THE EFFECT OF OXYGEN ON FLUORESCENCE INDUCTION IN ZEA MAYS CHLOROPLAST MEMBRANES

by

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The effect of oxygen on fluorescence induction in Zea mays chloroplast membranes

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ABSTRACT

During photosynthesis, light absorption initiates electron transport in the thylakoid membranes. The net result of the electron transport in the membrane is the transfer of electrons from water to the oxidized form of nicotinamide adenine dinucleotide phosphate(NADP) via photosystems I and II. However, a number of electrons will undergo a back reaction which results in so-called "variable fluorescence" from the antenna pigments. This fluorescence is highly dependent on the electron transport rate in the membranes.

The oxygen quenching effect on variable fluorescence was measured in order to study the functional differences of mesophyll and bundle sheath chloroplast membranes. The effect of oxygen on the distribution of light energy between photosystems I and II was studied using fluorescence emission spectra at 77K.

Oxygen was found to be a strong quencher of variable fluorescence in mesophyll chloroplasts but had very little effect in bundle sheath chloroplasts. These results indicate that oxygen quenching of variable fluorescence is induced by linear electron transport rather than cyclic electron transport. Also, oxygen affected the distribution of light energy between the two photosystems in bundle sheath chloroplasts but had much less effect on mesophyll chloroplasts. The observed state transitions in bundle sheath chloroplasts may be related to the conformational change induced by cyclic electron transport in the membranes.

To

My mother whose tears of joy has spurred me to gain knowledge.

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1.1 Photosynthesis

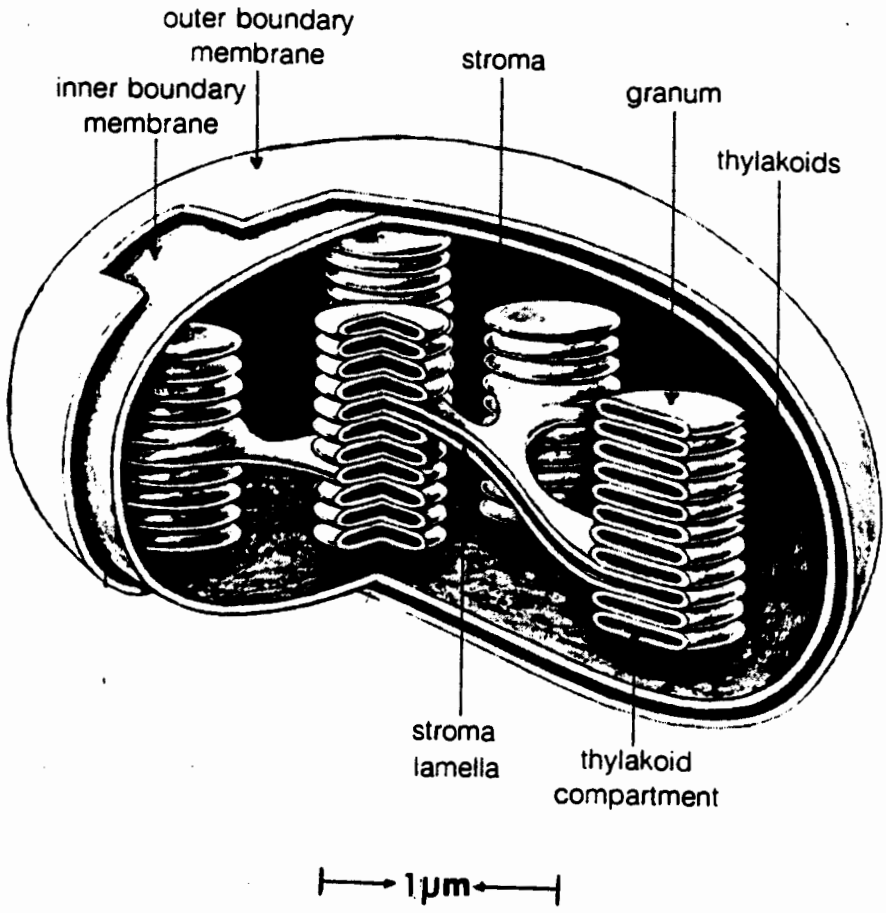
Green plants absorb photons with wavelengths between 400-700 nm and utilize the energy to promote chemical reactions, with the ultimate result that water and carbon dioxide are converted into oxygen and organic compounds. This is known as photosynthesis.

1.2 Photosynthetic apparatus

The photosynthetic apparatus of the higher plants is found inside organelles named chloroplasts. The membranes and internal compartments of a chloroplast are shown in figure 1.1. Chloroplasts have two outer membranes and extensive internal membranes, the thylakoid membranes. In most higher plants, chloroplast thylakoids are stacked to form grana (a single stack is a granum). The longer lamellae which connect one grana stack to another, extend throughout the chloroplast matrix (stroma) and are referred to as stroma lamellae.

Thylakoids contain pigments capable of trapping light energy and enzymes which convert this light energy into chemical energy via photochemical reactions using associated electron transport processes and energy coupling reactions involved in adenosine triphosphate (ATP) synthesis. The enzymes

Figure 1.1: Membranes and internal compartments of a chloroplast.
(after Wolfe, 1981)


outer boundary membrane
inner boundary membrane
stroma
granum
thylakoids
stroma lamella
thylakoid compartment
1 µm

responsible for the actual fixation of CO_2 and the synthesis of carbohydrates are water soluble proteins which are found in the stroma that surrounds the thylakoids.

1.3 Pigment systems

Pigment molecules in the thylakoid membranes initiate the process of photosynthesis by absorbing photons of visible light. There are three major classes of pigments participating in photosynthesis: chlorophylls, carotenoids, and phycobillins. Photosynthetic cells of higher plants always contain two types of chlorophylls, chlorophyll-a (chla) and chlorophyll-b (chlb). Chlorophyll-a plays an active role in photosynthesis, functioning as a photoenzyme (Rabinowitch and Govindjee, 1969), while the other pigments (including chlb) serve as physical energy suppliers and are referred to as accessory pigments. Chlorophyll-a absorbs red (band maxima at 680 nm) and blue (band maxima at 440 nm) light well, but not green. The accessory pigments absorb in the region where chla does not absorb strongly.

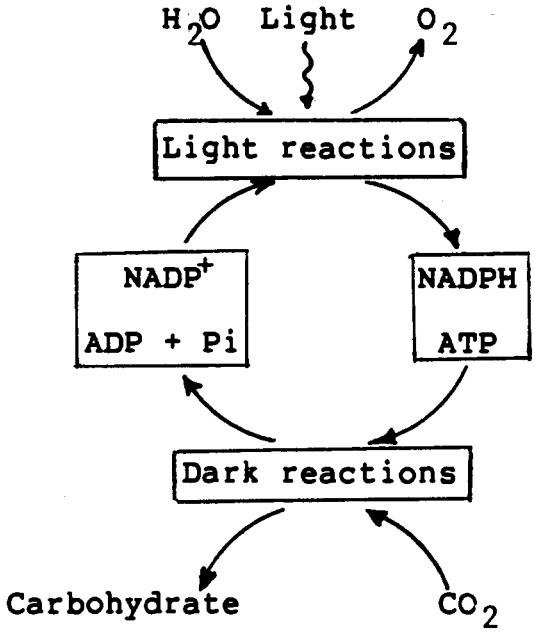
In higher plants and green algae, energy absorbed by other pigments is transferred to chla with high efficiency (Kirk, 1978). Energy transfer usually occurs with a small loss in energy (Colbow, 1973). Since chla has its absorption maximum at a longer wavelength than any of the accessory pigments, the energy absorbed by the pigments ends in chla.

1.4 Light reactions

The photosynthetic process can be divided into two parts, the light reactions and the dark reactions. In the light reactions, which take place in the thylakoid membranes, electrons are withdrawn from water and passed along a series of electron carriers to NADP (nicotinamide adenine dinucleotide phosphate). As a result, NADP is reduced to NADPH and oxygen is liberated. Associated with electron transport, there is a conversion of adenosine diphosphate (ADP) and inorganic phosphate (Pi) to ATP. Three molecules of ATP and two molecules of NADPH are believed to be necessary to convert CO_2 into carbohydrates. The light reactions can be written as follows:

$$n\ ADP + n\ Pi + 2H_2O + 2NADP^+ \longrightarrow n\ ATP + O_2 + 2NADPH + 2H^+$$

In the dark reactions of photosynthesis, the NADPH and ATP formed in the light reactions are used in the stroma to convert CO_2 to carbohydrates.



The series of enzyme reactions of the photosynthetic carbon reduction cycle (Calvin cycle) begin with the formation 3-phosphoglyceric acid (3-PGA) catalyzed by ribulose-1,5-bisphosphate carboxylase (RuBP carboxylase) (see appendix A).

1.5 Electron transport system

In the thylakoid membrane, the conversion of light energy into chemical energy is accomplished via a series of photoreactions. Thylakoid membranes have two different kinds of photosystems, which are complexes of light absorbing pigments, and are called photosystemI (PSI) and photosystemII (PSII). These are associated with the two photoreactions, NADP reduction and water splitting, respectively are connected by a series of electron transfer reactions. This widely accepted scheme of photosynthetic electron transfer, which was first suggested by Hill and Bendall (1960), is known as the "Z scheme" (Fig. 1.2).

Both PSII and PSI reaction centers contain chla together with specific protein complexes and are referred to as P680 and P700 respectively, corresponding to their absorption maxima in nm. Excitation of P680 generates a strong oxidant which oxidizes H_2O to O_2, while excitation of P700 generates a strong reductant which transfers electrons to NADP via electron carriers. Excitation energy produced from light absorbed by the light harvesting complex, which contains all antenna pigments and associated protein complexes of PSII, is transferred to P680,

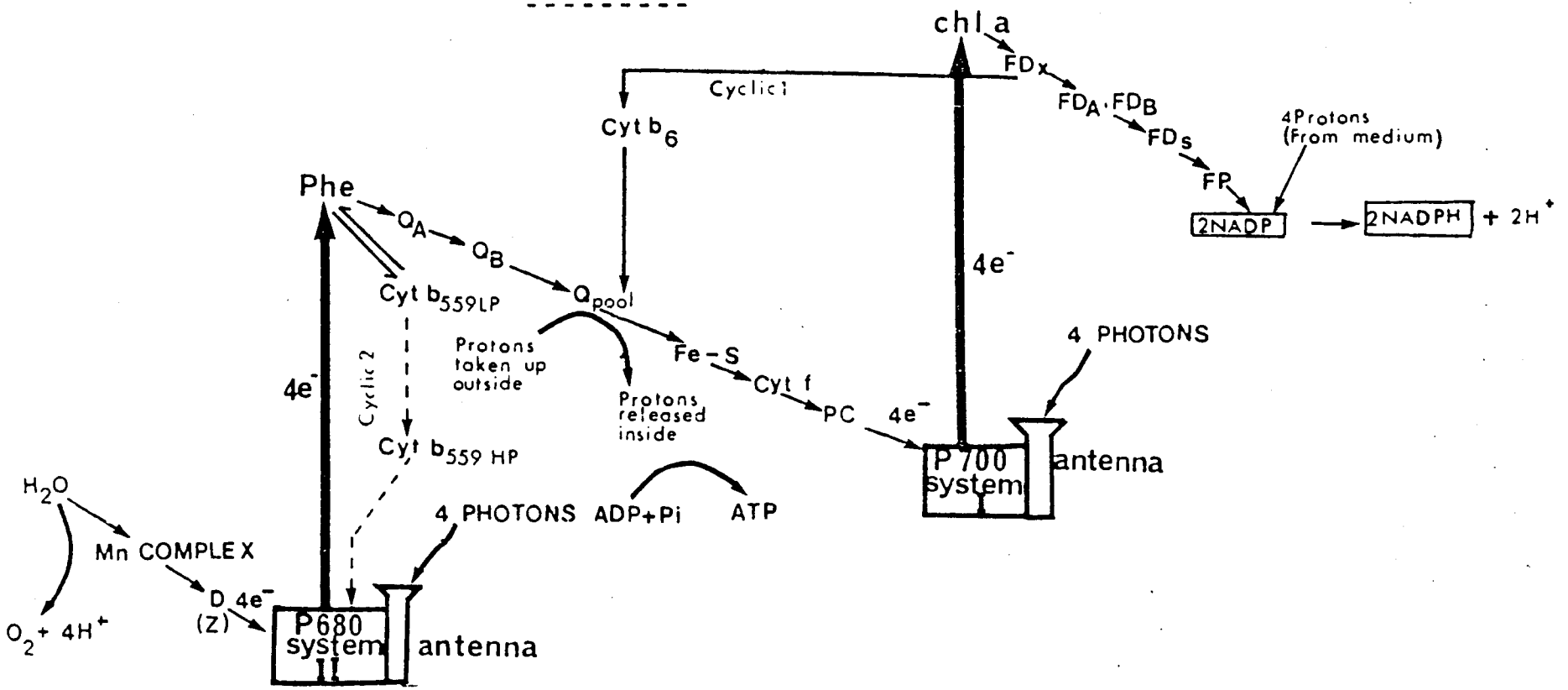
Figure 1.2: The Z scheme.

Abbreviations:

Z- the primary electron donor of PSII; P680 the reaction center of PSII; Phe pheophytin; Cyt-$b_{559_{LP\&HP}}$ - cytochrome-b_{559} (low and high potential); Q_A and Q_B - two molecules of plastoquinone; Q_{pool} - a large pool of plastoquinones; Fe-S iron-sulfur protein; Cyt-b_6 - cytochrome-b_6; Cyt-f- cytochrome-f; PC- plastocyanin; P700- the reaction center of PSI; Chla- chlorophyll a; FD_X, FD_A and FD_B- iron-sulfur proteins (bound ferredoxins); FD_S-soluble ferredoxin, FP- flavoprotein.

(after Zubay, 1983).

Z SCHEME


chl a
FDx
FDA·FDB
FDs
FP
4Protons
(From medium)
2NADP
2NADPH + 2H+
Cyclic1
Cyt b6
Phe
QA
QB
Qpool
Cyt b559LP
Cyt b559 HP
Cyclic 2
4e-
Protons
taken up
outside
Protons
released
inside
Fe-S
Cyt f
PC
4e-
4 PHOTONS
P700
system
antenna
H2O
Mn COMPLEX
D 4e-
(Z)
O2 + 4H+
4 PHOTONS
ADP+Pi
ATP
P680
system
antenna

promoting the primary photosynthetic act of reaction center oxidation. The water-splitting enzyme system reduces P680 and acts as the electron source for the electron transport chain. Electrons are transferred immediately(10^{-9} s) to the primary acceptor of PSII (pheophytin) (Klimov et al., 1977). From the primary acceptor of PSII, electrons are transferred via the electron transport chain, down the electrochemical gradient to P700. Light energy absorbed in the light harvesting complex of PSI is trapped at P700 and the charge separation at P700 leads to the oxidation of P700 and the reduction of the primary electron acceptor of PSI (Williams, 1977). Electrons from the primary acceptor of PSI are transferred to NADP via the ferredoxin-NADP reductase chain (see Fig. 1.2)

The electron transfer chain contains a number of components which include cytochromes, quinones and the copper-containing protein, plastocyanin (PC). The location of the various components in the electron transport chain is not entirely resolved. Cytochrome-b_6 (cyt-b_6) and cytochrome-f (cyt-f) are both located near PSI in the Z scheme. Plastoquinone (PQ) is functionally located between the two photosystems (Bishop, 1974). From the PQ pool electrons move to cyt-f via an iron-sulfur protein (Zubay, 1983). Cytochrome-f reduces the PC which in turn is oxidized by P700.

The electron flow from PSII to PSI is linked to transport of protons across the thylakoid membrane. Plastoquinone exists as a pool of about 4-7 molecules within one electron transfer

chain (Witt, 1979). This pool operates as a transmembrane H^+-pump so that the pool is functionally located between the outside and inside of the membrane (Witt, 1979). The number of protons which are translocated across the thylakoid membrane per electron varies between two and three. The number of protons translocated per electron (H^+/e) is two at saturating light intensity, corresponding to the maximum rate of electron flow (Rathenow and Rumberg, 1980). According to a hypothesis of Mitchell (1966), one H^+ is taken up by the reduced PQ and the other one by the proton-binding terminal electron acceptor at the outer surface of the thylakoid membrane. Furthermore, one H^+ is released with 1/2 H_2O oxidation and one with the oxidation of 1/2 PQH_2 (Plastohydroquinone) at the inside of the membrane. The electrochemical proton potential is used for ATP synthesis from ADP and Pi.

