESR detectable Mn(II) signal of thylakoid membranes (Fig. 4.23). Data described in section 4.2 (Fig. 4.6) and section 4.3 (Fig. 4.15) of this Chapter show that T_2^{-1} decreases as bound Mn is released. Data in Fig. 4.23 shows that in spite of the increase of free Mn(II) in the acidic pH range, there is



Figure 4.23 Effect of pH of the suspension medium on T_2^{-1} and ESR detectable free Mn(II) of thylakoid membranes. Chlorophyll concentration, 2 mg/ml. For details on T_2^{-1} and ESR measurements see the legend of Fig. 4.12.

no decrease in the T_2^{-1} . The absence of decrease in T_2^{-1} may be due to a compensatory increase in proton relaxation caused by an increase in the accessibility of the remaining tightly bound Mn. The released Mn may rebind to the membrane non-specifically and the increase in T_2^{-1} could be due to a change in the environment of the bound Mn. It is also likely that this enhancement in T_2^{-1} is due to the exposure of other paramagnetic species. As discussed earlier, the hidden Cu of plastocyanin may contribute to the relaxation of water protons only when membrane structure has been altered to expose the inaccessible copper.

4.5 Concluding Remarks

In summary, experimental results described in this chapter have provided us with new information and conclusions presented below.

(1) Parallel measurements of 0_2 evolution activity, T_2^{-1} and Mn content (free and bound) show that under certain specified conditions T_2^{-1} indeed monitors Mn related to 0_2 evolution. A good correlation between 0_2 evolution activity, T_2^{-1} and the Mn content of thylakoids is established when Mn, associated with the 0_2 evolving activity, is released by aging of thylakoids for different time periods at 35°C (Fig. 4.19).

(2) Addition of reductants (TPB, NH₂OH and H₂O₂) is suggested to convert higher oxidation states of Mn to Mn(II) as they cause an increase in T_2^{-1} . When thy lakoids are incubated with NH₂OH there is an increase followed by a decrease in T_2^{-1} indicating a two fold effect of NH₂OH -- reduction of manganese to Mn(II) and release of bound manganese. Increase in T_2^{-1} of thy lakoids upon the addition of H₂O₂ suggests that conversion of S₁ to S₋₁ is accompanied by reduction of higher oxidation states of Mn to Mn(II). Effect of TPB on T_2^{-1} suggests the existence of several distinct

pools of paramagnetic species in thylakoids. For further discussion see section 4.2.

(3) Presence of a non-functional (very loosely bound) pool of Mn is confirmed by the observation that O_2 evolution activity remains unaffected when Mn from this pool is released by displacement with low concentrations (10-20 mM) of MgCl₂ (Fig. 4.15).

(4) Measurement of the total Mn content of isolated LHC shows that there is 1 Mn/~11 molecules of LHC (Table 4.2). This accounts for about one-third of the total functional Mn present in thylakoid membranes. The Mn associated with the LHC may represent the tightly bound pool of Mn.

(5) Contribution from other paramagnetic species (e.g., Cu(II)) or change in the accessibility of Mn(II) to H₂O, may arise when thylakoid membranes are heat treated at 40-50°C (section 4.4.2) or suspended in media at very low and high pHs (section 4.4.3). Here, the release of Mn(II) is not accompanied by a decrease in T_2^{-1} probably because of an increase in accessibility of the usually inaccessible Cu(II) of plastocyanin to bulk water. The alternate explanation, that the accessibility of the remaining bound Mn is altered, cannot yet be excluded (see section 4.4.2 for further details).

Thus, our results emphasize that proton relaxation rate measurements monitor both the functional and non-functional pools of paramagnetic ions, particularly Mn(II). These pools include (a) a very loosely bound Mn pool unrelated to 0_2 evolution; (b) a loosely bound Mn pool related to 0_2 evolution; (c) a tightly bound pool of Mn contained in the LHC; and (d) perhaps plastocyanin Cu(II). These different pools can, however, be separately studied depending upon the experimental conditions.

CHAPTER 5

SUMMARY AND CONCLUDING REMARKS

The goal of this thesis was to study the involvement of bicarbonate and of manganese in PS II reactions. We established the site of bicarbonate action and characterized some aspects of the mechanism of its action on the electron transport from PS II to PS I. On the oxidizing side of PS II, we studied the involvement of Mn in the water splitting system using the techniques of NMR and ESR. The major conclusions of the present study are listed in Table 5.1 along with the figures and tables where supporting evidence is presented. In the following sections we briefly describe our major results.

5.1 Bicarbonate Requirement for PS II Reactions

The removal of bicarbonate from thyalkoid membranes has a direct effect on the Hill activity that is reversible specifically by the addition of bicarbonate. Warburg and Krippahl (1960) suggested that CO_2 requirement for the Hill reaction supported the concept of bicarbonate as the substrate for oxygen evolution. If CO_2 is indeed the source of O_2 evolved then the bicarbonate effect on the Hill reaction must be located on the oxidizing side of PS II. There is now overwhelming evidence that the major site of bicarbonate is located on the reducing side of PS II (see Chapter 3).

5.1.1 <u>A Few Characteristics of the Bicarbonate Effect</u>

The use of very high concentrations (10-20 mM) of bicarbonate to see the stimulation of electron flow may be criticized as it raises doubts about the physiological importance of the phenomenon. However, we observed that if a competing anion such as formate (used in the CO_2 depletion procedure) is absent in the reaction medium, then the stimulatory effect of bicarbonate can be observed even at 0.5 mM concentration; this is close to the concentration of bicarbonate <u>in vivo</u> (Ogren and Hunt, 1978).

Attempts to identify the active species $(HCO_3^- \text{ or } CO_2)$ involved have not been successful. The effect of bicarbonate as a function of external pH (5 to 9) shows a much larger stimulation of the Hill activity around pH 6 to 7, where the bicarbonate species predominates (Figs. 3.2 and 3.3). This could imply that the bicarbonate ion is the active species in stimulating the Hill reaction. However, the affinity of HCO_3^- to the membrane component(s) may be different at different pHs. No definite assertion can, therefore, be made regarding the species involved.

