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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE
PHOTOSYNTHETIC OXYGEN EXCHANGE MEASUREMENTS IN MARINE ALGAE

by

Sara Irene Swenson

B.Sc. University of Alaska, Fairbanks, 1976
M.Sc. University of Alberta, Edmonton, 1979

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
Physics

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SIMON FRASER UNIVERSITY
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Photosynthetic Oxygen Exchange

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ABSTRACT

Photosynthetic oxygen evolution occurs in the thylakoid membranes of chloroplasts as a result of water-splitting, which provides electrons for carbon dioxide assimilation in plants. The currently accepted model of oxygen evolution, the S state hypothesis, requires the generation and cooperation of four photochemically-formed oxidizing equivalents in individual photosystem II reaction centers. The major evidence for this model involves a linear four step oscillation in oxygen evolution observed during a series of saturating light flashes.

Oxygen exchange (evolution and uptake) was measured in marine algae using a bare platinum electrode in direct contact with the sample, and a silver/silver chloride counter electrode. Illumination of dark-adapted algae with five microsecond saturating light flashes produced a characteristic series of oxygen reduction current pulses at the platinum electrode. A "pile-up" of oxygen pulses was observed in marine algae when the decay time of the oxygen pulse was longer than the time interval between flashes.

These partially resolved experimental oxygen exchange pulses for the green algae, Ulva species, were numerically deconvoluted by fitting the experimental curve with the sum of time-shifted single pulses derived from the shape of the pulse
due to the third flash, proving dynamic linearity of the electrode system. By determining the amount of oxygen produced on the third flash, multiples of the reference curve were used to quantify the amount of oxygen produced for each flash in the flash sequence.

Plotting the oxygen yield as a function of flash number led to the familiar four step oscillatory pattern of oxygen exchange for *Ulva*. However, this oxygen exchange is a composite of oxygen evolution and endogenous oxygen uptake in the algae. Eliminating the oxygen evolution component and subtracting the resultant oxygen uptake from the oxygen exchange curve yielded the corrected oxygen evolution flash sequence. This showed a similar four step oscillation, but with negligible damping over the first three oscillations.

Ambient oxygen concentration affects oxygen exchange in *Ulva*. Oxygen uptake does not occur under anaerobic conditions, but oxygen evolution in *Ulva* is reversibly and partially inhibited.
Dedicated to my parents

for their continual support and encouragement
ACKNOWLEDGMENTS

I would like to thank the members of my supervisory committee, Dr. K. Colbow, Dr. M.L.W. Thewalt, and Dr. W.E. Vidaver, as well as Dr. A.H. Burr and Dr. R. Popovic, for their encouragement and advice over the past several years. My special thanks go to Silvia Wessel for her editing and critical input into this thesis and to Pascal Meunier for his involvement in systems analysis and circuit design. I would like to thank my fellow graduate students, especially Simon Watkins, Ken Urquhart, Doug Bruce, David Fraser, Peter Toivonen, Peter Sibbald, and Ursula Snyder, as well as Jim Whelan and Alexis Mackintosh for their help with various aspects of this thesis research. I would also like to thank Mireille Amat for instructing me in phycology and for showing me the best beaches to collect marine algae on a sunny day.

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CHAPTER 1. INTRODUCTION

Oxygen evolution is an integral component of photosynthesis in green plants, algae, and the cyanobacteria. Studies of oxygen evolution in isolated chloroplasts and plants have contributed greatly to the knowledge of photosynthetic processes (Emerson and Arnold, 1932; Joliot and Joliot, 1968; Kok et al., 1970; Joliot et al., 1971; Forbush et al., 1971; Radmer and Cheniae, 1977; Jursinic, 1981). However, the nature of the oxygen-evolving complex (OEC) and the detailed mechanism of oxygen evolution in plants is still largely unknown.

Research on photosynthetic oxygen evolution necessarily encompasses several scientific disciplines, such as physics, electrochemistry, plant biochemistry, and plant physiology, which are essential to the interpretation of $O_2$ exchange measurements. An overview of photosynthesis will be presented first, in order to present the scope of $O_2$ evolution and uptake within the field of photosynthesis before discussing the subject of study in Sec. 1.7. A detailed discussion of the oxygen-evolving complex and the currently accepted models for $O_2$ evolution and uptake will be presented in Chapter 2.
1.1 Photosynthetic systems

Photosynthetic bacteria, algae, and green plants absorb the electromagnetic energy of sunlight and convert it to chemical energy in the form of high energy bonds in adenosine triphosphate (ATP) and reduced (protonated) nicotinamide adenine dinucleotide phosphate (NADPH). ATP and NADPH are subsequently used to anabolize CO₂ to carbohydrates. In the photosynthetic process in plants and cyanobacteria, H₂O is the initial electron donor for electron transport.

The ability to oxidize water and evolve molecular oxygen is unique to plants, algae, and the cyanobacteria. The early work of Emerson (see Emerson, 1958) led to the discovery of two distinguishable pigment systems within the photosynthetic apparatus, and the determination that photosynthesis in these organisms requires the cooperation of two light reactions from Photosystem I (PS I) and Photosystem II (PS II) (Hill and Bendall, 1960; Duysens et al., 1961; Kok and Hoch, 1961). The two reaction centers of PS I and PS II (referred to as P₇₀₀ and P₆₈₀ respectively) are connected in series by electron- and proton-carrying components. Reaction centers are denoted by the letter P (for pigment) and the wavelength of maximum absorption of its lowest electronic transition, i.e., 700 nm for PS I and 680 nm for PS II.

The function of PS I is to generate a reductant in the form of NADPH, while the function of PS II is to provide the
oxidizing power necessary for water-splitting and to transfer electrons via electron carriers to PS I. Photosystem II photo-reactions remove electrons and protons from water, transfer electrons through the electron transport chain to reduce the PS I reaction centers, and yield molecular oxygen as a by-product. The NADPH and ATP molecules generated by light are then used to reduce CO$_2$ to carbohydrates by a series of dark reactions called the Calvin cycle.

Photosynthesis in all photosynthetic organisms consists of the following processes (after Foyer, 1984):

Light absorption:

The first step in photosynthesis is initiated when light is absorbed by an array of antennae pigment molecules (referred to as a light-harvesting complex or LHC) that are embedded in lipoprotein membranes (Junge, 1977). Some of the energy absorbed by the pigment molecules is transferred to a reaction center associated with either PS I or PS II that contains a type of chlorophyll molecule in a special environment.

Charge separation:

The absorption of a photon by a PS I or PS II reaction center chlorophyll molecule results in excitation of the molecule and the loss of an electron to an adjacent acceptor molecule (either a pheophytin or another
chlorophyll molecule). This initial charge separation is the first chemical change in the reaction sequence of photosynthesis (Sauer, 1979).

Electron transport.
The oxidized PS II reaction center chlorophyll becomes reduced by accepting an electron from an oxidizable substrate such as $\text{H}_2\text{O}$. The electron derived from the reaction center chlorophyll is removed from the reaction center by the primary electron acceptor. The transfer of electrons via the electron transport chain is coupled to the accumulation of protons which facilitates the synthesis of ATP (phosphorylation) by a membrane-bound ATPase (Avron, 1981).

Energy storage.
The chemical energy obtained from sunlight by the light reactions of photosynthesis is stored in the form of high energy bonds of nucleotides (ATP type) and in the reducing power of NADPH. ATP and NADPH are used to synthesize organic compounds via carbon metabolism (Robinson and Walker, 1981).

In green plants and algae, photosynthesis occurs in subcellular organelles called chloroplasts (Fig. 1.1) which have a double boundary membrane referred to as the chloroplast...
envelope. The area inside the envelope, called the stroma, contains the enzymes necessary for carbon metabolism. Within the stroma are inner membranes consisting of flattened sacs or discs, called thylakoids. Each chloroplast contains approximately $10^3$ thylakoids, each on the order of 500 nm in diameter (Witt, 1975). In higher plants, these are often arranged in stacks known as grana, which are linked by hollow tubes called stroma lamellae. This interconnected thylakoid membrane system contains the components necessary for the light-induced formation of ATP and NADPH (Fig. 1.2) and effectively separates the components required for the light and dark reactions of photosynthesis. The process of photosynthesis will be discussed in the following sections.

1.2 Light absorption and the transfer of energy

The photosynthetic pigments, associated with proteins in a light-harvesting complex in the thylakoid membrane, facilitate the absorption of light. Each thylakoid contains approximately $10^5$ pigment molecules (Witt, 1975), largely the primary photosynthetic pigment chlorophyll, carotenoids, and phycobilins. Higher plants contain chlorophyll-a and chlorophyll-b, while algae may contain other chlorophylls (c and d), in addition to chlorophyll-a. Chlorophyll and the various accessory pigments have absorption maxima at different
Fig. 1.1  Diagram of a higher plant or green algae chloroplast (after Salisbury and Ross, 1978)

The chloroplast is surrounded by a double membrane system comprised of lipids and proteins. The outer membrane serves as a physical barrier between the chloroplast and cytosol, while the inner membrane controls molecular traffic into and out of the chloroplast. The internal membranes of the chloroplast, which contain the photosynthetic pigments and associated proteins, are referred to as thylakoids (stacked to form grana) or stroma lamellae. These membranes extend throughout the chloroplast matrix (stroma).
This model of a thylakoid membrane shows the components of a photosynthetic system. Each thylakoid contains approximately 200 electron transfer chains and their associated pigment complexes (Witt, 1979b). The PS II and PS I reaction centers (P_680 and P_700) are situated near the intrathylakoid space. The primary acceptors for both photosystems are located near the stroma side of the membrane (Witt, 1979b).

Symbols:

PS I, Photosystem I; PS II, Photosystem II; NADP, nicotinamide adenine dinucleotide phosphate; Q_A, the primary electron acceptor; Q_B, the secondary electron acceptor; PQ, plastoquinone; FNR, ferredoxin-NADP reductase; Fd, ferredoxin; PC, plastocyanin; Fd-Th-red, Ferredoxin-thioredoxin reductase; CF_1 and CF_0, coupling factor components; ADP (ATP), adenine di(tri)nucleotide phosphate; Cyt b_6, cytochrome b_6; Cyt f, cytochrome f; Fe-S, iron-sulfur compound; P_i, phosphate; P, lipid molecule (in thylakoids, the lipid molecules are mainly galactolipids.)
wavelengths, ranging from 400 nm to 1100 nm. In higher plants, absorption peaks in both the blue (approximately 435 nm) and red (approximately 680 nm) portions of the spectrum.

Approximately 99% of the chlorophyll molecules in the photosynthetic membranes absorb light and channel excitation energy to the PS I and PS II reaction centers (Sauer, 1981). These antennae chlorophylls are associated with accessory pigments, such as carotenoids and phycobiliproteins, in a light-harvesting complex (Fig. 1.3). In green plants and green algae, antennae pigments are primarily chlorophylls (Fig. 1.3a), while cyanobacteria and the red algae contain specialized units called phycobilisomes which are composed of several different types of pigments (Fig. 1.3b). The light-harvesting complexes are in close proximity to the reaction centers and electron transport chains. Approximately 500-600 antennae pigment molecules are associated with each electron transport chain (Witt, 1975). The set of antennae molecules related to each electron transport chain is often referred to as a photosynthetic unit (PSU).

A small percentage of the total chlorophyll molecules, often found in dimers (Katz et al., 1979), are bound in special environments where they form the reaction centers or "traps" for the absorbed energy (P680 and P700). The PS II reaction center, P680, has been suggested to be either a dimeric chlorophyll-a aggregate (den Blanken et al., 1983) or a monomeric chlorophyll-a molecule (Davis et al., 1979). The
Fig. 1.3  PS II light-harvesting complex of a green alga

(after data by Nakatani, 1983a; Green and Camm, 1984; Nakatani et al., 1984) and a cyanobacterium or red alga (after Gantt et al., 1976)

The photosystem II (PS II) light-harvesting complex (LHC) in green algae (Chlorophyta) is associated with the reaction center polypeptide containing the PS II reaction center ($P_{680}$), Z (the primary electron donor), $Q_A$ (the primary electron acceptor), and PHEO (pheophytin). The light energy absorbed in the LHC is transferred to $P_{680}$ via resonance energy transfer ( Förster, 1965). Fluorescence occurs from the antenna chlorophyll molecules and from the back-reaction $Q_A \rightarrow P_{680}$.

The PS II light-harvesting complex for red algae (Rhodophyta) or cyanobacteria contains pigments embedded in a phycobilisome (Gantt and Conti, 1966) attached to the thylakoid membrane as shown for Porphyridium cruentum. Energy transfer is from phycoerythrin to phycocyanin to allophycocyanin to chlorophyll a (CHL). The protein at the center of the phycobilisome is most likely involved in anchoring it to the membrane (Gantt et al., 1976).
a

b  PHYCOSOMES

PHYCOERYTHRIN

ALLOPHYCOCYANIN

PHYCOCYANIN

PROTEIN

THYLAKOID MEMBRANE  CHL PS II
reaction centers are the sites of the initial photochemical events, which will be discussed in Sec. 1.4.

Absorption of a photon by a pigment molecule causes an electronic transition from its molecular ground state to an excited state. The ground state of chlorophyll molecules is generally the singlet state and thus excitation energy absorbed in the light-harvesting complex produces the first or second excited singlet state. This occurs on a time scale of $10^{-15}$ s (Govindjee and Govindjee, 1974; Pearlstein, 1982). This excitation energy can be transferred via exciton migration or resonance energy transfer through the antenna system to a reaction center (Franck and Teller, 1938; Förster, 1965) or be dissipated as light or heat. The loss of the acquired energy as light occurs through two different phenomena, fluorescence and phosphorescence. Fluorescence refers to emission of light that accompanies allowed radiative transitions from singlet excited states; these emissions usually have lifetimes less than $10^{-8}$ s. Radiative de-excitations from longer-lived metastable states (i.e. triplet states) are referred to as phosphorescence. However, due to the orientation of the light-harvesting complex with respect to the reaction center (see Fig 1.3 for PS II LHC), transfer of energy through the pigment array is very efficient (~98%) (Danks et al., 1983). Fluorescence from the antennae pigments is referred to as O-level or non-variable fluorescence.
1.3 Charge separation and the electron transport chain

Excitation energy is extracted from the light-harvesting complex (LHC) by the reaction center chlorophylls (P$_{680}$ or P$_{700}$). Energy transferred from the antennae pigments to the reaction center chlorophylls elevates the reaction center pigment to an excited state (P$_{680}^*$ or P$_{700}^*$). The chlorophyll molecule relaxes to its lowest excited state before a chemical change occurs. Within picoseconds of the electronic transition, an electron of the excited reaction center is captured by an adjacent acceptor molecule (A), which is then reduced (A$^-$). The acceptor molecule A is generally a dimeric chlorophyll (PS I) or pheophytin (PS II) (Foyer, 1984).

The oxidized PS II reaction center (P$_{680}^+$) is a strong oxidant which then captures an electron from the primary donor (Z) and returns to its ground state (P$_{680}^-$) leaving Z in an oxidized form (Z$^+$). The oxidized primary donor, Z$^+$, obtains an electron from the oxidation of H$_2$O. Water oxidation and donation of electrons to P$_{680}^+$ will be discussed in Sec. 2.3. The electron removed from P$_{680}$ is rapidly transferred to a second electron acceptor and then through a chain of carriers that are situated across the membrane. Electrons removed from P$_{680}$ are transferred to a plastoquinone (PQ) pool which reduces the oxidized PS I reaction center chlorophyll (P$_{700}^+$).
The series of electron transfers that occur in the light reactions of photosynthesis are represented by the Z-scheme (Hill and Bendall, 1960) as shown in Fig. 1.4.

1.4 Photophosphorylation

The flow of electrons through the electron transport chain is coupled to the synthesis of ATP (Arnon et al., 1954; Frenkel, 1954; Foyer, 1984). The membrane-bound enzyme complex responsible for the synthesis of ATP is ATPase or the coupling factor (CF) (Mitchell, 1976; McCarty, 1979). The chloroplast coupling factor is a reversible, light-activated ATPase that has two distinct components, a hydrophilic protein complex, CF\textsubscript{1}, and a hydrophobic protein complex, CF\textsubscript{0} (see Fig. 1.2). The protein complex, CF\textsubscript{0}, spans the thylakoid membrane and forms the binding site for CF\textsubscript{1}, which projects into the stroma. The site of ATP synthesis is located in CF\textsubscript{1}, while CF\textsubscript{0} contains a proton channel for translocation of protons across the thylakoid membrane (McCarty and Carmeli, 1982).

There are two sites of proton uptake on the stroma side of the thylakoid membrane and two sites of proton release into the intrathylakoid space. Protons are taken up from the stroma by the reduced plastoquinone pool (PQH\textsubscript{2}) and by the proton-binding terminal acceptor, NADP\textsuperscript{+}. Protons are released
Fig. 1.4 The Z scheme of photosynthesis (after Zubay, 1983)

The Z scheme is a model of PS I and PS II linear electron transfer given in terms of the redox potentials of the electron carriers. (Hill and Bendall, 1960). Electron transfer (indicated by solid lines) is coupled with proton translocation (dashed lines) across the thylakoid membrane.

**Symbols:**

- **M** = active site of the oxygen-evolving complex
- **Z** = primary electron donor to PS II (still unknown)
- **PHEO** = pheophytin
- **Q_A** and **Q_B** = protein-binding quinones
- **PQ POOL** = plastoquinone pool
- **Fe-S** = an iron-sulfur protein
- **CYT** = cytochrome
- **PC** = plastocyanin
- **Chla** = chlorophyll a
- **FD_X**, **FD_A**, **FD_B** = iron-sulfur proteins (bound ferredoxins)
- **FD_S** = soluble ferredoxin
- **FP** = ferredoxin-NADP-oxidoreductase
into the intrathylakoid space by oxidation of $H_2O$ and oxidation of the PQ pool. Plastoquinone serves as a "proton shuttle" which results in two protons translocated from the stroma into the thylakoids for every two electrons transferred through the electron transport chain (Trebst, 1978).

Charge separation generates an electrochemical potential difference $\Delta \psi$ and a localized electric field (Witt, 1979b) across the membrane. The translocation of protons in response to the electrochemical gradient generates a pH gradient across the membrane ($\Delta \rho H$) which produces a conformational change in the coupling factor (McCarty, 1979; McCarty and Carmeli, 1982; Ort and Melandri, 1982). Both $\Delta \psi$ and $\Delta \rho H$ drive ATP synthesis by "pumping" protons through a proteolipid channel in $CF_0$. This causes a structural transformation in the coupling factor complex ($CF_0-CF_1$), converting $CF_1$ into an ATP-synthesizing system. The $\Delta \rho H$ produced contributes significantly to the driving force for photophosphorylation, the proton motive force (pmf):

$$pmf = \Delta \psi - 2.3 \frac{RT}{F} \Delta \rho H$$

where $F$ is Faraday's constant, $T$ is temperature (Kelvin), $R$ is the gas constant (8.31 J/mole/K). This is the potential available for the synthesis of ATP by the chloroplast coupling
factor \( CF_0 - CF_1 \). At 25°C, \( 2.3RT/F \) has a value of 59 mV.

The synthesis of ATP from ADP is a dehydration reaction:

\[
\text{ADP} + \text{P}_i \rightarrow \text{ATP} + \text{H}_2\text{O}
\]

where \( \text{P}_i \) refers to a phosphate group, \( \text{PO}_4 \) (ortho-phosphate).

During photosynthesis, the \( \text{H}^+ \) and \( \text{OH}^- \) ions are separated as soon as they are removed from ADP and \( \text{P}_i \) due to the light-generated pH gradient across the membrane and water does not actually form. This ensures that an excess of \( \text{H}^+ \) ions are present inside the thylakoid membrane relative to outside the membrane (which is alkaline). Thus, the synthesis of ATP continually neutralizes the proton gradient that is generated by electron transport (Foyer, 1984).

1.5 Carbon Fixation

The energy stored in the phosphate linkage of ATP and in the reducing power of NADPH is utilized and consolidated in the dark reactions of photosynthesis. The energy temporarily stored in these compounds is used to drive the fixation of \( \text{CO}_2 \) to produce carbohydrates in the stroma of chloroplasts, via the Calvin cycle (Benson and Calvin, 1958; Calvin and Bassham, 1962) and to power biosynthetic reactions of the cell in the light.
The Calvin cycle, as it occurs in chloroplasts, is shown in Fig. 1.5. Six carbons (in the form of CO₂) react with 6 molecules of ribulose 1,5 bisphosphate (RuBP) to form 12 molecules of glyceraldehyde 3-phosphate (G3P), two of which give rise to one molecule of glucose (C₆H₁₂O₆), which is taken out of the cycle. Twelve molecules of H₂O are added into the cycle which are not shown in Fig. 1.5.

The first enzyme in the cycle, RuBP carboxylase, reacts with CO₂. The enzyme catalyzes a two-step reaction and has a high affinity for CO₂, but a low affinity for HCO₃⁻. This enzyme is present in the chloroplast in large quantities. It represents at least 15% of the total chloroplast protein and may be bound to the outer surface of the thylakoid membrane. RuBP carboxylase is allosteric and its enzymatic activity is controlled by light intensity, oxygen concentration, pH, and various metabolites. This enzyme is the main controlling factor of the Calvin cycle and also plays a role in photorespiration, which will be discussed in Sec. 1.6.

Each molecule of CO₂ fixed into glucose (1 complete cycle) requires 2 NADPH and 3 ATP molecules. The overall reaction for the biosynthesis of 1 glucose molecule is:
Fig. 1.5 The Calvin cycle (after Lehninger, 1982).