The electron carriers associated with the reducing side of PSI consist of iron-sulfur proteins. The first iron-sulfur protein is a soluble protein called ferredoxin (FD) which contains two iron atoms and two atoms of inorganic sulfide. The reducing side of PSI also contains three additional iron-sulfur proteins (FD_X, FD_A and FD_B) (Zubay, 1983), that are referred to as "bound" ferredoxins. Ferredoxins undergo a one electron oxidation-reduction reaction. The electrons from the primary acceptor of PSI are transferred to soluble ferredoxin, at a mid-point redox potential of E_m=-0.4 V relative to the hydrogen electrode, via FD_X(-0.7 V), FD_A(-0.54 V) and FD_B(-0.59 V). From

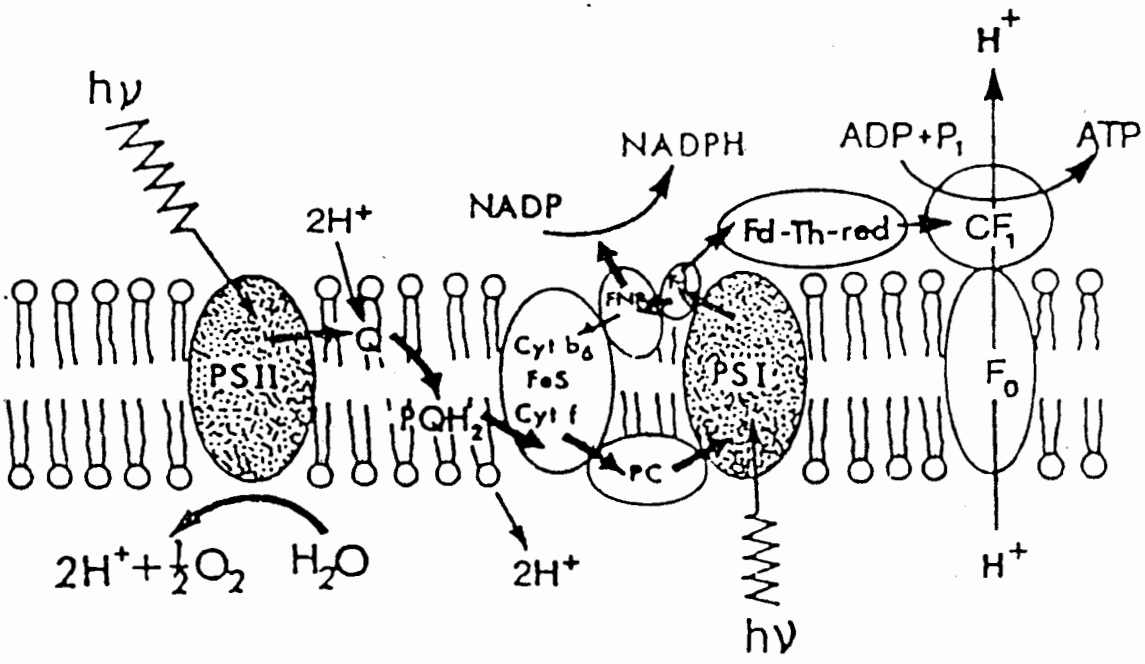
the soluble ferredoxin, electrons move to a flavoprotein (ferredoxin-NADP oxidoreductase) and then to NADP. The net result of the two systems is the transfer of electrons from H_2O to NADP. The overall reactions of the Z pathway yield the primary products of the light reactions, ATP and NADPH, releasing oxygen as a by-product. The overall process is known as linear electron transport.

It has been shown that electrons can be recirculated via cyt-b_6 to cyt-f and back to P700. This transfer is termed "cyclic electron transport". The net result of this system in vitro is direct conversion of light energy to chemical energy in the form of ATP, but not the reduction of NADP (Wolfe, 1981). In recent years some results suggest that a cyclic pathway around PSII involving cytochrome-b_{559} (cyt-b_{559}) also exists (Williams, 1977). Charge measurements on both sides of the thylakoid membrane showed that the primary electron acceptors are located at the outer surface and the primary donors at the inner surface (Witt, 1979). The location of the various electron transport components in the thylakoid membrane is shown schematically in Fig. 1.3.

According to the above Z scheme a minimum of 8 photons of light are required to oxidize two H_2O molecules and to reduce two NADP. Since two H_2O are required to release one O_2 and reduce one CO_2, 8 photons are required to fix one CO_2 molecule. Most measurements related to the quantum requirements of photosynthesis indicate that the number of photons required to

Figure 1.3: Thylakoid model showing components involved in the light reactions.

outer phase of thylakoid



inner phase of thylakoid

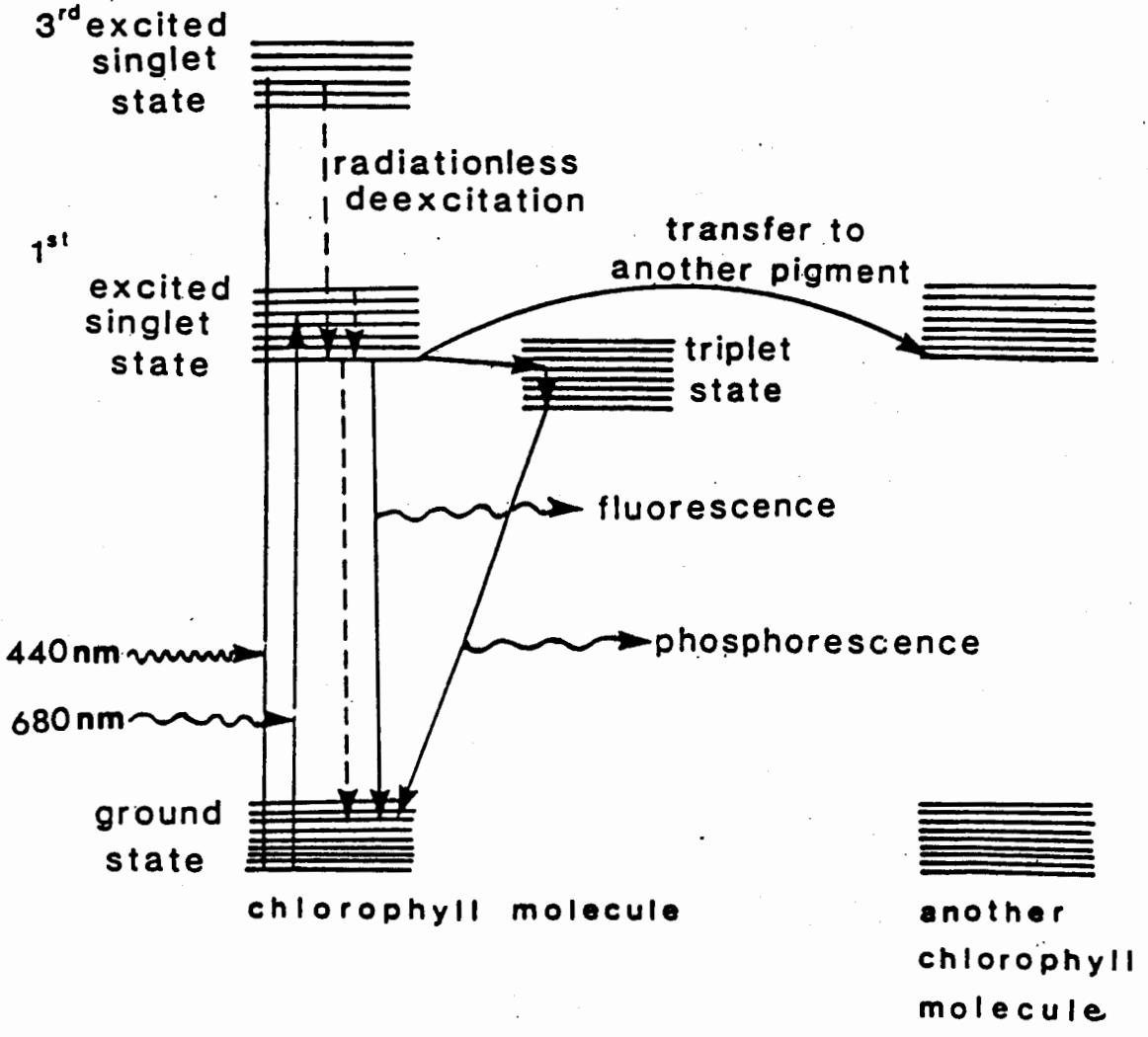
fix one CO_2 molecule is approximately 8-12 or even more. During continuous illumination, 2 NADPH and approximately 2-3 equivalents of ATP are probably obtained from linear electron transport, and a third equivalent of ATP may be obtained from cyclic electron transport (Zubay, 1983).

1.6 Fluorescence

Only part of the energy absorbed by the photosynthetic pigments is used in photosynthesis. The rest is lost as heat or radiated as fluorescence. Absorption of red light (band maxima at 680 nm) raises chla molecules to the first excited singlet state. Absorption of blue light (band maxima at 440 nm) raises the molecules to the third and higher excited singlet states (Govindjee and Govindjee, 1974). Fluorescence is emitted predominantly in a transition from the first excited singlet state of the chla molecule to the ground state (Fig. 1.4). Molecules which are in higher excited singlet state return to the first excited singlet state via internal conversion within 10^{-14} to 10^{-13} s.

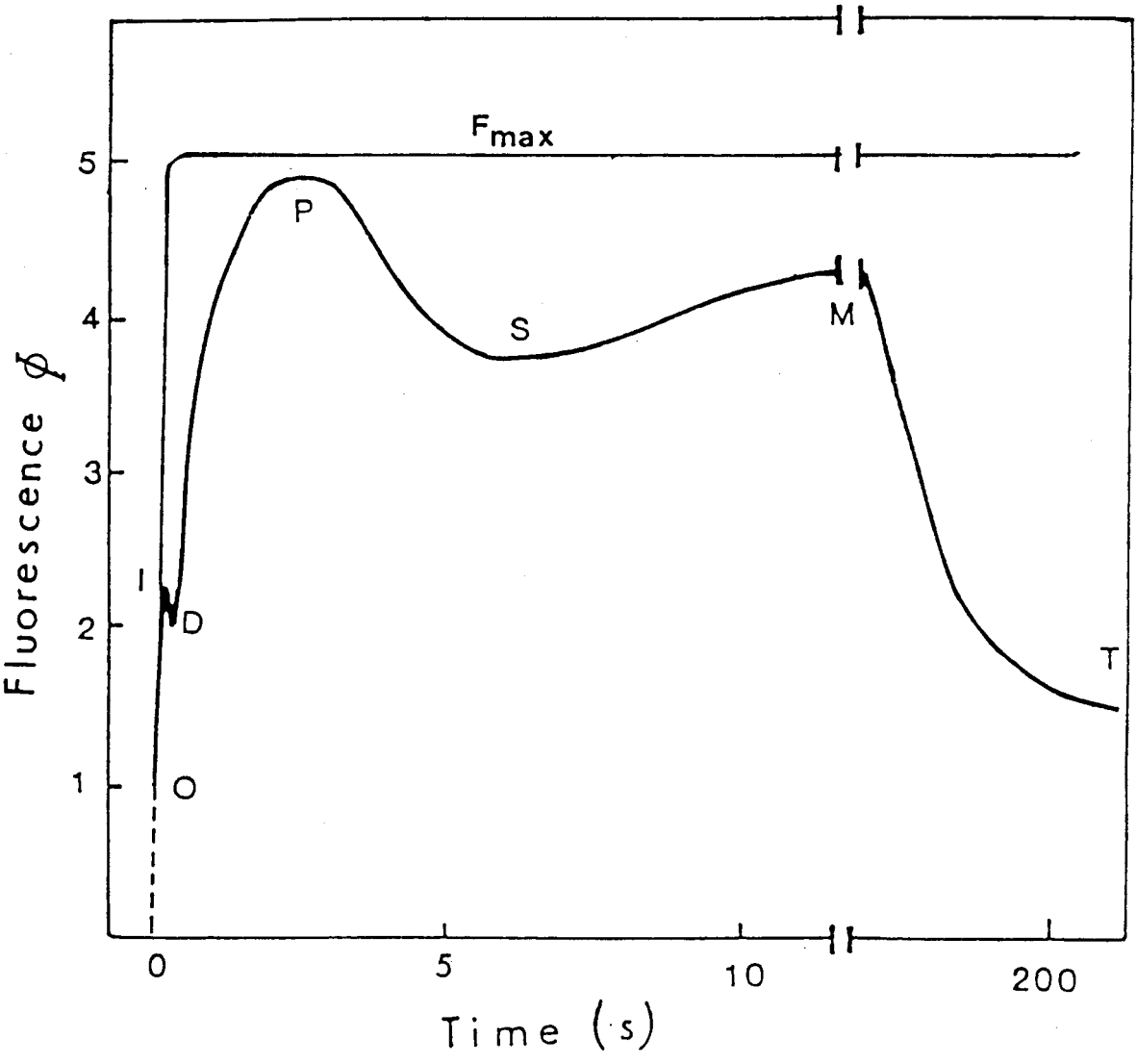
Fluorescence determinations have provided some of the most valuable data for the investigation of photosynthesis. The chla fluorescence which originates from the pigment molecules associated with PSII depends on the rate of electron transport through PSII reaction centers and on the oxidation-reduction of its primary acceptor. This is refered as "variable" fluorescence. In living cells, the intesity of

Figure 1.4: Excitation and deexcitation process of antenna chlorophyll.


3rd excited singlet state
radiationless deexcitation
1st excited singlet state
transfer to another pigment
triplet state
fluorescence
440nm
680nm
phosphorescence
ground state
chlorophyll molecule
another chlorophyll molecule

chla fluorescence varies with time for the first few minutes after onset of constant continuous illumination, following dark adaptation ($\sim$1/2 hr). The fluorescence induction passes through several transition stages. This induction phenomena was first observed by Kautsky (1931) and is known as "Kautsky effect". This consists of a fast change (5s) "O-I-D-P-S" (Fig 1.5) and the slow change "S-M-T", that achieves a steady state after several minutes. The fast change is related to the rate of electron flow through the electron transport chain and the slow change is related to temporary alterations in the thylakoid membranes, ATP formation, and CO_2. After commencement of excitation of the leaf, the fluorescence rises within nanoseconds to an initial O-level (F_0). This arises from the emission from chla molecules of the light harvesting antenna complex associated with PSII, and occurs prior to photochemical electron transport via the PSII reaction centers. The fluorescence rise from O to I is synchronous with the rise in the rate of oxygen evolution and is induced by the partial reduction of the primary acceptor of PSII (Papageorgiou, 1975). The fluorecence decay of from I to D is due to the oxidation of the PQ pool and reoxidation of the primary acceptor of PSII by PSI. The increase in fluorescence from D to P occurs primarily as a result of photoreduction of the PQ pool (Papageorgiou, 1975). The maximum level of fluorescence is observed

Figure 1.5: The Kautsky curve: the time course of fluorescence of a higher plant. The maximum level of fluorescence(F_{max}) was obtained by addition of DCMU which blocks the electron transport at plastoquinone.


Fmax
P
S
M
I
D
O
T
5
4
3
2
1
0
5
10
200
Time (s)
Fluorescence ϕ

in 3-(3,4-dichlorophenyl)-1,1'-dimethyl urea (DCMU) treated tissue, in which the electron transport is blocked at the reducing side of PQ. This shows that electron flow from the PQ pool to PSI prevents the PQ pool from becoming totally reduced when the inhibitor is absent (Baker and Bradbury, 1981).
The fluorescence decay from P to T can be affected by changes in:

1) the redox state of PQ

2) proton and other cation electrochemical gradients across the thylakoid membranes

3) ATP concentration in the external environment of the thylakoid

4) CO_2 fixation rate

Eventually, the rate of O_2 evolution and the quantum yield of chla fluorescence attain steady state values.

Fluorescence emission spectra provide other information about photosynthesis. At room temperature, fluorescence emanates mostly from chla associated with PSII, with a main band maximum at 685 nm. At liquid nitrogen temperature (77K), the fluorescence emission spectra show three bands at 685, 695 and 735 nm. The peak at 685 nm is thought to originate in the light harvesting chlorophyll a/b complex, and the peaks at 695 and 735 nm are believed to come from the antenna chlorophyll associated with PSII and PSI, respectively.