Bicarbonate has also been shown to cause enhancement of photophosphorylation (Punnett and Iyer, 1964; Batra and Jagendorf, 1965). The stimulatory effect of CO_2 on electron flow, investigated in this thesis, is not related to any indirect stimulation resulting from an enhancement of photophosphorylation by bicarbonate since the effect is present to its full extent even in the presence of uncouplers (NH₄Cl), gramacidin and methlamine HCl. Thus, the bicarbonate effect studied here is distinct from that discovered by Punnett and Iyer (1964).

5.1.2 Site of the Bicarbonate Effect is Between Q and the PQ Pool

Stemler and Govindjee (1973) suggested that the absence of bicarbonate blocks the oxidizing side of PS II. Wydrzynski and Govindjee (1975), on the basis of their data on chlorophyll <u>a</u> fluorescence transient, suggested a new site of bicarbonate action on the reducing side of PS II. Biochemical experiments described in section 3.3.1 clearly establish the major site of the bicarbonate effect on the rate of electron flow from Q to the PQ pool (also see Khanna <u>et al.</u>, 1976, 1977). We found no effect of bicarbonate depletion on the Hill reaction from water to SiMo (Table 3.2). Electron transport from water to DAD_{Ox} in the presence of DBMIB was largely depressed after bicarbonate depletion, and restored upon bicarbonate addition. Because DAD_{Ox} accepts electrons at the PQ pool and DBMIB is an inhibitor of electron flow from the PQ pool to cyt f, these results suggest that the major bicarbonate effect is located between Q and the PQ pool. In addition, the electron transport from DAD_{red} to MV in the presence of DCMU was not impaired by bicarbonate depletion, indicating that PS I dependent electron transport from the site of donation of DAD_{red} to MV is not influenced by bicarbonate depletion. Recently Izawa and Pan (personal communication) have confirmed the absence of bicarbonate effect on PS I dependent electron transport from duroquinone (donating at PQ) to MV.

The location of the site of action of bicarbonate is also indicated by absorption studies of the individual electron transfer reactions involving Q, R, PQ and P700 (see section 3.3 and a paper by the author in collaboration with U. Siggel; Siggel <u>et al.</u>, 1977). Absorption changes at 334 nm indicate the formation and decay of Q⁻ (Figs. 3.4 and 3.5). The formation of Q⁻ is very fast (< 1 μ s) and the decay of Q⁻ shows a t_{1/2} of 500 μ s. In the CO₂-depleted thylakoids, the time course of the absorption change at 334 nm shows two exponential phases of about equal magnitude with halftimes of 500 ± 100 μ s and 7 ± 3 ms. The biphasic relaxation in these thylakoids reflects heterogeneity of the sample introduced by the depletion procedure -- about 30% of the total sample is unaffected by the procedure of depletion, another 30% exhibits slow kinetics, the rest of the signal (40%) was, however, totally inactive. These experiments suggest that, in addition to the complexities noted above, the reoxidation of the reduced Q⁻ is inhibited by bicarbonate depletion since a large 500 μ s decay phase of Q⁻ is replaced by a large decay phase of 7 ± 3 ms.

Data on absorption changes at 265 nm indicate that the reduction of PQ by R^{2-} is slowed down from approximately a ms to 100-200 ms (for a detailed discussion, see section 3.3). This slow reaction time is apparent in both the absorption changes of P700 and PQ after a 20 µs flash (with an alteration from a predominantly ~50 ms phase to a predominantly ~200 ms phase), suggesting that the ~200 ms halftime corresponds to an electron transfer reaction included in the main pathway of the intersystem chain.

Analysis of the absorption changes at 265 nm by 85 ms repetitive flashes suggests that the 200 ms phase in CO_2 -depleted thylakoids corresponds to the reaction step $R^{2-} \Rightarrow PQ$.

5.1.3 Bicarbonate: A Regulator of Electron Flow Between Q and PQ?

Bicarbonate may regulate electron flow between Q and PQ by interacting with the protein component essential for the electron flow between Q and the PQ pool (see section 3.4). Support for this hypothesis comes from (a) the absence of bicarbonate stimulation in trypsin treated membranes, (b) the observation of a change in the affinity of atrazine binding in CO_2 -depleted thylakoids, and (c) the absence of proton uptake and release at the PQ pool in CO_2 -depleted thylakoids. Trypsin treatment has been shown to alter the protein covering the region Q-R-PQ (Renger, 1976). The absence of the bicarbonate effect in trypsinated thylakoids suggests that bicarbonate is ineffective, perhaps because its binding site on the protein component has been modified. Since trypsin treatment also alters the binding site for DCMU, a very close spatial arrangement between the bicarbonate binding site and the site of action of DCMU is suggested. Moreover, the affinity of atrazine (same site of action as DCMU) for its binding site on the protein is reduced in CO_2 -depleted membranes, again indicating a close spatial arrangement for the two sites. The absence of proton translocation at the PQ pool may indicate that a proper orientation of the protein component is also important for proton translocation across the membrane. This is consistent with the observation of Renger and Tiemann (1979) that in trypsin treated thylakoid membranes, there is no proton uptake and release at the PQ level.

5.2 Involvement of Manganese in Photosynthetic 02 Evolution

Manganese has been considered to be an important constituent of the O_2 evolving system (see Chapter 1). Recently, it has been shown that the relaxation rates of water protons can be used to study bound manganese in thylakoid membranes (Wydrzynski, 1977; Govindjee <u>et al.</u>, 1977; Wydrzynski <u>et al.</u>, 1978). Release of bound manganese results in the appearance of an ESR detectable signal typical for Mn(II) in an aqueous environment. We studied the involvement of Mn in the water splitting system using correlative measurements of NMR, ESR and O_2 evolution activity (see Chapter 4). A brief description of our major conclusions is presented in the following sections (also see Table 5.1).