The conversion of CO₂ into glucose during photosynthesis is represented by the Calvin cycle.

Symbols:

3PG = 3-phosphoglycerate
G3P = glyceraldehyde 3-phosphate
DHAP = dihydroxyacetone phosphate
FDP = fructose 1,6 diphosphate
F6P = fructose 6-phosphate
G6P = glucose 6-phosphate
E4P = erythrose 4-phosphate
X5P = xylulose 5-phosphate
SDP = sedoheptulose 1,7-phosphate
S7P = sedoheptulose 7-phosphate
R5P = ribose 5-phosphate
Ru5P = ribulose 5-phosphate
RuBP = ribulose 1,5-bisphosphate
Pᵢ = phosphate
The net reaction of carbon fixation is:

\[ 6 \text{CO}_2 + 18 \text{ATP} + 12 \text{NADPH} + 12 \text{H}^+ + 12 \text{H}_2\text{O} \rightarrow 6 \text{C}_6\text{H}_12\text{O}_6 + 18 \text{ADP} + 18 \text{P}_i + 12 \text{NADP}^+ \]

1.6 Photorespiration

Respiration is an ATP-generating process by which metabolites are oxidized by an inorganic compound, such as molecular oxygen. In the dark, this occurs in the mitochondria of plants, which are subcellular organelles that contain the enzymes necessary to catalyze the oxidation of organic cell nutrients (i.e. carbohydrates). In the mitochondria, NADH and CO$_2$ are produced by the tricarboxylic acid cycle and NADH is then reoxidized by an enzyme in the electron transport chain (Stryer, 1981). The electrons obtained from this oxidation are passed by the chain to reduce O$_2$ to give H$_2$O. The transfer of electrons from NADH to O$_2$ is coupled to the phosphorylation of ADP to ATP. Under normal conditions, mitochondrial respiration is not dependent to any extent on O$_2$ concentration since it is saturated at O$_2$ concentrations of 2% (Forrester et
al., 1966; D'Aoust and Canvin, 1974).

In the light, net $O_2$ uptake in plants and algae is superceded by $O_2$ released from the photosynthetic oxidation of $H_2O$. The rate of $O_2$ evolution during photosynthesis is generally much greater than the rate of $O_2$ consumption in the dark (Foyer, 1984). However, in some plants, the process of photorespiration occurs in the light and often exceeds dark respiration by as much as two to three times (Zelitch, 1968; Jackson and Volk, 1970). This light-dependent respiration is associated with glycolate biosynthesis in the chloroplast and subsequent glycolate metabolism in peroxisomes and mitochondria (Andrews et al., 1973; Berry et al., 1978; Tolbert, 1981). The process of photorespiration is depicted in Fig. 1.6. In contrast to mitochondrial respiration, photorespiration does not conserve energy, but consumes it. The term photorespiration is often used to refer to all forms of $O_2$ uptake in the light. Other types of $O_2$ consumption reactions that can occur in the light will be discussed in Sec. 2.6.

Photorespiration also oxidizes metabolites, releases $CO_2$, and requires ATP and NADPH. The majority of $O_2$ consumed in the light results from the oxygenation of RuBP via the oxygenase reaction of RuBP carboxylase:

$$\text{Mg}^{2+}$$

$$O_2 + \text{RuBP} \rightarrow 3\text{-phosphoglycerate} + 2\text{-phosphoglycollate}$$
Fig. 1.6 Photorespiration (after Foyer, 1984)

The process of photorespiration involves reactions that occur in the chloroplasts, mitochondria, and peroxisomes (which are subcellular microbodies that contain enzymes to form, utilize, and break down $H_2O_2$). Several products formed during photorespiration are transferred through the aqueous phase of the cytoplasm, the cytosol.

Photorespiration in chloroplasts results from the oxygenation reaction catalyzed by RuBP carboxylase/oxygenase. Phosphoglycerate (3PG) generated by this reaction is oxidized by $O_2$ to CO$_2$. The photorespiratory cycle ultimately recovers CO$_2$ in the mitochondria.
Both CO$_2$ and O$_2$ compete for the same active site on the membrane, and thus the rates of the two reactions depend on the relative concentrations of CO$_2$ and O$_2$. Since RuBP carboxylase also catalyzes the oxygenation reaction of RuBP, it is often referred to as RuBP carboxylase/oxygenase.

The rate of photorespiration depends on O$_2$ concentration and is absent at low O$_2$ concentrations (Jackson and Volk, 1970; D'Aoust and Canvin, 1974; Cornic, 1974). Photorespiration is initiated when the O$_2$ concentration increases in the presence of light since O$_2$ is a competitive inhibitor of RuBP carboxylase. Oxygen is consumed during photorespiration and H$_2$O$_2$ is formed which is metabolized by catalase. Ultimately, RuBP, the substrate for RuBP carboxylase, is formed. These pathways are presented in Fig. 1.6.

The process of photosynthesis generates a strong oxidant and a strong reductant. The strong reductant formed in PS I can reduce both O$_2$ and CO$_2$. If O$_2$ is reduced, the highly toxic superoxide anion (O$_2^-$) is formed which reacts readily with chloroplast components. This light-oxygen toxicity occurs when the supply of CO$_2$ is limited or there is an excess amount of O$_2$ present (Asada et al., 1977). The overall effect of photorespiration is to reduce the O$_2$ concentration and therefore relieve the inhibition of RuBP carboxylase, allowing reduction of CO$_2$ rather than O$_2$. Thus, photorespiration is most likely a physiological defense mechanism against light-oxygen toxicity (Björkman, 1973; Asada et al.,
1977). Under conditions of limiting CO$_2$ or excess light energy, the process of photorespiration wastes ATP and NADPH and thus enables transfer of electrons from the PS I reductant via ferredoxin to NADP$^+$. This also prevents photooxidation of chloroplast components by O$_2$ and other active forms of oxygen.

1.7 Subject of Study

Many problems still remain to be solved before the molecular mechanism of photosynthetic O$_2$ evolution is fully understood. There are many methods available to study this process, including ESR, NMR, fluorescence, light-induced absorbance changes, electron microscopy, mass spectroscopy, gas (CO$_2$ or O$_2$) exchange, biochemical isolation of lipid and/or protein complexes, etc. However, some of the breakthroughs in this field have come from kinetic studies of O$_2$ evolution in plants and chloroplasts which were illuminated by brief, saturating light flashes (Kok et al., 1970; Forbush et al., 1971; Joliot et al., 1971). These studies of O$_2$ evolution have been hampered largely by two problems; the difficulty of separating O$_2$ evolution and O$_2$ uptake components, and calibration of the electrode system.

Two types of electrode systems are currently used for O$_2$ exchange (evolution and/or uptake) measurements in plants or chloroplasts using a series of brief, saturating flashes. The
modulated polarographic electrode of Joliot and Joliot (1968) provides the first derivative of the $O_2$ signal, while the bare platinum electrode (Chandler and Vidaver, 1970; Swenson et al., 1986) provides the current due to $O_2$ reduction at the cathode as a function of time. Use of the bare Pt electrode has decreased in recent years, largely due to an inability to precisely calibrate this type of electrode system, but also due to the fact that it can provide little information on individual steps in the photosynthetic process. However, the bare Pt electrode is a valuable tool to study the effect of $O_2$ uptake which occurs in the light and to determine the relationship between ambient $O_2$ concentration and $O_2$ evolution. Neither of these two problems can be studied using the Joliot electrode, which attempts to discriminate between a fast light-induced response and any slower light-induced responses. The Joliot electrode minimizes, but does not eliminate, an $O_2$ uptake component.

The object of this research is to investigate the influence of $O_2$ concentration and internal $O_2$ consumption reactions on photosynthetic $O_2$ evolution. The electrode system was calibrated, and an attempt was made to separate the uptake and evolution components of the $O_2$ exchange measurements. Different genera and species of marine algae were used as experimental samples as they may resemble higher plants with respect to photosynthetic apparatus and reactions, are easily obtained, and are complete systems in themselves.
CHAPTER 2. THE OXYGEN EVOLVING COMPLEX

Photosynthetic oxygen evolution is carried out in the oxygen-evolving complex associated with Photosystem II. The components of the oxygen-evolving complex and the mechanism of water oxidation are discussed, beginning with the photochemical reactions of PS II.

2.1 Photosystem II

The primary photochemical reaction of PS II is an electron transfer from the excited PS II reaction center (P₆₈₀*) to an intermediate electron acceptor (Pheo) (Fig. 2.1). This charge separation (discussed in Sec. 1.4) can be written as:

\[ \text{hv} \quad P₆₈₀ \text{ Pheo} \rightarrow P₆₈₀^+ \text{ Pheo}^- \]  \hspace{1cm} 2.1

where Pheo refers to pheophytin (Klimov et al., 1977). Transfer of an electron from the lowest excited singlet state of P₆₈₀* to Pheo is believed to occur within 1 ps (Butler et al., 1983). The secondary electron transfer step (Fig. 2.1), which results in charge stabilization (Eckert and Renger, 1980), can be depicted as:
Fig. 2.1 Charge separation in PS II (after Govindjee et al., 1985).

The electron carriers of PS II that undergo oxidation-reduction reactions and charge accumulation are shown together with the approximate electron transfer times.
The primary electron acceptor, QA, is a plastoquinone (PQ) molecule which is associated with an iron molecule, and tightly bound to the reaction center polypeptide; this iron-associated plastoquinone is designated QA (Renger and Govindjee, 1985). QA is reduced to the unprotonated semiquinone anion, QA−, in less than 1 ns (Satoh, 1985). This charge transfer reaction, from P680* to QA, results in formation of an electrical potential difference across the thylakoid membrane (Junge and Witt, 1968).

QA is reoxidized by the secondary electron acceptor, QB (Fig. 2.1) which accepts two electrons to form QB2− (Bouges-Bocquet, 1973; Velthuys and Amesz, 1974; Velthuys, 1980). While the electron transfer from QA to QB is slow (200-300 µs), the electron transfer from QA to QB is even slower (0.6-0.8 ms) (Cramer and Crofts, 1985). The reduced secondary acceptor, QB2−, is subsequently protonated to form a bound plastoquinol (QBH2) which exchanges protons within 2 ms with a mobile plastoquinone molecule (PQ). The series of reactions is depicted with 70% and 30% of the PS II reaction centers in either of two oxidation states, QAQB or QAQB−, respectively, prior to the first, short (µs) light flash (Cramer and Crofts, 1982; Govindjee et al., 1985):
On the donor side of PS II, the oxidized PS II reaction center \( (P_{680}^+) \) is reduced by the redox component \( Z \) (Babcock et al., 1976) (see Fig. 2.1). Renger and Govindjee (1985) have suggested that \( Z \) is a special plastoquinol \( (PQH_2) \) that functions as a one-electron carrier between the oxygen evolving complex \( (OEC) \) and \( P_{680} \). The redox couple \( Z^{\text{ox}}/Z \) has been suggested to be \( PQH_2^+/PQH_2 \) (Ghanotakis et al., 1983). Electron transfer from \( Z \) to \( P_{680}^+ \) is dependent on the oxidation state or \( S \) state \( (S_n, n = 0,1,2,3,4) \) of the oxygen-evolving complex (Brettel et al., 1984; Schloeder et al., 1985). After one light flash, electron transfer from \( Z \) to \( P_{680}^+ \) occurs within 50 ns (van Best and Mathis, 1978).
Electron transfer from the manganese site in the OEC to $Z$ takes place in 50-800 $\mu$s depending on the oxidation state of the OEC (Govindjee et al., 1985). Reduction of $P_{680}^+$ occurs in 20 ns during transitions from $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ and in 250 to 300 ns during transitions from $S_2 \rightarrow S_3$ and $S_3 \rightarrow (S_4) \rightarrow S_0$ (Brettel et al., 1984; Schlodder et al., 1984). Oxygen evolution results from water-splitting in approximately 1 ms after the OEC has cycled to its most oxidized state ($S_4$) (Junge and Jackson, 1982; Govindjee et al., 1985). A detailed discussion of $O_2$ evolution will be presented in Sec. 2.4.

2.2 The oxygen evolving complex

2.2.1 Polypeptides

The oxygen-evolving complexes (OECs) of PS II are localized near the inner side of the thylakoid membrane (Akerlund et al., 1982; Larsson et. al, 1984) as shown in Fig. 2.2. Each OEC in the thylakoid membrane acts independently of the others (Kok et al., 1970; Forbush et al., 1971). Associated with the OEC are at least seven polypeptides. Four of these polypeptides are believed to be directly associated with the active site of the oxygen-evolving complex (Govindjee et al., 1985), labelled as 18, 24, 33, and 34 kD proteins in Fig. 2.2. The unit of molecular weight is a
Fig. 2.2 Model of the oxygen-evolving complex (after Govindjee, 1984; Govindjee et al., 1985)

Symbols:
M = 34 kD polypeptide containing active site for water-splitting
CYT b_{559} = cytochrome b_{559}
Z = primary electron donor to PS II
RC = reaction center complex
P_{680} = PS II reaction center
PHEO = pheophytin
Q_A = primary electron acceptor
LHC = light-harvesting complex
Q_B = secondary electron acceptor
PQ = plastoquinone
Q = lipid molecule
kD = kilodalton; 1 kD = 1 kg/mole
dalton (D), where 1 kD = 1000 g/mol. The other three polypeptides are labelled in Fig. 2.2 as CYT b\textsubscript{559}, RC, and 32kD. These polypeptides can be summarized as follows (Govindjee et al., 1985):

1. Photosystem II reaction center polypeptide (47-51 kD) containing a chlorophyll-a complex (P\textsubscript{680}); pheophytin (PHEO), the intermediate electron acceptor; a bound plastoquinol, the primary electron acceptor, (Q\textsubscript{A}); and a bound plastoquinol electron donor (Z).

2. Secondary bound plastoquinol electron acceptor (Q\textsubscript{B}) of 32 kD molecular weight

3. Cytochrome b\textsubscript{559} with 10 kD molecular weight

4. Intrinsic 27-34 kD polypeptide (labelled in Fig. 2.2 as M; referred to in text as 34 kD) associated with Mn

5. Peripheral, hydophilic 33 kD polypeptide, possibly associated with Mn (location in Fig. 2.1 is arbitrary)

6. Peripheral 23-24 kD polypeptide (Akerlund et al., 1982), referred to in text as 24 kD)

7. Peripheral 17-18 kD polypeptide (Akerlund et al., 1982)
The intrinsic 34 kD polypeptide is apparently the most essential protein for $O_2$ evolution, since removal of all 34 kD protein leads to complete inhibition of $O_2$ evolution (Murata et al., 1983). Membranes in which the 18 and 24 kD polypeptides are removed, but which contain the 34 kD polypeptide, can still evolve $O_2$ in the presence of $Cl^-$ and Ca$^{2+}$ ions, albeit with some loss of $O_2$ evolving capability (Nakatani, 1984; Ghanotakis et al., 1984b; Miyao and Murata, 1984). Oxygen evolution can be restored by rebinding one of the 24 kD proteins back to the membrane if the 34 kD protein is present at the binding site (Murata et al., 1983; Andersson et al., 1984b; Larsson et al., 1984). Optimal $O_2$ evolution is restored only if one 18 kD protein is rebound to the membrane or if the $Cl^-$ concentration is elevated to a greater concentration than is required for non-depleted PS II membranes (Akabori et al., 1984; Miyao and Murata, 1985). There is some controversy over the ratio of 18, 24, and 34 kD polypeptides present in the OEC. Murata et al. (1984) suggest that the 18, 24, and 34 kD polypeptides are required for optimal $O_2$ evolution in ratios of 1:1:1 per OEC, while Cammarata et al., (1984; Larsson et al., 1984) suggest that the ratio is 2:2:2 per OEC.

The molecular composition of the OEC and the specific function of the different polypeptides are still unknown, however current research on the isolation and purification of the polypeptide components of the OEC may soon enable
determination of the function and spatial relationships of the specific polypeptides.

2.2.2 Manganese

Manganese is known to be essential for the oxidation of $H_2O$ to $O_2$ (Cheniae, 1980; Sauer, 1980; Ono and Inoue, 1982; Dismukes, 1986). Studies done with mutant strains of the green algae _Chlamydomonas_, which have a reduced $O_2$ evolution, show a direct correlation between $O_2$ evolution, Mn content, and the abundance of a 24 kD polypeptide (Cammarata et al., 1984). Using chemical treatments to remove Mn from the membrane, four Mn atoms per PS II reaction center were found in membranes with normal $O_2$ evolution, while only 1–2 Mn atoms/PS II were released from the mutant strains. Therefore, 4 equivalent Mn atoms may be organized into a tetranuclear cluster bound to a 34 kD protein (labelled as $M$ in Fig. 2.1) and a 24 kD extrinsic protein (Cammarata et al., 1984).

Experimental evidence shows that in a variety of photosynthetic organisms, 4 Mn atoms are bound to the OEC (Schatz and Witt, 1984a,b; Bowes et al., 1983; Kuwabara and Murata, 1983; Cammarata et al., 1984; Ghanotakis et al., 1984a). PS II particles isolated from a red algae, _Porphyridium cruentum_, show that ~50 Chl molecules and 4 Mn atoms are bound together per reaction center (Schatz and Witt,
These preparations show an \( O_2 \) evolution rate of 2300 \( \mu \text{mol} \ O_2 \ (\text{mg Chl})^{-1} \ h^{-1} \) (Schatz and Witt, 1984a,b) under normal conditions and with saturating light intensity. In the many experiments which correlate the Mn content of thylakoid membranes with the \( O_2 \) evolution rate, considerable variations in \( O_2 \) evolution rates were found, but all evidence indicates that 4 Mn atoms per PS II reaction center are present in PS II membranes with optimal \( O_2 \) evolution (Kuwabara and Murata, 1983; Ghanotakis et al., 1984b; Cammarata et al., 1984). Lowered \( O_2 \) evolution rates have been correlated to a reduced Mn content in chloroplasts and PS II particles (Cammarata et al., 1984). However, variations in \( O_2 \) evolution rates could also reflect variations in the rate of reoxidation of the secondary quinone acceptor \( (Q_B) \) (Dismukes, 1986).

The requirement of 4 Mn atoms per PS II reaction center does not imply that all 4 atoms are essential for \( H_2O \) oxidation. Of the several bound Mn pools in chloroplasts (Cheniae and Martin, 1970; Sharp and Yokum, 1980; Khanna et al., 1981a,b), only one appears to be essential for \( O_2 \) evolution. Another pool of Mn is "very-loosely bound" and non-functional in \( O_2 \) evolution (Radmer and Cheniae, 1977; Yocum et al., 1981; Khanna et al., 1981b). However, a "loosely bound" pool appears to play a role in \( O_2 \) evolution (Radmer and Cheniae, 1977; Yocum et al., 1981; Khanna et al., 1983). The function of the "tightly bound" pool of Mn is not clear, but is essential for \( O_2 \) evolution (Khanna et al., 1983).
The localization of the Mn within the OEC has not been completely resolved. In the model of Cammarata et al. (1984), 4 Mn atoms lie in a cleft, bound to two 34 kD proteins and to the "Z" region of the reaction center protein. This also supports the model of Dismukes et al. (1983), which suggests that there are 2 Mn atoms per 34 kD protein (shown in Fig. 2.2).

2.2.3 Chloride ions

Chloride ions are known to be essential to the function of the OEC in that removal of Cl\(^-\) reversibly inhibits the O\(_2\) evolution mechanism (Hind et al., 1969; Kelley and Izawa, 1978; Govindjee et al., 1983; Izawa et al., 1983). The number of Cl\(^-\) ions per PS II reaction center has been estimated to be as high as 40 (Izawa et al., 1984). Chloride ions may be replaced by Br\(^-\), NO\(_3^-\), or I\(^-\) with decreasing order of effectiveness (Kelley and Izawa, 1978; Critchley et al., 1982). Other anions either inhibit O\(_2\) evolution (F\(^-\), OH\(^-\)) or have no effect (SO\(_4^{2-}\), ClO\(_4^-\), PO\(_4^-\)). Fluoride ions have been shown to reversibly inhibit the transitions S\(_0\) \(\rightarrow\) S\(_1\) \(\rightarrow\) S\(_2\) (Casey and Sauer, 1984a,b), while depletion of Cl\(^-\) in the dark apparently inhibits the S\(_2\) \(\rightarrow\) S\(_3\) transition (Theg et al., 1984; Itoh et al., 1984), resulting in inhibition of O\(_2\) evolution.

Since optimal O\(_2\) evolution requires the presence of the 18 kD protein or an elevated Cl\(^-\) concentration, it has been
suggested that the 18 kD protein functions in concentrating and delivering Cl\(^-\) to its functional site (Murata et al., 1983; Fukutata et al., 1983). If the 24 kD protein is removed, the concentration of Cl\(^-\) ions needs to be 5-10 times greater than if this protein is present. Andersson et al. (1984a) have suggested that the 24 kD protein provides binding sites which have a high affinity for Cl\(^-\), possibly the amino acid residues, arginine and lysine, which are abundant in this protein. However, since O\(_2\) evolution does proceed without the 24 kD polypeptide, its Cl\(^-\) binding site is not essential for O\(_2\) evolution.

The binding site for Cl\(^-\) has been determined to be localized within the OEC. The following evidence supports this conclusion:

1. Cl\(^-\) binding interferes with the initiation phase of hydroxylamine (NH\(_2\)OH) inhibition in thylakoids, indicating that the most likely binding site is Mn (Kelley and Izawa, 1978; Critchley et al., 1982).