1.7 O_2 quenching of fluorescence

Chla fluorescence can be quenched by molecular oxygen. Inter-system electron transport and cyclic electron transport appear to be subjected to regulation by O_2 (Vidaver et al., 1981a). Three distinct phases of chla fluorescence quenching were observed in green plants by applying O_2 pressure. These phases indicate three different mechanisms of O_2 quenching. The most sensitive phase (phase1) is dependent on inter-system electron transport. Phase1 is mainly related to the quenching of the P-level in the induction curve and is characterized by a large component of DCMU-sensitive electron transport dependent activity. Phase2 (quenching of the maximum level of fluorescence, F_{max}) is related to quenching of PSII reaction centers or one molecule of PQ (Q_A)(see Fig 1.2). Phase3 (quenching of the O-level) is related to quenching of antenna chlorophyll molecules. O_2 appears to be an electron acceptor in photosynthetic electron transport in the vicinity of PC (Vidaver et al., 1981b) and at ferredoxin (Allen, 1977).

1.8 State transitions

In photosynthetic electron transport, PSI and PSII are considered to work in series; they occur in separate locations and have different accessory pigments associated with them. PSI and PSII do not have identical absorption spectra so they can be unequally excited. In this event, the redistribution of excitation energy may occur between the two photosystems to

attain the optimum photosynthetic electron transfer rate for a particular light condition. Under conditions where PSII is preferentially excited, the photosynthetic apparatus is converted to stateII. In this state, the organism slowly adjusts to the imbalance by allowing some of the excitation energy to be directed, by energy transfer (spillover), to PSI. This is characterized by a relative decrease in fluorescence emitted by chla associated with PSII and a relative increase in fluorescence emission from chla associated with PSI. When illumination conditions are altered so that PSI is preferentially excited, the photosynthetic system is converted to stateI. In this state, spillover is maintained at a minimum, resulting in an increase in PSII and a decrease in PSI fluorescence. The changes between stateI and stateII are presumed to maximize the quantum efficiency of photosynthetic electron transport under limiting intensities for different spectral qualities of the incident light.

Many processes are related to state transitions. Barber et al. (1974) suggested that the transport of Mg^{2+}across the thylakoid membranes is involved in the state transitions. Phosphorylation of the light harvesting chlorophyll a/b protein complex (LHCP), which is regulated by the redox level of PQ, was found to decrease the PSII fluorescence and this phosphorylation was proposed to influence to the state I-II transition (Horton and Black, 1980). Recently Fork et al. (1983) found that in the blue-green algae, dark adapted cells have fluorescence spectra

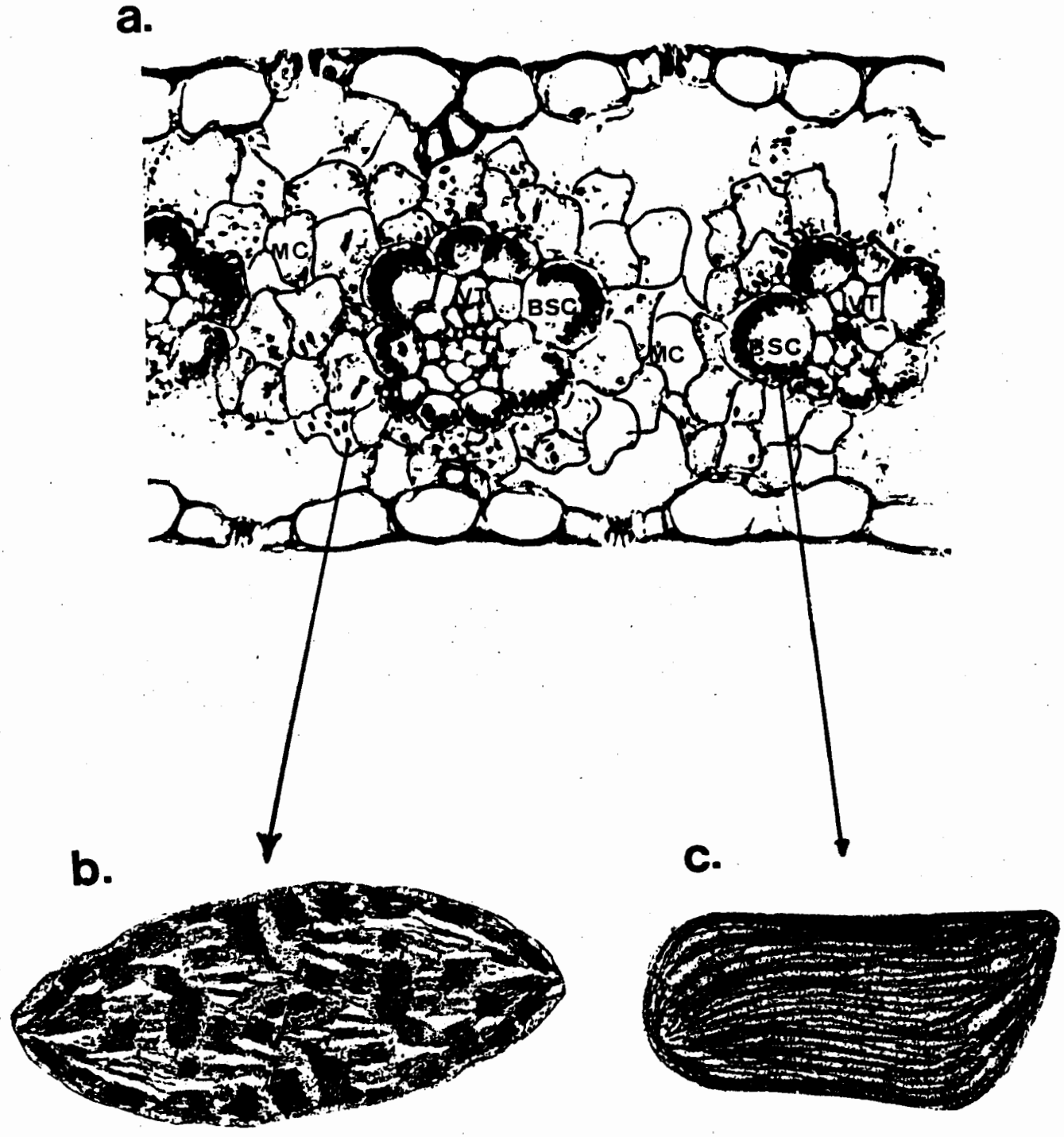
similar to stateII spectra and that the state I-II transition is induced by a cyclic electron flow around PSI. However there are many other factors which affect fluorescence, such as dark to light transitions and quenching effects.

1.9 The subject of study

Higher plants can be divided into two major groups, C_3 and C_4 species, based on the initial products of photosynthetic CO_2 fixation. In C_3 plants such as soyabean and tobacco, CO_2 is initially fixed into 3-PGA by RuBP carboxylase. In C_4 species such as corn and crabgrass, atmospheric CO_2 is initially fixed into oxaloacetate by phosphoenolpyruvate (PEP) carboxylase. Another characteristic of C_4 plants which distinguishes them from C_3 plants is the presence of two major chloroplast-containing leaf cell types: the bundle sheath cells which tightly surround the vascular tissue, and the mesophyll cells which in turn surround the bundle sheath layer (Fig. 1.6 a).

In C_3 plants, CO_2 is fixed into carbohydrates by the Calvin cycle. The assimilation of CO_2 in the C_4 plants is performed by co-ordination of two types of cells in the leaf, the mesophyll and bundle sheath cells. Most of the atmospheric CO_2 is initially fixed by PEP carboxylase in the mesophyll cells , with the resultant oxaloacetate being predominantly reduced to malate. The malate is transported to the bundle sheath cells and decarboxylated to pyruvate and CO_2 by the "malic" enzyme.

Figure 1.6: (a) Cross-section of a Zea mays leaf.
(b) Mesophyll chloroplast.
(c) Bundle sheath chloroplast.
(after Ray and Black, 1979).

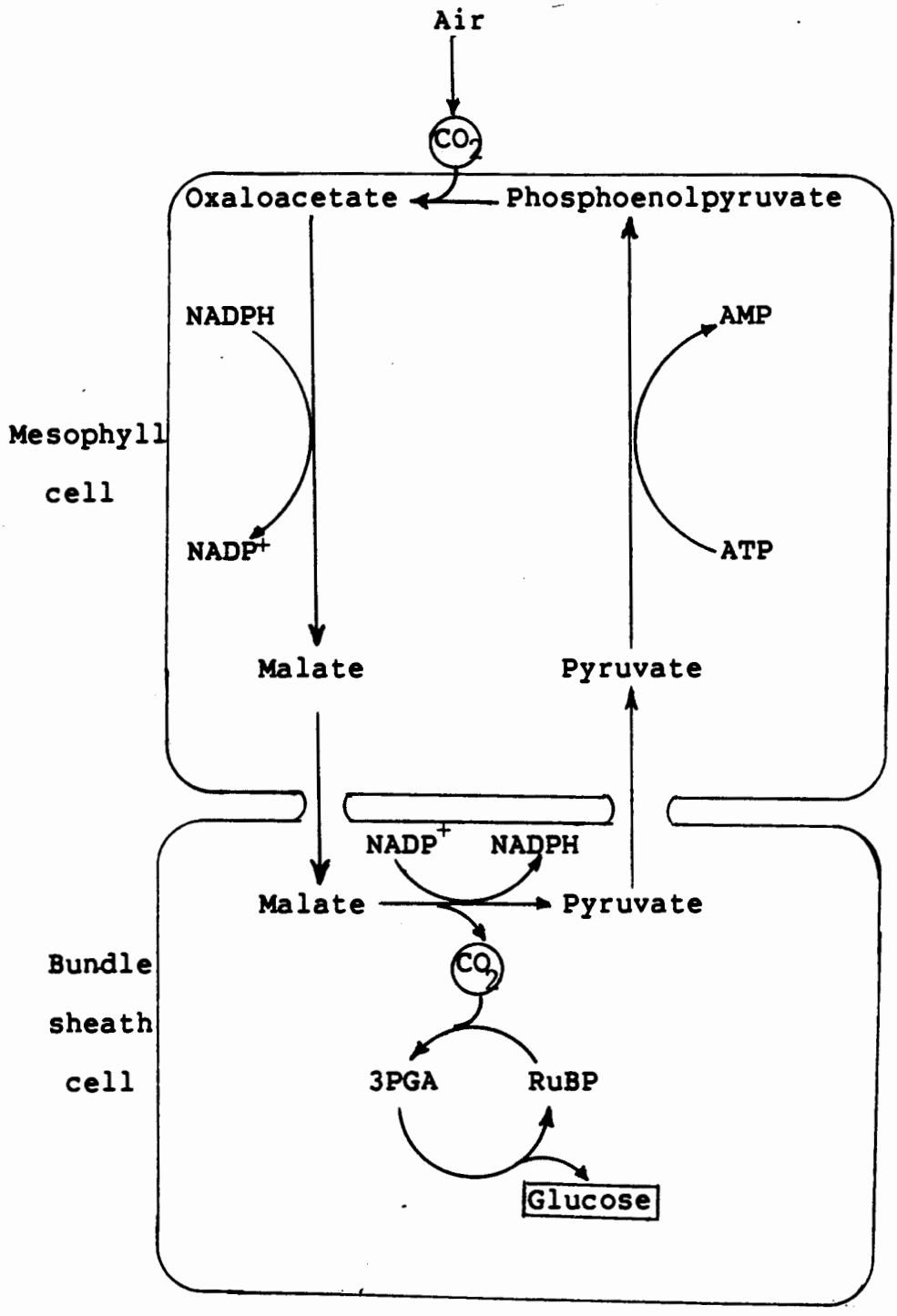


VT : vascular tissues

MC : mesophyll cells

BSC: bundle sheath cells

This CO_2 is refixed by RuBP carboxylase and further metabolized through the Calvin cycle. The pyruvate in the bundle sheath cells is transferred back to the mesophyll cells where it is converted into PEP by phosphorylation (Kanai and Edwards, 1973).



Chloroplasts of mesophyll cells contain grana (Fig. 1.6 b), but those of the bundle sheath cells exhibit varying degrees of grana development depending on the species, age, and growth conditions. The appearence of PSII activity in developing higher plant chloroplasts appears to be correlated with the formation of grana (Andersen et al., 1972). Bishop (1974) demonstrated that chloroplasts of young bundle sheath cells from some C_4 plants (including corn) contain grana that are lost during ontogeny (development) with concomitant loss of PSII activity. The developed bundle sheath chloroplasts from maize lack grana and only show extensive stroma-exposed thylakoids (Fig. 1.6 c).

Mayne et al. (1974) concluded that in maize there is only 15-17% as much PSII activity in bundle sheath chloroplasts as in the mesophyll chloroplasts based on fluorescence emission spectra and delayed light emission. Without PSII, the bundle sheath chloroplasts would be unable to generate NADPH (i.e. incapable of linear electron flow) using water as the electron donor and would have to import it from the mesophyll chloroplasts (Downtown et al., 1970). The organization of the electron transport components in mesophyll and bundle sheath chloroplasts of maize suggested that mesophyll chloroplasts possessed the full complement of various electron transport components, comparable to chloroplasts from C_3 plants. These studies also suggested that agranal (i.e. no grana) bundle sheath chloroplasts contained the full complement of PSI and cyt-f but lacked a major portion of PSII and its associated

chlorophyll a/b light harvesting complex (LHC), and most of the cyt-b_{559} (Ghiradi and Melis, 1983). Thus, the profile of the structural and functional relation in mesophyll and bundle sheath chloroplasts appears to be one of grana-facilitated generation of reducing power (NADPH) in the mesophyll chloroplasts only, and stroma thylakoid-facilitated ATP formation in mesophyll and bundle sheath chloroplasts.

The object of the present research is to study photosynthetic activities and differences in the effects of O_2 in both types of cells. Studies including the effects of O_2 were made of the state transitions in both types. In addition to that, PSII activity was studied in the top, middle and bottom part of the corn leaf (Fig. 2.1 a) in stateI and in stateII.

II. Materials and methods

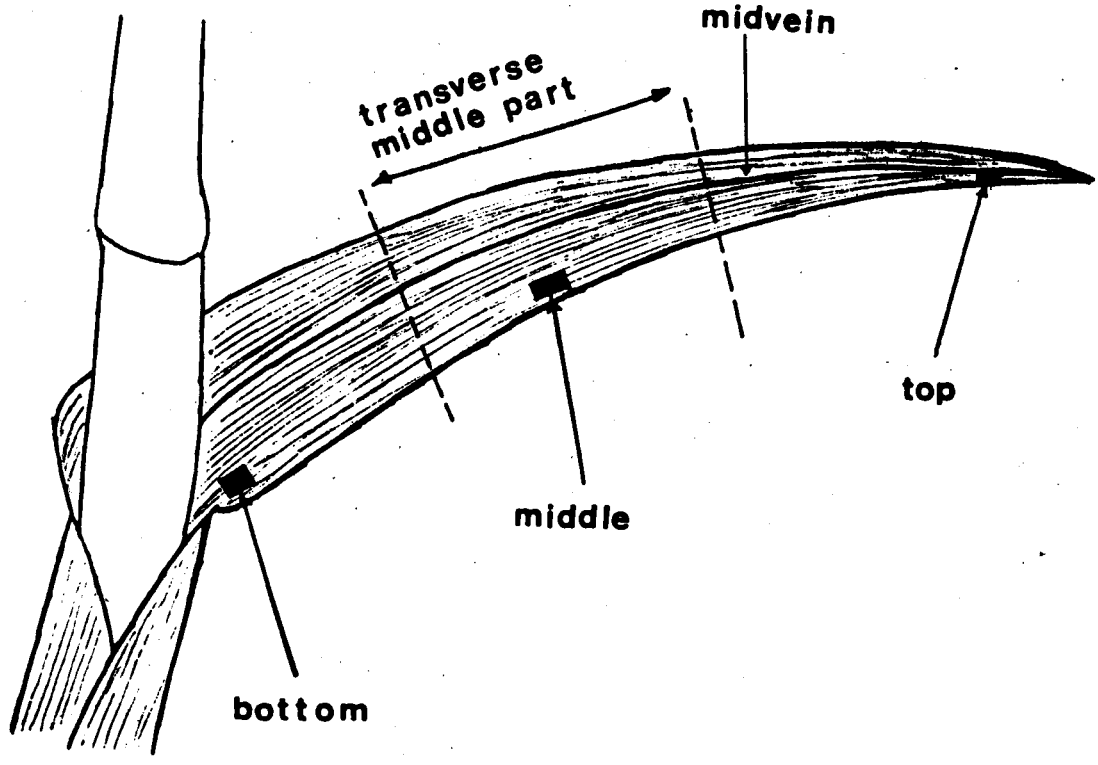
2.1 Plant culture

The Zea mays plants were grown on a 5 cm thick bed of soil-sand-peat moss mixture in the green house under a constant cycle of 16 hr light and 8 hr dark. The second and the third leaves from the bottom of plants harvested after 4 to 6 weeks were used in the experiments.