5.2.1 Effect of TPB, NH₂OH and H₂O₂ on T_2^{-1} of Thylakoid Membranes

If redox reagents added to thylakoid membranes shift the oxidation state of bound manganese then we expect T_2^{-1} of water protons to change. When TPB is added to thylakoid membranes T_2^{-1} increases as a function of added TPB showing four distinct plateaus which may be related to the successive reduction of different pools of Mn or other paramagnetic species (Fig. 4.1). TPB has been shown to interact with the oxidizing (or water) side of PS II (Homann, 1972; Erixon and Renger, 1974). Since TPB donates electrons in competition with H_20 there is an inhibition of 0_2 evolution. To explain our results on T_2^{-1} and O_2 evolution activity as a function of [TPB], we suggest that TPB donates electrons at two different sites depending upon its concentration (see section 4.2.1). At low (< 2 mM) concentrations of TPB there is no increase in T_2^{-1} suggesting that at these concentrations TPB is not interacting with the Mn of thylakoid membranes. As the concentration is increased further (> 2 mM) there is an enhancement in T_2^{-1} probably due to the conversion of oxidized form of Mn to Mn(II), implying an interaction of TPB with the various pools of Mn in thylakoid membranes. However, we cannot rule out the possibility that TPB induced increase in T_2^{-1} may include contributions from other paramagnetic species such as copper.

Effect of NH_2OH on the Mn of thylakoids appears two fold: conversion of higher oxidation states of Mn to Mn(II) and release of bound Mn. Bouges (1971) showed that O_2 yield as a function of flash number is altered in thylakoid membranes treated with low concentrations of NH_2OH . The oxygen yield pattern in NH_2OH treated thylakoids shows that the

first peak of 0_2 evolution is shifted from the 3rd to the 5th flash. Bouges proposed that the shift is caused either due to the binding of two molecules of NH_2OH or due to the presence of two electrons (donated by NH₂OH). Our measurements of T_2^{-1} enhancement in the presence of NH₂OH (section 4.2.2, Fig. 4.6) suggest that NH_2OH acts by increasing the concentration of Mn(II); $NH_{2}OH$ donates electrons and causes the reduction of higher oxidation states of Mn to Mn(II). At higher concentrations of $NH_{2}OH$ there is a progressive loss of 0_2 evolution which is correlated with the release of bound Mn (Chennae and Martin, 1969). We note that the release of Mn(II), as monitored by ESR spectroscopy, becomes apparent even at very low concentrations and increases at higher concentrations (Fig. 4.7). The small amount of manganese released at low concentrations may be from the very loosely bound pool of Mn that is not related to 0_2 evolution. However, the release of Mn is not complete even at very high concentrations (100 mM) of NH2OH confirming the presence of a tightly bound pool of Mn (Fig. 4.6).

An enhancement in T_2^{-1} is also observed (Fig. 4.13) when thylakoids are treated with H_2O_2 which favors the formation of more reduced S states. At high pH (8.8), H_2O_2 converts S_1 to S_{-1} which is quite stable in the dark (Velthuys and Kok, 1978). Since S_{-1} is a more reduced state it suggests that H_2O_2 increases T_2^{-1} by reducing oxidized Mn to Mn(II). 5.2.2 <u>Different Pools of Manganese in Thylakoid Membranes</u>

There are at least three pools of Mn in thylakoid membranes: (a) a very loosely bound pool of Mn which is non-functional in O_2 evolution; (b) a loosely bound pool of Mn related to O_2 evolution; and (c) a tightly bound pool of Mn to which no definite role had been assigned in the past.
Proton relaxation rate measurements monitor the functional as well as the non-functional manganese. The presence of very loosely bound nonfunctional manganese in confirmed by parallel measurements of T_2^{-1} , ESR detectable Mn(II) and O_2 evolution activity. This pool of Mn is released by the addition of 10-20 mM MgCl₂ (section 4.3.1, Fig. 4.15), as indicated by a decrease in T_2^{-1} and an increase in ESR detectable free Mn(II) signal, without any parallel effect on O_2 evolution activity. Since Mg can cause structural changes (e.g., grana stacking), such changes may lead to a change in the accessibility of Mn to bulk H₂O thus affecting T_2^{-1} . To obtain an insight into this problem, the effect of MgCl₂ was examined in trypsinated membranes which do not show Mg induced grana stacking. The effect of MgCl₂ on T_2^{-1} and ESR detectable free Mn(II) persists in trypsinated membranes (Fig. 4.17 and Table 4.1), implying that these effects are not related to structural changes involved in grana stacking (see section 4.3.2).

We have identified (section 4.3.3, Table 4.2) a tightly bound pool of Mn associated with the light harvesting complex, which constitutes about one-third of the functional manganese. The Mn associated with the LHC may have an important structural role in chloroplasts.

The removal of the functional pool of Mn by aging at 35°C is correlated well with a decline in 0_2 evolution activity. T_2^{-1} also shows a decrease as the concentration of Mn in thylakoids is decreased. This confirms the conclusion that T_2^{-1} can indeed monitor bound Mn related to 0_2 evolution.

5.2.3 <u>Contribution to Proton Relaxation Rates from Paramagnetic Species</u> Other than Mn(II)

Experiments described in sections 4.4.2 and 4.4.3 suggest that proton relaxation rates may also be affected by paramagnetic species other than Mn(II). This seems to occur when the membrane structure is altered by heat treatment at 40-50°C or by suspending thylakoids at extremely low and high pHs. Under these conditions, release of Mn(II) is not correlated with a decrease in T_2^{-1} ; this may be due to an increase in the accessibility of usually inaccessible Cu(II) of plastocyanin to bulk water. It is likely that heat treatment also makes the remaining tightly bound Mn more accessible to water protons. Another possibility is that the released Mn rebinds to the membranes non-specifically and the increase in T_2^{-1} is due to a change in the environment of the bound Mn. Sufficient evidence is not yet available to choose between either possibility.