2. F\(^-\) and OH\(^-\) (Kelley and Izawa, 1978) and amines (Sandusky and Yocum, 1983, 1984) compete with Cl\(^-\) for binding sites and inhibit O\(_2\) evolution. This occurs at a binding site which has been localized at or between the site for H\(_2\)O oxidation and photooxidation of artificial electron donors (Kelley and Izawa, 1978).
3. Binding of Cl\(^-\) stabilizes the OEC against thermal damage leading to the release of Mn atoms from the active site and irreversible inactivation (Coleman et al., 1984).

In Fig. 2.2, Cl\(^-\) is shown to be bound to the 34 kD protein in the cleft which contains 2 Mn atoms and is the site of H\(_2\)O oxidation and H\(^+\) release.

Chloride appears to exert its effect by binding to the membrane in a pH dependent manner (Critchley et al., 1982; Theg and Homann, 1982; Govindjee et al., 1983). At pH values above 7.2-7.4, more Cl\(^-\) is required for optimal O\(_2\) evolution (Kelley and Izawa, 1978; Critchley et al., 1982) and Critchley et al. (1984) suggest that OH\(^-\) and Cl\(^-\) ions compete for binding sites. Experimental evidence in inverted thylakoid membranes shows that irreversible inactivation of O\(_2\) evolution is suppressed by high Cl\(^-\) concentration and is not dependent on the presence or absence of the 24 kD protein.

Depletion of Cl\(^-\) ions causes the OEC to become more sensitive to inhibition by Tris (hydroxymethyl)aminomethane buffers (Tris) (Izawa et al., 1983), NH\(_2\)OH (Kelley and Izawa, 1978), and heat treatment (Coleman et al., 1984). This indicates that Cl\(^-\) is involved in maintaining photooxidation of the OEC by the reaction center (Sandusky and Yocum, 1983).

Two models have been described which are consistent with these observations. However, neither of these two models
completely explains all experimental evidence concerning the effect of Cl⁻ on O₂ evolution. In the first model, Cl⁻ is assumed to bind in one of the lower S-states (S₀ or S₁) to the active site containing Mn in the OEC. In the dark, Cl⁻ stabilizes the S₁ state against deactivation to S₀ and allows formation of a long-lived S₂ state. The active site would then directly control binding of H₂O at the Mn atoms (Dismukes, 1986).

In the second model, a single Cl⁻ ion binds to the Mn site in order to neutralize each positive charge accumulated in response to the differential extraction of protons and electrons in the S-state transitions. This would result in the proper conformation for O₂ evolution by coupling the OEC to the PS II reaction center (Johnson et al., 1983; Govindjee, 1984; Dismukes, 1986). Thus, both uptake of Cl⁻ during one S-state transition, and release of Cl⁻ during a different S-state transition will take place. If charge neutrality is maintained by binding Cl⁻, it could be predicted that one Cl⁻ is taken up on the S₁ → S₂ transition, and one Cl⁻ is released during the S₃ → S₀ transition (Dismukes, 1986). This agrees with the observed inhibition loci for F⁻ inhibition and Cl⁻ depletion, respectively (Dismukes, 1986).
2.2.4 Calcium binding

The role of Ca\(^{2+}\) in PS II and O\(_2\) evolution is still uncertain, although it is known to be essential for O\(_2\) evolution (Dismukes, 1986). Ca\(^{2+}\) can restore O\(_2\) evolution in membranes which are missing the 24 kD polypeptide (Miyao and Murata, 1984). Treatment of PS II particles which releases the 18 and 24 kD proteins results in inhibition of O\(_2\) evolution and shows a reduced (40%) Ca\(^{2+}\) content (Miyao and Murata, 1984). At physiological (in vivo) salt concentrations, Ca\(^{2+}\) and the 18 and 24 kD proteins are all required for O\(_2\) evolution. The 18 and 24 kD proteins provide high affinity binding sites for Ca\(^{2+}\) (Ghanotakis et al., 1984b). Calcium appears to be involved in the binding of essential protein subunits within the OEC or in the binding of the 34 kD polypeptide to the PS II reaction center protein. Ono and Inoue (1983a,b) have proposed that both Mn and Ca\(^{2+}\) must be bound to the "resting" OEC before it can be photoactivated.

2.3 Water oxidation

Water oxidation to molecular oxygen in photosynthetic systems may be represented by the overall equation:

\[ 2 \text{H}_2\text{O} \rightarrow \text{O}_2 + 4 \text{H}^+ + 4 \text{e}^- \]
The effective pH near the water-splitting site in chloroplasts is determined to be approximately 7 (Sauer, 1980). In chloroplasts, water oxidation occurs in four steps with an energy average of 0.81 eV for each step in the coupling of H₂O with the OEC. However, the energy requirements for the individual steps are very different. Figure 2.3 shows the redox energies for free intermediates of each of the oxidation steps for water-splitting. Figure 2.4 compares one-electron energy steps for free intermediates and the corresponding steps for intermediates associated with an oxygen-evolving complex (Renger, 1978; Renger and Eckert, 1980).

The charge-accumulating intermediates which function in the oxidation of H₂O are most likely Mn complexes (Renger, 1977; Govindjee et al., 1978; Radmer and Cheniae, 1977; Wydryzynski, 1982; Renger and Govindjee, 1985). The highest energy barrier in the oxidation of H₂O is the first step as shown in Fig. 2.4 (Renger, 1978). The water-splitting enzyme (labelled M in Fig. 2.2) must function in lowering this potential barrier, thus reducing the energy needed to undergo the first electron transfer. Complexes can be formed with Mn in the oxidation states Mn(II), Mn(III), and Mn(IV) (Harriman et al., 1978) and thus Mn complexes are attractive for charge-storing. Mn(II) complexes are generally stable, while Mn(IV) complexes usually form oxygen-bridged bimolecular structures (Harriman et al., 1978). In principle, this would allow an overall four electron oxidation as a Mn(II) complex undergoes
This diagram shows the redox energy of the one-electron steps for the oxidation of $H_2O$ to $O_2$. The solid line (---) indicates the pathway via $H_2O_2$ as the two-equivalent reduction state. The dashed line (-----) indicates the reduction pathway via $H_2O + O$ as the two-equivalent reduction stage. The dotted line (......) shows the path of minimum energy.
NUMBER OF ELECTRONS REMOVED FROM WATER AT pH = 7.0

REDOX ENERGY (eV)

1.0  2.0  3.0  4.0

Minimum energy path
(0.81 eV/electron)

$H_2O + H^+ + e^- \rightarrow H^+ + H_2O$

$H_2O + O_2 \rightarrow H_2O_2 + H^+ + e^-$

$O + H_2O \rightarrow O_2^- + 2H^+ + e^-$

$O_2^- = O_2 + e^-$
Fig. 2.4 Comparison of redox energies for oxidation of H$_2$O via free intermediates and via the catalytic pathway in photosynthesis (after Renger, 1978).

Energetics of the four step water oxidation via:

a. free intermediates

b. intermediates complexed with Mn in the oxygen-evolving complex

The zero points of the energy scale, H$_2$O and (H$_2$O)$^*$, are arbitrary and the absolute energies of both states do not have the same value. The symbol $^*$ is used to denote a complexed intermediate, which is not the same as the corresponding free intermediate.
FREE ENERGY CONTENT (ARBITRARY UNITS)

\[ \begin{align*}
(1) & : 2H_2O \\
(2) & : H_2O_2 + 2e^- + 2H^+ \\
(3) & : H_2O + OH^- + e^- + H^+ \\
(4) & : O_2 + 3e^- + 4H^+ \\
(5) & : O_2 + 2(H_2O)^* + 4e^- + 4H^+ \end{align*} \]
a two electron oxidation to form a bimolecular Mn(IV) complex (Harriman et al., 1978).

The water-splitting enzyme must also ensure that the highly reactive water oxidation intermediates are stabilized until the oxidation of H₂O is completed. Manganese has a variable coordination number (Cheniae, 1970; Lawrence and Sawyer, 1978) and thus a variable number of groups can form bonds with the central Mn atom in the Mn complex. Thus bonds can be formed which stabilize the reactive intermediates formed during H₂O oxidation (Renger, 1978). Compared to all the other transition metal ions, Mn has the largest thermal equilibrium constant for the following reaction (Wells, 1965):

\[
\text{M(III)(H}_2\text{O)} + \text{H}_2\text{O} \rightarrow \text{M(III)(OH)}^- + \text{H}_3\text{O}^+ .
\]

This indicates that Mn(III)OH⁻ is very stable in neutral aqueous solutions (Wells, 1965).

The interaction of H₂O molecules with the OEC is still undetermined. Based on experimental studies with H₂O analogs (i.e. NH₂OH and NH₂NH₂), it was proposed that the ability of a molecule to interact with the Q₂ evolving site correlates with the shape of the molecule, rather than its chemical properties (Radmer, 1983; Radmer and Ollinger, 1983). Radmer determined that the H₂O binding site lies in a cleft approximately 4 Å wide and 2.5 Å deep, with two Mn atoms binding two H₂O molecules 1.47 Å apart (the length of an
O-N bond). Radmer suggests that the existence of a cleft around the binding site may help prevent the decay of S states by allowing H₂O oxidation intermediates to react with other molecules in the binding site, but not thylakoid membrane components outside of the cleft.

2.4 Kinetics of oxygen evolution

Oxygen evolution results from Photosystem II water-splitting activity in the oxygen-evolving complex (OEC). The currently accepted model of water-splitting, the S state hypothesis (Kok et al., 1970; Forbush et al., 1971) requires the generation and cooperation of four photochemically formed oxidizing equivalents (OEC → OEC⁴⁺) in individual PS II reaction centers. The major evidence for this model involves a linear four step oscillation in O₂ evolution observed during a series of short saturating light flashes (Kok et al., 1970; Joliot et al., 1971). Every light flash oxidizes each reaction center by releasing one electron, which results in oxidation of the oxygen-evolving centers. One electron is transferred to Z, which in turn transfers an electron to P₆₈₀⁺, and protons are released. This event is referred to as a single turn-over of the OEC. The turn-over time of the OEC is the time which is required for the OEC to recover from photoexcitation. In terms of kinetic studies, it is the time
interval between two successive saturating light flashes which give half of the maximum $O_2$ yield on the second flash as on the first flash (Diner and Mauzerall, 1973b). The $O_2$ yield per light flash oscillates with a periodicity of four which is compatible with chemical intermediates or oxygen-evolving centers successively cycling through five different oxidation states, $S_0$ through $S_4$, as shown in Fig. 2.5. The subscript $n = 0, 1, 2, 3, 4$ indicates the number of oxidizing equivalents stored in the system, in excess of that in $S_0$. Each $S_n$ state differs from the preceding state, $S_{n-1}$, by the loss of one electron. When the intermediates reach the most oxidized state, $S_4$, $O_2$ is released in the final step as $S_4$ returns to $S_0$ in the dark.

Each $S$ state transition occurs in two stages, i.e. $S_n \rightarrow S_n' \rightarrow S_{n+1}$. The prime (') represents the excited state created immediately after light absorption in the reaction center $P_{680}$. The transition from $S_n' \rightarrow S_{n+1}$ is a dark reaction during which one positive charge equivalent is accumulated. One electron is transferred to the photooxidized PS II reaction center ($P_{680}^+$) and transferred through the electron transport chain while the state advances. The $S_4$ state, which has accumulated four oxidizing equivalents, is highly unstable, and rapidly regenerates $S_0$ by evolving $O_2$. The state advancement reactions are rate-limited by the reoxidation of the primary acceptor $Q_A^-$, except for $S_3 \rightarrow (S_4) \rightarrow S_0$, which is rate-limited by reactions leading to
Fig. 2.5 The S-states of the oxygen-evolving complex (after Radmer and Cheniae, 1977).

This schematic diagram represents the model of $O_2$ evolution proposed by Kok et al. (1970). Each OEC undergoes the cyclic series of reactions which are depicted in this figure. The S-states denote the oxidation state of the OEC and are indicated by the subscripts $n = 0-4$. The reactions $S_n \rightarrow S_n^*$ are phototransitions and the subsequent dark relaxations are the steps $S_n^* \rightarrow S_{n+1}$. The approximate time constants for the S-state transitions are indicated for each transition. The deactivation reactions which occur are represented by dashed lines (-----) and labelled with the corresponding rate constants.
The release of O$_2$ (Dekker et al., 1984c).

The S state transitions, the H$^+$ release pattern, and the time constants for electron transfer to the primary acceptor Z may be listed as follows (Junge and Jackson, 1982):

Each of the transitions $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, and $S_3 \rightarrow (S_4) \rightarrow S_0$ constitute one step in the oxidation of H$_2$O.

The step pattern for H$^+$ release in dark-adapted thylakoids is 1,0,1,2 (Wille and Lavergne, 1982) for the sequential S state transitions with the corresponding H$^+$ release times (Förster et al., 1981). The Z reduction times were determined by Babcock et al. (1976). Recently Brudvig et al. (1984) have shown that the OEC exists in two different states, resting (following long dark-adaptation) and active. Thus, the H$^+$ release pattern may actually be different for an active OEC.

In dark adapted chloroplasts, the only S states present are $S_0$ and $S_1$. $S_2$ and $S_3$ deactivate in the dark to $S_1$ within 5 s in chloroplasts and within 60 s in isolated
thylakoids (Joliot and Kok, 1975). Dark adaptation of plants, algae, and chloroplasts essentially synchronizes the oxygen-evolving centers, in that only $S_0$ and $S_1$ are present after a few minutes. Short, saturating light flashes with a frequency of approximately 1 to 4 Hz will result in single turn-overs of each oxygen-evolving complex. Deactivation of $S_2$ and $S_3$ in PS II membranes at room temperature has been determined to be $T_{1/2} = 20$ s and 100 s, respectively (Beck et al., 1985). The deactivation of S states is dependent on the redox state of the electron acceptor side of PS II (Rutherford et al., 1984; Vermaas et al., 1984; Govindjee et al., 1985). Decay of $S_2$ centers to the $S_1$ state associated with $Q_B$ had a halftime of 20-30 s, while those associated with $Q_B$ had a halftime of ~150 s (Rutherford et al., 1984). The decay of $S_3 \rightarrow S_2$ was determined to be relatively independent of the redox state of the PQ pool (Vermaas et al., 1984).

The distribution of S states in dark adapted chloroplasts has been suggested to range from 100% $S_0$ (Bader, 1984) to 100% $S_1$ (Vermaas et al., 1984). The most probable initial S-state distribution in dark-adapted chloroplasts is $S_0:S_1:S_2:S_3 = 0.25:0.75:0:0$ (Velthuys and Kok, 1978; Lavergne, 1986). However, Vermaas et al. (1984) showed that in long-time dark adapted thylakoids, the distribution is 100% $S_1$, with a fraction of the centers (15-25%) possessing a one-electron donor capable of reducing $S_2$ or $S_3$ with fast kinetics ($T_{1/2} = 1.0-1.5$ s). This is equivalent to an
initial S state distribution of 75-85% $S_1$ and 15-25% $S_0$.

The $O_2$ flash yield sequence produced by a series of single turn-over saturating flashes shows a periodicity of four which is eventually damped to a steady-state value. Maximum $O_2$ yield in most photosynthetic systems occurs on the third flash, with successive maxima on the seventh, eleventh, etc. flashes. After 20-30 flashes, the $O_2$ yield has reached its steady-state value ($Y_i$). The general behavior of the $O_2$ flash yield sequence indicates that all PS II reaction centers act independently. The periodicity of four is generally consistent with experimental results and with the S-state model, but the damping to a steady-state value after a number of flashes is still the subject of debate (Forbush et al., 1971; Thibault, 1978; Lavorel, 1978; Jursinic, 1981; Delrieu, 1983).

The damping to a steady-state value was explained by Forbush et al. (1971) as a phase loss due to double hits or misses at the PS II reaction center. "Misses" are randomly distributed failures of S-state transitions or quantum absorption. "Double hits" occur when there are two successive S-state transitions within a single light flash, i.e. when two photons are absorbed by the same $P_{680}$ reaction center. Thus, misses retard the phase of the cycle, while double hits advance it, and both contribute to the phase loss. However, the damping can also partly be explained by a light-induced endogenous $O_2$ uptake which occurs in green plants and algae.
during a series of saturating flashes and may influence single flash yields to the extent that measured yields are not equivalent to actual water-splitting yields.

2.5 The role of oxygen uptake

Green plants and algae show a light-dependent $O_2$ uptake (Hoch et al., 1963; Vidaver and French, 1965; Ried, 1968; Jackson and Volk, 1970; Sargent and Taylor, 1972; Radmer and Kok, 1976; Radmer et al., 1978; Gerbaud and Andre, 1979; Canvin et al., 1980; Watanabe et al., 1980; Fock et al., 1981).

Oxygen uptake in the light may occur by direct photoreduction of $O_2$, referred to as the Mehler reaction (Mehler, 1951a,b; Radmer and Kok, 1976; Radmer et al., 1978), the oxygenase reaction of RuBP carboxylase-oxygenase and the subsequent photorespiratory metabolism of glycolate (see Sec. 1.6) (Andrews et al., 1971; Bowes et al., 1971; Badger and Andrews, 1974; Berry et al., 1978), mitochondrial respiration (Jackson and Volk, 1970), and possibly chlororespiration (Bennoun, 1982).

Light-induced $O_2$ uptake has been studied in many photosynthetic organisms. In the green alga, *Chlorella*, this $O_2$ uptake was shown to be wavelength dependent; an increase in $O_2$ consumption occurred with short wavelength light (maxima at 370 nm and 460 nm) (Miyachi et al., 1980; Pickett and
French, 1967; Ried, 1968; Sargent and Taylor, 1971), while red light (maximum at ~680 nm) caused a decrease in the rate of $O_2$ uptake (Ried, 1968; Sargent and Taylor, 1972). The increase in $O_2$ consumption has been associated with a blue light enhancement of dark respiration during photosynthesis.

Mitochondrial respiration has been shown to be both inhibited (Hoch et al., 1961) and uninhibited (Peltier and Thibault, 1985b) by light. In cyanobacteria, which have two functioning photosystems, but no membrane-bound organelles (i.e. chloroplasts or mitochondria), plastoquinone has been determined to be a common link between the respiratory and photosynthetic electron transport chains (Hirano et al., 1980). In the cyanobacteria and photosynthetic bacteria, inhibition of dark respiration is suggested to be due to an interaction between photosynthetic and respiratory chains which are present in the same membrane (Sandmann and Malkin, 1984; Vermeglio and Carrier, 1984). However, in green algae, the photosynthetic and respiratory apparatus are found in different subcellular organelles, the chloroplasts and mitochondria, respectively. Thus any interaction between the two pathways must be via metabolic shuttles (Evans and Carr, 1979).

Light-dependent $O_2$ uptake which is sensitive to $CO_2$ concentration was found to be due to the photorespiratory glycolate pathway (Sec. 1.6) (Canvin et al., 1980; Gerbaud and Andre, 1979; Peltier and Thibault, 1985a). With saturating $CO_2$
concentrations, the oxygenase activity of RuBP carboxylase-oxygenase should be completely inhibited in the light, but O$_2$ uptake still occurs (Canvin et al., 1980; Gerbaud and Andre, 1980; Peltier and Thibault, 1985a). However, the mechanism of this uptake is still unknown. In green algae, under conditions in which photorespiration has been shown to be absent (i.e. 0.8% CO$_2$, 8% O$_2$), no light inhibition of O$_2$ uptake was found (Brown, 1953; Peltier and Thibault, 1985b). Peltier and Thibault (1985b) have suggested that in the green alga, *Chlamydomonas reinhardii*, Mehler reactions (direct photoreduction of O$_2$) do not occur during CO$_2$ fixation, and that this O$_2$ uptake is due to mitochondrial respiration.

Radmer and Kok (1976) have reported that illuminated algae reduce O$_2$ at a high rate when the CO$_2$ concentration is not limiting, and when CO$_2$ fixation does not occur (i.e. during the lag in CO$_2$ reduction following a dark-light transition or induction phase). During the first 20 s of continuous illumination of dark-adapted *Scenedesmus*, no net O$_2$ evolution or CO$_2$ uptake was detected using a mass spectrometer (with response time of 2-3 s). However, O$_2$ evolution during this interval was normal, and thus was completely compensated for by the O$_2$ uptake.

In the presence of inhibitors of CO$_2$ fixation, O$_2$ evolution was maximal, but a high rate of O$_2$ uptake was also observed. In the presence of cyanide, a potent inhibitor of
both the oxygenase and carboxylase activity of RuBP carboxy-
lase/oxygenase (Lorimer et al., 1973a,b), O₂ uptake was
inferred to be due to the photoreduction of O₂ in place of
CO₂. Radmer and Kok (1976) suggested that a special high
capacity oxidase, distinct from RuBP oxidase, exists in whole
cells, and that CO₂ and O₂ directly compete via Mehler
reactions for the light-generated reducing power of PS I.
This oxidase may be associated with ferredoxin, which is known
to mediate O₂ reduction by illuminated chloroplasts (Arnon
et al., 1967). During the induction phase and when CO₂
reduction does not keep pace with O₂ evolution, O₂ is the main
electron acceptor (Radmer and Kok, 1976; Radmer et al., 1978).
This O₂ uptake was observed in several different plants and
algae (Ozbun et al., 1964; Lex et al., 1972; Volk and Jackson,
1972), suggesting that rapid direct photoreduction of O₂ is
common to all photosynthetic organisms.

