# THE ROLE OF OXYGEN IN THE REGULATION OF ELECTRON TRANSPORT AND PHOSPHORYLATION IN PHOTOSYNTHESIS

by

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**The role of oxygen in the regulation of electron transport and phosphorylation in photosynthesis.**

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## ABSTRACT

The effect of $O_2$ on electron transport and phosphorylation was studied by investigating the influence of $O_2$ on chlorophyll-a fluorescence and the electric field indicating absorbance change at 518nm ($\Delta A_{518}$).

Chlorophyll-a fluorescence induction in brown algae, green algae and higher plants show differences in relative fluorescence intensity which characterize each plant. Differences were observed only in the absence of 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and are therefore electron transport dependent. The peak in fluorescence induction ($F_p$) was always lower in algae. In all plants $O_2$ strongly quenched $F_p$ indicating the low $F_p$ in algae may be partially accounted for by endogenous $O_2$ quenching. This idea is supported by the high rates of light induced $H_2O_2$ formation seen in chloroplasts of brown algae. Various electron transport inhibitors were used in lettuce chloroplasts to localize the site of $O_2$ quenching. Electron transport through plastoquinone and plastocyanin is required to observe $F_p$ quenching by $O_2$.

The kinetics of $\Delta A_{518}$ *in vivo* was dependent on $O_2$ concentration. Decreased $O_2$ or red background light accelerated the $\Delta A_{518}$ decay while far-red light induced a transient

acceleration. The red light accelerated decay could be restored to the dark rate by increased $O_2$. A linear relationship exists between intensity of red light and $O_2$ pressure required for restoration. N,N$^1$-dicyclohexylcarbodiimide or KCN eliminated all $O_2$ and background light effects. It was concluded that $O_2$ competed with electron transport to photosystem I acceptors to influence the $\Delta A_{518}$ decay. A mechanism involving the $O_2$ sensitive ferredoxin-thioredoxin-reductase activation of ATPase is discussed.

The light induced acceleration of $\Delta A_{518}$ decay in leaves was inhibited by DCMU or by removal of $O_2$. In chloroplasts with added ADP and phosphate and/or reconstructed electron transport, the acceleration was also inhibited by DCMU or lack of $O_2$. The anaerobic inhibition was not observed when hydroxylamine replaced water-splitting as electron donor to photosystem II. Anaerobiosis also inhibited FeCN reduction in chloroplasts and the slow rise of millisecond delayed fluorescence. These results suggest anaerobic inhibition is associated with water-splitting.

## Dedication

This thesis is dedicated to Rom, whom I will never be able to beat at ping-pong.

## ACKNOWLEDGEMENTS

Photosynthetic research is necessarily interdisciplinary and this work would not have been possible without the joint cooperation of Professor William Vidaver and Professor K. Colbow as my co-senior supervisors. I wish to thank them both for their expertise, support, confidence and patience with me over the past few years.

I wish to thank Dr. Radovan Popovic for sharing his experience with me and who was instrumental in "convincing" me of the importance in taking "just one more sample" at 3:30 a.m. Sunday morning.

I thank Dr. Lister for his often sought advice and for serving on my Committee, as well as Dr. Jay Burr, Dr. Mike Thewalt and Dr. Michael Gresser for their assistance and many fruitful discussions.

I am grateful to my fellow graduate students and also to the Machine Shop, Electronics Shop and technical staff in biology and physics for their ideas and assistance.

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# Chapter 1

## Photosynthesis

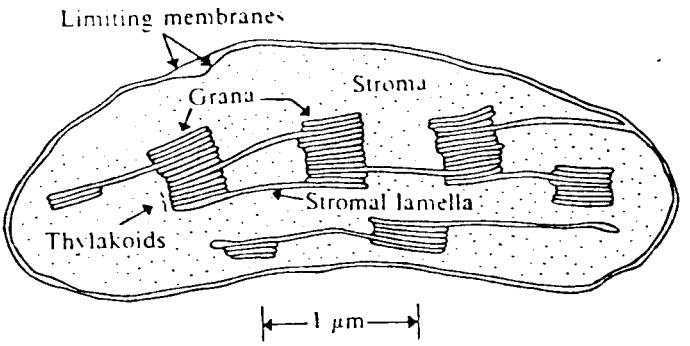
Sunlight energy absorbed by plants is converted into biochemical energy mainly in the form of ATP and NADPH which are used in the assimilation of $CO_2$ and production of carbohydrates [see Zubay (1983) for a recent review]. These carbohydrates are the primary energy storage product of the biosphere and in this way photosynthesis provides fuel for all living states of the biosphere. Aerobic organisms recover energy originating from photosynthesis through oxidative phosphorylation which is roughly the reverse of photosynthesis. In eukaryotic organisms the conversion of light energy and oxidative phosphorylation occur in specialized subcellular organelles, chloroplasts and mitochondria respectively. Photosynthetic organisms possess both organelles.