Since proton relaxation rates monitor both functional and non-functional paramagnetic species, it seems important to maintain proper conditions so that the contribution from other paramagnetic species, not related to 0_2 evolution, is minimal. It appears that aging of thylakoids at 35°C (section 4.4.1, Fig. 4.19) does not involve any drastic changes in the membrane structure so as to increase the contribution of other paramagnetic species. In these experiments, as noted earlier, there is a perfect correlation between the amount of functional Mn, 0_2 evolution activity and T_2^{-1} , thus confirming the conclusion that water proton relaxation rates can indeed monitor bound Mn.

166

TABLE 5.1

Conclusions Made in the Thesis

	Conclusions	Location of Supporting Evidence
1.	As CO ₂ -stimulation of electron flow in thylakoid membranes occurs even with 0.5 mM added bicarbo- nate, it may have a physiological significance.	Fig. 3.1
2.	The CO ₂ stimulation of electron flow is unrelated to the CO ₂ effect on photophosphorylation.	Table 3.1
3.	The site of CO ₂ effect is between Q and the PQ pool; the reduction of PQ becomes the bottleneck reaction ($t_{1/2} = 100-200$ ms) in CO ₂ -depleted thylakoids; in normal thylakoids, the bottleneck step is the reoxidation of PQH ₂ ($t_{1/2} = 25-50$ ms).	Table 3.2 Figs. 3.6, 3.10
4.	It appears that the mechanism of action of CO_2 is through a protein covering the Q, R and PQ region: the CO ₂ effect is absent in trypsin-treated thy- lakoids; bicarbonate depletion changes the affin ity of atrazine to the membrane; and bicarbonate depletion blocks the proton uptake, and hence the release at the PQ level.	Figs. 3.14, 3.15, 3.16, 3.17, 3.18
5.	Addition of TPB, NH ₂ OH and H ₂ O ₂ is suggested to convert higher oxidation states of Mn to Mn(II) as they cause an increase in T_2^{-1} ; addition of NH ₂ OH also releases bound Mn thus causing a decrease in T_2^{-1} .	Figs. 4.1, 4.6 4.7, 4.13
6.	A very loosely bound pool of Mn, associated with thylakoids, is released by 10-20 mM MgCl ₂ with- out affecting O ₂ evolution activity.	Fıg. 4.15
7.	The isolated light harvesting complex contains ~1/3 of the total functional Mn pool; this may be the tightly bound pool of Mn in chloroplasts.	3 Table 4.2
8	Removal of Mn by aging at 35°C shows a parallel decrease both in O2 evolution activity and T21, thus showing that T21 can indeed be a good monitor of functional Mn of thylakoids.	Fig. 4.19
9.	Contribution of Cu(II) to water proton relaxation rates may become significant when membrane struc- ture is altered by specific treatments.	Figs. 4.20, 4.21, 4.22 4.23

LITERATURE CITED

Abeles, B., A. H. Brown and B. C. Mayne (1961) Plant Physiol. 36, 202-207. Allen, F. and J. Franck (1955) Arch. Biochem. Biophys. 58, 124-143. Anderson, J. M. and N. A. Pyliotis (1969) Blochim. Biophys. Acta. 189, 280-293. Armstrong, J. M. (1964) Biochim. Biophys. Acta. 86, 194-197. Arnon, D. I. (1949) Plant Physiol. 24, 1-15. Arntzen, C. J. (1978) In Current Topics in Bioenergetics (Edited by D. R. Sanadi and L. P. Vernon), Vol. 8, pp. 111-160. Academic Press, New York. Arntzen, C. J. and J. M. Briantais (1975) In Bioenergetics of Photosynthesis (Edited by Govindjee), pp. 52-113. Academic Press, New York. Ausländer, W. and W. Junge (1974) Biochim. Brophys. Acta 357, 285-298. Ausländer, W. and W. Junge (1975) FEBS Lett. 59, 310-315. Babcock, G. T. and K. Sauer (1975a) Biochim. Biophys. Acta 376, 315-328. Babcock, G. T. and K. Sauer (1975b) Blochim. Blophys. Acta 376, 329-344. Babcock, G. T., R. E. Blankenship and K. Sauer (1976) FEBS Lett. 61, 286-289. Barber, J. (1976) In The Intact Chloroplast, Topics in Photosynthesis (Edited by J. Barber), Vol. 1, pp. 89-134. Elsevier Scientific, Amsterdam. Batra, P. P. and A. T. Jagendorf (1965) Plant Physiol. 40, 1074-1079. Binder, A., E. Tel-Or and M. Avron (1976) Eur. J. Biochem. 67, 187-196. Blankenship, R. E. and K. Sauer (1974) Blochim. Biophys. Acta 357, 252-266. Blankenship, R. E., G. T. Babcock, J. T. Warden and K. Sauer (1975a) FEBS Lett. 51, 287-293. Blankenship, R. E., G. T. Babcock and K. Sauer (1975b) <u>Biochim</u>. <u>Biophys</u>. Acta 387, 165-175.

Blumberg, W. E. and J. Peisach (1966) Blochim. Blophys. Acta 126, 269-273. Böhme, H., S. Reimer and A. Trebst (1971) Z. Naturforsch. 26b, 341-352. Bose, S. and C. J. Arntzen (1978) Arch. Biochem. Biophys. 185, 567-578. Bouges, B. (1971) Biochim. Biophys. Acta 234, 103-112. Bouges-Bocquet, B. (1973a) Biochim. Biophys. Acta. 292, 772-785. Bouges-Bocquet, B. (1973b) Biochim. Biophys. Acta. 314, 250-256. Bouges-Bocquet, B. and R. Delosme (1978) FEBS Lett. 74, 173-181. Bowes, J. M. and A. R. Crofts (1978) Z. Naturforsch. 33c, 271-275. Burke, J. J., C. L. Ditto and C. J. Arntzen (1978) Arch. Biochem. Biophys. 187, 252-263. Carr, H. Y. and E. M. Purcell (1954) Phys. Rev. <u>94</u>, 630-638. Chen, K. Y. and J. H. Wang (1974) Bioinorg. Chem. 3, 367-380. Cheniae, G. M. (1970) Ann. Rev. Plant. Physiol. 21, 467-498. Cheniae, G. M. and I. F. Martin (1966) Brookhaven Symp. Biol. 19, 406-417. Chennae, G. M. and I. F. Martin (1968) Biochim. Biophys. Acta 153, 819-837. Cheniae, G. M. and I. F. Martin (1969) Plant Physiol. 44, 351-360. Cheniae, G. M. and I. F. Martin (1970) Biochim. Biophys. Acta 197, 219-239. Cheniae, G. M. and I. F. Martin (1971) Plant Physiol. 47, 568-575. Cheniae, G. M. and I. F. Martin (1972) Plant Physiol. 50, 87-94. Cheniae, G. M. and I. F. Martin (1973) Photochem. Photobiol. 17, 441-459. Cheniae, G. M. and I. F. Martin (1978) Biochim. Biophys. Acta 502, 321-344. Delrieu, M. J. (1974) Photochem. Photobiol. 20, 441-454.