The O₂ uptake by isolated, illuminated chloroplasts in
the absence of added electron acceptors occurs via a Mehler
reaction (Mehler, 1951). Electrons which are transferred from
the water-splitting system (OEC) through the electron trans-
port chain of PS II and PS I may be available for reduction of
a suitable mediator. At some point in the electron transfer
chain, the electrons are transferred from the autooxidizable
mediator to O₂. This may proceed by three different mecha-
nisms, depicted in Fig. 2.6. The photosynthetic reduction of
O₂ results in a net consumption of O₂ since H₂O₂ is usually
Fig. 2.6 Photoreduction of O$_2$ via the Mehler reaction (after Allen, 1977).

The transfer of electrons to O$_2$ occurs via the reaction:

\[
2e^- + O_2 + 2H^+ \xrightarrow{\text{mediator}} H_2O_2
\]

a. Photosynthetic O$_2$ reduction is mediated by a compound (R), such as methyl viologen, which transfers electrons singly to oxygen. Superoxide may dismutate catalytically or spontaneously to produce H$_2$O$_2$.

b. A mediator accepts two electrons per molecule from the electron transport chain, donating one electron to oxygen and one to superoxide (Elstner and Heupel, 1974).

c. Oxygen reduction is mediated by the electron carrier, ferredoxin, and involves reduction of both oxygen and superoxide (Allen, 1975).
the product, rather than H₂O (Mehler, 1951a, b; Good and Hill, 1955).

Reduction of O₂ by electron acceptors associated with PS I (Asada et al., 1974; Harbour and Bolton, 1975; Jursinic, 1978) yields the superoxide free radical, O₂⁻. The rate of O₂⁻ production has been estimated from the superoxide dismutase inhibition rate of cytochrome c reduction in spinach chloroplasts to be ~15 μmol (mg Chl)⁻¹ hr⁻¹ (Asada et al., 1974). This rate corresponds to ~5-10% of the total electron flow usually observed in chloroplasts (Asada et al., 1977). Addition of ferredoxin increases the rate of O₂ uptake since ferredoxin is rapidly reduced by PS I and reduced ferredoxin reacts quickly with O₂ (Allen, 1975). Generally, reduced ferredoxin will donate an electron to NADP⁺, generating NADPH. However, under conditions of limiting CO₂ concentration, more O₂⁻ is generated (Radmer and Kok, 1976; Marsho et al., 1979; Egneus et al., 1975). This radical is highly toxic and its conversion to O₂ and H₂O₂ is catalyzed by a family of enzymes: the superoxide dismutases. Superoxide dismutase, present in chloroplasts, catalyzes the reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

in order to prevent conversion of O₂⁻ into more reactive species, such as the hydroxyl radical OH⁻ (Fridovich, 1978; Halliwell, 1978). Hydrogen peroxide may diffuse to the
peroxisomes where it is scavenged by catalase, although the chloroplasts may have another enzyme present to break down H$_2$O$_2$ (Asada et al., 1977; Halliwell et al., 1981). Ascorbate (Foyer and Halliwell, 1976) and glyoxylate (Elstner et al., 1975) have been proposed as scavengers of H$_2$O$_2$ in chloroplasts.

The superoxide anion is also scavenged by interaction with other chloroplast components, such as cytochrome f (Fe$^{3+}$ → Fe$^{2+}$), plastocyanin (Cu$^{2+}$ → Cu$^{+}$), ferredoxin (Fe$^{2+}$ → Fe$^{3+}$), ascorbate, reduced glutathione, and Mn$^{2+}$ (Mn$^{2+}$ → Mn$^{3+}$). Superoxide is converted to inactive molecular oxygen only when O$_2$ oxidizes reduced ferredoxin, plastocyanin, and cytochrome f (Asada et al., 1977).

Oxygen uptake has been observed during flash illumination of algae and chloroplasts (Weiss and Sauer, 1970; Jursinic, 1978; Schmid and Thibault, 1979; Jursinic, 1980), but the sites of this uptake are still uncertain. Mitochondrial respiration in the light does not vary on a time scale of seconds (Jackson and Volk, 1970), and for this reason, it most likely does not contribute to a flash-induced O$_2$ uptake.

Schmid and Thibault (1979) have shown that in tobacco chloroplasts, a PS I O$_2$ uptake affects the O$_2$ yield in a sequence of short (5 µs) saturating flashes. This O$_2$ uptake in chloroplasts was greatly enhanced by the presence of an exogenous electron acceptor such as p-benzoquinone or ferri-cyanide but its order of magnitude was not determined. The O$_2$ uptake was evident on the first two flashes, but was
swamped by the oscillating $O_2$ evolution pattern after the second flash. In the presence of the electron transport inhibitor 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the $O_2$ uptake was present when an exogenous electron acceptor was added, indicating that this was a PS I-mediated $O_2$ uptake. Using mutant tobacco chloroplasts (with an exogenous electron acceptor) exhibiting only PS I photoreactions, the steady-state $O_2$ uptake was shown to be dependent on flash frequency.

Recently, Beck et al. (1985) have shown that a biphasic $O_2$ consumption occurs in untreated PS II membranes after continuous illumination. The fast phase of $O_2$ consumption was highly variable and dependent on the intensity and length of the illumination period and lasted approximately 2 min. This fast $O_2$ uptake was suggested to be due to dissipation of reactive intermediates formed during illumination. The slow phase of $O_2$ consumption lasted about 1 h, was dependent on the amount of PS II OEC in the sample preparation and was not inhibited by the electron transport inhibitor, DCMU. The maximum rate of $O_2$ consumption in the slow phase was proportional to the maximum rate of $O_2$ evolution with saturating illumination. The authors suggest that the slow phase may be a property of the PS II OEC. Beck et al. (1985) suggest that reduced endogenous plastoquinone (PQ) or reduced endogenous 2,5-dichloro-p-benzoquinone (DCBQ) is the electron donor for
the slow consumption of O$_2$ catalyzed by the OEC.

Oxygen consumption was found to occur in both PS I and PS II of photosynthetic organisms through several different processes and is an integral component of photosynthesis (Mehler, 1951a,b; Vidaver and French, 1965; Jackson and Volk, 1970; Glidewell and Raven, 1975; Radmer et al., 1978; Schmid and Thibault, 1979; Peltier and Thibault 1985a,b; Beck et al., 1985). Oxygen consumption appears to play a role in the dissipation of excess reducing power in PS I and may occur at the level of the photosynthetic electron transport chain via a Mehler reaction or at the level of CO$_2$ metabolism. In the light, the PS II OEC is apparently in an active state which is able to catalyze the reduction of O$_2$ (Beck et al., 1985). During dark-adaptation of PS II particles, a structural change in the Mn site of the OEC occurs, changing the electron transport properties of the donor side of PS II and causing a conversion from the active state to a resting state which does not consume O$_2$ (Brudvig, 1984; Brudvig et al., 1984).
CHAPTER 3. MATERIALS AND METHODS

3.1 Marine algae

Different genera of multicellular marine algae from three divisions of eukaryotic algae were used to study the kinetics of O$_2$ exchange in photosynthesis. Green algae (Chlorophyta) contain chloroplasts and are similar to higher plants in their photosynthetic apparatus and function. Brown algae (Phaeophyta) also contain chloroplasts, but have different thylakoid arrangements and pigment/protein complexes and slightly different photosynthetic mechanisms than green algae and higher plants. Red algae (Rhodophyta) are eukaryotic, but have a photosynthetic structure similar to the prokaryotic cyanobacteria. The characteristics of the three divisions will be discussed in Chapter 4, along with O$_2$ exchange measurements for various genera within the divisions.

One genera of green algae, *Ulva*, was used predominantly for photosynthetic O$_2$ exchange measurements due to its local availability and similarity in photosynthetic structure and apparatus to higher plants.

3.1.1 Sample preparation

The algal genera used for this research were subject to
local availability. Red algae (such as *Irííäeã sp.* and *Porphyra sp.*) were predominantly collected during the winter and early spring (January through April), while green algae (such as *Ulva sp.* and *Enteromorpha sp.*) were collected during the late spring and summer months (April through September). Brown algae (*Laminaria* and *Alaria*) were collected at various times throughout the year.

Marine algae were collected from specific sites in order to minimize sample variability. *Ulva sp.* was found in low intertidal zones at Brockton Point in Stanley Park, Vancouver, B.C. *Enteromorpha sp.* and *Porphyra sanjuanensis* were found in high intertidal zones in Ambleside Park in West Vancouver, B.C. and Belcarra Park in Port Moody, B.C. *Porphyra perforata, P. sanjuanensis, Irííäeã sp., Laminaria,* and *Alaria* were collected from Brockton Point in Vancouver, B.C. and Barnet Marine Park in Burnaby, B.C. Whenever possible, entire living plants were collected, however, in some cases, living algae which were cast up on the beach were used.

Algae were maintained in Millipore filtered (0.22 μm) seawater in aerated containers in a Percival (model 1.35LL) incubator at 10 ± 1°C for periods up to a week. A 14 h photoperiod was provided by cool-white fluorescent tubes with an intensity of 0.3-0.4 mW/cm². Generally, most experiments were performed within 1-3 days after collecting algae. A reduction in overall O₂ exchange was noticed in algae which had been maintained in an artificial environment for 5-7 days.
3.1.2 Determination of chlorophyll content

The total chlorophyll content in green algae was measured using the method of Arnon (1949). Twenty samples of algae were cut with a 6 mm diameter cork borer and then placed into a mortar. After the addition of fine sand particles and a few ml of acetone to the mortar, the algae disks were finely ground. Two to three more ml of acetone were added to the mortar and the chlorophyll/acetone solution was poured into a flask. The mortar was continually rinsed with acetone until all the chlorophyll was extracted. The flask was filled to provide 10 ml of solution which was centrifuged for 5-10 min at 6000-7000 rpm. Sand, lipids, proteins and extraneous material precipitated out of solution, while the extraction layer contained chlorophyll and acetone. Five ml of the extract was pipetted into 25 ml acetone, resulting in a 1:6 dilution for the chlorophyll absorbance measurements.

The absorbance (in µg chlorophyll/ml solution) was measured in a Beckman spectrophotometer and determined by the following equation (Arnon, 1949):

$$A = \frac{20.2 A_{645} + 8.02 A_{663}}{A_{663}}$$

where $A_{645}$ and $A_{663}$ are the absorbances of chlorophyll at 645 nm and 663 nm, respectively. Since 10 ml of solution (algae in acetone) was used, the total chlorophyll content
per algal thallus (μg Chl) was found by multiplying eqn. 3.1 by 10 ml × 6/20, where 6 is the dilution factor and 20 samples were used.

3.2 The electrode system

Oxygen exchange (uptake and/or evolution) measurements on marine algae were made using a bare Pt and Ag/AgCl electrode system (Haxo and Blinks, 1950; Chandler and Vidaver, 1971; Swenson et al., 1986) shown in Fig. 3.1. The basic components of the electrode system include the Pt and Ag/AgCl electrodes and a dialysis membrane separating the electrodes and electrolyte solution from the environment in the sample holder. A thin layer of electrolyte was in direct contact with the cathode and anode. This type of electrode system is often referred to as a membrane-covered polarographic detector (MPOD) (Clark, 1956; Hitchman, 1978).

The electrode assembly is housed in a stainless steel pressure cell (Morita, 1970; Chandler and Vidaver, 1971) with a Lucite window at one end. The electrodes are seated in a plastic mounting in the top of the sample holder and the algae sample is placed on top of the Pt electrode. The top of the sample holder screws into the metal base.

The electrode system with Pt as the cathode and Ag/AgCl as both counter and reference electrode was used in order to
Fig. 3.1 Diagram of the platinum and silver electrode holder

a. The platinum and silver electrodes are indicated in this exploded perspective view of the electrode holder (after Chandler and Vidaver, 1971). The bottom of the sample holder is not shown.

b. The relationship of the Pt and Ag electrodes to the algae sample, dialysis membrane and electrolyte solution is depicted (not drawn to scale).
avoid voltage fluctuations due to the polarization. The components and chemical reactions of the electrode system will be discussed in the following sections.

3.2.1 Membranes and electrolyte solutions

A membrane separating the algal thallus and electrolyte solution from the surrounding environment provides a finite diffusion layer of controlled thickness at the electrode. The membrane is the largest barrier to diffusion of the \( \text{O}_2 \) molecules to the Pt cathode (Hitchman, 1978).

When the Pt electrode is biased at \(-0.7\) V vs Ag/AgCl, \( \text{O}_2 \) is reduced at the cathode. This results in a lowered \( \text{O}_2 \) concentration at the Pt electrode, and thus \( \text{O}_2 \) in the sample holder diffuses through the membrane and into the thin layer of electrolyte solution at a faster rate than in the absence of the applied potential. Approximately 15-20 min were required for equilibration of the system.

The criteria for a suitable membrane for polarographic \( \text{O}_2 \) detectors is adequate physical strength, permeability characteristics which do not vary with time, high permeability to \( \text{O}_2 \), and availability in thin sheets (Hitchman, 1978). Many different membranes have been used for polarographic \( \text{O}_2 \) detectors, such as fluorinated plastics, polyethylene, silicone rubber, natural rubber, polyvinyl chloride, cellophane, mylar,
and teflon (Hoare, 1968; Hitchman, 1978). The thickness of the most commonly used membranes is 10-25 μm (Hitchman, 1978).

For these experiments, a dialysis membrane was used, which consists predominantly of cellulose, and has a high permeability to O₂. The dialysis membrane was soaked in seawater and held tightly in place on top of the electrodes with an O-ring. Both the thickness of the dialysis membrane and the resultant shape of the O₂ exchange curve depend on how tightly the membrane is stretched over the electrodes.

The electrolyte solution provides the electrochemical contact between the anode and cathode and must contain the anion(s) necessary to provide the reference couple for the anode. Seawater, the natural habitat of marine algae, provides the best choice for the electrolyte solution since it contains a high concentration of Cl⁻ ions. The environmental concentration of Cl⁻ ions in the seawater varied over the period of this research from 18.8-22.9 g Cl⁻/l seawater.
3.2.2 The Platinum Cathode

Oxygen produced by the algae may diffuse through the dialysis membrane into the ambient atmosphere and it may diffuse to the Pt electrode where it is reduced. A Pt cathode potential of \(-0.7\) V with respect to \(\text{Ag}/\text{AgCl}\) provides four electrons in total for the reduction of each \(O_2\) molecule to \(\text{OH}^-\) according to the equations:

\[
O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^- \quad 3.1
\]
\[
H_2O_2 + 2e^- \rightarrow 2OH^- \quad 3.2
\]

if all the hydrogen peroxide is reduced (Davies et al., 1962). \(H_2O_2\) dissociates to the peroxide anion by:

\[
H_2O_2 \rightarrow HO_2^- + H^+ \quad 3.3
\]

where:

\[
\log \frac{[\text{HO}_2^-]}{[H_2O_2]} = \text{pH} - 11.63 \quad 3.4
\]

Equation 3.4 indicates that in the vicinity of the Pt electrode, the electrolyte solution is alkaline as indicated by the pink-red color of phenolphthalein added to the electrolyte (seawater); the pH at the Pt has been determined to be approximately 12.3 (Hitchman, 1978). The proposed reaction
scheme for the reduction of $O_2$ at the Pt electrode in alkaline media (Hoare, 1968) is:

$$\begin{align*}
Pt & \quad O_2 \rightarrow O_2^- \rightarrow HO_2 \rightarrow HO_2^- \\
\quad e^- & \quad H_2O \quad OH^- \quad e^-
\end{align*}$$

The peroxide anion is catalytically decomposed at the cathode:

$$2HO_2^- \rightarrow 2OH^- + O_2$$

forming an $O_2$ molecule which may also undergo the electron transfer steps of Eq. 3.5. Thus the overall reaction can be written as:

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$$

Four electrons are produced for each $O_2$ molecule reduced at the cathode and thus the net amount of $O_2$ produced by the algae can be calculated from the integrated oxygen current pulse. This will be discussed in Sec. 3.4.

The reduction of $O_2$ at the Pt electrode obeys the relation (Hoare, 1968; Hitchman, 1978):
\[ \frac{dN_{O_2}}{dt} = \frac{j}{nF} = k_1 [O_2]_{el} - k_2 [OH^-]_{el} \]  

where \( N_{O_2} \) represents the number of moles of \( O_2 \) reduced at the cathode per unit area per unit time, \( n \) is the number of electrons transferred from one \( O_2 \) molecule, \( j \) is the current density (amps/m\(^2\)), \( F \) is Faraday's constant, \( [O_2]_{el} \) and \( [OH^-]_{el} \) are the concentrations of the two species at the surface of the electrode, and \( k_1 \) and \( k_2 \) are rate constants for the forward and backward reactions of eqn. 3.7, i.e. \( O_2 \) reduction and \( OH^- \) oxidation, respectively.

3.2.3 The silver electrode

The silver electrode serves two functions: it acts as both counter and reference electrode. The reference electrode in this system is the redox couple \( Ag/AgCl \) (0.222 V vs. NHE at 25 °C), which is prepared by operating the anode in seawater. The \( Ag \) oxidization in the presence of \( Cl^- \) ions in the electrolyte solution is given by:

\[ Ag + Cl^- \rightarrow AgCl + e^- \]  

Depletion of \( Cl^- \) ions in the electrolyte solution will occur
over a period of time since Cl\textsuperscript{−} is required by both the electrode system and for participation in the process of photosynthetic water-splitting in the algae. However, in all of the experiments performed, the duration of the experiments was short enough to minimize the depletion of Cl\textsuperscript{−} ions and the resultant decrease in the electrolyte strength.

3.3 Experimental methods and apparatus

3.3.1 Experimental methods

Samples were cut from algae with a 6.0 mm diameter cork borer and tightly held on the bare Pt electrode by a thin dialysis membrane (cellulose) soaked in seawater. The membrane was stretched to cover the Ag/AgCl electrode and secured with an O-ring. For all measurements, the Pt electrode was biased at \(-0.700 \pm 0.001\) V vs Ag/AgCl, and was maintained at constant potential by the circuits shown in Fig. 3.2 c and d.

For all experiments, the voltage was applied across the electrodes after the sample had dark-adapted for approximately 10 min. Approximately 20-30 min was required to obtain a stable baseline.

All experiments were repeated two to three times at room temperature using disks cut from the same algal thallus. If
Fig. 3.2 Measuring circuit for the data acquisition system.

a. The circuit used for preliminary O$_2$ exchange measurements is depicted.

b. The power supply for the measuring circuits shown in c and d replaced the 1.5 V battery used in part a.

c. The O$_2$ reduction current at the cathode is measured across a load resistor, $R_L$ (1 or 10 kΩ).

d. The O$_2$ reduction current at the cathode is measured across a decade box resistor, ($R_L$ can be varied from 1 Ω to 99.999 kΩ).

In all three measuring circuits, the voltage across $R_L$ was input to the Tracor Northern signal averager. The Pt electrode was maintained at $-0.70 \pm 0.05$ vs Ag/AgCl in circuit a, and $-0.700$ V with respect to Ag/AgCl in circuits c and d. The symbol $\bigcirc$ represents shielded cable.
samples were used for a second experiment, they were dark-adapted for ten minutes, which was sufficient for reproducibility. Most experiments were repeated up to ten times using algae collected over the growing season, using 2-3 samples from each thallus, yielding 20-30 experiments for each species. This was necessary to compensate for diurnal and seasonal variations. Much variability was found among a species, thus an estimation of error in the measurements is difficult to achieve. Data which is presented in this thesis represents individual experiments which are representative of the average response for a given species. To compensate for seasonal variations, only \( \text{O}_2 \) exchange measurements which were reproducible over a long period of time are presented.

The physiological state of the algae at the time of the \( \text{O}_2 \) exchange measurements is of paramount importance. Control \( \text{O}_2 \) exchange curves were taken prior to each experiment. If the control curve did not fall within the expected range of \( \text{O}_2 \) exchange values, the experiment was repeated until this occurred. In some cases, optimization of the sample placement within the electrode was sufficient, in other cases, a new control was eventually established.

Establishing control \( \text{O}_2 \) exchange curves for different genera of marine algae was essential to interpretation of the data. Long-term control \( \text{O}_2 \) exchange measurements, encompassing seasonal variations, were done for several different genera of marine algae and are presented in Chapter 4.
3.3.2 Electron transport inhibitors

The electron transport inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), was used to inhibit \( \text{O}_2 \) evolution by blocking the photosynthetic electron transport chain. Discs cut from algae were kept in seawater containing 5 \( \mu \text{M} \) DCMU for 1 h in the dark prior to measurement.

Antimycin A was used to block mitochondrial electron transport, and thus inhibit respiration (Izawa et al., 1967). Algal discs were kept in 10 \( \mu \text{M} \) Antimycin A for 3 h in the dark prior to measurement.

3.3.3 Apparatus for oxygen exchange measurements

The apparatus for all \( \text{O}_2 \) exchange measurements consisted of the electrode system (Fig. 3.1), the measuring circuit (Fig. 3.2), a light source (and pulse generator for flash illumination), a signal averager, and a monitor (computer or chart recorder). For flash illumination, the apparatus is depicted in Fig. 3.3. For experiments using continuous light, the flash source and frequency generator were replaced by a light source and a 4.7 cm thick layer of water to absorb the infrared portion of the spectrum (depicted in Fig. 3.4 for anaerobic conditions.)
Fig. 3.3 Apparatus for oxygen exchange measurements with flash illumination

The light was channeled to the sample holder by a light pipe from the xenon flash lamp or reflected by mirrors from the excimer-pumped dye laser. Early experiments were recorded on a chart recorder; later experiments were input to a microcomputer.
PULSE GENERATOR 

LIGHT PIPE OR MIRRORS

PULSED LIGHT SOURCE

XENON FLASH OR LASER

(5 µs or 10 ns)

SAMPLE HOLDER

ELECTRODES (Pt, Ag)

REFERENCE ELECTRODE

AND MEASURING CIRCUIT

INPUT VOLTAGE

TRIGGER

COMPUTER (CHART RECORDER)

PULSE (1-10 Hz)

TRACOR NORTHERN SIGNAL AVERAGER
Fig. 3.4 Apparatus for oxygen exchange measurements under low O₂ concentrations.