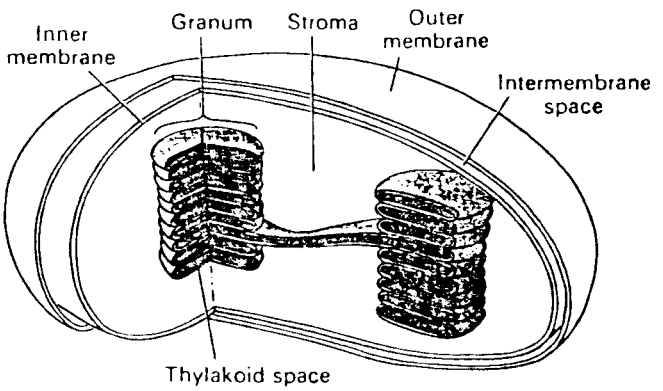
Energy transduction in chloroplasts and mitochondria is dependent on vectorial proton transport across an asymmetric membrane, tightly coupled to electron transport through both intrinsic and extrinsic membrane proteins. In chloroplasts electron transport is driven by light and in mitochondria by NADH. Vectorial proton transport contributes to the proton electrochemical potential ($\Delta\mu_{H^+}$) across the membrane, the energy stored in which is transferred to the terminal phos-

phate ester linkage of ATP by a multisubunit membrane protein (ATPase). ATPase is a proton pump which uses the energy released from hydrolysis of ATP to ADP and phosphate to pump protons across the membrane. However, $\Delta\mu_{H^+}$ can drive ATPase backwards, and ATP formation accompanies the $\Delta\mu_{H^+}$ driven transport of protons across the membrane through a membrane bound subunit of ATPase. Thus in chloroplasts and mitochondria $\Delta\mu_{H^+}$ formed by light induced or respiratory electron transport, respectively, powers the phosphorylation of ADP to ATP catalysed by ATPase. The molecular mechanism by which ATPase couples proton transport to phosphorylation is unknown.

Chloroplasts have two outer membranes and an extensive internal thylakoid membrane (Figure 1.1). The thylakoid membrane is topologically closed with a definite inside and outside effectively dividing the interior of the chloroplast between the intrathylakoid space and the stroma. In higher plants and some green algae the highly folded thylakoid membrane is organized into tight membrane stacks, the grana, which are interconnected to a large extent by stroma lamellae. All components involved in the light induced formation of ATP and NADPH are found closely associated with the thyla-

Figure 1.1 Schematic diagram of a higher green plant chloroplast.
[After; Salisbury and Ross (1978).]




# Diagram of a chloroplast

koid membrane. The enzymes responsible for the fixation of $CO_2$ and carbohydrate formation are soluble proteins of the stroma.

In the light, proton transport causes the pH of the intrathylakoid space to be up to three pH units lower than that of the stroma. The $\Delta pH$ ($pH_{stroma}$ - $pH_{intra}$) and the electrical potential ($\Delta\phi$, negative stroma side) across the membrane are the two components of $\Delta\mu_{H^+}$, the driving force of phosphorylation (Nicholls, 1982), i.e.

$$\Delta\mu_{H^+} = \Delta\phi + \frac{2.3RT}{F}\Delta pH$$

where R = gas constant = 8.31 J $mole^{-1}$ $K^{-1}$

T = temperature (K)

F = Faraday constant = $9.64 \times 10^{10}$ coul $mole^{-1}$ $°K^{-1}$

or expressed in mV at 30°C

$$\Delta\mu_{H^+} = \Delta\phi + 60\,\Delta pH$$

$\Delta\mu_{H^+}$ is the potential available to chloroplast coupling factor ($CF_o$-$CF_1$) ATP synthetase to phosphorylate ADP. On a

molecular level the efflux of at least three protons from the intrathylakoid space to the stroma through $CF_o$-$CF_1$ ATP synthetase is believed to be necessary for the formation of one ATP from ADP and phosphate (Hauska and Trebst, 1977).