169

Den Haan, G. A., H. G. DeVries and L. N. M. Duysens (1976) Biochim. Biophys. Acta 430, 265-281. Döring, G., H. H. Stiehl and H. T. Witt (1967) Z. Naturforsch. 22b, 639-644. Doschek, W. W. and B. Kok (1972) <u>Biophys. J.</u> 12, 832-838. Dwek, R. A. (1973) <u>Nuclear Magnetic Resonance (NMR) in Biochemistry</u>: Application to Enzyme Systems. Clarendon, Press, Oxford. Early, J. E. (1973) Inorg. Nucl. Chem. Lett. 9, 487-490. Eckert, H. J., H. E. Buchwald and G. Renger (1979) FEBS Lett. 103, 291-295. Emerson, R. and W. Arnold (1932a) J. Gen. Physiol. 15, 391-420. Emerson, R. and W. Arnold (1932b) <u>J. Gen. Physiol. 16</u>, 191-205. Erixon, K., and G. Renger (1974) Biochim. Biophys. Acta 333, 95-106. Farrar, T. C. and E. D. Becker (1971) Pulse and Fourier Transform NMR: Introduction to Theory and Methods. Academic Press, New York. Fogg, G. E. (1968) <u>Photosynthesis</u>, pp. 13-23. American Elsevier Publ. Co., Inc. New York. Forbush, B., B. Kok and M. McGloin (1971) Photochem. Photobiol. 14, 307-321. Fowler, F. (1977) <u>Biochim. Biophys. Acta</u> 462, 414-421. Fowler, C. F. and B. Kok (1974) Biochim. Biophys. Acta 357, 299-307. Foyer, C. H. and D. O. Hall (1979) <u>FEBS</u> <u>Lett.</u> 101, 324-328. Garrett, B. B. and L. O. Morgan (1966) <u>J. Chem. Phys</u>. <u>44</u>, 890-897. Gerhardt, B. (1966) Ber. Dtsch. Bot. Ges. 79, 63-68. Geske, D. H. (1959) J. Chem. Phys. 63, 1062-1070. Geske, D. H. (1962) J. Chem. Phys. 66, 1743-1744. Giaquinta, R. T. and R. A. Dilley (1975) Brochim. Brophys. Acta 387, 288-305. Giaquinta, R.T., R. A. Dilley, B. R. Selman and B. J. Anderson (1974) Arch. Biochem. Biophys. 162, 200-209.

Good, N. E. (1963) Plant Physiol. 38, 298-304.

Good, N. E., S. Izawa and G. Hind (1966) <u>Curr. Top. Bioenerg</u>. <u>1</u>, 75-112.

Govindjee (1979) Plant. Biochem. J. (In press).

- Govindjee and P. A. Jursinic (1979) <u>Photochemical</u> and <u>Photobiological</u> <u>Reviews</u> 4, 125-205.
- Govindjee and R. Khanna (1978) In <u>Photosynthetic</u> <u>Oxygen</u> <u>Evolution</u> (Edited by H. Metzner), pp. 269-282. Academic Press, London.
- Govindjee and J. J. S. van Rensen (1978) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> 505, 183-213.
- Govindjee, M. P. J. Pulles, R. Govindjee, H. J. van Gorkom and L. N. M. Duysens (1976) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>449</u>, 602-605.
- Govindjee, T. Wydrzynski and S. B. Marks (1977) In <u>Bioenergetics of</u> <u>Membranes</u> (Edited by L. Packer, G. C. Papageorgiou and A. Trebst), pp. 305-316. Elsevier Biomedical Press, Amsterdam.
- Govindjee, P. Mathis, C. Vernotte, D. Wong, S. Saphon, T. Wydrzynski and J. M. Briantais (1979) Z. Naturforsch. (In press).
- Gribova, Z. P., N. I. Zakharova and L. I. Murza (1978) <u>Molekulyarnaya</u> <u>Biologia</u> 12, 157-164.
- Hardt, H. and B. Kok (1976) Biochim. Biophys. Acta 449, 125-135.

Hardt, H. and B. Kok (1977) Plant Physiol. 60, 225-229.

Harnischfeger, G. (1974) Z. Naturforsch. 29c, 705-709.

Harriman, A. and J. Barber (1979) In <u>Photosynthesis in Relation to Model</u> <u>Systems, Topics in Photosynthesis</u> (Edited by J. Barber), Vol. 3, pp. 243-280, Elsevier Scientific, Amsterdam.

Henriques, F. and R. B. Park (1976) <u>Biochim. Biophys. Acta</u> 430, 312-320.

Hill, R. and F. Bendall (1960) <u>Nature</u> (London) <u>186</u>, 136-137.

Hill, R. and R. Scarisbrick (1940) <u>Nature</u> 146, 61-62.

Hoch, G. and I. F. Martin (1963) <u>Arch. Blochem</u>. <u>Biophys</u>. <u>102</u>, 430-438.

Homann, P. H. (1967) <u>Plant Physiol</u>. <u>42</u>, 997-1007.