2.2 Bundle sheath strand isolation

Bundle sheath strands were isolated by following the method of Chollet and Ogren (1973). Removing the midveins, the transverse middle part (see Fig. 2.1) of the leaves (second and third only) were cut into small strips (2 mm wide and 1 cm long) by using a razor blade. These leaf strips (15 grams) were ground in a blender (Osterizer, Sunbeam corporation (Canada) Ltd., Model no. LR 18259) with 100 ml of grinding medium containing 0.5 M sorbitol, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM $Mgcl_2$, and 10 mM 2(N-Morpholino) ethane sulfonic acid (MES) (pH 5.5). The blender was operated at its highest speed for three fifteen second bursts. The homogenate was filtered through 500, 250 and 80 μm nylon nets in series. The residue on the 80μm net was thoroughly rinsed with resuspending medium containing 0.6 M

Figure 2.1: A corn leaf.


midvein
transverse
middle part
top
middle
bottom

sorbitol, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM $MgCl_2$ 5 mM N-2-hydroxyethylpiperazine-N-ethane sulfonic acid (HEPES) (pH 7.8). Material of size between 80 μm and 250 μm was collected and resuspended in the same medium. The isolation procedure was carried out at 5°C.

2.3 Mesophyll protoplast(cell contents excluding the wall) isolation

The same part of the leaves as above were used for the preparation of mesophyll cells. Leaves were washed and cut longitudinally into two parts (both sides of midveins) and peeled very carefully by using a sharp razor blade (without destroying any cells). These peeled leaves were laid in a 9 cm diameter petri dish with 20 ml of enzyme medium containing 0.5 M sorbitol, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM MES, 0.5% potassium dextran sulfate (KDS) (weight/volume, W/V), 0.2% bovine serum albumin (BSA) (W/V), 0.2% pectinase (R-10) (W/V) and 2% cellulase (Onozuka) (W/V). Enzyme digestion was continued and samples were gently shaken every fifteen minutes during an incubation period of 1.5 hr at 35°C under low fluorescent illumination.

After incubation the enzyme medium was gently decanted and filtered through a 80 μm nylon net, then centrifuged at 125g for 3 min. The supernatant was discarded, the pellet resuspended and washed twice in the resuspending medium (described earlier) by centrifugation. The pellet was then resuspended in 5 ml of the

medium, placed on ice and used within 2 hr after isolation. Microscopic examination showed less than 5% contamination with bundle sheath cells. Fast isolation is necessary to prevent cell destruction by endogenous enzymes.

2.4 Isolation of bundle sheath chloroplasts

Chloroplasts were isolated from bundle sheath cells prepared as described above. Cells were ground in a glass tissue grinder in a medium containing 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM HEPES adjusted to pH 7.5 with KOH. Chloroplasts were then sedimented by centrifugation for 5 min at 2000g. This procedure was repeated twice, and the chloroplasts were then resuspended in 5 ml medium containing 0.3 M sorbitol, 10 mM EDTA and 50 mM N-tris(hydroxymethyl) methyl glycine (Tricine) (pH 8.0). These chloroplasts were used to measure electron transport activity.

2.5 Isolation of mesophyll chloroplasts

The mesophyll protoplasts were suspended in the suspending medium (described in 2.4). The protoplasts were passed three times through a syringe with narrow needle (0.25 mm). Chloroplasts released from the cells were centrifuged at 1000g for four minutes and the supernatant was discarded. The pellet was resuspended in the resuspending medium.

All chloroplast isolations were carried out at 5°C.

2.6 Light microscopy

The mesophyll protoplasts and bundle sheath strands suspended in medium containing 0.6 M sorbitol, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM $MgCl_2$ and 5 mM HEPES were examined and photographed with a Wild Heerbrugg Model 20-68723 microscope equipped with a 35 mm camera. Mesophyll protoplasts were photographed with a clearance of approximately 0.5 mm between the cover slip and slide to prevent protoplast breakage.

2.7 Oxygen evolution

Oxygen evolution was measured by using a Clarke electrode (Delieu and Walker, 1972). This electrode consists of a silver anode and a platinum cathode, linked by a KCl bridge, which are separated from the reaction medium by a semipermeable membrane (see Fig. 2.2 a). Voltage (0.7 V) is applied across these electrodes so that the platinum is made negative with respect to the silver. At this voltage, oxygen is reduced at the platinum surface, and the current flowing is a measure of the oxygen consumed at the cathode. In a well-stirred solution this depends on diffusion across the membrane which is, in turn, related to the concentration of O_2 in solution. The electrode was calibrated before each experiment using a standard value for the concentration of oxygen in air saturated water at 20°C (0.280 µmols/ml). The zero O_2 calibration was obtained

Figure 2.2: (a) Diagramatic representation of electrode reactions.

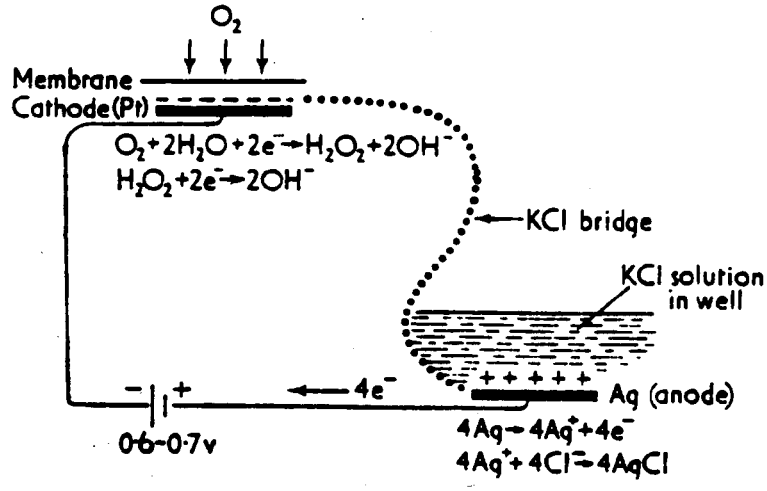
(b) Oxygen evolution measuring apparatus.

1- lamp, 2- lens, 3- magnetic stirrer

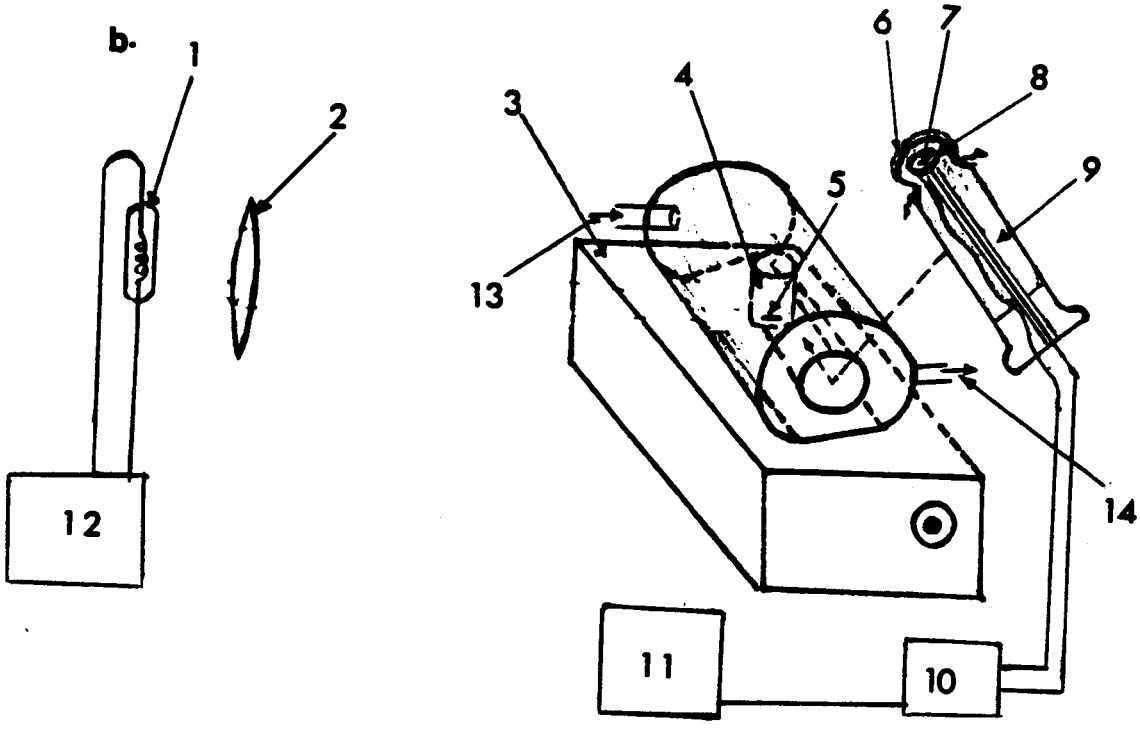
4- sample cuvette, 5- magnetic stirrer bar , 6- semipermeable membrane, 7- Pt cathode, 8- Ag anode, 9- potasium chloride, 10- amplifier, 11- strip chart recorder, 12- power supply, 13- water inlet, 14- water outlet.

The active electrode (6-9) was inserted in the hole as indicated by the dotted arrow and brought close to the sample cuvette.

a.


O_2
Membrane
Cathode(Pt)
$O_2+2H_2O+2e^-\rightarrow H_2O_2+2OH^-$
$H_2O_2+2e^-\rightarrow 2OH^-$
KCl bridge
KCl solution in well
$4e^-$
Ag (anode)
0·6-0·7v
$4Ag\rightarrow 4Ag^++4e^-$
$4Ag^++4Cl^-\rightarrow 4AgCl$

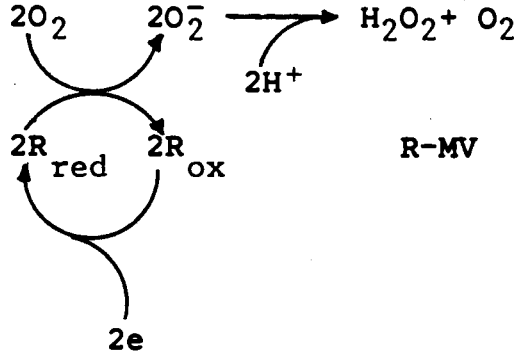
b.


1
2
3
4
5
6
7
8
9
10
11
12
13
14

by adding sodium dithionite($Na_2S_2O_4$).

$$Na_2S_2O_4 + O_2 + H_2O \longrightarrow NaHSO_4 + NaHSO_3$$

Total electron flow was measured by using methylviologen (MV) as the terminal electron acceptor. During the illumination of chloroplast suspensions MV takes the electrons from the chloroplast (from the primary acceptor of PSI) and transfers them to oxygen, forming superoxide. The superoxide (O_2^-) may subsequently form H_2O_2 either spontaneously or catalytically by using superoxide dismutase.



The above reaction is known as the Mehler reaction and it is written as:

$$2O_2 + 2H^+ \longrightarrow O_2 + H_2O_2$$

On addition of catalase the amount of O_2 released ($2H_2O_2 \longrightarrow 2H_2O + O_2$) is directly proportional to the total electron flow (Popovic et al., 1983).

The oxygen evolution measuring apparatus is shown in Fig. 2.2 b. The 1 ml reaction medium contained 20 µg of chl, 2 mM NH_4Cl and 2 mM MV. This was added to the cuvette of the Clarke electrode apparatus. The water circulation around the cuvette was maintained at 20°C. Saturating light was obtained with a 150

W tungsten projector lamp and condenser lens to focus light on the sample cuvette. O_2 reduction under 1 min illumination was recorded. Then the sample was maintained in the dark to reach a steady state. Release of O_2 from H_2O_2 was measured by adding 300units (1 unit decomposes 1 μmole of H_2O_2/min at 25°C, pH 7.0) of commercial catalase after 1 min in the dark.

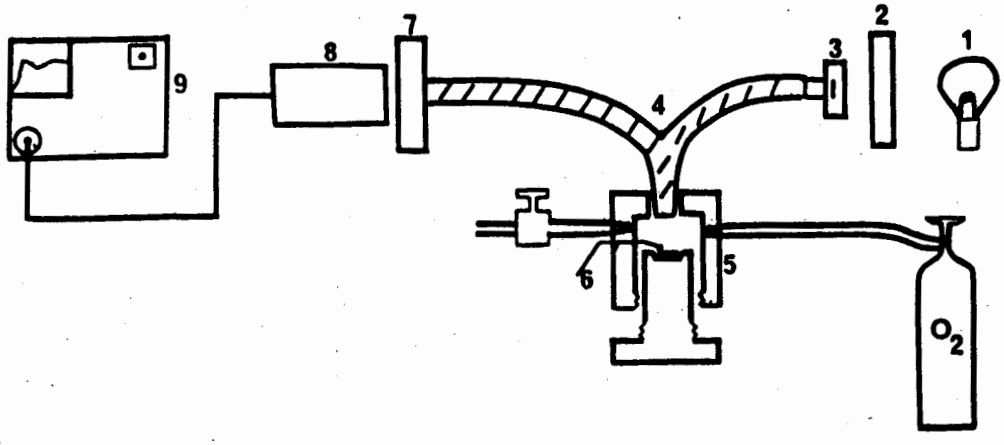
The total chlorophyll concentration was measured using the method of Arnon (Arnon, 1949).

2.8 Fluorescence measurements

The apparatus for fluorescence measurements is shown in figure 2.3. The corn leaf segments or protoplasts were excited by blue light and fluorescence was measured in the red region.

A 120 V, 650 W General electric DWY quartzline lamp was used as a light source. The light was passed through an infrared absorbing water filter. A blue Corning filter (4-96) which transmits between 400-600 nm light (see Fig. 2.4) was placed between a lens system and a shutter. The lens system was used to focus the light onto one arm of a bifurcated fibre optics system to the sample. Fluorescence was detected by an EMI 9558 QB photomultiplier after passing through the other arm of the bifurcated light fibre and a red transmitting Corning filter (2-64) (see Fig. 2.4). This system minimizes the excitation light entering the photomultiplier. The photomultiplier was operated at 1200 V and the signal was transmitted to a Tracor Northern 1710 multichannel analyzer with signal averager. The

Figure 2.3: Fluorescence measuring apparatus.



1 Lamp
2 Blue Filter
3 Shutter
4 Light Pipe
5 Sample Cell
6 Sample
7 Red Filter
8 Photomultiplier
9 Tracor Northern Signal Averager

signal display with time can be easily controlled by this setup. The fluorescence signal stored on the Tracor Northern was plotted using a Hewlett-Packard X-Y recorder. Light flashes were controlled using a electronic shutter control model ES-217-A with a shutter opening time of 6 ms, permitting reasonably precise F_o level extrapolations at low light intensities.

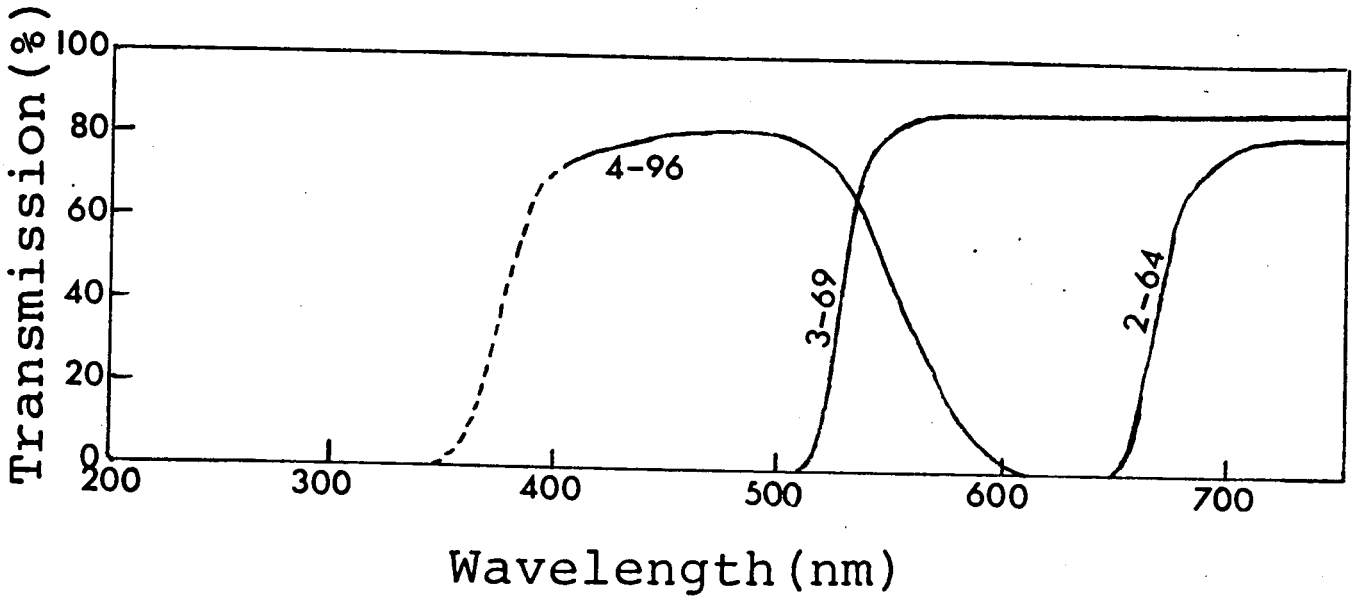
The sample cell and connecting tubing were made out of stainless steel. The top of the sample cell contained a plexiglas window for light transmission (Morita,1970).