Light driven proton transport is coupled to electron transport through both membrane bound and soluble thylakoid proteins. Light energy is initially captured by pigments (chlorophyll-a, chlorophyll-b and carotenoids in higher plants) believed to be associated with three major types of protein complexes. In higher plants these are the P700-chl-a-protein complex, the P680-chl-a-protein complex and the chl-a/chl-b light harvesting protein complex (Prézelin, 1981). Only a very small amount of the total chlorophyll is photochemically active. Two highly specialized chlorophyll-a protein complexes or reaction centres, P680 and P700 undergo reversible photooxidation when excited. These reaction centers form part of photosystem II and photosystem I respectively. The rest of the chlorophyll, approximately 400 molecules per P680 and P700, serve to increase the effective absorbance cross section of P680 and P700 and are termed antennae pigments. When promoted to an excited state by photon absorption antennae chlorophylls lose excitation energy by resonant energy transfer to neighboring chlorophylls. This process competes successfully with fluorescent and non-radiative energy dissipation pathways, and much of

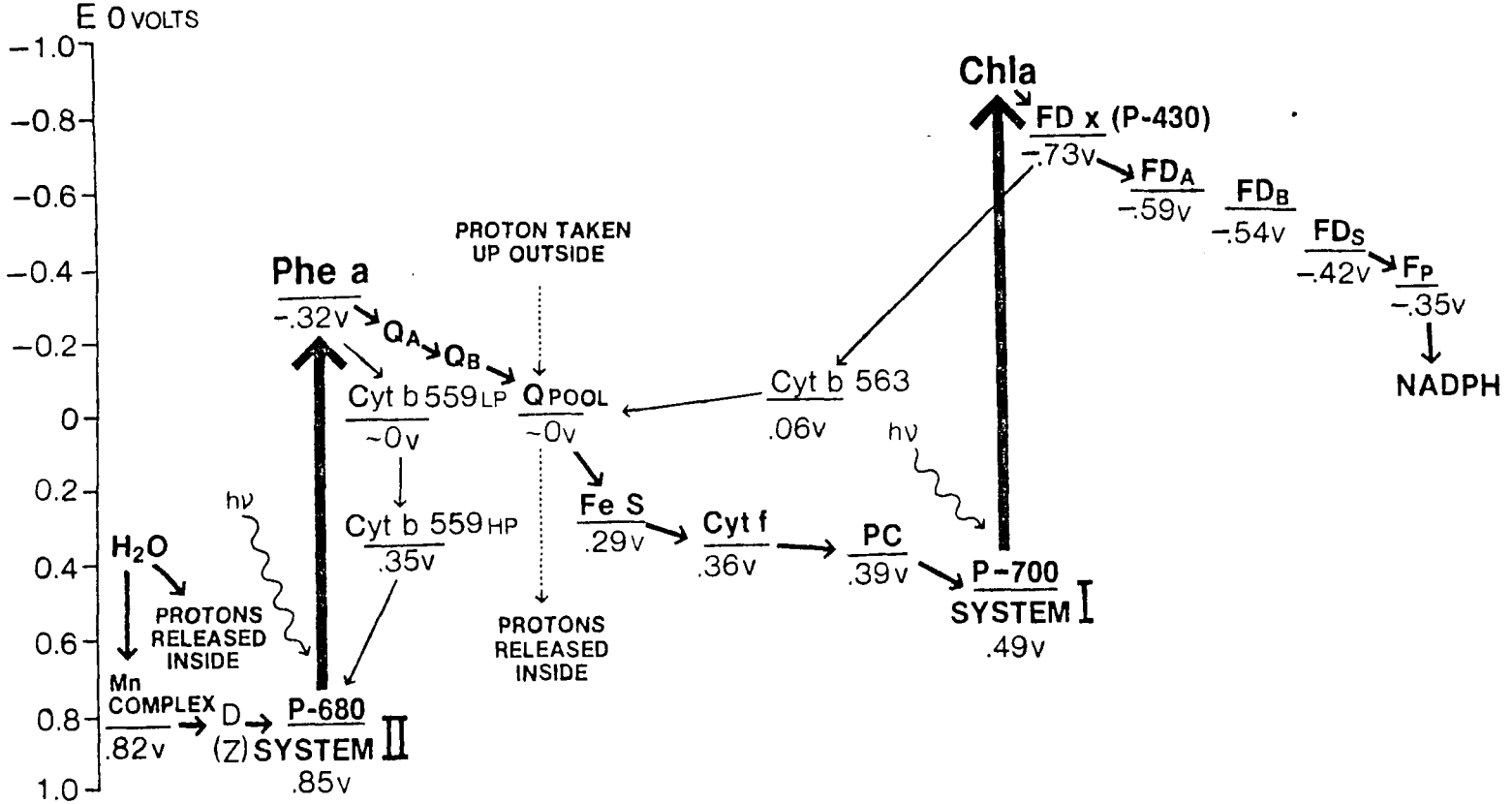
the excitation energy in the antennae pigment bed eventually reaches the reaction centers. P680 and P700 are believed to be chlorophyll-a dimers (Katz et al., 1979), and when excited are strong reducing agents which donate an electron from the aromatic $\pi$ electron system of chlorophyll-a to an electron acceptor. The oxidized chlorophyll-a complex ($P680^+$ or $P700^+$) is a powerful oxidant which extracts an electron from an electron donor molecule. The reactive chlorophyll of photosystem II (P680), differs from that of photosystem I (P700) in that it forms a stronger oxidant on photooxidation. The energy gap from the ground state to the first excited state is also greater in P680 than in P700, therefore photosystem I can utilize longer wavelength light ( > 700nm) than photosystem II.