Homann, P. H. (1968) <u>Biochem</u>. <u>Biophys</u>. <u>Res</u>. <u>Commun</u>. <u>33</u>, 229-234. Homann, P. H. (1972) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>256</u>, 336-344.

- Huzisige, H., M. Isimoto and H. Inoue (1968) In <u>Comparative Biochem</u>. <u>and Biophys. of Photosynthesis</u> (Edited by A. T. Jagendorf and R. C. Fuller), pp. 170-178. Univ. of Tokyo Press, Tokyo.
- Inoue, Y., Y. Kobayashi, E. Sakamoto and K. Shibata (1975) Plant Cell Physiol. 16, 327-336.
- Izawa, S. (1962) Plant Cell Physiol. 3, 221-227.
- Izawa, S. and N. E. Good (1966) Plant Cell Physiol. 41, 544-553.
- Izawa, S. and R. L. Pan (1978) <u>Biochem. Biophys. Res. Commun.</u> 83, 1171-1177.
- Izawa, S., J. M. Gould, D. R. Ort, P. Felker and N. E. Good (1973) Biochim. Biophys. Acta 305, 119-128.
- Joliot, P. (1961) J. Chim. Phys. 58, 584.
- Joliot, P. (1965) <u>Biochim. Biophys. Acta</u> 102, 116-134.
- Joliot, P. (1967) <u>Brookhaven Symp. Biol.</u> <u>19</u>, 418-433.
- Joliot, P. (1968) Photochem. Photobiol. 8, 451-463.
- Joliot, P. and B. Kok (1975) In <u>Bioenergetics of Photosynthesis</u> (Edited by Govindjee), pp. 387-412. Academic Press, New York.
- Joliot, P., G. Barbieri and R. Chabaud (1969) <u>Photochem</u>. <u>Photobiol</u>. <u>10</u>, 309-329.
- Joliot, P., A. Joliot, B. Bouges and G. Barbieri (1971) <u>Photochem</u>. <u>Photobiol</u>. <u>14</u>, 287-305.
- Jones, L. and B. Kok (1966) Plant Physiol. 41, 1037-1043.
- Junge, W. and W. Ausländer (1973) <u>Blochim</u>. <u>Blophys</u>. <u>Acta</u> <u>333</u>, 59-70.
- Junge, W. and H. T. Witt (1968) Z. Naturforsch. 23b, 244-254.
- Junge, W., G. Renger and W. Ausländer (1977) FEBS Lett. 79, 155-159.
- Junge, W., W. Ausländer, A. J. McGeer and T. Runge (1979) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>546</u>, 121-141.
- Jursinic, P., J. Warden and Govindjee (1976) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>440</u>, 322-330.

- Katoh, S. and A. San Pietro (1967) <u>Arch. Biochem</u>. <u>Biophys</u>. <u>122</u>, 144-152.
- Kessler, E., W. Arthur and J. E. Brugger (1957) <u>Arch. Blochem</u>. <u>Biophys.</u> <u>71</u>, 326-335.
- Khanna, R., P. A. Jursinic, T. Wydrzynski and Govindjee (1976) <u>American</u> <u>Society of Photobiology Abstract #WAM-C12</u>, pp. 67-68.
- Khanna, R., Govindjee and T. Wydrzynski (1977) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>462</u>, 208–214.
- Khanna, R., S. Rajan, Govindjee and H. S. Gutowsky (1979) <u>Biophys</u>. J. <u>25</u>, Biophysical Society Abstract #T-AM-E8, p. 147a.
- Kimimura, M. and S. Katoh (1972) Plant Cell Physiol. 13, 287-296.
- Klimov, K. V., A. V. Klevanik, V. A. Shuvalov and A. A. Krasnovsky (1977) FEBS Lett. 82, 183-186.
- Kok, B. and G. M. Cheniae (1966) In <u>Current Topics in Bioenergetics</u> (Edited by R. Sanadi), Vol. I, pp. 1-47. Academic Press, New York.
- Kok, B., B. Forbush and M. McGloin (1970) <u>Photochem</u>. <u>Photobiol</u>. <u>11</u> 457-475.
- Kok, B., R. Radmer and C. F. Fowler (1974) In Proc., <u>3rd Intl. Congr.</u> <u>Photosynthesis</u> (Edited by M. Avron), Vol. I, pp. 485-496. Elsevier, Amsterdam, The Netherlands.
- Laemmli, U. K. (1979) Nature (London) 227, 680-685.
- Lagoutte, B. and J. Duranton (1975) FEBS Lett. 51. 21-23.

Lavorel, J. (1976) FEBS Lett. 66. 164-168.

- Lemasson, C. and G. Barbieri (1971) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>245</u>, 386-397.
- Lozier, R., M. Baginsky and W. L. Butler (1971) <u>Photochem</u>. <u>Photobiol</u>. <u>14</u>, 323-328.
- Lumsden, J. and D. O. Hall (1975) <u>Biochem</u>. <u>Biophys</u>. <u>Res. Commun</u>. <u>64</u>, 595-602.
- Malkin, R. and H. B. Posner (1978) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>501</u>, 552-554.
- Mar, T. and Govindjee (1972) J. Theor. Biol. 36, 427-446.
- Margulies, M. (1972) <u>Biochim. Biophys. Acta</u> <u>267</u>, 96-103.

Meiboom, S. and D. Gill (1958) <u>Rev. Sci. Intrum.</u> 29, 688-691.

Metzner, H. (1966) Naturwissenschaften 53, 141-150.

Metzner, H. (1976) Bioelectrochem. Bioenergetics 3, 573-581.

Mildvan, A. S. and M. Cohn (1970) Adv. Enzyml. 33, 1-70.

Mohanty, P., T. Mar and Govindjee (1971) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>253</u>, 213-221.

Ogren, W. L. and L. D. Hunt (1978) In <u>Photosynthetic Carbon Assimilation</u> (Edited by H. W. Siegelman and G. Hind), pp. 127-138. Plenum Publishing Corporation, New York.

Olson, J. M. (1970) Science 168, 438-446.

Ouitrakul, R. and S. Izawa (1973) Biochim. Biophys. Acta 305, 105-118.