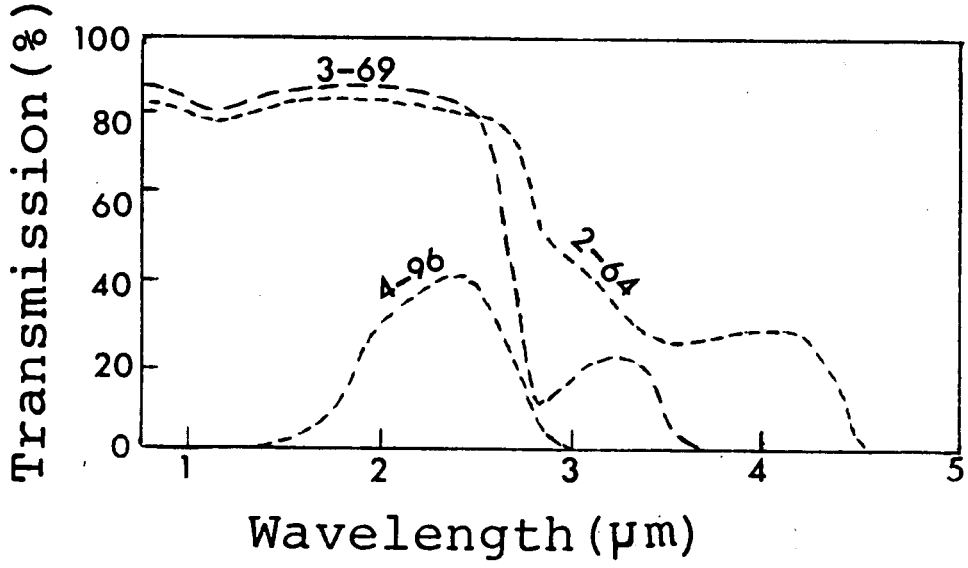
To avoid an adiabatic temperature increase in the sample compartment during experiments the pressure was applied slowly and steadily (15 atm/min) to the required value. Pressure in the sample cell was adjusted using a set of valves and pressure gauges. All measurements were taken after reaching the desired pressure which took about 3 min.

The light intensity on the sample was adjusted by a power supply. The light intensity was measured by a Tektronix J16 Digital Photometer with a J6502 radiometer probe.

In the first set of experiments the fluorescence time course and maximum level of fluorescence were measured in mesophyll and bundle sheath protoplasts. The procedure was: 1) dark adaption for 10 min; 2) a flash of light (60 ms) to determine the O-level fluorescence; 3) after 2 min darkness 5 s illumination to determine the P-level fluorescence; 4) adding a drop of 5 µM DCMU to the sample; 5) after 5 min darkness 5 s illumination to determine the maximum level of fluorescence (F_{max})

Figure 2.4: Transmission spectra of Corning filters (4-96, 2-64 and 3-69).


Transmission (%)
100
80
60
40
20
0
200
300
400
500
600
700
4-96
3-69
2-64
Wavelength (nm)


Transmission (%)
100
80
60
40
20
0
1
2
3
4
5
3-69
4-96
2-64
Wavelength (μm)

Oxygen quenching experiments were performed on leaves, mesophyll and bundle sheath protoplasts. Experiments were repeated 3-5 times by using a fresh sample for each determination. The procedure was: 1) dark adaption for 5 min; 2) a flash (60 ms) to determine the fluorescence O-level; 3) after 2 min darkness 5 s illumination to determine the P-level fluorescence; 4) dark adapt 5 min, apply O_2 pressure and determine the O-level; 5) after 2 min dark interval determine the P-level fluorescence.

2.9 Low temperature fluorescence emission spectra

2.9.1 In protoplasts

All measurements of fluorescence were carried out on 50 µl samples of protoplast suspension frozen to liquid nitrogen temperature (77K) in 2 mm diameter glass tubes in a Dewar system. The pellets were resuspended in 3 ml resuspending medium (described earlier) and 30 µl fluorescein (10 µM final concentration) was added. The fluorescein fluorescence was used as a normalising standard. The chlorophyll concentration in the suspension was 10-20 µg/ml. Samples (50µl) were placed in the bottom of the glass tube without touching the walls.

The sample were preilluminated for 4 min with far-red light (lightI) with an intensity of 10 W/m^2, using a 709 nm

interference filter, in order to place the cells in "stateI". During the illumination the tubes were continuously shaken to avoid any settling of chloroplast suspension. Immediately after preillumination at room temperature, the samples were rapidly chilled by dipping the glass tube into liquid nitrogen in the dark. "StateII" was obtained by preilluminating the sample with a red light (lightII) of 10 W/m^2 intensity, using a 680 nm interference filter (Hodges and Barber, 1983). Similarly, samples were preilluminated with lightI and lightII under 3 atm O_2 pressure and then were frozen to 77K.

Samples were excited with 480 nm light (half width 10 nm) and fluorescence spectra were taken using a Perkin-Elmer MPF-44B dual wavelength fluorescence spectro photometer, with a resolution of 1 nm. In this fluorometer a 150 W xenon lamp was used as incident light source and a R928 (Hamamatsu TV Co., Ltd.) photomultiplier as detector. A Corning cut off filter (3-69) (see Fig 2.4) was placed between the sample and the detector to prevent excitation light from reaching the detector. The scanning speed in the experiments was 120 nm/min. Fluorescence spectra were normalised using the 535 nm band fluorescence of fluorescein. Three emission spectra were taken for each sample for three different sample positions. During the experiments the detector gain was kept at a constant value.

2.9.2 In leaves

For this experiment 40 cm long corn leaves (developing) were chosen. A leaf segment (2 mm wide, 1 cm long) was cut from top of a leaf and attached to the outside of the previously described glass sample tube. The sample was then preilluminated for 2 min with far red light (700 nm, 10 nm band width) at room temperature to establish stateI, and then was frozen to 77K by pouring liquid nitrogen around the vertical cylindrical glass tube in the dewar while the illumination continued (Ley and Bulter, 1980). The samples were excited with 480 nm light (20 nm band width) and fluorescence emission spectra were taken, using the fluorometer described above with slit width corresponding to 2 nm resolution. Another, similar leaf segment was chosen from the top (other side of the midvein) and was adapted to stateII using 680 nm light (10 nm band width) and then frozen to 77K. The fluorescence emission spectrum was then obtained, and normalized to the previous one at 735 nm by changing the gain on the signal amplifier.

Similar measurements were taken for the leaf segments from the middle and bottom part of the same leaf. For comparison similar experiments were done with spinach and lettuce leaves.

2.10 Chlorophyll determination

Chlorophyll concentration in the various extracts were determined by the method of Arnon (1949). The absorbance of the chlorophyll extract in 80% acetone was measured at 663 and 645 nm using a Beckman spectro photometer. The concentration was calculated using the following empirical formula:

Chl concentration in μg/ml= $20.2 \times A645 + 8.02 \times A663$

This determination was used for the calculation of O_2 evolution per mg chl and also for low temperature emission spectra measurements.

III. Results

3.1 Cell preparations

Twenty to fifty percent of the total leaf chlorophyll can be retained in the form of protoplasts using the enzymatic digestion of leaves with the conditions employed. Figure 3.1 a, b shows a preparation of isolated mesophyll protoplasts and the bundle sheath strands from maize leaf. Light microscopy confirmed that the mesophyll protoplast fractions were free from contaminating bundle sheath cells and vice versa.

3.2 Electron transport activity

The fluorescence induction curves for O-level (F_o), P-level (F_p) and F_{max} are shown in figure 3.2 for dark adapted mesophyll and bundle sheath cells. The sudden rise from F_o to F_{max} in the presence of DCMU is indicative of maximum PQ reduction. The values of $f_p=(F_p-F_o)/F_o$ and $f_m=(F_{max}-F_o)/F_o$ for mesophyll cells are 1.06 and 1.69 respectively, and 0.35 and 0.40 for bundle sheath cells. These results indicate important differences of the electron transport system between the two types of cells.

The effect of O_2 pressure on f_p of corn leaves is plotted in figure 3.3 at various light intensities of 5, 10 and 20 W/m^2. The values of f_p in air vary between 2.9 and 3.1 at these light intensities which was normalized to 3.0. O_2 quenching of f_p in

beans was studied by Bruce et al. (1983) and half-quenching was observed at 7 atm O_2. My results of O_2 quenching in corn were similar to the bean data and half quenching was observed at 5, 10 and 13 atm for light intensities of 5, 10 and 20 W/m^2 repectively, suggesting a similar mechanism for O_2 quenching. Each phase of O_2 quenching differed by about an order of magnitude in its sensitivity to O_2. The effect of O_2 on the O-level (phase3) fluorescence yield was negligible up to 40 atm O_2 compared to phase1 quenching. Since linear electron transport is not blocked in these experiments, phase2 quenching by O_2 can be ruled out. Therefore, in this case, the quenching of fluorescence by O_2 is related to the oxidation of intersystem electron transport carriers.

The quenching of fluorescence by O_2 was studied in isolated mesophyll and bundle sheath cells and the results are shown in figure 3.4. The values of f varied slightly in different preparations so that the average value is considered. The average value of f in air was found to be 1.3 and 0.4 for mesophyll and bundle sheath cells respectively. The quenching curves of mesophyll cells were similar to leaves and half-quenching was observed at 7, 10 and 13 atm for varying light intensities. Due to lack of linear electron transport activity, bundle sheath cells did not show much quenching of fluorescence by oxygen and variations of light intensity also had little effect. From inhibitor studies Vidaver et al. (1981b) have found that linear electron transport through PQ and PC was

required to observe f_p quenching by O_2. These results indicate that linear electron transport through PQ and PC is present in mesophyll cells and that it is missing in bundle sheath cells.

In leaves and mesophyll cells, increased light intensity partially overcame the quenching effect by O_2. More light intensity is required at higher O_2 pressure, to obtain one half of the f value in air. Increasing light intensity would cause more electrons to flow through the electron transport chain, keeping electron carriers in a more reduced state and consequently increasing the value of f_p.

Total electron transport activity in the mesophyll and bundle sheath chloroplast membrane was measured by using methylviologen as the terminal electron acceptor (Fig. 3.5). Mesophyll chloroplasts from corn gave results similar to spinach or lettuce chloroplasts (Popovic et al.,1983). During illumination O_2 is reduced to H_2O_2. The equivalent amount of O_2 was then released by addition of catalase, which converts H_2O_2 back into O_2. The amount of O_2 released after addition of catalase in the dark was negligible in bundle sheath chloroplasts compared to mesophyll chloroplasts. These results indicate that the electron transport activity around PSII is negligible in bundle sheath chloroplasts. They also indicate that little O_2 is reduced by the PSI-Mehler reaction in bundle sheath cells.

3.3 State transitions and grana development

Fluorescence emission spectra were measured in cells frozen into stateI and stateII at 77K. Emission spectra of mesophyll cells in air and O_2 frozen in stateI and stateII are shown in figure 3.6 a, b. These spectra were normalised to the 535 nm peak which corresponds to the fluorescein peak at 77K. Oxygen did not effect the fluorescein fluorescence (data not shown). The ratios of fluorescence at 735 nm to 685 nm are similar in stateI and stateII in the presence of air (1 atm) and 3 atm O_2 pressure. However oxygen decreases the fluorescence intensity slightly.

Similar measurements were made on bundle sheath cells and the results are shown in figure 3.7 a, b. These spectra show little fluorescence emission at 685 nm compared to mesophyll cells and high fluorescence at 735 nm. Table 3.1 shows the peak ratios of mesophyll and bundle sheath cells in stateI and stateII in the presence of air (1 atm) and 3 atm O_2. The errors were calculated using the standard deviation method.

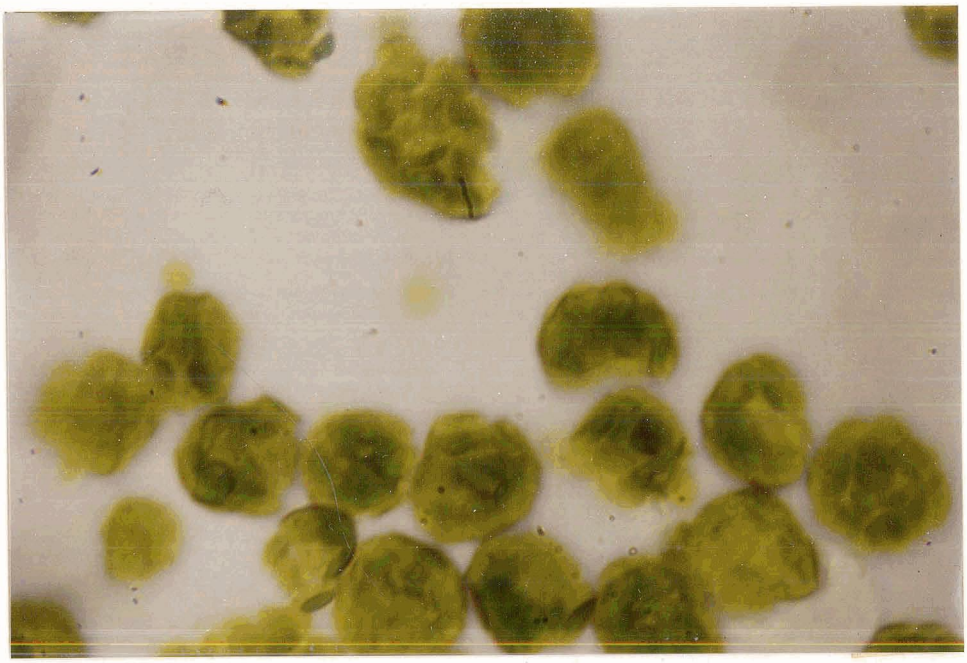
StateI and stateII transitions were also studied in different parts of corn leaves using fluorescence emission spectra at low temperature. The small leaf segments were chosen from the top, middle and bottom part of the leaf and exposed to lightI and lightII in order to place the cells in stateI and stateII respectively. Figures 3.8 a, b, c show the fluorescence emission spectra of top, middle and bottom part of the leaf in stateI and stateII. The spectra were normalized at the 735 nm

peak. The variation of intensity of fluorescence at 685 nm from bottom to top part of the leaf, shows the variation of PSII activity along the leaf.

For comparison leaf segments from spinach and lettuce were exposed to lightI and lightII and fluorescence emission spectra were measured at low temperature (see Fig. 3.9). The leaf segments from spinach and lettuce contain developed grana as does the middle part of the corn leaf. All the spectra except those from the top part of the corn leaf confirmed that a larger fraction of the excitation energy is delivered to PSII in stateI. These results agree with earlier literature about stateI and stateII transitions.

Figure 3.1: Light micrograph of a field of
(a) isolated mesophyll protoplasts
(b) isolated bundle sheath strands
from corn.
Magnification= 100.

a.



b.