The orientation of the two photosystems results in a bulk charge separation across the thylakoid membrane when the reaction centers are photooxidized. The $P680^+$ and $P700^+$ are located near the intrathylakoid space side and the reduced primary acceptor molecules near the stroma side of the membrane (Witt, 1979). This asymmetry contributes to $\Delta\mu_{H^+}$ (through $\Delta\phi$) and physically separates the strong reductants and oxidants formed by photooxidation of the reaction centers. The arrangments of photosystem I, photosystem II and the various electron carriers, donors and acceptors with

respect to both redox potential and localization in the thylakoid membrane is shown in Figure 1.2, an interpretation of the classic Hill-Bendall Z scheme. The Z scheme is based on the supposition that photosystem II and photosystem I operate in series. There is much evidence indicating that photosystem II reduces and photosystem I oxidizes the same pool of "intersystem" electron carriers (Duysens and Amesz, 1962). The ultimate source of electrons in this non-cyclic or linear electron transport pathway is the oxidation of water driven by photosystem II and the ultimate sink, reduction of $NADP^+$ to NADPH via photosystem I. Three sites contribute to proton gradient formation in linear electron transport. Oxidation of water driven by $P680^+$ occurs by a four step charge accumulation process (Forbush et al., 1971) on the intrathylakoid side of the thylakoid membrane. Release of one $O_2$ molecule follows the stepwise withdrawal of four electrons from water by intermediates oxidized by $P680^+$ and the concomitant release of four protons to the intrathylakoid space. The reduction of $NADP^+$ to NADPH by electron carriers reduced via photosystem I requires two electrons and one proton which is taken from the stroma. Subsequent reduction of stroma constituents by NADPH will remove one more proton from the stroma. The third site of proton translocation involves the pool of plastoquinone molecules (5-10 per P680) which are ultimately reduced by photosystem II and

Figure 1.2 The Z scheme of photosynthesis.
[After; Zubay (1983).]

# "Z SCHEME" OF PHOTOSYNTHETIC ELECTRON TRANSFER




ABBREVIATIONS

Phe = pheophytin *a*; $Q_A$ and $Q_B$ = two molecules of plastoquinone; $Q_{pool}$ = a large pool of plastoquinones; Fe-S = an iron-sulfur protein (sometimes called M); cyt-b = cytochrome $b_{563}$; cyt-*f* = cytochrome *f*; PC = plastocyanin; Chl = chlorophyll *a*; $FD_x$, $FD_A$, and $FD_B$ = iron-sulfur proteins (bound ferredoxins); $FD_s$ = soluble ferredoxin; FP = flavoprotein (ferredoxin-NADP oxidoreductase). $FD_x$ is sometimes called X or $A_2$; D is sometimes called Z. The component called D has not been identified. Electron transport is coupled with proton translocation across the thylakoid membrane (*dashed arrows*).

oxidized by photosystem I. Reduction of plastoquinone to plastohydroquinone requires two electrons and two protons which are supplied by the stroma. Subsequent oxidation by plastoquinol-plastocyanin oxidoreductase releases two protons to the intrathylakoid space. Plastoquinone is believed to operate as a "proton shuttle" in this way and linear electron transport results in the net "pumping" of four protons into the thylakoids from the stroma per every two electrons transferred from water to $NADP^+$ (Trebst, 1978).