The data acquisition system is the same as shown in Fig. 3.2. The mixing tanks allow any ambient O₂/N₂ ratio to be obtained. To obtain an anaerobic environment, N₂ is flushed through the system.
MIXING TANK → VALVES → SAMPLE HOLDER → MIXING TANK

PRESSURE GAUGE

PRESSURE GAUGE

STEEL TUBING → \( N_2 \) → \( O_2 \)
3.3.4 Oxygen exchange measurements under low oxygen partial pressures or under anaerobic conditions

Oxygen exchange measurements were made under conditions of low ambient O₂ concentrations and under anaerobic conditions. Two different systems were used for these measurements. In order to measure O₂ exchange under low O₂ partial pressures or under anaerobic conditions, the sample holder was connected to O₂ and/or N₂ tanks. The O₂ and N₂ were supplied to the sample cell by two high pressure Linde specialty gas mixture tanks, one containing O₂ with 280 μl CO₂ per liter, and the other containing N₂ with 285 μl CO₂ per liter. A 1.5 l stainless steel chamber was arranged in parallel with the sample cell to allow mixing of the O₂ and N₂ to any desired partial pressure (Fig. 3.4).

An anaerobic environment was also obtained by connecting the sample holder with plastic tubing to a small Pittman air pump as shown in Fig. 3.5. Air was circulated through a flask of alkaline pyrogallol solution and throughout the closed system at a flow rate of approximately 100 cm³/min until the O₂ in the system was removed. The pyrogallol solution was prepared by adding 2.8 g pyrogallic acid in 10 ml distilled water to 5 g KOH in 10 ml distilled water (Gabb and Latchem, 1967). Generally, two to three hours were required to obtain a completely anaerobic environment. In order to ensure that the algae did not become dessiccated by the circulating air,
Fig. 3.5  Apparatus for oxygen exchange measurements under anaerobic conditions.

Continuous illumination was used for this series of experiments. Algae was placed in the sample holder and the system closed to the atmosphere. The gas in the system was pumped through pyrogallol to remove $O_2$. The data acquisition system is the same as for flashing light experiments.
I/O INPUT VOLTAGE
-RL
J
I
REFERENCE ELECTRODE
COMPUTER
I
FOWR SUPPLY
I
001
PYROGALLOL
I
I
PUMP
I
UV SHUTTER FILTER
I
LIGHT PIPE
I
LIGHT SOURCE
I
SAMPLE HOLDER
I
ELECTRODES
I
FOCUSING SOURCE AND MEASURING CIRCUIT
I
INPUT VOLTAGE
I
REFERENCE ELECTRODE AND MEASURING CIRCUIT
I
COMPUTER (CHART RECORDER)
I
TYGON TUBING
I
WATER FLASK
I
TRACOR NORTHERN SIGNAL AVERAGER
a flask of water was connected in series with the flask of pyrogallol solution.

3.3.5 Light sources

Both pulsed and continuous light were used to illuminate the sample, depending on the type of experiment performed. Continuous illumination was provided by a Quartzline EGH lamp (Canadian General Electric, 120V, 300W) operated at voltages up to 110 V. Light was passed through a 4.7 cm thick layer of water to absorb the infrared portion of the spectrum. Pulsed light was obtained by a xenon flash (4 or 5 µs pulse) or an excimer-pumped dye laser with flash duration less than 10 ns. Both xenon flash and continuous excitation light were led to the sample through fiber optic bundles. Mirrors were used to reflect the laser light into the sample holder.

For the first series of flash illumination O₂ exchange experiments, an EG&G FX 224 xenon flash lamp operated at 1.0 kV with a 10 µF discharge capacitor produced a pulse width (FWHM) of 4 µs. For later experiments, an EG&G FX 249 xenon flash lamp operated at 1.0 kV with a 10 µF discharge capacitor resulted in a pulse width of 5 µs. Each pulse must be intense and sufficiently long to provide at least one turn-over of each oxygen-evolving complex (OEC) for a low probability of misses, but must be short enough to minimize double hits in
the reaction centers (two turn-overs of the OEC). The light was increased until the maximum net O$_2$ evolution was achieved. However, it is difficult to saturate the S$_2$ $\rightarrow$ S$_3$ transition (Delrieu, 1980), and thus there still may have been a high proportion of misses for OECs in the S$_2$ state prior to a light flash.

The flash frequency was set by a Hewlett Packard 3301B function generator, which also triggered the signal averager simultaneously with the first flash. The flash frequency for all control experiments was set to 3.3 Hz, which kept the time interval between flashes short enough to minimize deactivation of the S-states of the OEC (Joliot et al., 1971; Kok et al., 1971), but long enough to allow the S-state transitions to occur.

The energy output per pulse of the (1 kV) xenon flash at 3.3 Hz was determined to be 3.0 ± 0.3 mJ using a Scientech 361 power meter with detector model VPH-2 (Newport Research Corporation). Using 450 nm as the average wavelength of the xenon lamp (reference), the emission was calculated to be $\sim 2 \times 10^{15}$ photons per flash.

An excimer-pumped dye laser was used to provide flash illumination when a short pulse of a specific wavelength was required. Far red light was obtained by using oxazine 725 perchlorate dissolved in methanol as the laser dye, which has maximal fluorescence at approximately 720 nm. Red light was obtained by using DCM dissolved in methanol, which has a
fluorescence maximum at 650 nm.

White light from a Quartzline EGH lamp, operated at voltages up to 110 V, was used to provide illumination times of up to 2 min. A simple mechanical shutter controlled the duration of illumination. The light intensity was measured with a Tektronix J16 photodetector with J6502 irradiance probe.

3.3.6 Data acquisition system

The current produced by $O_2$ reduction at the Pt cathode was measured across a load resistor, $R_L$ (which ranged from 1 to 40 kΩ), and input to a Tracor Northern Model TN 1710 signal averager. Three measuring circuits were used during this research. Preliminary measurements were performed using the circuit depicted in Fig. 3.2a. Later measurements used the circuit shown in Fig. 3.2c, while the final circuit is shown in Fig. 3.2d. Figure 3.2 b depicts the power supply used for circuits 3.2c and d.

Early experiments were recorded on a Moseley 7001 AR X-Y recorder. For later experiments, the signal averager was interfaced to a microcomputer and output to a Hewlett Packard 7470A plotter.
3.4 Data analysis

To correct for the pile-up of pulses, the data were fitted with the Simplex algorithm (Nedler and Mead, 1965; Caceci and Cacheris, 1980) on an IBM PC. The data were read into the PC by means of a Houston Instruments HIPAD digitizer to a maximum of 1000 equidistant horizontal and vertical data points. Curve fitting, as a function of the total number of data points (N) showed that the number of data points in the curve did not affect the values of the parameters used to fit the curve.

The O₂ exchange curve was generated by adding pulses with the same shape as the third pulse (the reference response), shifted along the time axis with the appropriate time interval between pulses. The amplitude of each pulse was determined to give the best fit to the experimental curve for the total O₂ exchange. This fitting procedure assumes that the time course of the O₂ current from a single flash delivered at different times changes in scale, but not shape. This is a reasonable assumption since O₂ is always produced at the same locations within the algae, the evolution of O₂ follows four-step kinetics which only change in magnitude as a function of flash number (Kok et al., 1970), and the half-time for O₂ evolution is independent of flash number (Joliot et al., 1966; Dekker et al., 1984b).

The error function for the algorithm computed the sum of the squares of the distances from the experimental curve to
the computer-generated curve for one point in ten (approximately 100 equidistant points). The fractional error per pulse for the $i^{th}$ flash can be expressed by taking the square root of the error value for the $i^{th}$ flash divided by the number of points analyzed for each flash ($N'$, where $N' = N/10x$ and $x$ is the total number of flashes), and dividing by the relative amplitude of the $i^{th}$ flash multiplied by the amplitude of the reference response, expressed in number of points. The fractional error for the $i^{th}$ flash is thus:

$$E = \sqrt{\frac{\text{output error}}{N'}} \times \frac{Y_i(\text{rel})}{Y_3}$$

where $Y_i(\text{rel})$ is the relative amplitude of the $i^{th}$ pulse (with respect to the amplitude of the reference response) and $Y_3$ is the net $O_2$ yield for the third flash expressed in number of data points.

The net $O_2$ produced by the algae can be determined for $O_2$ exchange curves which have been fitted by the Simplex algorithm by multiplying the relative yield for the $i^{th}$ pulse, $Y_i(\text{rel})$, by the amount of $O_2$ produced during the reference response.
CHAPTER 4. OXYGEN EXCHANGE MEASUREMENTS IN MARINE ALGAE

Much of the early work on $O_2$ exchange (evolution and uptake) and light-induced $O_2$ uptake was first measured in algae (Brown and Weiss, 1959; Hoch et al., 1963; Vidaver and French, 1965). Algae, in general, represent very diverse groups, but the processes of $O_2$ evolution and uptake in different algal divisions have many similarities to each other and to those of higher plants.

An analysis of $O_2$ exchange curves is difficult because of the inter-relationship between production and consumption of $O_2$ by the algae and its detection by the electrode system. Thus, a discussion of photosynthetic $O_2$ exchange cannot be complete without considering the mechanism of $O_2$ reduction at the Pt electrode and how it affects the measured $O_2$ exchange in the algae. However, in order to investigate kinetics of $O_2$ exchange, both in algae and in higher plants, it is essential to determine the "normal" (control) $O_2$ exchange. Control $O_2$ exchange curves represent $O_2$ exchange in untreated, freshly collected algae, dark-adapted for 10 min in air prior to $3.3 \pm 0.1$ Hz flash illumination.

For flash illumination, relatively little information exists in the literature on $O_2$ exchange measurements using the bare Pt electrode; for this reason, control $O_2$ exchange curves were established for different genera of marine algae.
Oxygen exchange measurements were taken every 6-8 weeks over the period of this research in order to establish the controls. Genera in three divisions were studied, Rhodophyta (red algae), Phaeophyta (brown algae), and Chlorophyta (green algae). A detailed discussion of the different kinetics for the genera studied is beyond the scope of this thesis.

4.1 The Red Algae (Rhodophyta)

Rhodophyta can be distinguished from other groups of eukaryotic algae by several characteristics, including the absence of aggregated thylakoids and the presence of certain accessory photosynthetic pigments, the phycobilins (Bold and Wynne, 1985). Phycoerythrin is generally the predominant accessory pigment and imparts the red coloration to many Rhodophyta, thus giving this genera the common name, red algae. However, many red algae do not appear reddish, and in fact a full range of coloration is found (Bold and Wynne, 1985).

The light-harvesting complex in the red algae consists largely of aggregated phycobiliproteins which are found on the external surface of the thylakoids. These are referred to as phycobilisomes (see Fig. 1.3).

The control $O_2$ exchange curves for dark-adapted Porphyra perforata and Iridaea sp. are shown in Fig. 4.1a and Fig. 4.2, respectively. The variation between the two $O_2$ exchange
Fig. 4.1 Oxygen exchange in *Porphyra*

a. The $O_2$ exchange curve for dark-adapted *Porphyra perforata* in air at 3.2 Hz flash frequency is depicted. The first flash occurs at $t = 0$. Oxygen is produced on the third flash.

b. The same sample of *Porphyra perforata* used in Fig. 4.1a was dark-adapted for 30 min in 5% $O_2$ prior to illumination. This shows more distinct current pulses than Fig. 4.1a.
Fig. 4.2 Oxygen exchange in *Iridaeae*

The O₂ exchange curve for dark-adapted *Iridaeae* at 3.2 Hz flash frequency is shown. Eleven light flashes were given to the sample, with the first flash at \( t = 0 \), and the last flash at \( t = 3.2 \) s.
curves suggests that the mechanisms of \( O_2 \) evolution and/or uptake may be quite different between the two red algal genera. The mechanisms of \( O_2 \) exchange in red algae is unknown, however, there is no evidence to indicate that the process of \( O_2 \) evolution is different in red algae than in the green algae. Brechignac and Andrè (1984) found that in the red alga, *Chondrus crispus*, photorespiration is absent and thus is not responsible for a light-induced \( O_2 \) uptake. Photoreduction of \( O_2 \) has been found in all photosynthetic organisms examined (Bunt and Heeb, 1971; Glidewell and Raven, 1975; Radmer and Kok, 1976; Radmer and Ollinger, 1980; Shelp and Canvin, 1980; Fock et al., 1981; Behrens et al., 1982; Furbank et al., 1983) and thus it likely occurs in red algae.

The \( O_2 \) exchange curve for *Porphyra* in air (Fig. 4.1a) does not fit the S-state model depicted by Kok et al. (1970). The lack of a four step pattern of \( O_2 \) exchange in *Porphyra* under normal conditions may be due to differences in \( O_2 \) uptake or \( O_2 \) evolution or both. The structure and/or function of the oxygen-evolving complex in *Porphyra* may be different than other red algal genera (e.g. *Iridaea*). The possibility that *Porphyra* is photoinhibited under lower light intensities than other red algal genera should be investigated. In order to determine if the variation in \( O_2 \) exchange is due to different \( O_2 \) uptake processes, samples of *Porphyra* were dark-adapted in 5% \( O_2 \) for 30 min prior to illumination. With a reduced ambient \( O_2 \) concentration, \( O_2 \) consumption processes which occur in *Porphyra*
will be decreased. The O\textsubscript{2} exchange curve for \textit{Porphyra} in 5% O\textsubscript{2} (Fig. 4.1b) does show four step O\textsubscript{2} exchange kinetics. The significance of this result is difficult to interpret since a decrease in ambient O\textsubscript{2} concentration will probably inhibit the process of O\textsubscript{2} evolution as well as decrease O\textsubscript{2} consumption processes. However, since a four step O\textsubscript{2} exchange pattern is evident from Fig. 4.1b, this indicates that the structure and function of the OEC is \textit{Porphyra} cannot be radically different from other marine algae which show a four step O\textsubscript{2} exchange pattern under normal conditions.

4.2 The Brown Algae (Phaeophyta)

The division Phaeophyta consists of diverse organisms which range in size from microscopic to the largest of marine plants (\textit{Macrocystis}). One common factor among brown algae is their unique thylakoid arrangement. The thylakoids occur in groups of three with some interconnections between the lamellae (Bold and Wynne, 1985). The brown coloration of many members of Phaeophyta is due primarily to the pigment fucoxanthin.

Studies on photosynthetic water-splitting in the brown algae have indicated that both copper and manganese play a role in the OEC (Holdsworth and Arshad, 1977), however this has not yet been conclusively proven.

Oxygen exchange curves for flash illumination of two genera of brown algae are shown in Fig. 4.3 for \textit{Alaria} and in
Fig. 4.3 Oxygen exchange in *Alaria*

a. The O$_2$ exchange curve for dark-adapted *Alaria* at 3.2 Hz flash frequency is similar to that of *Ulva*, but is lower in magnitude. This may be due to the thickness of the algae. There is no evidence to indicate that the O$_2$ exchange mechanisms in *Alaria* are different than in the green algae.

b. Three light flashes given to the sample (with the first flash at $t = 0$) produce O$_2$ on the third flash, which represents the characteristic response of the system to O$_2$ production by *Alaria*. 
Fig. 4.4 and 4.5 for Laminaria. Different samples of Laminaria showed two distinct O₂ exchange curves; the variation may be due to two different species or to seasonal or diurnal variations within the same species.

4.3 The Green Algae (Chlorophyta)

Oxygen exchange has been widely studied in the green algae due to the similarity of photosynthetic structure and function to higher plants. Two genera of green algae were studied, Ulva and Enteromorpha, which both belong to the same family, Ulvaceae. Figure 4.6 shows the O₂ exchange curve for Enteromorpha, which is similar to the O₂ exchange curve shown for Ulva in Fig. 4.7.

Oxygen exchange curves for Ulva were obtained for a large number of samples; a representative O₂ exchange curve is depicted in Fig. 4.7. The O₂ exchange curve of Fig. 4.7, taken for dark-adapted Ulva, shows O₂ was released on the second flash. This is consistent with the findings of Jursinic (1978), who observed that O₂ was released on the second flash for physiologically fresh samples. This indicates that a certain number of OECs undergo double turnovers of some of the PS II reaction centers on either the first or second flash.

The O₂ exchange curve for Ulva illuminated with 680 nm
Fig. 4.4 Oxygen exchange in *Laminaria*

The O$_2$ exchange curve for dark-adapted *Laminaria* at 3.1 Hz flash frequency shows distinct O$_2$ pulses and includes both O$_2$ uptake and evolution components as observed in *Ulva*. Thirteen light flashes were given to the sample. The first flash occurred at $t = 0$, and the last flash occurred at $t = 3.9$ s.
Fig. 4.5 Oxygen exchange in *Laminaria*.

a. The O$_2$ exchange curve for dark-adapted *Laminaria* at 3.3 Hz flash frequency is quite different from that of Fig. 4.4 for a different sample of *Laminaria*, but is similar to that of *Alaria* in Fig. 4.3. The "dip" in the oscillation at the sixth and eleventh flashes in Fig. 4.4 is not evident in this figure. The O$_2$ current pulse at the second flash indicates the number of double turnovers of the OEC during either the first or second flash. This is common in freshly collected algae (Jursinic, 1978).

b. Three short light flashes (with the first flash at $t = 0$) given to depict the reference response for *Laminaria*. 
Fig. 4.6 Oxygen exchange in *Enteromorpha*.

The $O_2$ exchange curve for dark-adapted *Enteromorpha* at 3.0 Hz flash frequency is similar to that for *Ulva* in Fig. 4.7.
Fig. 4.7. Oxygen exchange in Ulva

The O₂ exchange curve for dark-adapted Ulva at 3.3 Hz flash frequency is similar to that for Laminaria in Fig. 4.4. The first flash occurs at t = 0. The presence of an O₂ signal for the second flash indicates double hits on either the first or second flash.
light is shown in Fig. 4.8. The ratio of $Y_3/Y_4$ ($Y_n$ is the net $O_2$ yield on the nth flash) is larger for white light illumination (Fig. 4.7) than for 680 nm illumination. Dark-adapted Ulva was illuminated with light of different wavelengths, ranging from 602 to 680 nm. The flash yield sequences for these $O_2$ exchange curves are shown in Fig. 4.9.

Britz (1976) has reported that Ulva undergoes a circadian rhythm of chloroplast movement. This would almost certainly alter $O_2$ exchange measurements. In fact, Ulva has been found to undergo large diurnal variations of light-saturated photosynthetic capacity in situ, which is manifest by minima and maxima in the rate of net $O_2$ evolution (Mishkind et al., 1976; Mishkind and Mauzerall, 1977). Freshly collected samples had a maximum net $O_2$ evolution under saturating light at noon, and a minimum at midnight (Mishkind et al., 1976). At low light intensities, however, the rate of net $O_2$ evolution remained constant. Mishkind et al. (1976) found that freshly collected Ulva, kept under continuous illumination ($2 \text{ mW/cm}^2$), maintained these oscillations for up to 36 h. These variations in $O_2$ exchange for one sample give an indication of the difficulty inherent in obtaining control $O_2$ exchange curves. For this reason, an error analysis for the control curves presented in this chapter was not attempted.

The experimental results presented in this chapter represent "normal" $O_2$ exchange for three divisions of algae. A comparison of $O_2$ yield per flash for the different algae is
Fig. 4.8 Oxygen exchange in *Ulva* illuminated with 680 nm light

The $O_2$ exchange curve for dark-adapted *Ulva* illuminated with 680 nm light at 3.2 Hz flash frequency is different from that in Fig. 4.7 for white light illumination. The absence of $O_2$ on the second flash (indicated by ↑) indicates that the duration of the nanosecond laser flash was short enough to minimize double turn-overs of the OECs.
Fig. 4.9 Oxygen exchange flash yield sequences for *Ulva*
iilluminated at different wavelengths

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>602</td>
<td>•------•</td>
</tr>
<tr>
<td>620</td>
<td>■——■——■</td>
</tr>
<tr>
<td>640</td>
<td>Δ......Δ</td>
</tr>
<tr>
<td>660</td>
<td>▲——▲——▲</td>
</tr>
<tr>
<td>680</td>
<td>○——○——○</td>
</tr>
</tbody>
</table>
depicted in Fig. 4.10. This figure suggests that some of the mechanisms of uptake and evolution may be quite different between the divisions. The difference in thallus thickness of the different algal genera should be considered, as well as the location of chloroplasts within the cell.

In order to begin to interpret the kinetics of $O_2$ exchange in marine algae, one genus of algae, *Ulva*, was studied. The analysis presented in Chapter 5 for the green algae, *Ulva*, can be adapted to analyze $O_2$ exchange kinetics for the genera of marine algae discussed in this chapter.
Fig. 4.10 Comparison of oxygen exchange flash yield sequences for the different genera of algae

**Iridaea** (Fig. 4.2)  \[ \Delta \cdots \Delta \]

**Alaria** (Fig. 4.3)  \[ \ast \cdots \ast \]

**Laminaria** (Fig. 4.4)  \[ \bullet \cdots \bullet \]

**Laminaria** (Fig. 4.5)  \[ \circ \cdots \circ \]

**Enteromorpha** (Fig. 4.6)  \[ \Delta \cdots \Delta \]

**Ulva** (Fig. 4.7)  \[ \circ \cdots \circ \]
CHAPTER 5. RESULTS AND DISCUSSION

OXYGEN EXCHANGE MEASUREMENTS IN ULVA

The bare Pt electrode system described in Chapter 3 records the current due to $O_2$ reduction at the cathode as a function of time and is linear over a wide range of ambient $O_2$ concentrations during steady-state measurements. Dynamic measurements of $O_2$ evolution and/or uptake in biological systems can be made with the bare platinum electrode, however, these measurements are limited by the response time of the electrode system.