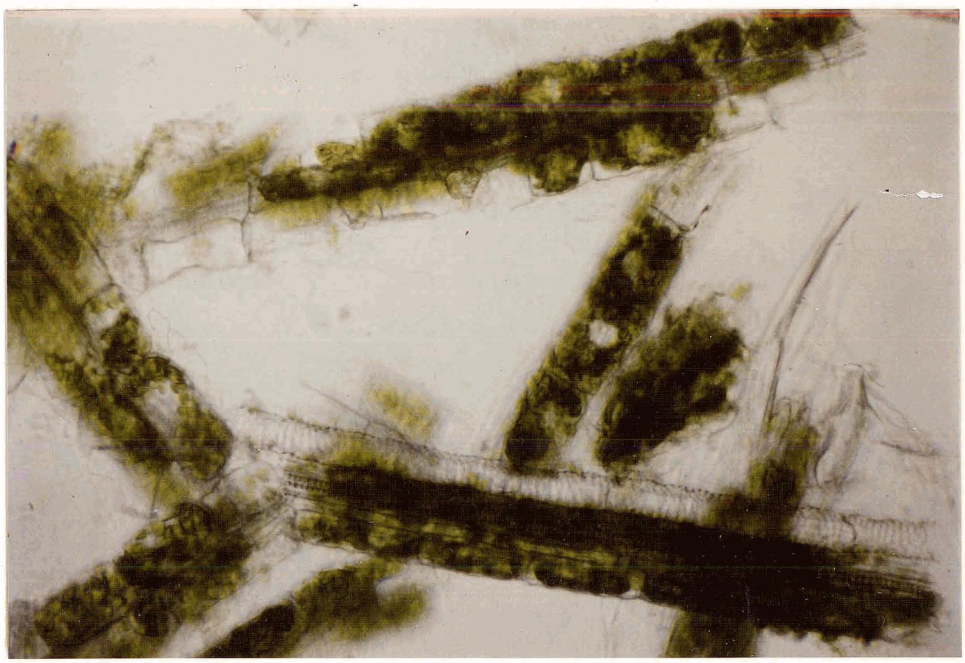


Figure 3.2: Time course of relative fluorescence induction of mesophyll and bundle sheath cells.
The initial rise to the O-level was measured with a time scale in ms.
The P-level was measured with a time scale in s. F_{max} was measured in the presence of DCMU with a time scale in s.

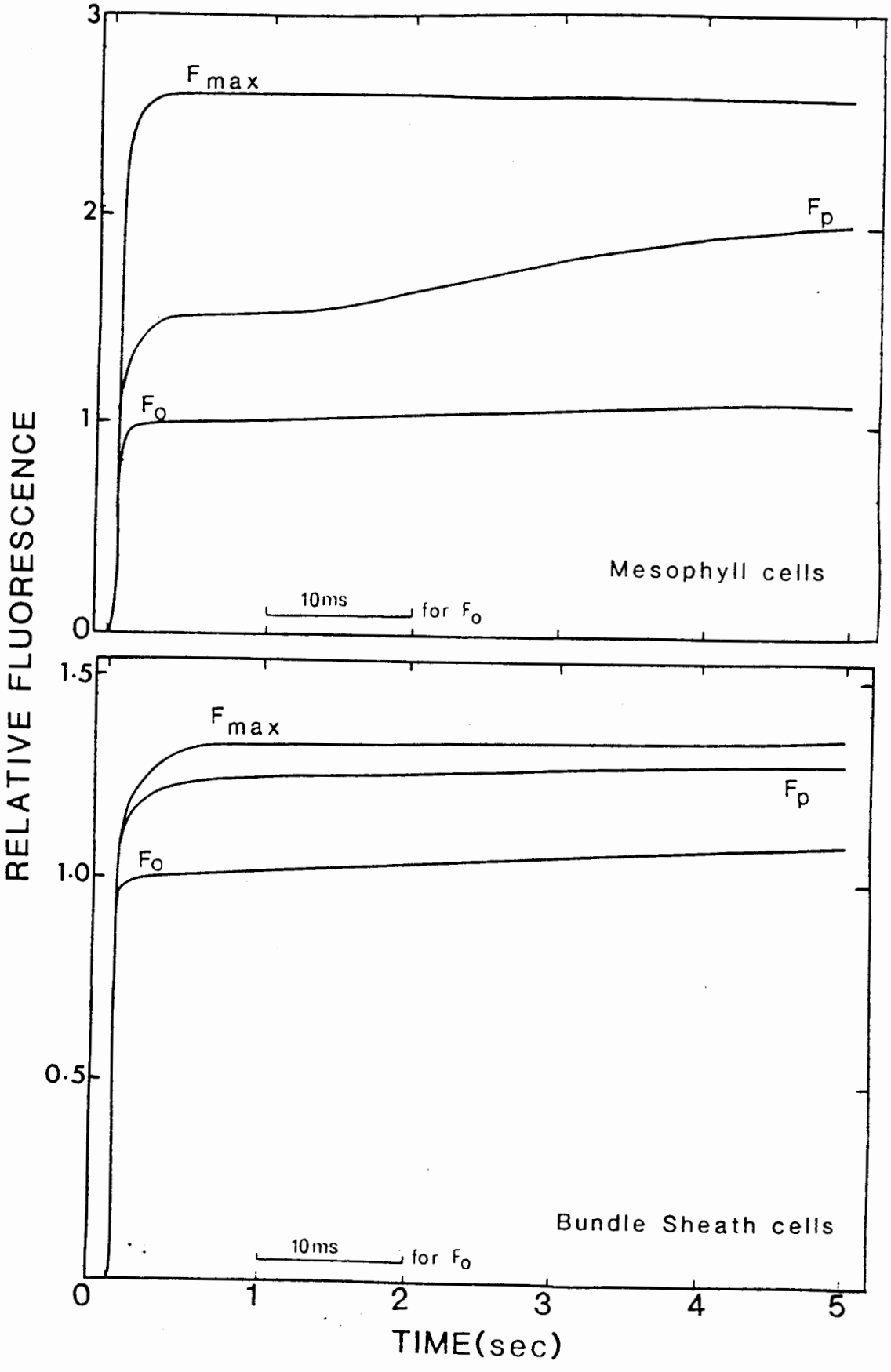

RELATIVE FLUORESCENCE
3
2
1
0
F max
Fp
Fo
Mesophyll cells
10ms
for Fo
1·5
1·0
0·5
F max
Fp
Fo
Bundle Sheath cells
10ms
for Fo
0
1
2
3
4
5
TIME(sec)

Figure 3.3: O_2 quenching of relative fluorescence(f_p) in corn leaves at various light intensities. ⊙- 5 W/m^2, ◇- 10 W/m^2 and ⊡- 20 W/m^2. Dotted lines indicate half-quenching values.

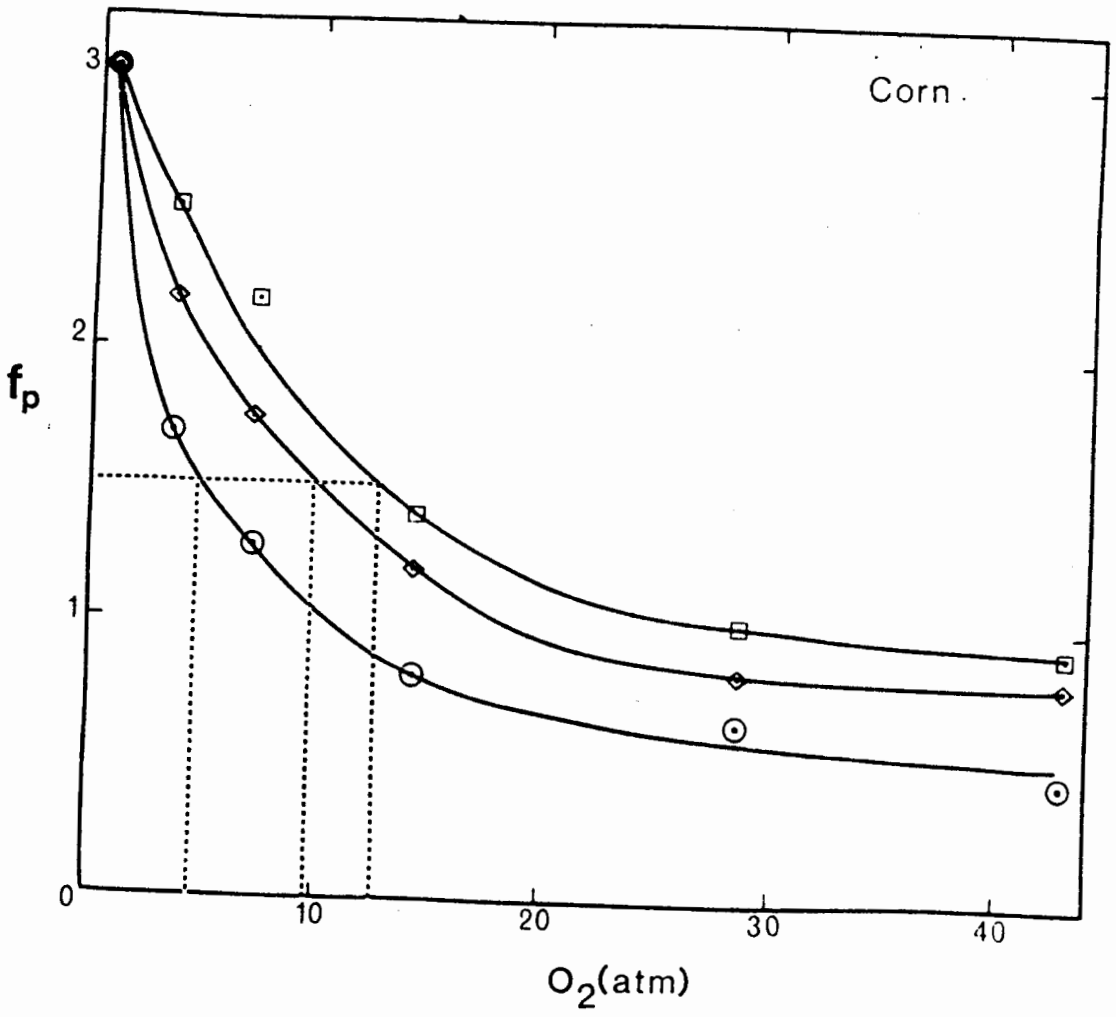

Corn.
f_p
3
2
1
0
10
20
30
40
O_2(atm)

Figure 3.4: O_2 quenching of relative fluorescence(f_p)

(a) mesophyll cells

(b) bundle sheath cells

at light intensities ⊙- 5 W/m^2,

◈- 10 W/m^2 and ⊡- 20 W/m^2.

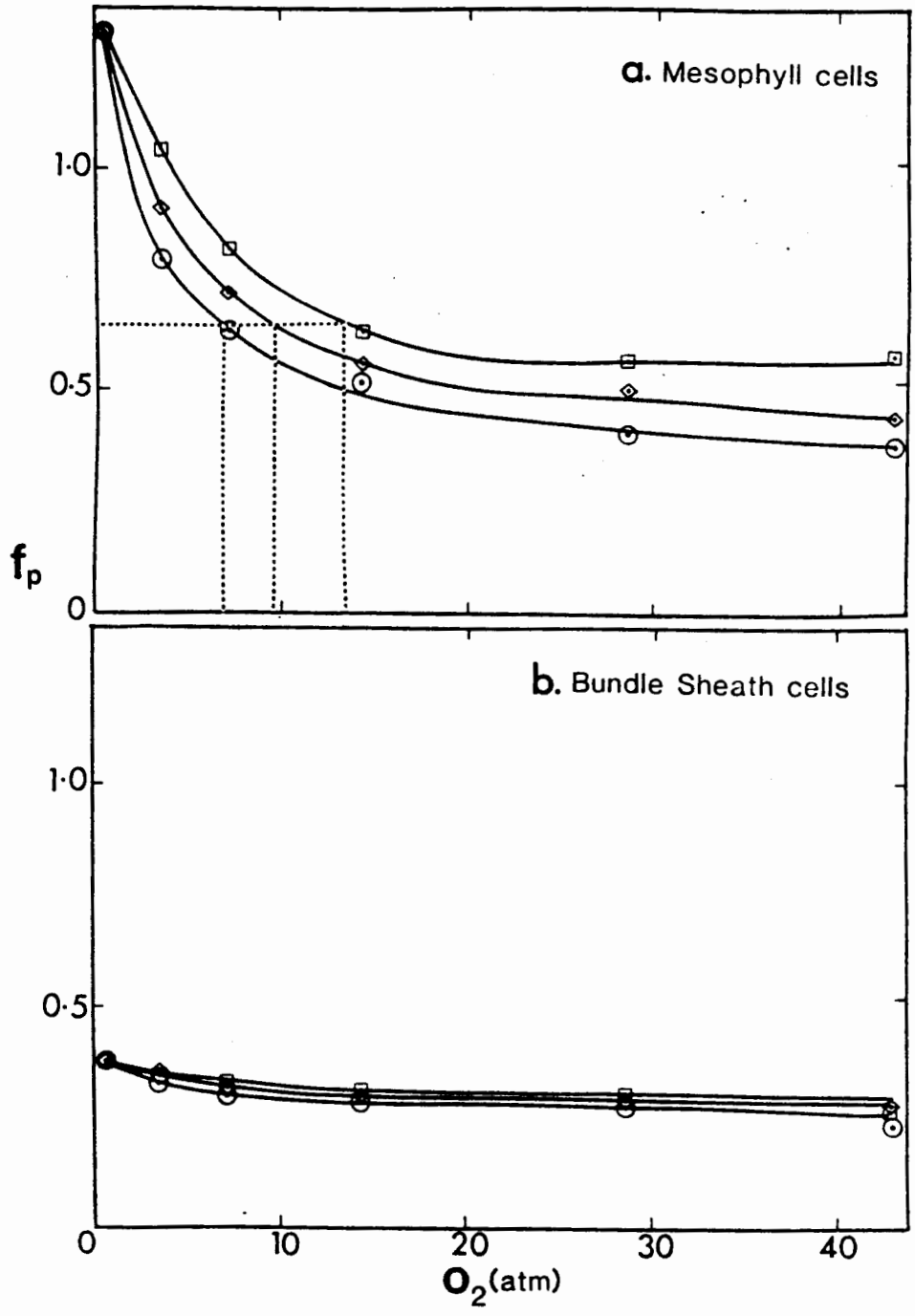

a. Mesophyll cells
1·0
0·5
0
f_p
b. Bundle Sheath cells
1·0
0·5
0
10
20
30
40
O_2(atm)

Figure 3.5: Light dependent O_2 reduction in the presence of methylviologen (MV) and subsequent O_2 release after the addition of catalase for mesophyll and bundle sheath chloroplasts.

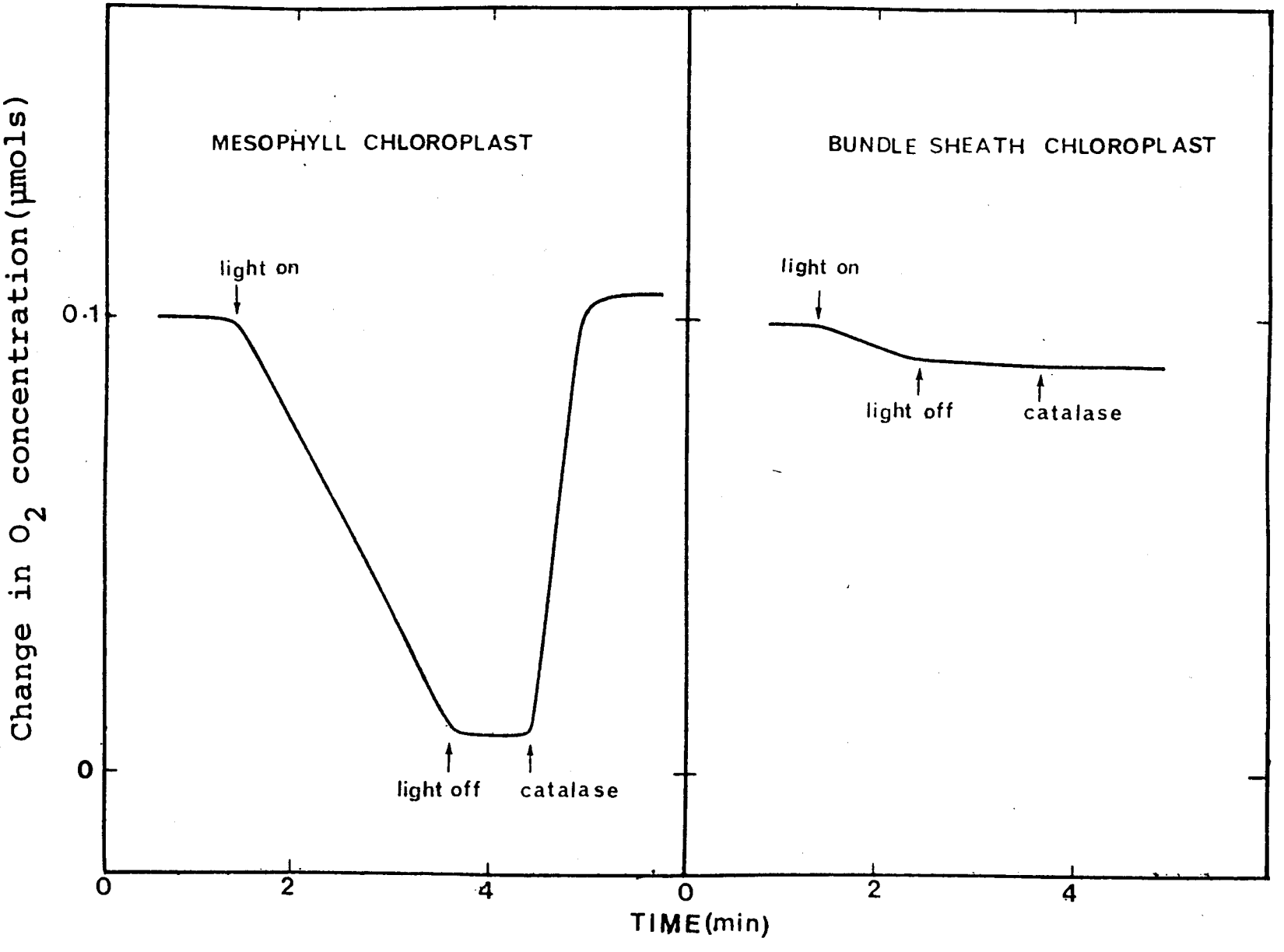

MESOPHYLL CHLOROPLAST
BUNDLE SHEATH CHLOROPLAST
light on
light off
catalase
light on
light off
catalase
Change in O_2 concentration (µmols)
0·1
0
0
2
4
0
2
4
TIME (min)

Figure 3.6: Fluorescence emission spectra at 77K of mesophyll cells in stateI (——) and in stateII (----). StateI and stateII were obtained by illuminating the cells with lightI (709 nm) and lightII (680 nm) for 4min after which the cells were cooled to 77K.
(a) In air (1atm) (b) In 3 atm O_2
The curves were normalised with respect to fluorescein peak at 535 nm.