Park, R. B. and N. G. Pon (1963) J. Mol. Biol. 6, 105-114.

Pfister, K. and C. J. Arntzen (1979) Z. <u>Naturforsch</u>. (In press).

- Pfister, K., S. R. Radosevich and C. J. Arntzen (1979) <u>Plant Physiol</u>. (In press).
- Phung-Nhu-Hung, S., B. Houlier and A. Moyse (1974) <u>C. R. Acad. Sci</u>. (Paris) <u>279</u>, 1669-1672.

Pirson, A. (1937) Z. Bot. 31, 193-267.

Pirson, A., C. Tichy and G. Wilhelmi (1952) Planta 40, 199-253.

Possingham, J. V. and D. Spencer (1962) <u>Aust. J. Biol. Sci. 15</u>, 58-68.

- Possingham, J. V., M. Vesk and F. V. Mercer (1964) <u>J. Ultrastructure</u> <u>Res. 11</u>, 68-83.
- Pulles, M. P. J., H. J. van Gorkom and J. G. Willemsen (1977) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>449</u>, 536-540.

Punnett, T. and R. V. Iyer (1964) <u>J.Biol. Chem.</u> 239, 2335-2339.

Radmer, R. (1979) <u>Biochim. Biophys. Acta</u> 546, 418-425.

Radmer, R. and G. M. Cheniae (1977) In <u>Primary Processes of Photosyn-</u> <u>thesis, Topics in Photosynthesis</u>, (Edited by J. Barber), Vol. 2, pp. 303-348. Elsevier Scientific, Amsterdam.

Radmer, R. and B. Kok (1973) Biochim. Biophys. Acta 314, 28-41.

- Renger, G. (1970) Z. <u>Naturforsch</u>. 25b, 966-971.
- Renger, G. (1972a) Physiol. Veg. 10, 329-345.
- Renger, G. (1972b) Eurp. J. Biochem. 27, 259-269.
- Renger, G. (1972c) Biochim. Biophys. Acta 256, 428-439.
- Renger, G. (1976) <u>Biochim. Biophys. Acta</u> 440, 287-300.
- Renger, G. (1977) FEBS Lett. 81, 223-228.
- Renger, G. and Tiemann (1979) <u>Biochim. Biophys. Acta</u> 545, 316-324.
- Renger, G. and Ch. Wolff (1976) Blochim. Biophys. Acta 423, 610-614.
- Ruben, S., M. Randall, M. Kamen and J. L. Hyde (1941) <u>J. Am. Chem. Soc.</u> <u>63</u>, 877-879.
- Saphon. S. and A. R. Crofts (1977) Z. Naturforsch. 32c, 617-626.
- Sauer, K., P. Mathis, S. Acker and J. A. van Best (1978) <u>Biochim</u>. <u>Biophys. Acta</u> 503, 120-134.
- Schliephake, W., W. Junge and H. T. Witt (1968) Z. <u>Naturforsch</u>. 23b, 1561-1578.
- Schmidt, R. and W. Junge (1975) Biochim. Biophys. Acta 394, 76-92.
- Shuvalov, V. A., B. Ke and E. Dolan (1979) FEBS Lett. 100, 5-8.
- Siderer, Y., S. Malkin, R. Poupko and Z. Luz (1977) Arch. Biochem. Biophys. 179, 174-182.
- Siggel, U. (1976) Bioelectrochem. Bioenerg. 3, 302-318.
- Siggel, U., R. Khanna, G. Renger and Govindjee (1977) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>462</u>, 196-207.
- Steinback, K. E., J. J. Burke and C. J. Arntzen (1979) <u>Arch. Biochem.</u> <u>Biophys</u>. <u>195</u>, 546-557.
- Stemler, A. (1977) <u>Biochim. Biophys. Acta</u> 460, 511-522.
- Stemler, A. (1979) Biochim. Biophys. Acta 545, 36-45.
- Stemler, A. and Govindjee (1973) Plant Physiol. 52, 119-123.
- Stemler, A. and R. Radmer (1975) <u>Science</u> 190, 457-458.

Stemler, A., G. T. Babcock and Govindjee (1974) Proc. Natl. Acad. Sci. U.S. 71, 4679-4683. Stern, B. K. and B. Vennesland (1960) J. Biol. Chem. 235, PC 51. 237, Stern, B. K. and B. Vennesland (1962) J. Biol. Chem. 596-602. Stiehl, H. H. and H. T. Witt (1968) Z. Naturforsch. 23b, 220-224. Stiehl, H. H. and H. T. Witt (1969) Z. Naturforsch. 24b, 1588-1598. Takahashi, M. and K. Asada (1976) Eur. J. Biochem. 64, 445-452. Takahashi, M. and K. Asada (1977) Plant and Cell Physiol. 18, 807-814. Tel-Or, E. and M. Avron (1974) In Proc. 3rd Int. Photosynthesis Congr. (Edited by M. Avron), Vol. I, pp. 569-578. Dr. N. V. Junk Publishers, The Hague. Tel-Or, E. and W. D. Stewart (1975) Nature (London) 258, 715-716. Thibault, P. (1978) J. Theor. B101. 73, 271-284. Tiemann, R., G. Renger, P. Gräber and H. T. Witt (1979) Biochim. Biophys. <u>Acta</u> <u>546</u>, 498-519. Tischer, W. and H. Strotmann (1977) <u>Biochim. Biophys. Acta</u> 460, 113-125. Tolbert, N. E. (1973) Plant Physiol. 51, 234-244. Trebst, A. (1974) Ann. Rev. Plant Physiol. 25, 423-458. Vaklinova, S. (1964) C. R. Acad. Bulg. Sci. 17, 265-268. Van Niel, C. B. (1931) Arch. Microbiol. 3, 1-112. Velthuys, B. R. (1976) Ph.D. Thesis, The State University, Leiden, The Netherlands. Velthuys, B. R. and J. Amesz (1974) Biochim. Biophys. Acta 333, 85-94. Velthuys, B. R. and B. Kok (1978) Biochim. Biophys. Acta 502, 211-221. Velthuys, B. R. and J. W. M. Visser (1975) FEBS Lett. 55, 109-112. Warburg, O. and G. Krippahl (1960) Z. Naturforsch. 15b, 367-369. Warden, J. T., R. E. Blankenship and K. Sauer (1976) Blochim. Biophys. <u>Acta 423</u>, 462-478.