The bare Pt electrode has been used extensively for research on $O_2$ exchange in marine algae under continuous illumination (French et al., 1961; Fork, 1963; Vidaver and French, 1965; Chandler and Vidaver, 1970; Chandler and Vidaver, 1971). However, little or no research has been done using this electrode system for $O_2$ exchange measurements with short, saturating light flashes. In the past 15 years, research has focussed on elucidating the molecular components and the mechanism of photosynthetic water-splitting and concomitant $O_2$ evolution. The Joliot electrode (Joliot and Joliot, 1968) has been used predominantly for flashing light studies since it minimizes the $O_2$ uptake component.

However, both the mechanism of $O_2$ evolution and the sites of light-induced $O_2$ uptake in photosynthesis are still
unknown. Much speculation has focussed on the role of light-induced O₂ consumption in photosynthesis and its effect on O₂ evolution, but no one has yet been able to separate the O₂ evolution and uptake components in the kinetic measurements. In order to determine the mechanism and kinetics of O₂ evolution, it seems reasonable that the various processes of O₂ uptake must be separated from O₂ evolution and not just minimized or neglected. In fact, Vidaver et al. (1984) have suggested that the "in vivo functioning" of the water-splitting system depends on the presence of partial O₂ pressures (≤0.01 atm). Brudvig (1984) has shown that thylakoids with an active OEC take up O₂ at the rate of 1-2 μmol O₂/mg Chl/h. Govindjee et al. (1985) proposed that an acceptable model of O₂ evolution must include the possibility that OECs may be capable of converting O₂ → H₂O, i.e. "run in reverse". This suggests the incorporation of an O₂ uptake component in the S state model of photosynthetic water-splitting.

In this research, the bare Pt electrode system was used in order to study both O₂ evolution and O₂ uptake, since both components are provided in the O₂ exchange measurements. The magnitude of the competing processes determines whether the net current response will be positive or negative. Thus any transient O₂ consumption occurring in the algae in response to a light flash will be superimposed on an O₂ evolution component and measured as O₂ exchange.

Since the bare Pt electrode system depicted in Fig. 3.1
has not been used previously to study $O_2$ exchange under flash illumination, it was essential to determine the best method to analyze $O_2$ exchange (evolution and uptake) measurements, including determination of system linearity, how to interpret and calibrate the $O_2$ reduction curves, calibration of the electrode system, and attempting to separate $O_2$ uptake and evolution components from the exchange measurements. A large part of this thesis research focused on setting up the system, optimizing and calibrating it (Chapter 5), and determination of control responses in different genera of marine algae (Chapter 4).

5.1 Interpretation of oxygen exchange curves for *Ulva*

An analysis of $O_2$ exchange curves must consider the processes leading to the production and consumption of $O_2$ in the algae as well as the diffusion of $O_2$ out of the algal thallus and reduction of $O_2$ at the Pt electrode. Oxygen produced by the algae in the sample holder can diffuse through the dialysis membrane into the environment in the sample holder and diffuse toward the Pt electrode where it is reduced. The recorded $O_2$ exchange curves will reflect the rate-limiting steps which occur during any of these processes.

Some of the earliest $O_2$ flash yield studies by Joliot and co-workers indicated that the two principal limiting
processes occurring between the photochemical acts in the chloroplasts and O₂ reduction at the cathode are a first order thermal reaction which occurs after the primary photochemistry, but prior to O₂ evolution (with a rate constant of 800 s⁻¹ at 20°C) and diffusion of O₂ from the algae to the Pt cathode (Joliot et al., 1966). This rate-limiting process prior to the production of O₂ in the algae (i.e. the S₃ → (S₄) → S₀ transition) has been determined by means of flash-induced UV absorbance spectroscopy (Dekker et al.; 1984a) to have a half-time of 1300 μs, which is comparable to the time constant of 1.25 ms determined by Joliot et al. (1966). Thus, O₂ evolution occurs on a time scale which is much longer than the duration of a microsecond flash, and within a few ms after the onset of the flash, no more O₂ is evolved by the plant.

An estimate of the diffusion time of O₂ from the algae to the cathode may be determined by considering diffusion across a uniform sample of thickness d, where the time constant is given by \( \tau \sim 4d^2/\pi^2D \) (Rubinow, 1975; Crank, 1975). Thickness of the Ulva samples was determined to be ~10 μm by weighing samples of known area on a Mettler H2O balance, and assuming that algae has the same density as water. Estimating the thickness of the electrolyte layer is difficult, but for this approximate calculation, the electrolyte layer is assumed to be less than the thickness of the algae, i.e. ~5 μm. The diffusion constant D for O₂ in biological materials is
D \simeq 1.3 \times 10^{-9} \text{ m}^2/\text{s} \ (\text{Fischkoff and Vanderkooi, 1975}) \text{ and in water } D \simeq 1.8 \times 10^{-9} \text{ m}^2/\text{s} \ (\text{Boynton and Brattain, 1929}). \text{ Using an average value of } D \text{ to include diffusion through both the algae and electrolyte, the diffusion time is calculated to be } \tau \sim 30 \text{ ms}. \text{ This diffusion time is manifested in the rise time of the } \text{O}_2 \text{ current pulse for each flash.}

The \text{O}_2 \text{ pulse for each flash indicates the rate at which } \text{O}_2 \text{ is reduced at the Pt cathode from the onset of a flash to the beginning of the next flash. Production of } \text{O}_2 \text{ by the algae increases the rate of } \text{O}_2 \text{ reduction at the cathode, while uptake of } \text{O}_2 \text{ by the algae removes } \text{O}_2 \text{ from the system, which results in a decrease in the rate of } \text{O}_2 \text{ reduction at the Pt electrode. This cathodic } \text{O}_2 \text{ reaction depends on the ambient } \text{O}_2 \text{ concentration in the sample holder (discussed in Sec. 5.3)). A series of three short (4 \mu s) light flashes given to an algal thallus produces the characteristic response of the system, with } \text{O}_2 \text{ produced on the third flash, as shown in Fig. 5.1. The } \text{O}_2 \text{ current pulse will decay back to the original baseline if no successive flash is given. The integrated curve is a measure of the } \text{O}_2 \text{ produced by the algae due to one complete cycle of the oxygen-evolving complex (i.e. } S \text{ state transitions from } S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow (S_4) \rightarrow S_0), \text{ with } \text{O}_2 \text{ evolved on the } S_3 \rightarrow (S_4) \rightarrow S_0 \text{ transition.}
Fig. 5.1 Reference curve for *Ulva* sp.

The $O_2$ reduction current pulse due to the third 4 μs light flash at 3.3 Hz flash frequency (the reference curve) represents the characteristic response of the electrode system. The decay of the pulse represents the decay of the system response to $O_2$ produced by *Ulva* by the third flash. The integrated curve gives a measure of the net amount of $O_2$ evolved as a result of three light flashes.
5.1.1 Variation of flash frequency

Flash frequencies, varying from 1-3.3 Hz for optimal response, have been used by different research groups (Kok et al., 1970; Forbush et al., 1971; Joliot et al., 1971; Diner, 1975). A series of experiments was performed with *Ulva* to determine the flash frequency for optimum O$_2$ exchange. The result of varying the flash frequency for *Ulva* is shown in Fig. 5.2. The same sample was used for each of the four flash frequencies in order to minimize sample variability. Between each series of flashes, the samples were dark-adapted for 10 min. A variation in flash frequency significantly altered the O$_2$ exchange pattern. Part of this change is due to the different total light energy received by the algal thallus during the same time interval. The small spikes on the curves are due to the response of the electrode to the light pulse and are constant in size in all experiments using the same flash intensity.

The O$_2$ exchange curve at 1.65 Hz flash frequency showed no O$_2$ produced on the first pulse, a small amount on the second (which can be attributed to double turnovers of the OECs on either the first or second flash), and the largest amount of O$_2$ on the third pulse. At 6.6 Hz, the individual O$_2$ pulses are absent, but a dip in the O$_2$ exchange curve is observed approximately 1.3 s after the first pulse. The initial slope of the O$_2$ current pulse resulting from O$_2$
Fig. 5.2 Oxygen exchange curves for *Ulva* at various flash frequencies

$O_2$ exchange measurements in air as a function of time for 10 min dark-adapted *Ulva* are given for flash frequencies of 1.65, 3.3, 6.6, and 13.2 Hz.
exchange in the algae caused by the third saturating flash at all flash frequencies is approximately the same, this indicates that the rise of the pulse is proportional to the amount of O₂ produced by the algae. The maximum slope of the rising O₂ signal for each flash is thus a measure of the change in O₂ exchange by the alga. Plotting the slopes of the rising O₂ curve for each pulse as a function of flash number will give the net O₂ yield per flash (the flash yield sequence).

At 3.3 Hz, the individual pulses in the O₂ exchange pattern were distinct, and the dark interval between flashes was short enough to minimize deactivation of the S states. This flash frequency is comparable to that used most often by other researchers (Joliot et al., 1971; Weiss et al., 1971; Diner, 1975; Zeinalov and Litvin, 1979; Schmid and Thibault, 1979). For these reasons, 3.3 Hz was used as the control.

5.1.2 Extrapolation of decay curves

Oxygen exchange (evolution and uptake) in Ulva as a function of flash number at 3.3 Hz flash frequency is shown in Fig. 5.3. At a flash frequency of 3.3 Hz, the rate of O₂ reduction at the cathode does not stabilize after each flash and the baseline steadily increases due to a "pile-up" of individual O₂ pulses. This occurs because the relaxation time of the system to a steady-state value is much longer than the
Fig. 5.3  Oxygen exchange for *Ulva* in air with 3.3 Hz flash frequency

O₂ exchange for 5 min dark-adapted *Ulva sp.* in seawater under atmospheric conditions during 4 μs saturating light flashes at 3.3 Hz (300 ms between flashes). The letters A, B, C, and D are used to calculate the flash yields as discussed in the text. The beginning of each 4 μs flash is labelled on the horizontal axis.
time between flashes. The pile-up of \( \text{O}_2 \) pulses at the bare Pt electrode is difficult to avoid during \( \text{O}_2 \) exchange measurements using light flashes since the flash frequency must be high enough (>1 Hz) to minimize decay of the \( S_2 \) and \( S_3 \) states during the dark interval between flashes (Kok et al., 1970; Joliot et al., 1971). To eliminate the effect of the pile-up of pulses, each \( \text{O}_2 \) pulse is corrected for the \( \text{O}_2 \) evolved from the previous flash. However, this correction does not account for \( \text{O}_2 \) evolved by the plant which is consumed in a PS I or PS II reaction without diffusing to the Pt cathode.

The linear four-step flash yield sequence of \( \text{O}_2 \) evolution (exchange) may be deduced by plotting the change in \( \text{O}_2 \) current due to each light pulse. For example, in Fig. 5.3, point C denotes the rate of \( \text{O}_2 \) being reduced at the cathode at the beginning of the fourth flash, whereas point D denotes the maximum rate of \( \text{O}_2 \) reduction at the cathode for this flash. The difference \( D - C = 0.49 \mu \text{A} \) is the change in \( \text{O}_2 \) current due to \( \text{O}_2 \) exchange on the fourth flash. However, it is preferable to extrapolate the actual maximum rate of \( \text{O}_2 \) reduction at the cathode for a given flash by correcting for the decay of the \( \text{O}_2 \) signal due to the preceding flash. For example, in Fig. 5.3, the decay of the \( \text{O}_2 \) signal after the third flash is approximated by the line CE, where point E is the extrapolated value of the residual \( \text{O}_2 \) from the third flash at the time of the maximum \( \text{O}_2 \) signal from the fourth
flash (point D). The difference $D - E$, which is $0.69 \mu A$ in Fig. 5.3, is the $O_2$ signal from the fourth flash after accounting for the residual $O_2$ from the third flash. This difference was calculated for each flash in the sequence and plotted as a function of flash number. The expanded $O_2$ exchange curve of Fig. 5.3 is shown in Fig. 5.4a, and the flash yield sequence for this curve is shown in Fig. 5.4b. Actual decay curves were taken for each flash in the sequence (as shown in Fig. 5.1 for the third flash) and in all cases, the linearly extrapolated decay for a given pulse (e.g. line CE) is the same to within $0.5\%$ as the measured decay to the point indicating residual $O_2$ for the following flash (e.g. point E). If the slopes of the linear part of each rising $O_2$ signal are plotted as a function of flash number as discussed previously, a similar four step pattern as shown in Fig. 5.4b is obtained. The integrated individual $O_2$ current pulses plotted as a function of flash number also give the same result. These flash yield sequences, however, represent $O_2$ exchange or net $O_2$ evolution, rather than actual $O_2$ evolution.

To facilitate comparison between our results and those of other researchers, all values of $O_2$ per flash were normalized to the difference $B - A = 0.90 \mu A$ for the third flash in Fig. 5.3. Flash yield sequences obtained with intact algae have been found to be notably more damped than those with chloroplasts (Lavorel, 1978), however, as can be seen in Fig. 5.5, the $O_2$ exchange data for *Ulva* show good agreement
Fig. 5.4 Oxygen exchange curve and the flash yield sequence at 3.3 Hz for dark-adapted Ulva

a. The O₂ exchange curve of Fig. 5.3 is expanded to show the pile-up of pulses.

b. The net O₂ yield per flash shows a damped oscillatory pattern with maxima at the third, eighth, twelfth, etc. flashes. This type of flash yield sequence is the basis for the four step model of O₂ evolution (Kok et al., 1970).
Fig. 5.5  Comparison of oxygen flash yield sequences for
dark-adapted samples under atmospheric conditions
as a result of saturating light flashes:

1) Our data from Fig. 5.3 for Ulva sp. using 4 μs
   flashes at 3.3 Hz (○—○○○○). 

2) Data of Joliot et al. (1971) for chloroplasts
   using 2 μs pulses 320 ms apart (●—●—●). 

3) Data of Forbush et al. (1971) for isolated
   spinach chloroplasts with 1 s between flashes
   (▲..▲). 

4) Data of Diner (1975) for Chlorella cells using
   4 μs pulses 320 ms apart (○-----○).
with that of Forbush et al. (1971) and Joliot et al. (1971) for chloroplasts, but are lower in comparison to that of Diner (1975) for *Chlorella*, a unicellular green algae. The flash yield sequence of Forbush et al. (1971) for spinach chloroplasts was obtained by using a flash frequency of 1 Hz, while the others used 3.1 Hz.

A similar, damped four-step oscillation was observed by Dekker et al. (1984b) by measuring UV flash-induced absorbance changes in dark-adapted PS II membranes. This oscillation has been attributed to the reduction of Mn(IV) ions in the oxygen-evolving complex (OEC) to Mn(III) during the 1 ms $S_3 \rightarrow (S_4) \rightarrow S_0$ transition. The oscillation in absorbance changes in Mn is independent of electrode/O$_2$ measurements and thus may reflect the actual O$_2$ evolution pattern (Dekker et al., 1984b). However, this method still does not separate the processes of O$_2$ uptake and O$_2$ evolution. Beck et al. (1984) reported that the OEC takes up O$_2$ and this may have some bearing on the Mn oxidation states. This absorbance change sequence gives a fit to the Kok model with initial distribution of $S_0 = 25\%$, $S_1 = 75\%$, and with 9% misses and 9% double hits on all transitions (Dekker et al., 1984b).
5.1.3 Fitting with the Simplex algorithm

If the bare platinum electrode is assumed to be a linear system, then the total output function due to a series of excitations is simply the sum of all individual O₂ current pulses, modified by their time decay. This appears to be a reasonable assumption since the O₂ diffusion time from the plant to the electrode is short compared to the decay time of the O₂ reduction current (~50 ms compared to 1.5-2.0 s), and the O₂ reduction rate at the cathode is most likely linearly proportional to O₂ concentration. Therefore, it should be possible to describe the waveform of the entire flash sequence as a superposition of the O₂ signals from each flash in the sequence.

To prove this hypothesis, different amplitudes of the oxygen current pulse for the reference curve (as shown in Fig. 5.1), were time-axis shifted and added together to give the best fit to experimental oxygen exchange measurements. The characteristic electrode response (the reference curve, shown in Fig. 5.1) was determined by giving three 5 µs saturating flashes to samples of dark-adapted Ulva. No O₂ was produced on the first and second flashes by the dark-adapted Ulva. The O₂ signal due to the third short, saturating flash was recorded until the signal had decayed back to the original baseline as seen in Fig. 5.1. Pulses having the same shape as this reference curve, but shifted along the time
axis by the time between flashes, were added together to obtain the best fit to the experimental $O_2$ exchange sequence. A comparison of the experimental $O_2$ exchange sequence, the best computer fit, and the reference curve are shown in Fig. 5.6.

The control $O_2$ exchange curve in Fig. 5.6 shows that similar amounts of $O_2$ are produced on both the third and fourth flashes. This control curve is different from the control curve shown in Fig. 5.3, which shows a larger amount of $O_2$ produced on the third flash than on the fourth flash (Fig. 5.3). These different control curves may be due to seasonal variations within the same species, or to two different species of Ulva. The control curve in Fig. 5.6 may indicate a high number of misses for the $S_3 \rightarrow S_4$ transition in Ulva, which results in a decrease in the $O_2$ yield for the third flash ($Y_3$) and an increase in $O_2$ yield for the fourth flash ($Y_4$).

The computer fit uses the Simplex algorithm, thus it is difficult to use statistical theories to compute the error (Caceci and Cacheris, 1984). For this reason, the sum of squares of the vertical distances (in number of points) for one horizontal point in ten was used to determine maximum error (discussed in Sec. 3.4). As can be seen from Table 5.1, the largest error for fitting any of the pulses was 7.25% for the fifth pulse. Most error values were in the range of 3-5%.

The computer fit determines the maximum rate of oxygen
Fig. 5.6 Oxygen exchange curve and fit for *Ulva*

$O_2$ exchange in *Ulva* (upper trace ———) using a bare Pt electrode is shown to be sum of multiples of the reference curve (lower trace ———). The fitted curve is given by the dashed line (-----).
Table 5.1 Relative Amplitude, Position, and Maximum Error for each Flash Determined by Computer Fitting of the Experimental Oxygen Exchange Curve

The relative amplitude with respect to the reference pulse and the maximum error is given for each O₂ pulse in the flash sequence. Net O₂ produced per pulse can be obtained by multiplying the amount of O₂ produced during the reference response by the relative amplitude of each pulse.

<table>
<thead>
<tr>
<th>FLASH NO.</th>
<th>ELAPSED TIME (s)</th>
<th>RELATIVE AMPLITUDE</th>
<th>ERROR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.031508</td>
<td>0.0031660</td>
<td>----</td>
</tr>
<tr>
<td>2</td>
<td>0.26782</td>
<td>0.0062441</td>
<td>----</td>
</tr>
<tr>
<td>3</td>
<td>0.57239</td>
<td>0.98370</td>
<td>3.10</td>
</tr>
<tr>
<td>4</td>
<td>0.87696</td>
<td>0.99156</td>
<td>2.51</td>
</tr>
<tr>
<td>5</td>
<td>1.1815</td>
<td>0.68305</td>
<td>7.25</td>
</tr>
<tr>
<td>6</td>
<td>1.4861</td>
<td>0.36928</td>
<td>5.42</td>
</tr>
<tr>
<td>7</td>
<td>1.7907</td>
<td>0.53418</td>
<td>3.61</td>
</tr>
<tr>
<td>8</td>
<td>2.0953</td>
<td>0.71800</td>
<td>3.29</td>
</tr>
<tr>
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</tr>
<tr>
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<td>3.99</td>
</tr>
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</tr>
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</tr>
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<td>15</td>
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</tr>
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<td>4.5266</td>
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<td>4.77</td>
</tr>
<tr>
<td>17</td>
<td>4.8312</td>
<td>0.68153</td>
<td>5.18</td>
</tr>
</tbody>
</table>
reduction at the cathode for each flash, relative to the reference curve, and is also shown in Table 5.1. Plotting the relative amplitudes (Table 5.1) as a function of flash number gives the flash yield sequence for the computer-generated oxygen exchange curve. Figure 5.7 compares the amplitudes of the computer-generated curve to the flash yield sequence obtained by plotting the change in the $O_2$ reduction rate for each flash and extrapolating (i.e. to point E in Fig. 5.3 for the fourth flash) to account for residual $O_2$ from the previous flash as discussed in Sec. 5.1.1.

The computer-generated flash yield sequence (Fig. 5.7) shows essentially no difference, within the fitting error, in the amount of oxygen produced during the third and fourth flashes. The ratio of $O_2$ yields for the third and fourth flashes ($Y_3$ and $Y_4$, respectively) gives a value of $Y_3/Y_4 = 0.9920$. Extrapolating the decay of the $O_2$ current pulse to account for residual $O_2$ shows only slightly more $O_2$ produced on the third pulse than on the fourth pulse, with $Y_3/Y_4 = 1.053$. The variation between the values of $Y_3/Y_4$ for both methods of determining the $O_2$ yield is within experimental error. Thus $Y_3/Y_4$ is ~1 for this control $O_2$ exchange curve.