Soluble ferredoxin, an electron acceptor on the reducing side of photosystem I, can reduce intersystem electron carriers via a cytochrome-$b_6$ dependent pathway (Trebst and Avron, 1977; Crofts and Wood, 1978). This pathway forms the basis of cyclic electron transport around photosystem I. Plastoquinone is involved and proton pumping by the "plastoquinone shuttle" is believed to accompany cyclic electron flow. Cyclic electron flow therefore contributes to ATP formation, but not NADPH production. There is some uncertainty in the role of plastoquinol-plastocyanin oxidoreductase in both linear and cyclic electron flow. Plastoquinol-plastocyanin oxidoreductase is a protein complex consisting of cytochrome f, cytochrome $b_6$, a Rieske FeS centre and a bound plastoquinol (Hurt and Hauska, 1982). There is an oxidant induced reduction of cytochrome $b_6$, dependent on the bound plastoquinol, which may indicate the presence of a Q cycle as proposed for the cytochrome b/c complex of mito-

chrondria (Selak and Whitmarsh, 1982; Olsen et al., 1980). A Q cycle (Mitchell, 1976) would involve the sequential or concerted two step oxidation of quinol by components in the plastoquinol-plastocyanin oxidoreductase resulting in the translocation of two protons across the membrane for every electron transferred to $P700^+$.

Carbon assimilation in a $C_3$ plant (Calvin-Benson cycle) requires an input of three ATP and two NADPH per $CO_2$ fixed. The reduction of two $NADP^+$ to two NADPH takes four electrons eventually derived from water and as the translocation of at least three protons is required per ATP produced, three ATP necessitate translocation of nine protons. The required ratio of protons pumped per electron reducing $NADP^+$ ($H^+/e^-$) is therefore at least 9/4 = 2.25. To increase $H^+/e^-$ above the 2 predicted by linear electron transport, the operation of cyclic electron transport and/or a Q cycle have been invoked (Crowther and Hind, 1980). An operative Q cycle in linear electron transport would result in a $H^+/e^-$ of 3 and variable rates of cyclic electron transport in conjunction with linear electron transport could give $H^+/e^-$ from 2 on up.

Another mechanism of increasing the $H^+/e^-$ ratio and still maintaining the one $CO_2$ taken up per one $O_2$ evolved stoichiometry of steady state photosynthesis is by pseudocyclic electron transport (Arnon et al., 1967). In pseudocyclic

electron transport, $O_2$ acts as terminal electron acceptor and $O_2$ uptake equals $O_2$ evolved, hence the terminology "pseudo-cyclic". Electron transport through plastoquinone contributes to proton gradient formation and depending on which side of the membrane $O_2$ is reduced, and the enzymes involved, the uptake of protons associated with reformation of water from $O_2^-$ may or may not contribute to $\Delta\mu_{H^+}$. Pseudo-cyclic electron transport increases $H^+/e^-$ by decreasing the number of electrons reaching $NADP^+$ rather than increasing the proton pumping efficiency and thus differs dramatically from the cyclic electron transport and Q cycle mechanisms.

Photoreduction of $O_2$ by photosystem I resulting in the production of the superoxide anion radical ($O_2^-$) has been shown by Asada et al. (1974). Superoxide dismutase (SOD) catalyses the dismutation of $O_2^-$ to $H_2O_2$ (Elstner, 1982), i.e.