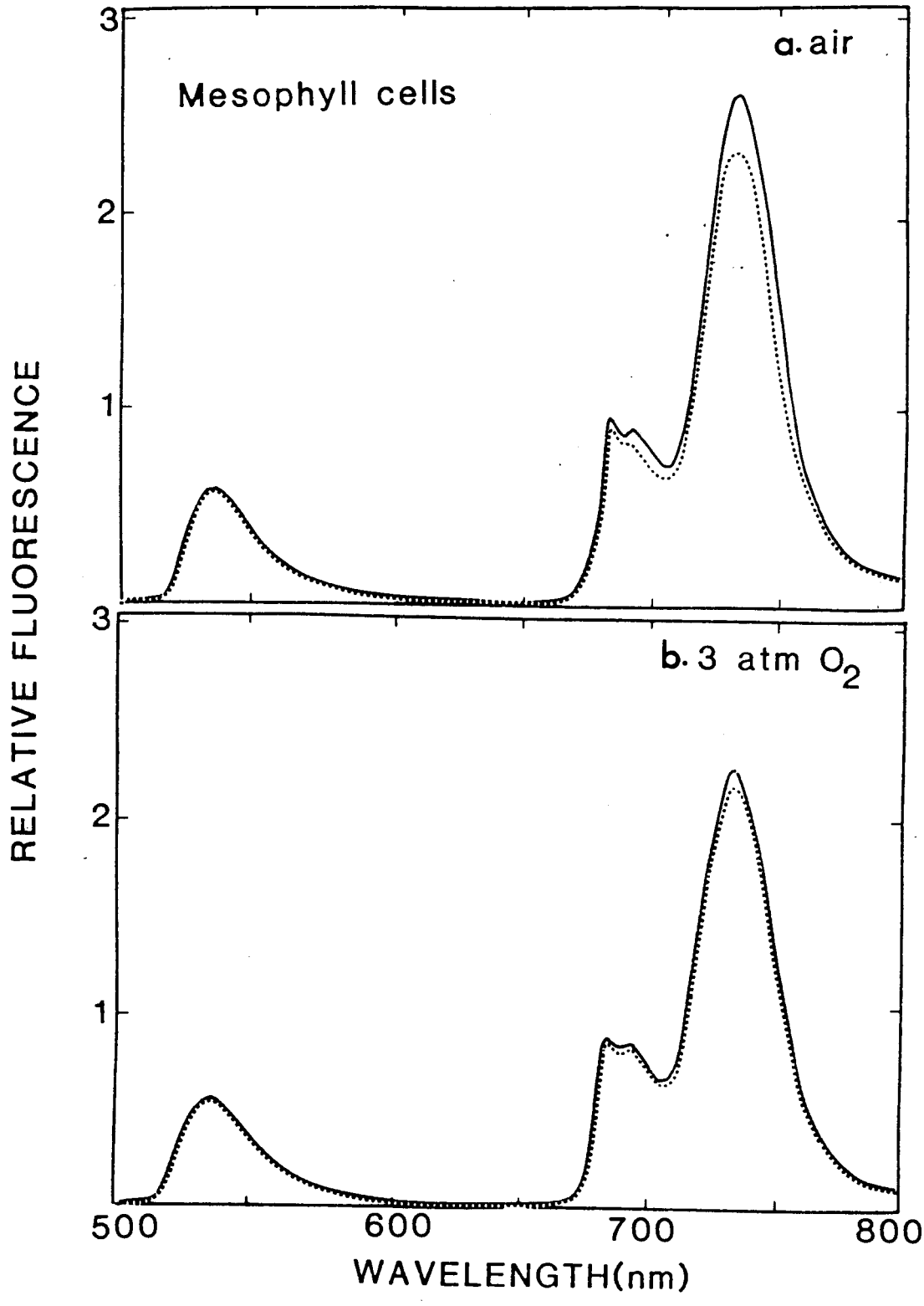

Mesophyll cells
a. air
b. 3 atm O_2
RELATIVE FLUORESCENCE
WAVELENGTH(nm)
3
2
1
500
600
700
800

Figure 3.7: Fluorescence emission spectra at 77K of bundle sheath cells in stateI (——) and in stateII (----). StateI and stateII were obtained by illuminating the cells with lightI (709 nm) and lightII (680 nm) for 4min after which the cells were cooled to 77K.
(a) In air (1atm) (b) In 3 atm O_2
The curves were normalised with respect to fluorescein peak at 535 nm.

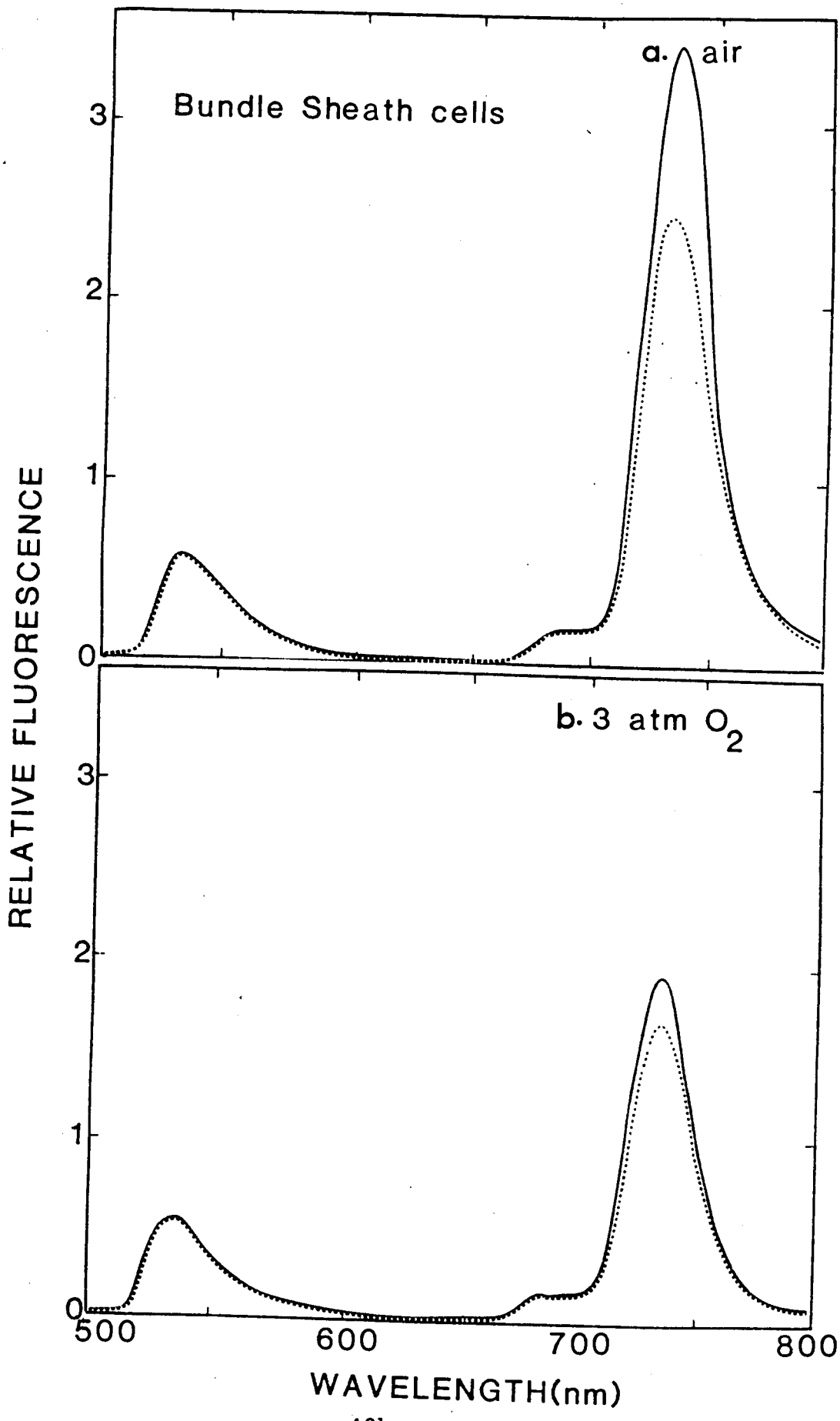

Bundle Sheath cells
a. air
b. 3 atm O_2
RELATIVE FLUORESCENCE
0
1
2
3
500
600
700
800
WAVELENGTH(nm)

Figure 3.8: Low temperature emission spectra of corn leaf segments frozen to 77K in stateI (——) during the illumination with 700 nm (10 nm band width) light or frozen in stateII (---) during the illumination with 680 nm (10 nm band width) light.
(a) top (b) middle (c) bottom parts of the leaf.
Curves were normalised to the 735 nm emission peak.

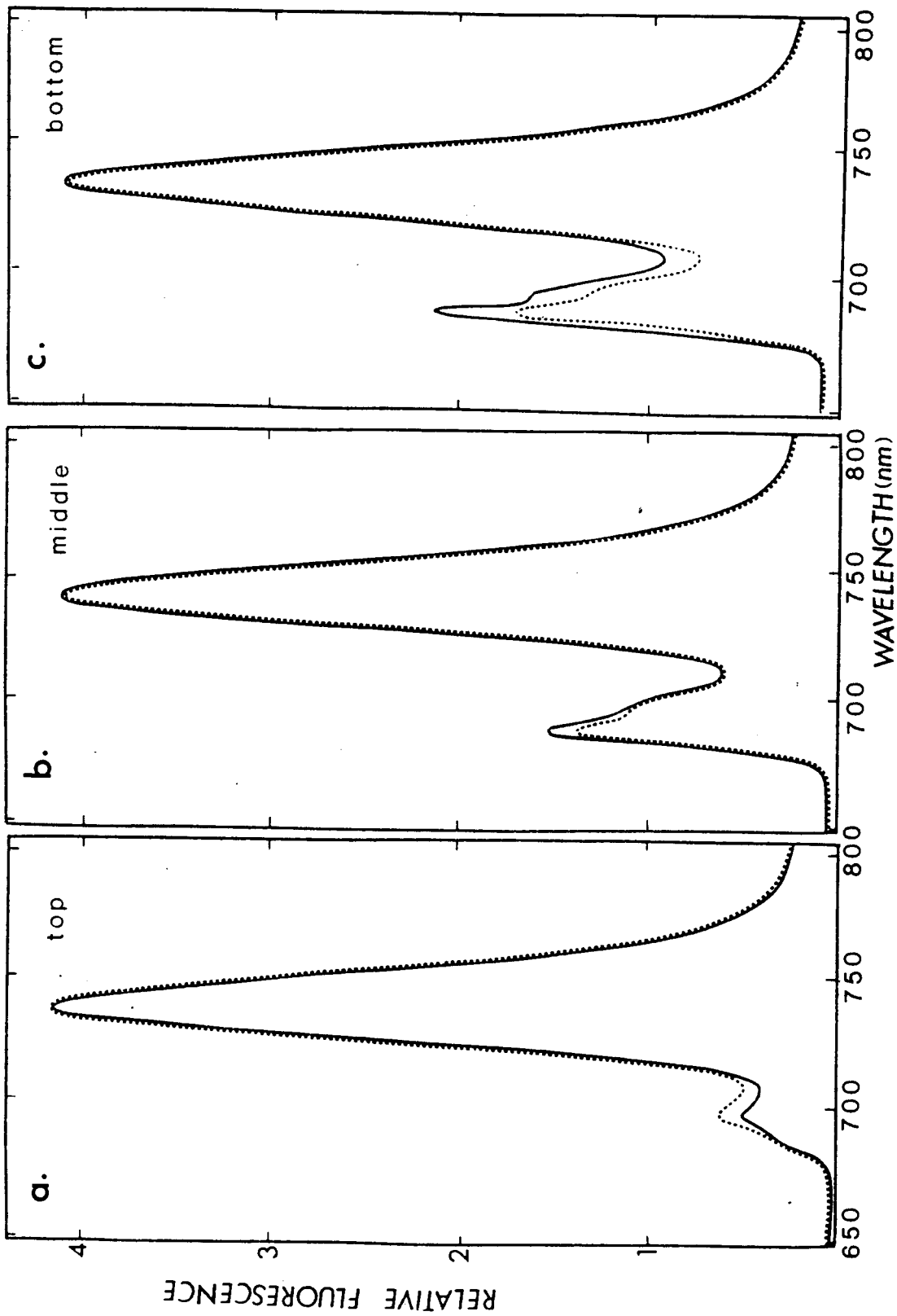

a.
top
b.
middle
c.
bottom
RELATIVE FLUORESCENCE
WAVELENGTH (nm)
650
700
750
800
1
2
3
4

Figure 3.9: Low temperature emission spectra of spinach and lettuce leaf segments frozen to 77K in stateI (——) and stateII (----). Curves were normalised to the 735 nm emission peak.