The first two oscillations in yield are similar for both flash yield sequences in Fig. 5.6. However, the third oscillation in yield for the computer-fitted curve is much larger than was previously obtained by extrapolating the decay of the
Fig. 5.7 Oxygen exchange and evolution flash yield sequences for *Ulva*.

Using the two methods of determining the net $O_2$ yield, the $O_2$ yield as a function of flash number is plotted for the $O_2$ exchange curve of Fig. 5.6:

- Extrapolating the decay of the previous pulse to the intersection of a vertical line with the maximum reduction rate for the given pulse.
- The computer fit.
current pulses. This could be due to an increased error in the fitting at the minima and maxima of the O₂ exchange curve after the twelfth flash (t > 3.2 s) as can be seen in Fig. 5.6 or may indicate that the O₂ yield for Ulva may not be damped to a steady-state value as quickly as was determined previously.

The net amount of O₂ evolved per flash can be calculated from the relative amplitudes determined during the computer fitting if the amount of O₂ produced by the third flash can be calculated. The O₂ yield due to the third flash (the reference curve) was integrated to give the net charge produced by O₂ reduction at the cathode. This calculation does not account for a flash-induced O₂ uptake which has been shown to occur (Weiss and Sauer, 1970; Schmid and Thibault, 1979; Jursinic, 1980; Swenson et al., 1986), but will only enable determination of net O₂ produced per flash. Integration of the reference curve (Fig. 5.1) gives a net charge of 4.15 × 10⁻⁷ Coulombs.

Using 4 as the number of electrons which result from the reduction of O₂ at the cathode (as discussed in Sec. 3.2.2), the amount of O₂ produced by Ulva during the reference response (the third flash), is then calculated to be 1.1 × 10⁻¹² moles. The net amount of O₂ produced per flash can then be determined by multiplying this value by the relative amplitudes given in Table 5.1. This method of quantifying the O₂ reduction during a series of short saturating flashes takes into account the pile-up of pulses which occur during O₂
This information can also be used to determine the photosynthetic unit (PSU) size for \textit{Ulva}. The chlorophyll content of the sample was determined to be 4.2 \(\mu\)g chl, or \(2.8 \times 10^{15}\) chlorophyll molecules \((5 \times 10^{-9}\) mole\). From Table 5.1, the relative amplitudes for \(O_2\) produced on the first four flashes are 0.0031660, 0.0062441, 0.98370, and 0.99156. Multiplying these amplitudes by the amount of \(O_2\) produced during the reference response \((1.1 \times 10^{-12}\) moles\) gives the number of moles \((\text{molecules})\) of \(O_2\) produced by a complete cycle of the OEC (which includes OECs in both the \(S_0\) or \(S_1\) states prior to the series of light flashes). Adding the amounts of \(O_2\) produced during the first four light flashes gives a value of \(2.2 \times 10^{-12}\) mole, or \(1.3 \times 10^{12}\) molecules. Thus, \(2.8 \times 10^{15}\) chlorophyll molecules cooperate to produce \(1.3 \times 10^{12}\) \(O_2\) molecules. This is equivalent to a PSU size of 2290 \(\pm\) 150 chlorophyll molecules, close to the value of 2360 \(\pm\) 160 chl/\(O_2\) calculated for \textit{Ulva lactuca} by Mishkind and Mauzerall (1977).
5.2 Separation of Oxygen Uptake and Evolution Components

5.2.1 Flash Illumination

The magnitude of the uptake component of $O_2$ exchange was determined by inhibiting photosynthetic water-splitting by DCMU, which prevents the oxidation of $Q_A$ (the first stable electron acceptor in PS II) by electron transport. Addition of DCMU produced a large $O_2$ uptake as shown in Fig. 5.8 for varying flash frequencies. The $O_2$ uptake occurs either during the $\mu$s flash or in the subsequent dark interval and was larger for higher flash frequencies. As seen in Fig. 5.8, a flash frequency of 6.6 Hz resulted in a shift in the onset of $O_2$ uptake to earlier times and also produced a larger uptake than at lower flash frequencies. The uptake at 3.3 and 6.6 Hz reached its maximum rate in less than 2 s after the first flash, which coincides with the positions of the fifth and sixth flashes at 3.3 Hz, and the ninth and tenth flashes at 6.6 Hz. Since the amplitude of the uptake is approximately inversely proportional to flash frequency, and thus proportional to the total light intensity, this uptake may reflect the oxidation in the light of a limited pool of reductants, most likely plastoquinols and other intersystem carriers, by PS I.

Antimycin A was added to seawater with samples of Ulva to inhibit the mitochondrial respiratory chain and determine if
Fig. 5.8 Oxygen uptake in air for DCMU-treated *Ulva* with varying flash frequencies.

The DCMU-mediated $O_2$ uptake in *Ulva* appears to be dependent on total light intensity since the uptake is larger at higher flash frequencies.
this uptake could be due to respiration. Addition of Anti-
mycin A also inhibited O₂ evolution, and thus it is not
possible to eliminate mitochondrial respiration. However, any
variation in mitochondrial respiration is much longer than 5 s
(Jackson and Volk, 1970) and this suggests that the O₂
uptake is not due to respiration.

The results of the DCMU experiments (Fig. 5.8) suggest
that the first minimum in the O₂ flash yield in Fig. 5.4 is
not only due to a four-step O₂ evolution, but contains a
superimposed O₂ uptake component. The light-driven O₂
uptake which is not inhibited by DCMU has been associated with
PS I activity (Vidaver and French, 1965; Satoh et al., 1976).
This well-known O₂ uptake transient is generally masked by
high rates of O₂ evolution.

Oxygen which is taken up by the algae concurrently with
O₂ evolution may not be detected at the Pt electrode if the
magnitude of the uptake is less than that of the O₂ evolved
and is not sufficient to alter the shape of the signal. If
the only effect of an O₂ uptake is to reduce the amount of
O₂ detected at the Pt cathode, plotting the O₂ yield as a
function of flash number cannot distinguish O₂ exchange from
actual O₂ evolution by water-splitting. Since a light-
dependent O₂ uptake occurs, some of the features of the
flash yield sequence could be associated with the O₂ uptake
as well as some of the reaction centers receiving misses or
double hits. Although the occurrence of misses and double
hits in the PS II OECs is well-known, the existence of an O₂ uptake should be incorporated into the S state theory.

The O₂ uptake in Fig. 5.8 is clearly a PS I photo-reaction since O₂ uptake associated with PS II would be suppressed by DCMU. Oxygen uptake during flash illumination has been shown to exist in tobacco chloroplasts (Schmid and Thibault, 1979) and was greatly enhanced by the presence of an exogenous electron acceptor such as p-benzoquinone or ferri-cyanide. Since Schmid and Thibault (1979) did not present a quantitative estimate of the magnitude of the O₂ uptake, their results are not directly comparable to the uptake shown in Fig. 5.8.

Because of the magnitude of this DCMU-mediated O₂ uptake, it seems reasonable that an analysis of the O₂ exchange curve in terms of the S state hypothesis can not be concerned solely with O₂ evolution, but must consider O₂ uptake as well. Oxygen consumption has been shown to occur in both PS I and PS II (Vidaver and French, 1965; Beck et al., 1985), and thus it is unlikely that the O₂ uptake will be exactly the same with PS II active or inhibited. As well, flash yield sequences obtained with PS II membranes containing exogenous electron acceptors may not be equivalent to O₂ evolution which occurs when both photosystems are functional. However, PS I O₂ uptake is most likely larger than that of PS II, thus a first approximation to actual O₂ evolution would determine the magnitude of the O₂ uptake and "correct"
for this effect by adding it to the $O_2$ exchange curve. The corrected $O_2$ evolution curve is shown in Fig. 5.9a together with the $O_2$ exchange curve of Fig. 5.4 for comparison. The $O_2$ evolution curve can then be plotted in terms of the $O_2$ yield as a function of flash number as described for Fig. 5.3. Figure 5.9b compares the $O_2$ exchange flash yield sequence of Fig. 5.4 with the corrected $O_2$ evolution curve. The corrected $O_2$ evolution flash yield is greater than that of the measured $O_2$ exchange, suggesting the actual rates of $O_2$ evolution may be greater than measured $O_2$ exchange rates. The damping which is evident in the $O_2$ exchange flash yield sequences (Figs. 5.4, 5.5, and 5.9) is not present in the $O_2$ evolution flash yield sequence. Removal of the DCMU-induced PS I $O_2$ uptake component from the measured $O_2$ exchange results in suppression of the damping in the $O_2$ yield without invoking the miss and double hit parameters.

A flash yield sequence with deep sustained oscillations, similar to those in Fig. 5.4b but lower than the one in Fig. 5.9b, was obtained by Kok and Velthuys (1976), who added oxidized benzoquinone to *Anacystis* cells (cyanobacteria) to inhibit respiratory $O_2$ uptake. Their results clearly indicate that the flash yield sequence can be altered by a process which inhibits $O_2$ uptake. Thus if the $O_2$ exchange curve of Fig. 5.4 contains a significant $O_2$ uptake component, then in terms of an $S$ state model, with or without misses and double hits, the calculated initial distribution of $S$ states
Fig. 5.9 Measured oxygen exchange in *Ulua* compared to corrected oxygen evolution

a) Comparison of Measured $O_2$ exchange in air (-----) and corrected $O_2$ evolution (.....) as a function of flash number for 10 min dark-adapted *Ulua*.

b) Comparison of measured $O_2$ exchange (●——●) and corrected $O_2$ evolution (o.....o) flash yield sequences for the above curves.
will be different for \( \text{O}_2 \) evolution and \( \text{O}_2 \) exchange (evolution and uptake).

Using the experimental \( \text{O}_2 \) exchange curve and the corrected \( \text{O}_2 \) evolution curve of Fig. 5.9a, the area under the curve to the baseline for each of the first four flashes will give a measure of the initial \( S \) state distribution \( (S_0(0), S_1(0), S_2(0), S_3(0)) \). Since \( \text{O}_2 \) is observed on the second pulse for fresh dark-adapted algae, consistent with the findings of Jursinic (1981), the area under the curve of the second pulse, to the beginning of the third pulse, is interpreted as a measure of the number of \( S_1 \) states in the dark (i.e. \( S_1(0) \)) which undergo a double hit on either the first or second flash. No \( \text{O}_2 \) was evolved on the first pulse, indicating that \( S_2(0) = 0 \). The miss factor can be determined by monitoring the \( \text{O}_2 \) yield for the third flash as a function of light intensity. Many researchers consider that there is an equal probability of misses for each of the \( S \) state transitions (Thibault, 1978; Jursinic, 1981; Wydrzynski, 1982), but Delrieu (1980, 1983, 1984) has shown that the \( S_2' \rightarrow S_3 \) transition has the greatest probability of misses. Calculating the area under the curve for both the third and fourth pulses, to the beginning of the fourth and fifth pulses respectively, and taking into account the miss and double hit factors, the initial \( S \) state distribution for both measured \( \text{O}_2 \) exchange and corrected \( \text{O}_2 \) evolution was determined to be 34\% \( S_0(0) \) and 66\% \( S_1(0) \) for \( \text{O}_2 \) exchange and 38\% \( S_0(0) \) and 62\% \( S_1(0) \) for
corrected $O_2$ evolution. There is considerable variation in published initial $S$ state distributions calculated for both algae and chloroplasts. Our initial $S$ state distribution for *Ulva* is most comparable to that of another green algae, *Chlorella*, also under xenon flash illumination, where $S_0^{(0)}$ and $S_1^{(0)}$ were found to be 33% and 67% respectively (Juršin, 1981). For all of the above cases, $S_2^{(0)}$ and $S_3^{(0)}$ were zero.

5.2.2 Continuous Illumination

Many attempts have been made to interpret photosynthetic induction phenomena which are manifest as changes in the $O_2$ exchange rates in dark-adapted algae and higher plants upon illumination (Blinks and Skow, 1938; Hill and Wittingham, 1953; Brown and Good, 1955; Vidaver and French, 1965; Chandler and Vidaver, 1970; Schmid and Thibault, 1979). The induction transients are produced by the competing processes of evolution and uptake. The light-induced $O_2$ uptake transient during continuous illumination has been well-documented as discussed in Sec. 2.5., and is shown in Fig. 5.10 using DCMU to inhibit PS II $O_2$ evolution. The DCMU-induced $O_2$ uptake is compared with the corresponding $O_2$ exchange curve for a sample cut from the same algal thallus.

Since the $O_2$ exchange curve represents the sum of $O_2$
Fig. 5.10  Oxygen exchange and oxygen uptake in Ulva under continuous illumination

a. The $O_2$ exchange for Ulva under continuous illumination is composed of both evolution and uptake components. The magnitude of the PS I $O_2$ uptake in comparison to total $O_2$ exchange can be seen from this figure.

b. The DCMU-induced $O_2$ uptake is shown for continuous illumination.

Arrows indicate when the light was turned on (↑) and off (↓). The baseline is shown as output from the signal averager. This may be arbitrarily shifted to zero.
uptake and \( \text{O}_2 \) evolution, subtracting the DCMU-mediated \( \text{O}_2 \) uptake (PS I) from the measured \( \text{O}_2 \) exchange should yield the corrected \( \text{O}_2 \) evolution curve (discussed for flash illumination in Sec. 5.2.1). The corrected \( \text{O}_2 \) evolution curve is shown in Fig. 5.11, together with the \( \text{O}_2 \) exchange curve and the inverse of the uptake curve from Fig. 5.11. Variation in the \( \text{O}_2 \) evolution curve occurred at the point labelled A in Fig. 5.11; in some cases, a rapid increase in \( \text{O}_2 \) evolution at the onset of illumination would taper off, decrease, and then increase to the steady-state value, in other cases, there was a sharp decrease followed by a steady increase to a steady-state value of \( \text{O}_2 \) evolution.

The approximation of actual \( \text{O}_2 \) evolution is affected by the same considerations that apply to the approximation of \( \text{O}_2 \) evolution by flash illumination. The \( \text{O}_2 \) uptake shown in Fig. 5.10 is solely a PS I reaction, since PS II is inhibited, and it is unlikely that the corrected \( \text{O}_2 \) evolution will be the same with PS II active or inhibited. It is interesting to speculate, though, that the "anomaly" shown in Fig. 5.11 at Point A represents the PS II uptake by the OEC reported by Beck et al. (1985), which is not corrected for by subtracting the PS I \( \text{O}_2 \) uptake.
Fig. 5.11 Oxygen exchange, uptake, and evolution for Ulva under continuous illumination.

An approximation to actual O₂ evolution in Ulva can be obtained by correcting the O₂ exchange curve to account for the PS I O₂ uptake shown in Fig. 5.10. The significance of point A is discussed in the text.
O$_2$ YIELD (arbitrary units)

O$_2$ EVOLUTION

O$_2$ EXCHANGE

O$_2$ UPTAKE

TIME (s)
5.3 Variation of Oxygen Concentration

5.3.1 Effect of Ambient Oxygen Concentration on Oxygen Exchange

In order to determine the effect of ambient \( O_2 \) concentration on \( O_2 \) exchange, the sample holder was flushed with different partial pressures of \( O_2 \), with the remaining pressure due to \( N_2 \) (discussed in Sec. 3.3.4). Carbon dioxide was added to the pure gases in order to ensure that \( CO_2 \) concentration was not limiting and that any variations in \( O_2 \) exchange were due to variations in \( O_2 \) concentration, rather than \( CO_2 \). This was done using both continuous and flash illumination.

The \( O_2 \) exchange curves for *Ulva* with flash illumination (3.3 Hz) are shown in Fig. 5.12. The control curve in Fig. 5.12, obtained under atmospheric conditions both in air and at 20% \( O_2 \), gave identical \( O_2 \) exchange curves. A variation in the \( O_2 \) concentration from 17% to 24% showed little difference in the resultant \( O_2 \) exchange curves from those in air.

The \( O_2 \) exchange curve at 100% \( O_2 \) (1 atm. \( O_2 \) pressure) showed a similar pattern to the control curve at 20% \( O_2 \) (1 atm. total pressure), but with a decreased net \( O_2 \) evolution. Oxygen partial pressures over 0.85 atm. gave similar results. At high \( O_2 \) partial pressures, the minimum in the \( O_2 \) exchange curve at the seventh flash is still present.
Fig. 5.12  Oxygen exchange flash yields in *Ulva* as a function of ambient oxygen concentration.

O₂ exchange in 1.5% and 100% O₂ at 1 atmosphere is compared with O₂ exchange in air (20% O₂). All samples were dark adapted for 10 min and illuminated with xenon flashes (4 µs FWHM) at 3.3 Hz.
however successive minima are not apparent. The slope of the initial rise of each individual $O_2$ pulse for each flash is not as steep as with 20% $O_2$, and the individual $O_2$ current pulses are less distinct. This observation may be due to a saturation effect at the Pt cathode, resulting in a decrease in the $O_2$ reduction rate. This could also be due to an increased $O_2$ uptake, since an increase in ambient $O_2$ partial pressures resulted in an increase in $O_2$ uptake, which could be due to direct photoreduction of $O_2$. The $O_2$ exchange curve with 1 atm $O_2$ is most likely affected by both saturation of the Pt electrode and an increase in $O_2$ uptake by the algae.

Decreasing the amount of $O_2$ in the sample cell to 1.5% $O_2$ gave a different $O_2$ exchange curve than did 20% $O_2$ as shown in Fig. 5.12. No $O_2$ was evolved on either the first or the second flash, and less $O_2$ was detected on the third flash than on the fourth, in contrast to the results obtained at 20% or 100% $O_2$. The results in Fig. 5.12 indicate that variations in ambient $O_2$ concentration affect the measured rate of $O_2$ exchange. Plotting the peak heights as a function of flash number does not yield a four step pattern of net $O_2$ evolution as shown in Fig. 5.13. After each flash, the rate of $O_2$ reduction at the cathode decreases almost to the original baseline. The later flashes in the sequence yield a higher cathodic $O_2$ reaction rate than the third flash, which is usually larger at higher $O_2$ concentrations.

The lack of a four step pattern of $O_2$ exchange under low
Fig. 5.13 Oxygen exchange flash yield sequences for *Ulva* at 1.5% O$_2$ and under anaerobic conditions

A comparison of the O$_2$ exchange flash yield sequence between low ambient O$_2$ partial pressure (1.5% O$_2$ at 1 atm) (●— — — ●) and under anaerobic conditions (▲— — — ▲) is shown. At low O$_2$ concentrations, the four step pattern is not evident.
ambient $O_2$ concentrations may be due to variations in either the $O_2$ evolution or uptake kinetics, or both. Several explanations have been postulated to explain the altered $O_2$ exchange curves under anaerobic conditions and these are also applicable to $O_2$ exchange under low $O_2$ partial pressures. These explanations will be discussed in the following section.

5.3.2 Effect of Anaerobiosis on Ulva

For many years, there has been considerable debate on the effects of anaerobiosis on photosynthetic water-splitting in the measured rate of $O_2$ exchange (Rabinowitch, 1956). The presence of environmental $O_2$ is believed to be essential (Burk and Warburg, 1951; Warburg, 1952; Vidaver et al., 1984) or not essential (Franck, 1953; Allen and Franck, 1955) for $O_2$ evolution. It is widely accepted that lack of $O_2$ inhibits the OEC, but the sites and extent of the inhibition are still not certain.

Part of the controversy appears to result from the difficulty of obtaining a completely anaerobic environment. Different techniques have been used to deplete the system of $O_2$; some of these techniques may result in an irreversible inhibition of $O_2$ which is not directly due to anaerobiosis. Many of the experiments were most likely not carried out in anaerobic environments, but rather under conditions of low $O_2$
partial pressures. The sensitivity of the system is of paramount importance in order to detect minute quantities of $O_2$, on the order of $10^{-15}$-$10^{-16}$ moles.

Some of the earliest experiments on the effect of anaerobiosis on $O_2$ exchange measurements showed that green algae (Chlorella and Scenedesmus) which were illuminated in an anaerobic environment still evolved small quantities of $O_2$ (Franck et al., 1945). This inhibition of $O_2$ evolution has been attributed to the slow metabolic production of poison(s) (Franck et al., 1945), the requirement of $O_2$ for water-splitting (Burk and Warburg, 1951; Warburg, 1952), or reduction of the plastoquinone (PQ) pool (Diner, 1975, 1977).

Diner (1974) has suggested that Chlorella cells placed in an anaerobic environment (argon containing 5 ppm $O_2$) undergo an immediate reduction of the plastoquinone (PQ) pool in the dark. The redox state of the OEC is dependent on the oxidation state of the PQ pool. Under decreasing $O_2$ concentrations, the PQ pool becomes more reduced (Diner and Mauzerall, 1973a). The increasing reduction of the PQ pool with decreasing $O_2$ concentration is due to a balance between the pool reduction by an endogenous reductant and its oxidation by $O_2$ (Diner and Mauzerall, 1973a).