Weaver, E. C. (1968) <u>Ann. Rev. Plant Physiol.</u> 19, 283-294.

Weiss, C. and K. Sauer (1970) Photochem. Photobiol. 11, 495-501.

West, J. and R. Hill (1967) Plant Physiol. 42, 819-826.

Whittingham, C. P. and P. M. Bishop (1963) <u>Natl. Acad. Sci. Natl. Res.</u> <u>Counc.</u>, Publ. 1145, pp. 371-380.

Whittingham, C. P. and A. H. Brown (1958) <u>J. Exptl. Bot.</u> 9, 311-319.

- Williams, W. P. (1977) In <u>Primary Processes of Photosynthesis</u>, <u>Topics in</u> <u>Photosynthesis</u> (Edited by J. Barber), Vol. 2, pp. 49-147. Elsevier Scientific, Amsterdam, The Netherlands.
- Willstäter, R. and A. Stoll (1918) <u>Untersuchungen über die Assimilation</u> <u>der Kohlensäure</u>. Springer, Berlin.
- Winget, D. and M. Spector (1979) <u>Proc. Nat. Acad. Sci</u>. (U.S.A.) (In press)
- Witt, H. T. (1975) In <u>Bioenergetics of Photosynthesis</u> (Edited by Govindjee), pp. 493-554. Academic Press, New York.
- Wong, D. and Govindjee (1979) FEBS Lett. 97, 373-377.
- Wong, D., H. Merkelo and Govindjee (1979) FEBS Lett. 104, 223-226.
- Wurmser, R. (1930) In <u>Oxidation et Reductions</u>, p. 30. Presses Univ. de France, Paris.
- Wydrzynskı, T. (1977) Ph.D. Thesis, University of Illinoıs at Champaıgn-Urbana.
- Wydrzynski, T. and Govindjee (1975) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>387</u>, 403-408.
- Wydrzynski, T. and K. Sauer (1979) Bjochim. Biophys. Acta (In press).
- Wydrzynski, T., N. Zumbulyadis, P. G. Schmidt and Govindjee (1975) <u>Biochim. Biophys</u>. <u>Acta</u> <u>408</u>, 349-354.
- Wydrzynski, T., Govindjee, N. Zumbulyadıs, P. G. Schmidt and H. S. Gutowsky (1976) <u>ACS Symp. Ser</u>. <u>34</u>, 471–481.
- Wydrzynski, T., S. B. Marks, P. G. Schmidt, Govındjee and H. S. Gutowsky (1978) <u>Biochemistry</u> <u>17</u>, 2155–2162.

Yamashita, T. and W. L. Butler (1968) <u>Plant Physiol</u>. <u>43</u>, 1978-1986. Yamashita, T. and G. Tomita (1974) <u>Plant Cell Physiol</u>. <u>15</u>, 69-82. Yamashita, T., J. Tsuji and K. Tomita (1971) <u>Plant Cell Physiol.</u> <u>12</u>, 117-126.

Yamashita, T., J. Tsuji-Kaneko, Y. Yamada and G. Tomita (1972) <u>Plant</u> <u>Cell Physiol</u>. <u>13</u>, 3539-364.

Zilinskas, B. A. and Govindjee (1975) <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> <u>387</u>, 306-319.

Zilinskas, B. and Govindjee (1976) Z. <u>Pflanzenphysiol</u>. <u>77</u>, 302-314.

Zilinskas-Braun, B. and Govindjee (1974) Plant Sci. Lett. 3, 219-227.

Rita Khanna was born on March 7, 1950 in Delhi, India where she received her primary and secondary education. She obtained her Bachelor of Science degree in Biology and her Master of Science in Botany (both in the First Class) from the University of Delhi in 1971 and 1973, respectively. She was a University Grants Commission scholar from 1970 to 1972. In 1973 she completed a Diploma in Molecular Biochemistry at the Indian Institute of Science, Bangalore on an Institute Fellowship. From July 1973 to August 1974, under a Research Fellowship from the Council of Scientific and Industrial Research, she isolated and characterized specific t-RNAs and synthetases in developing chick embryo. She joined the University of Illinois in 1974, where she has held teaching and research assistantships (1975-79); she was a receptent of the University of Illinois graduate fellowship during 1974-75, and in the Summer of 1976. During the summer of 1976 she worked at the Max Volmer Institute for Physical Chemistry and Molecular Biology, Technical University, West Berlin.

She is a member of the American Society of Plant Physiologists, the Biophysical Society and Sigma Xi, and has coauthored the following publications:

- 1. Studies on the Role of Cyclic AMP on Lipid Metabolism. Project Report submitted in partial fulfillment of the requirement for the Diploma in Molecular Biochemistry, Indian Institute of Science, Bangalore, India, July 1973, 51 pp.
- Investigation of the Absorption Changes of the Plastoquinone-System in Broken Chloroplasts: The Effect of Bicarbonate-Depletion. <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u> (1977) 462, 196-207.

VITA

4. Bicarbonate - Its Role in Photosystem II. In <u>Photosynthetic</u> <u>Oxygen Evolution</u>. Edited by H. Metzer (1978), pp. 269-282. Academic Press, London.

Ł

- 5. Excitation Energy Transfer in Photosystem I and II from Grana and in Photosystem I from Stroma Lamellae, and Identification of Emission Bands with Pigment-Protein Complexes at 77[°]K. Zeitschrift fur Pflanzenphysiologie (1979). In press.
- 6. Photosynthesis in Green Plants. In <u>Handbook Series in Nutrition</u> <u>and Food</u>. Edited by M. Recheigl, Jr. CRC Press, Inc., Cleveland, Ohio (In press).