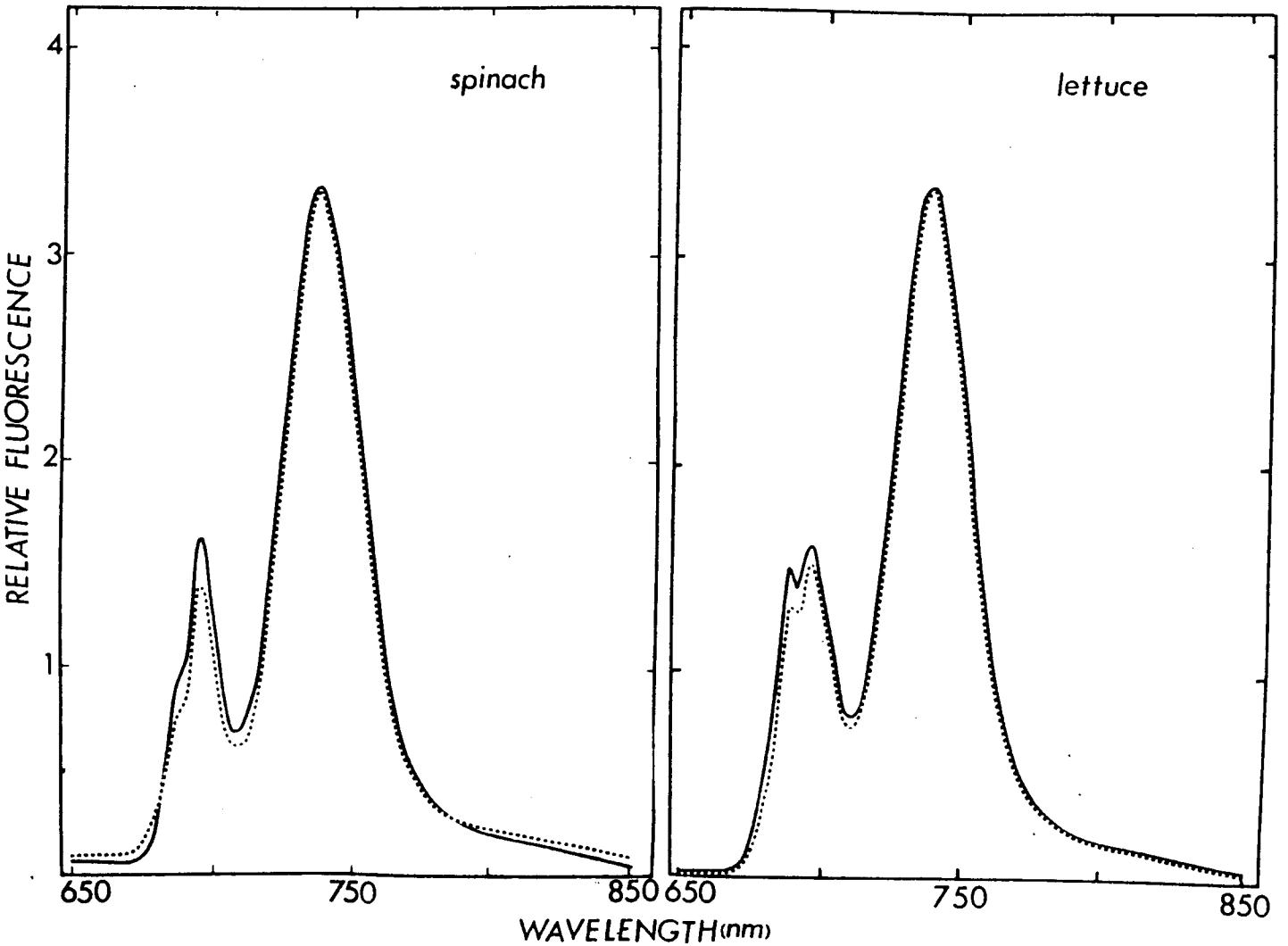

spinach
lettuce
RELATIVE FLUORESCENCE
4
3
2
1
650
750
850
650
750
850
WAVELENGTH(nm)

Table 3.1: PSI/PSII fluorescence ratio of stateI and stateII under air (1 atm) and 3 atm O_2 in mesophyll and bundle sheath cells.

Mesophyll cells:

	air F735/F685	O_2 F735/F685	(air) - (O_2) F735/F685
StateI	2.69±0.02	2.63±0.06	≈0
StateII	2.62±0.02	2.56±0.07	≈0

Bundle sheath cells:

	air F735/F685	O_2 F735/F685	(air) - (O_2) F735/F685
StateI	17.1±0.5	11.2±0.2	5.9±0.7
StateII	14.1±0.3	10.8±0.3	3.3±0.6

Table 3.2 a: PSI/PSII fluorescence ratio of different parts of the corn leaves (developing) which were adapted to stateI and stateII.

	Top F735/F685	Middle F735/F685	Bottom F735/F685
StateI	16.7±0.4	2.7±0.1	1.9±0.1
StateII	14.8±0.2	2.9±0.1	2.5±0.2

Table 3.2 b: PSI/PSII fluorescence ratio of spinach and lettuce leaves were adapted to stateI and stateII.

	Spinach F735/F685	Lettuce F735/F685
stateI	3.5±0.1	2.3±0.1
stateII	4.3±0.2	2.5±0.1

IV. Discussion

4.1 Electron transport activity

During the induction of photosynthesis(5-10 sec) after illumination the variable fluorescence yield is determined mainly by changes in the redox state of the primary electron acceptor (Q) (Bradbury and Baker, 1981). Thus f_p is a measure of the relative number of closed PSII reaction center traps (Q^-/Q). If the number of electrons derived from water splitting is greater than the number of electrons delivered to PSI through electron transport, the variable fluorescence increases. The addition of DCMU blocks inter-system electron transport at PQ, and causes variable fluorescence to reach the maximum value(F_{max}).

The value of relative fluorecence f_p that was observed in both isolated maize mesophyll and bundle sheath protoplasts indicates differences in electron transport in the two types of cells. The value of f_p is a relative measure of the number of electrons that flow through the linear electron transport chain. Therefore the high value of f_p (1.06) in mesophyll cells suggests that there is a high rate of linear electron transport activity. The low value of f_p(0.35) in bundle sheath protoplasts suggests a low rate of linear electron transport activity. The value of f_m (1.69 for mesophyll and 0.40 for bundle sheath cells) also

suggests that PSII activity is low in bundle sheath cells.

Since O_2 always decreased variable fluorescence, it must either act to prevent Q reduction or promote its oxidation. Vidaver et al. (1981b) suggested that a quenching site is located between PQ and PSI chlorophyll(P700) and may possibly be at the copper enzyme PC. O_2 may act as an electron acceptor in the electron transport chain. O_2 quenching of variable fluorescence in maize (C_4) gives results similar to beans (Bruce et al., 1983) which suggests a similar mechanism of O_2 quenching exists in C_3 and C_4 plants.

In C_4 plants, grana-containing mesophyll chloroplasts possess the full complement of various electron transport components, comparable to chloroplasts from C_3 plants. Therefore, O_2 should affect mesophyll cells in a way similar to C_3 chloroplasts. Agranal bundle sheath chloroplasts lack a major portion of PSII reaction centers and its associated chl a/b LHC. In mesophyll cells, there is approximately one Q molecule per 300 chl molecules (both a and b), but in bundle sheath chloroplasts the Q content is lower. In bundle sheath cells, little O_2 quenching of variable fluorescence was observed. These results support the conclusions of Bruce et al. (1983) that the electron transport through PQ and PC is required to observe the f_p quenching by O_2.

Chloroplasts have two different types of PSII reaction center complexes, $PSII_\alpha$ and $PSII_\beta$ (Melis and Homann, 1978). Mesophyll chloroplasts contain both $PSII_\alpha$ and $PSII_\beta$ while bundle

sheath chloroplasts contain only $PSII_{\beta}$(Ghiradi and Melis, 1983) which provides the few electrons needed for the operation of the cyclic electron flow through the cyt b_6-f complex and PSI (Arnon and Chain, 1975). The $f_p$quenching in mesophyll and bundle sheath cells suggests that $PSII_{\alpha}$ plays an active role in linear electron transport.

Increased light intensity appears to partially overcome the quenching effect by O_2. At low light intensity, the electron transport rate through each PSI and PSII reaction center depends on their respective antenna size and is proportional to the incident light intensity. Under saturating light, the electron transport rate is limited by the reoxidation of PQ pool (Ghiradi and Melis,1984). The intensities used in my experiments were less than the saturating light intensity. The O_2 quenching of fluorescence in both maize leaves and mesophyll cells (Figs. 3.3 and 3.4 a) shows that $f_p$increases with increasing light intensity at a given O_2 concentration. Since increasing light intensity causes more electrons to flow through the electron tranport chain, which keeps the PQ pool in a more reduced state, $f_p$increases. In bundle sheath cells, the variation of light intensity did not have much effect on fluorescence quenching due to lack of linear electron transport.

The amount of O_2 released by addition of catalase to chloroplasts is directly proportional to total electron flow through the electron transport system (Popovic et al.,1983). Here, the methylviologen which accept the electrons from the

primary acceptor of PSI, is used as terminal electron acceptor. The amount of H_2O_2 generated in bundle sheath chloroplasts suggested that little O_2 is reduced by the PSI-Mehler reaction. The cyclic electron flow around PSI is present in both mesophyll and bundle sheath chloroplasts. The amount of O_2 released after addition of catalase is negligible in bundle sheath chloroplasts compared to mesophyll chloroplasts. This suggests that the Mehler reaction may not be the main source of H_2O_2 in mesophyll chloroplasts and also suggests that the total electron flow via linear electron transport chain is negligible in bundle sheath chloroplasts.

4.2 State transitions and grana development

StateI is obtained after illumination in lightI (light that is mainly absorbed by PSI) and in this state the transfer of light energy from PSII to PSI is reduced. StateII has been defined as the state obtained after illumination with lightII (absorbed mainly by PSII) and in this state energy transfer from PSII to PSI is enhanced (Wang and Mayers, 1974). The light distribution between PSI and PSII in stateI and in stateII was studied using fluorescence emission spectra at 77K.

Three mechanisms are related to state transitions in the literature. Phosphorylation of the light harvesting chlorophyll a/b protein complex (LHCP), which is controlled by the redox state of PQ, was proposed to induce the state I-II transitions (Chow et al., 1981). The phosphorylation of LHCP in thylakoids

is catalyzed by a protein kinase and the dephosphorylation by a phosphatase. Phosphorylation of LHCP was found to increase the spillover of excitation energy from PSII to PSI, whereas dephosphorylation had the reverse effect. The redox state of PQ controls the activity of the protein kinase. Fork et al. (1983) concluded that the state II-I transition is induced by a cyclic electron flow around PSI in the blue green alga Synechococcus lividus. They also found that the redox level of PQ had no relation to the state I-II transitions in the blue green alga. Biggins et al.(1984) have proposed that a small conformational change in the thylakoid membranes decreases the energy transfer rate from PSII to PSI upon transition to stateI in phycobilisome- containing organisms. This conformational change may be driven by local gradients of proton and counterions, or changes in redox state of charged electron carriers.

State transitions were studied in intact mesophyll and bundle sheath cells which contain different electron transport systems. Grana-containing mesophyll cells have a fluorescence emission spectrum at 77K with maxima at 685, 695 and 735 nm. Mesophyll cells did not show state transitions and the ratio of F735/F685 (Table 3.1) is approximately the same in both states. In mesophyll cells, 3 atm O_2 did not change the fluorescence emission spectra at 77K for either state. In bundle sheath cells, light emitted in the 735 nm band accounts for 90% of the total fluorescence emission. These fluorescence emission spectra suggest that the agranal bundle sheath cells are deficient in

PSII pigment assemblies. State transitions were observed in bundle sheath cells and the ratio of F735/F685 changes remarkably (Table 3.1). These results indicate that linear electron transport need not be necessary to observe the state transitions. Oxygen decreases the fluorescence of PSI in the same manner in both states in both types of cells (Figs. 3.6 and 3.7). The cells in stateI, under 3 atm O_2 give the same fluorescence emission spectra as the cells in stateII obtained with 1 atm air. Thus, oxygen produces the lightII effect when the cells were exposed to lightI in air. The mechanism by which lightI induces the state II-I transition is not clear. It is possible that a cyclic electron flow around PSI is part of the mechanism producing the state transitions. There is no explanation for the ratio of F735/F685 found in bundle sheath cells but it may also be caused by other mechanisms which affect fluorescence intensity.

When different parts chosen from developing maize leaves were exposed to lightI and lightII, the fluorescence emission spectra showed appreciable differences with respect to state transitions. The top part, which contains fully developed mesophyll and bundle sheath cells, produced a small fluorescence peak at 685 nm, while the bottom part which contains young mesophyll and bundle sheath cells gave a large fluorescence peak at 685 nm. It has been demonstrated that chloroplasts of young bundle sheath cells from maize contain grana that are lost during ontogeny with a concomitant loss of PSII activity

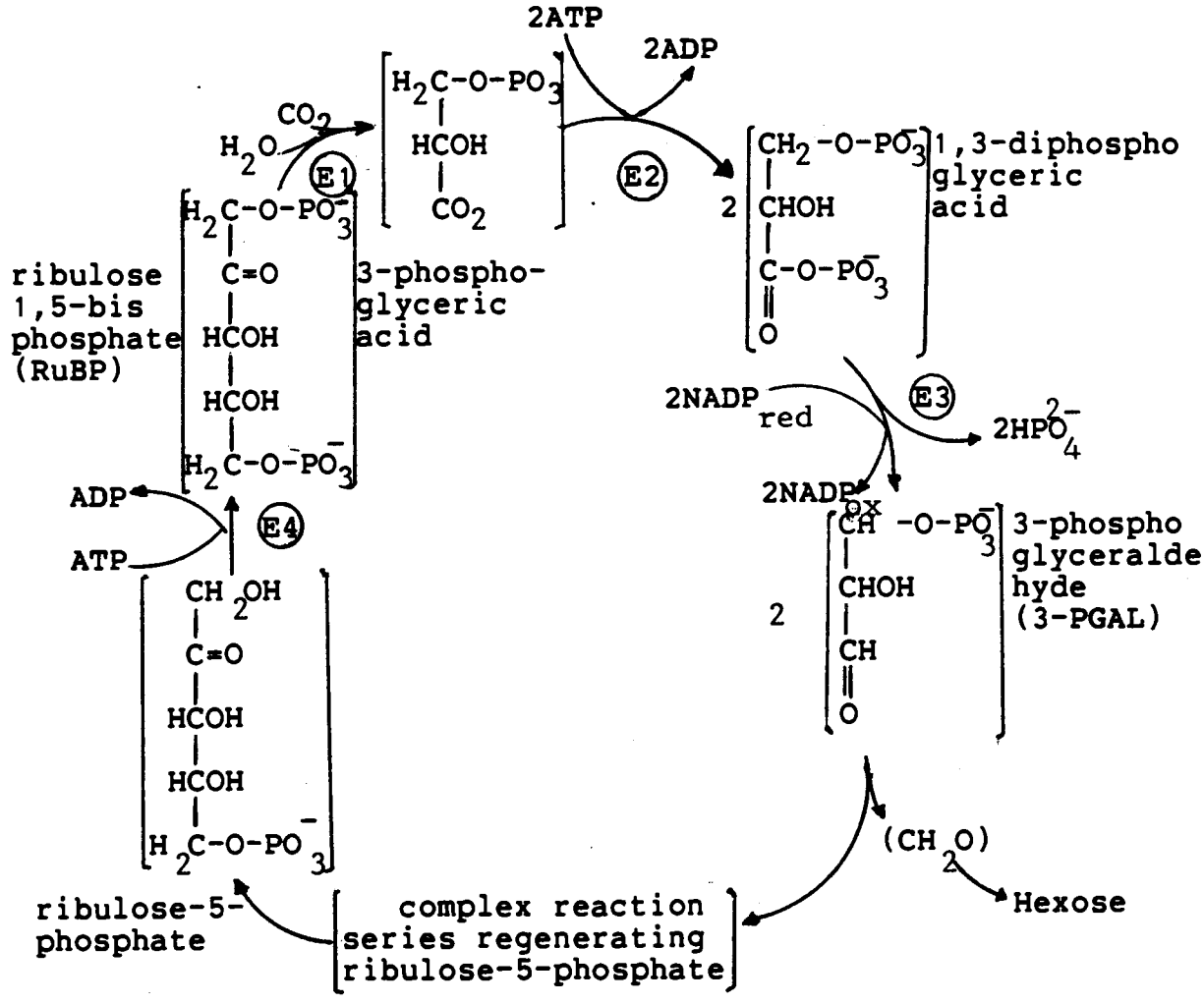
(Bishop, 1974). The fluorescence emission band at 685 nm clearly showed the varying PSII activity which correlates with the degree of grana development along the maize leaf. The ratio of F735/F685 (Table 3.2a) indicate that state transitions can be clearly observed in young parts of leaves of C_4 plants. These results were compared with grana-containing spinach and lettuce leaves. The middle and bottom parts of maize leaves showed state transitions similar to those of spinach and lettuce leaves, which suggests a similar mechanism for state transitions in C_3 and C_4 plant leaves.

V. Conclusion

This thesis presents the results of two fluorescence studies in mesophyll and bundle sheath cells. O_2 quenching of variable fluorescence in grana-containing mesophyll cells and agranal bundle sheath cells suggest that O_2 acts as an oxidant of electron transport carriers which participate in linear electron transport, but not in cyclic electron transport. Increasing light intensity partially overcomes the effect of O_2 quenching of variable fluorescence emitted from PSII.

The state transition was observed in bundle sheath cells. In the state transition process, the relative fluorescence intensity at low temperature associated with PSI changes remarkably compared to fluorescence associated with PSII. Therefore, state transitions may be produced partially by cyclic electron transport around PSI. Fluorescence emission spectra studies may reflect varying degrees of grana structure depending on the development of maize leaves.

Calvin cycle



E 1 = ribulose-1,5-bisphosphate carboxylase

E 2 = 3-phosphoglyceric acid kinase

E 3 = 1,3-diphosphoglyceric acid dehydrogenase

E 4 = ribulose-5-phosphate kinase

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