Under anaerobic conditions, the reduction of the PQ pool leads to a transfer of the rate-limiting step for a single turn-over of the OEC to the acceptor side of PS II. Diner (1975) has suggested that the OEC exists in the following
redox state in response to the first light flash:

\[
S_1Q_AQ_B^{2-} \xrightarrow{\text{hv}} S_2Q_AQ_B^{2-} \xleftrightarrow{\text{hv}} S_2Q_AQ_B^{-} \xrightarrow{\text{hv}} S_2Q_AQ_B^{2-} \quad 5.1
\]

where the symbols are defined as in Sec. 2.1. The OECs in the dark are thus almost exclusively in the \( S_1Q_AQ_B^{2-} \) state. Following one light flash, the OECs exist in the \( S_2Q_AQ_B^{2-} \) state and cannot undergo another photoreaction until \( Q_A \) is reoxidized. The reoxidation of \( Q_A \) requires two steps, the transfer of two electrons from \( Q_B^{2-} \) to \( PQ \), and the transfer of one electron from \( Q_A^{-} \) to \( Q_B \). If the \( PQ \) pool is completely reduced, the transfer from \( Q_B^{2-} \) to \( PQ \) cannot occur until the \( PQ \) pool is reoxidized. Following the second and third flashes the following transitions are believed to occur (Diner, 1975):

\[
S_2Q_AQ_B^{-} \xrightarrow{\text{hv}} S_3Q_AQ_B^{-} \xleftrightarrow{\text{hv}} S_3Q_AQ_B^{2-} \xrightarrow{\text{hv}} S_0Q_AQ_B^{2-} \quad 5.2
\]

Under anaerobic conditions, if most OECs are in the \( Q_AQ_B^{2-} \) state, then the \( S_2' \rightarrow S_3 \) transition is independent of the redox state of the \( PQ \) pool and thus should be accelerated, which was found to be the case in *Chlorella* (Diner, 1975).
The lowered net O\textsubscript{2} evolution in \textit{Ulva} at low O\textsubscript{2} partial pressures (Fig. 5.12) is partially due to the effect of anaerobiosis, which results in reduction of the PQ pool in the dark. The PQ pool reduction causes an increase in the "miss" parameter, i.e. the number of OECs which do not have a single turnover in response to a light flash, and thus results in a decrease in the net O\textsubscript{2} yield.

The O\textsubscript{2} exchange curve under anaerobic conditions (Fig. 5.14) also shows a decrease in net O\textsubscript{2} yield. The net O\textsubscript{2} yield, plotted as a function of flash number, does not fit the four step S state model (Fig. 5.13). This may be due to a higher rate of misses on some of the S state transitions, reduction of the PQ pool, or possibly be due to an autocatalytic effect of O\textsubscript{2} on the OEC.

Illumination of \textit{Ulva} under anaerobic conditions does not allow elimination of the O\textsubscript{2} uptake component. Any O\textsubscript{2} which has been evolved by the algae, may also be consumed. However, at least the O\textsubscript{2} pulse for the first flash should represent O\textsubscript{2} evolution. The Simplex algorithm was not used to fit this O\textsubscript{2} evolution curve, since the anaerobic flash yield experiments preceded development of the fitting technique.

Diner and Mauzerall (1973a) determined that continuous far red light superimposed on 0.25 Hz red light flashes increased the O\textsubscript{2} yield under anaerobic conditions by 7-fold over the O\textsubscript{2} yield without background illumination. The increased O\textsubscript{2} yield was attributed to oxidation of electron carriers on
Fig. 5.14 Oxygen exchange for *Ulva* under anaerobic conditions with flash illumination

The $O_2$ exchange curve is shown for *Ulva* which has been dark-adapted for 2 h under anaerobic conditions prior to a series of 3.3 Hz xenon light flashes.
the acceptor side of PS II by far red light.

Since far red light apparently oxidizes electron carriers (e.g. O$_B$ and the PQ pool) that are reduced in an anaerobic environment, it is possible that far red light flashes given to algae in the absence of O$_2$ will alter the anaerobic inhibition of O$_2$ exchange. The control O$_2$ exchange curve (data not shown) in air showed smaller O$_2$ pulses for the third and fourth flashes in the sequence than with the xenon flash, however the O$_2$ yield per pulse gradually increased with increasing flash number. The oscillations in O$_2$ which are evident with white light or red light flashes (680 nm) were greatly diminished with far red light flashes. This decrease in net O$_2$ evolution is most likely due to a higher rate of misses on all S state transitions, which indicates that the far red light flashes are not intense enough to saturate the PS II reaction centers. A series of far red light flashes, given to 2 h dark-adapted and anaerobic Ulva, showed the same result as the control, but the O$_2$ pulses were not visible until the fourth flash. This observation supports the anaerobic inhibition of O$_2$ evolution, but is not conclusive since the light intensity effect cannot be eliminated.
5.3.3 Oxygen Requirement for Water-Splitting and Concomitant Oxygen Evolution

The dependence of \( O_2 \) evolution on the presence or absence of \( O_2 \) has been widely debated. Zeinalov and Litvin (1979) suggested that \( O_2 \) may be bound to the S states. Beck et al. (1985) have shown that the resting PS II OEC takes up \( O_2 \). Vidaver et al. (1984) have suggested, on the basis of absorbance change measurements under anaerobic conditions, that the functioning of the water-splitting system requires the participation of \( \leq 0.01 \text{ atm.} \ O_2 \).

Oxygen exchange measurements were performed over a period of three years to determine if the water-splitting system could be reversibly and completely inhibited by the absence of \( O_2 \), as measured by \( O_2 \) exchange. These experiments were performed over a long time period to eliminate any seasonal variations in overall \( O_2 \) exchange that occur within a species. The discussion in Sec. 5.3.2 supported a reversible inhibition of \( O_2 \) evolution, but did not explore the possibility that the environment in the sample holder was not completely anaerobic. For *Chlorella*, Kessler (1973) observed that \( 20 \text{ h} \) of dark inactivation were required for maximum inhibition of \( O_2 \) evolution. However, for *Ulva*, \( 6 \text{ h} \) was the longest time that anaerobic conditions could be maintained and still obtain recovery of \( O_2 \) evolution when air was added to the system.

The sample holder was made anaerobic by flushing the
sample holder with $N_2$ (containing $CO_2$) for periods of time up to 6 h. The first results (Fig. 5.15) appeared to indicate that a complete, reversible inhibition occurred. However, the measuring system was redesigned (Fig. 3.5) to allow approximately a ten-fold amplification of the signal, and the experiments were repeated. Using two different methods for obtaining an aerobic environments, flushing with $N_2$ and pumping air through pyrogallol (Fig. 3.3 and 3.4, respectively), $O_2$ evolution was always observed, even after 6 h anerobic exposure (in the dark) as shown in Fig. 5.16. This seems to indicate, at least for Ulva, that ambient $O_2$ is not required for water-splitting to occur. However, the possibility that $O_2$ may be bound to the $S$ states, and released at the onset of illumination to catalyze water-splitting cannot be excluded.
Fig. 5.15 Oxygen exchange for *Ulva* under aerobic and anaerobic conditions with continuous illumination.

The O\textsubscript{2} exchange curve is shown for *Ulva* which was dark-adapted in air for 1 h as compared to *Ulva* which was dark-adapted for 1 h under anaerobic conditions prior to a continuous illumination. After anaerobiosis, air was let in to the sample holder and the sample was dark-adapted for 10 min. The recovery curve is shown in the upper right corner of the figure.

Arrows indicate when the the light was turned on (↑) or off (↓).
Fig. 5.16 Oxygen exchange for Ulva under aerobic and 6 h anaerobic conditions with continuous illumination.

The $O_2$ exchange curve is shown for Ulva which was dark-adapted in air for 20 min as compared to Ulva which was dark-adapted for 6 h under anaerobic conditions prior to a continuous illumination. After anaerobiosis, air was let in to the sample holder and the sample recovery was similar to that shown in Fig. 5.15.

Arrows indicate light on (↑) or off (↓).
CHAPTER 6 SUMMARY AND CONCLUSIONS

Kinetic studies of \( \text{O}_2 \) exchange (evolution and uptake) in isolated chloroplasts and plants have contributed greatly to the knowledge of photosynthetic processes. Oxygen evolution occurs in the thylakoid membranes of chloroplasts as a result of water-splitting in photosystem II, while \( \text{O}_2 \) uptake occurs through both PS I and PS II photoreactions. In marine algae, \( \text{O}_2 \) uptake in the light occurs primarily through direct photoreduction of \( \text{O}_2 \) by photosynthetic electron carriers (the Mehler reactions) (Mehler 1951a,b; Radmer and Kok, 1976). Mitochondrial respiration in both the light and dark also contributes to \( \text{O}_2 \) consumption, although respiration in the light occurs on a longer time scale than do the Mehler reactions (Jackson and Volk, 1970). Photorespiration does not appear to play a large role (if any) in light-induced \( \text{O}_2 \) consumption in marine algae (Glidewell and Raven, 1976; Shelp and Canvin, 1980; Osmond, 1981; Brechignac and Andre, 1984; Peltier and Thibault, 1985a,b).

Several major problems still remain to be resolved in the area of photosynthetic water oxidation, including isolation of the oxygen-evolving complex (OEC) and delineation of its arrangement in the thylakoid membrane, elucidation of the molecular components of the OEC and definition of their specific biochemical functions, and determination of the mechanism and molecular intermediates (e.g. plastoquinone, Mn complexes,
of water oxidation and concomitant \( \text{O}_2 \) evolution. The consumption of \( \text{O}_2 \) by various processes in photosynthetic organisms complicates the interpretation of \( \text{O}_2 \) exchange measurements which are aimed at understanding the mechanism of water oxidation. Many researchers have attempted to isolate the \( \text{O}_2 \) evolution and uptake components from the measured rates of \( \text{O}_2 \) exchange, but without much success. Although most research in the past few years has focussed on isolating the molecular components of the OEC, and on the process of water oxidation itself, the ability to distinguish \( \text{O}_2 \) uptake from \( \text{O}_2 \) evolution in \( \text{O}_2 \) exchange measurements would greatly advance the understanding of the kinetics of both processes.

Both water oxidation and the intrinsically energy-wasteful process of direct photoreduction of \( \text{O}_2 \) by electron transport carriers (which diverts electrons from the final PS I electron acceptor, NADP\(^+\)) are important to the formation and regulation of NADPH and ATP which are used to power the dark reactions of \( \text{CO}_2 \) fixation.

Different genera of marine algae in the divisions Chlorophyta, Phaeophyta, and Rhodophyta were used to study the flash yield kinetics of photosynthetic \( \text{O}_2 \) uptake and evolution. A "pile-up" of \( \text{O}_2 \) current pulses was observed in all genera of marine algae which were studied under conditions where the time interval between the flashes was shorter than the decay time of the \( \text{O}_2 \) pulse. This pile-up of pulses occurred since the flash frequency had to be chosen to allow a continuous
cycling of the oxidation states of the oxygen-evolving complex (OEC) with minimal deactivation of the S states.

The response of a bare platinum electrode to $\text{O}_2$ exchange in marine algae (and more generally to any plant sample in an electrolyte medium) is characteristic of the electrode system. The reference response of the electrode/algae system is given by the current pulse due to the third saturating microsecond light flash for dark-adapted algae, including decay of the pulse to the original baseline. The amount of $\text{O}_2$ produced by the algae during the reference response is determined by calculating the area under the curve, which represents the total charge produced by reduction of $\text{O}_2$ at the cathode. Since four electrons are produced for each molecule of $\text{O}_2$ reduced (i.e., $n = 4$ electrons/$\text{O}_2$), the net $\text{O}_2$ evolved by photosynthetic water oxidation is given by:

$$\text{Net } \text{O}_2 \text{ (moles)} = \frac{Q_{\text{O}_2}}{4q_eN_A}$$

where $Q_{\text{O}_2}$ is the area under the $\text{O}_2$ curve (in Coulombs), $q_e$ is the electronic charge, and $N_A$ is Avogadro's number. For the area under the $\text{O}_2$ exchange curve to be expressed in Coulombs, the vertical and horizontal axes must be expressed in terms of $\text{O}_2$ reduction current (generally in $\mu$A) and time, respectively.
The partially resolved experimental \( \text{O}_2 \) exchange pulses for the green algae, *Ulva sp.*, were numerically deconvoluted by fitting the experimental curve with the sum of time-shifted single pulses derived from the shape of the reference response, proving dynamic linearity of the electrode system. This allows quantification of the net amount of oxygen produced per flash by multiplying the relative amplitude of each pulse by the amount of \( \text{O}_2 \) produced during the reference response (eqn. 6.1). This value, however, represents net \( \text{O}_2 \) evolution, and does not account for \( \text{O}_2 \) produced by water oxidation, but consumed in Mehler reactions before diffusing out of the algae. The photosynthetic unit size can also be determined by calculating the net \( \text{O}_2 \) evolved for one complete cycle of the OEC (i.e. considering the \( \text{O}_2 \) produced from four saturating light flashes). For *Ulva sp.*, this was determined to be \( 2290 \pm 150 \text{ chl/} \text{O}_2 \text{ molecule} \), similar to that of *Ulva lactuca* with \( 2360 \pm 160 \text{ chl/} \text{O}_2 \text{ molecule} \) (Mishkind and Mauzerall, 1977).

The \( \text{O}_2 \) consumption which is evident in *Ulva* when DCMU is used to inhibit electron transfer from \( Q_A \) to the PQ pool, reaches its maximum value at ~2 s after the onset of 3.3 Hz flash illumination. This means that the \( \text{O}_2 \) current pulses in the measured \( \text{O}_2 \) exchange curve which are due to the first few light flashes do not contain a significant \( \text{O}_2 \) uptake component. By the fourth and fifth flashes, however, the light-induced \( \text{O}_2 \) consumption reactions in *Ulva* do contribute
significantly to the measured $O_2$ exchange rates. The form of the $O_2$ uptake suggests that a limited pool of reductants, e.g. plastoquinols and other intersystem carriers, is available for oxidation by $O_2$. The charge (in Coulombs) produced by $n$ flashes could be determined by taking the area under the DCMU-induced $O_2$ uptake curve, and dividing by the charge per electron which gives the number of electrons taken up by $O_2$. In the Mehler reactions, molecular oxygen requires one electron to produce superoxide ($O_2^-$) and another electron to produce $H_2O_2$. Thus the number of $O_2$ molecules (moles) consumed in Mehler reactions could be determined.

If the net $O_2$ yield is plotted as a function of flash number, a four-step sequence of $O_2$ exchange was observed in the genera *Iridaea*, *Laminaria*, *Alaria*, *Ulva*, and *Enteromorpha*. Sufficient evidence for a transient endogenous uptake in algae and chloroplasts has been demonstrated to justify including a correction for $O_2$ uptake in the four-step flash sequence. Subtracting the DCMU-induced $O_2$ uptake component from the measured $O_2$ exchange results in a four-step flash yield sequence with sustained oscillations in yield. However, $O_2$ consumption occurs in both PS I and PS II, and thus the flash yield sequence which has been corrected for PS I uptake is not likely the same as if PS II $O_2$ uptake is also considered. As well, flash yield sequences obtained with PS II membranes containing exogenous electron acceptors may not be equivalent to $O_2$ evolution which occurs when both photosystems are
functional. However, PS I $O_2$ uptake is most likely larger than that of PS II, thus subtraction of the PS I $O_2$ uptake should give a reasonable approximation to actual $O_2$ evolution. If it were possible to inhibit PS I $O_2$ uptake without affecting $O_2$ evolution, the effect of $O_2$ consumption reactions on the measured rates of $O_2$ exchange and the flash yield sequence could be determined. Vidaver (1969; 1972) has shown that in *Ulva lobata* under continuous illumination, high hydrostatic pressures (~5000-10,000 psi) result in inhibition of PS I $O_2$ uptake with little inhibition of $O_2$ evolution. Using short, saturating light flashes to illuminate *Ulva sp.* under 5000-10,000 psi hydrostatic pressure, it may be possible to experimentally inhibit PS I $O_2$ uptake without significantly altering the process of $O_2$ evolution. This may result in further insights into the effect of $O_2$ uptake on the four-step flash yield sequence.

Many studies have been performed with *Ulva* to investigate the transient light-induced $O_2$ uptake using continuous illumination (Vidaver and French, 1965; Chandler and Vidaver, 1970). If the transient DCMU-induced $O_2$ uptake is subtracted from the $O_2$ exchange curve, the large dip attributed by Vidaver to PS I $O_2$ uptake is absent (Vidaver and French, 1965). The results shown in Fig. 5.11 essentially verifies his assumption that the large dip in $O_2$ exchange after the $"O_2 gush"$ must be mostly due to a PS I-mediated $O_2$ uptake. The effect of anaerobiosis on the mechanism of $O_2$
evolution is still being widely debated. Generally, however, most researchers concur that lack of $O_2$ inhibits $O_2$ evolution, but the extent of the inhibition and the sites of inhibition are not clear. Diner and Mauzerall (1973a,b) have shown that anaerobiosis results in reduction of the PQ pool in the dark, but this may not completely account for the inhibition of $O_2$ evolution in the absence of $O_2$. Since continuous far red light superimposed on 0.25 Hz red flashes increased the $O_2$ yield under anaerobic conditions, Diner and Mauzerall (1973a) attributed this increase to the oxidation of reduced PS II electron carriers by far red light. Thus, it is possible that far red light flashes given to algae in the absence of $O_2$ will alter the anaerobic inhibition of $O_2$ evolution.

However, an attempt was made to do this using far red laser pulses (705-720 nm), but the intensity was not sufficient to alter the anaerobic response from the control response in air.

The $O_2$ exchange curves for Ulva under anaerobic conditions always show a small amount of $O_2$ produced, even after 6 h of anaerobic exposure. For Ulva, it appears that $O_2$ is not required for water-splitting to occur. However, the possibility that $O_2$ could be bound to the $S$ states and released at the onset of illumination to catalyze the production of $O_2$ can not be disregarded. Experiments by Radmer and Ollinger (1980a) using mass spectrometry have led them to suggest that under aerobic conditions in the dark, the $S_1$ state of spinach chloroplasts does not contain a bound intermediate.
oxidation product of $\text{H}_2\text{O}$. The authors also state that any association between the $S_0$ and $S_1$ states and water intermediates would be short-lived. However, it is difficult to extrapolate experimental results obtained in air to those obtained under anaerobic conditions, where plants are under $O_2$ stress, and thus this possibility should be investigated more thoroughly under anaerobic conditions.

Further studies should be made on the flash yield kinetics of red algae, e.g. *Porphyra*, which show different kinetics than the green or brown algae. It would be interesting to determine if the lack of a four step $O_2$ exchange pattern in *Porphyra* is due to variations in $O_2$ evolution or uptake. The appearance of a four-step pattern of net $O_2$ exchange in *Porphyra* under low ambient $O_2$ concentration suggests that different mechanisms (or lack) of $O_2$ uptake may be occurring under normal conditions. Determination of the light intensity which is sufficient to saturate oxygen-evolving centers should also provide information on $O_2$ exchange in *Porphyra* and enable determination of whether photoinhibition is occurring under light intensities which provide normal responses in green and brown algae.
APPENDIX

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenine trinucleotide phosphate</td>
</tr>
<tr>
<td>CF₀, CF₁</td>
<td>coupling factor components</td>
</tr>
<tr>
<td>CHL, Chl</td>
<td>chlorophyll molecule</td>
</tr>
<tr>
<td>Cyt</td>
<td>cytochrome</td>
</tr>
<tr>
<td>DCMU</td>
<td>3-(3,4-dichlorophenyl)-1,1-dimethylurea</td>
</tr>
<tr>
<td>Fd-Th-red</td>
<td>ferredoxin-thioredoxin reductase</td>
</tr>
<tr>
<td>Fd</td>
<td>ferredoxin</td>
</tr>
<tr>
<td>FD</td>
<td>bound ferredoxin usually subscripted, e.g. FDXₖ, FDXₐ, or FDX₈</td>
</tr>
<tr>
<td>Fdₛ</td>
<td>soluble ferredoxin</td>
</tr>
<tr>
<td>Fe-S</td>
<td>iron-sulfur protein</td>
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<tr>
<td>FNR</td>
<td>ferredoxin-NADP reductase</td>
</tr>
<tr>
<td>Fp</td>
<td>ferredoxin-NADP oxidoreductase</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton, 1 kD = 1000 g/mole</td>
</tr>
<tr>
<td>LHC</td>
<td>light-harvesting complex</td>
</tr>
<tr>
<td>M</td>
<td>active site of the oxygen-evolving complex</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>OEC</td>
<td>oxygen-evolving complex</td>
</tr>
<tr>
<td>Qₐ</td>
<td>primary electron acceptor for photosystem II</td>
</tr>
<tr>
<td>Qₐ</td>
<td>secondary electron acceptor for photosystem II</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PC</td>
<td>plastocyanin</td>
</tr>
<tr>
<td>PHEO, Pheo</td>
<td>pheophytin</td>
</tr>
<tr>
<td>$P_i$</td>
<td>phosphate group ($P_{O_3}$)</td>
</tr>
<tr>
<td>PS I</td>
<td>photosystem I</td>
</tr>
<tr>
<td>PS II</td>
<td>photosystem II</td>
</tr>
<tr>
<td>PSU</td>
<td>photosynthetic unit</td>
</tr>
<tr>
<td>$P_{680}$</td>
<td>photosystem II reaction center pigment</td>
</tr>
<tr>
<td>$P_{700}$</td>
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<tr>
<td>PQ</td>
<td>plastoquinone</td>
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<tr>
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<td>RuBP</td>
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<tr>
<td>$Y_i$</td>
<td>net oxygen yield for flash number $i$</td>
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<tr>
<td>$Z$</td>
<td>primary electron donor to photosystem II</td>
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<tr>
<td>$\Delta\Psi$</td>
<td>electrochemical potential difference</td>
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<tr>
<td>$\Pi$</td>
<td>lipid molecule</td>
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LIST OF REFERENCES


Burk, D., O. Warburg (1951) Reaction involving 1 quantum, and energy cycle in photosynthesis. Z. Naturforsch. 6B:12-122.