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

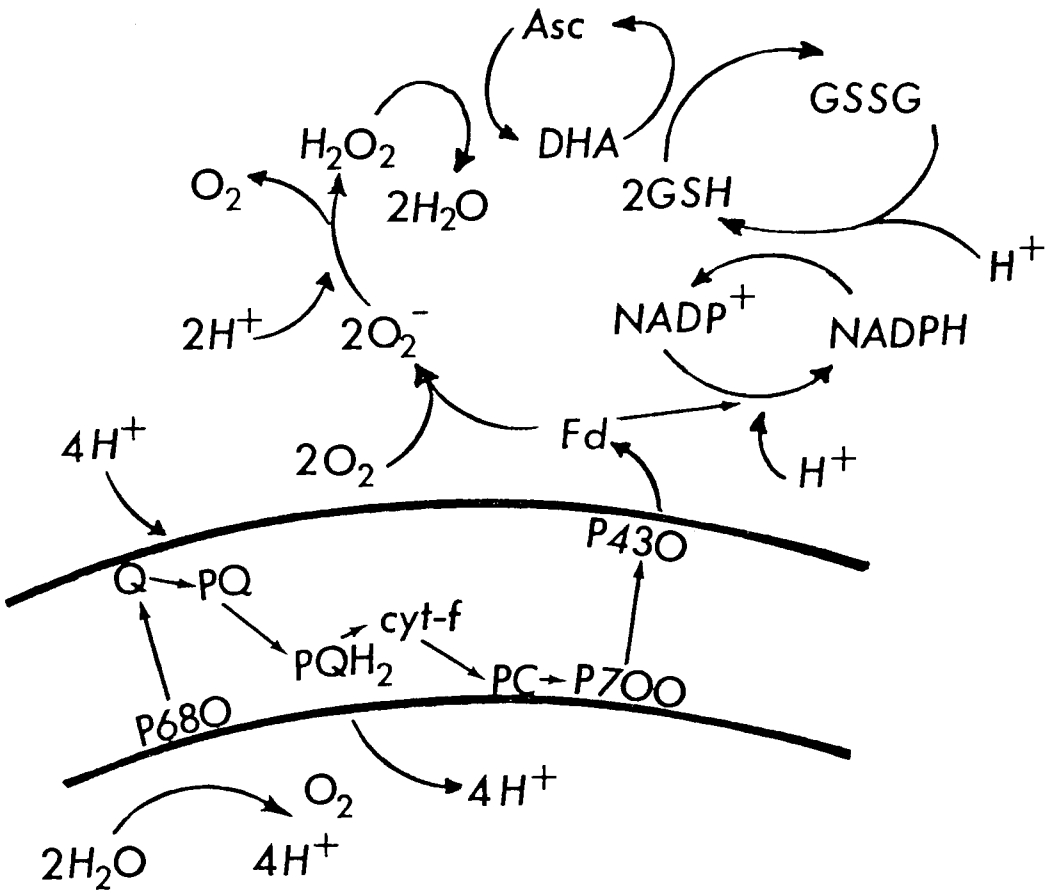
SOD is present in chloroplasts at about 10μM (Asada, 1973). $O_2^-$ and $H_2O_2$ are active species of $O_2$ and their interaction with chloroplast components produces other reactive $O_2$ species, such as the hydroxyl radical (OH•) and excited singlet state $O_2$ ($^1O_2$). These highly reactive molecules oxidize many cell components damaging chloroplasts and leaf tissues

(Nakano and Asada, 1980). $O_2^-$ is converted effectively to $H_2O_2$ by SOD in chloroplasts and $H_2O_2$ can be reduced to $H_2O$ at the expense of ascorbate or by the action of catalase. A pathway for the regeneration of ascorbate from dehydroascorbate via glutathione coupled to light induced NADPH formation has been described (Nakano and Asada, 1981; Halliwell et al., 1981) (Figure 1.3). In addition to the scavenging of active oxygen species it is seen that this pathway would contribute to ATP formation (but not to net $NADP^+$ reduction).

The operation of pseudocyclic phosphorylation has been demonstrated in vitro to be dependent on the concentration of soluble ferredoxin, and can approach rates of ATP formation coupled to $NADP^+$ reduction at physiological concentrations of ferredoxin (Furbank and Badger, 1983). High rates of $O_2$ uptake concomitant with $O_2$ evolution have been measured in chloroplasts, cells and leaves (Canvin et al., 1980; Egneus et al., 1975; Furbank et al., 1982), suggesting a role for pseudocyclic electron transport in vivo. Woo (1983) showed cyclic electron transport to be more important than pseudocyclic electron transport for $CO_2$ fixation in intact chloroplasts, although the conditions used (low chlorophyll concentration, $<3\mu g$ chl $ml^{-1}$) insured that the $O_2$ concentration remained low. At present there is still much controversy over the relative activities of linear, cyclic and pseudocyclic electron transport, as well as the possible operation of a Q cycle, in vivo.

Figure 1.3 Pseudocyclic electron transport incorporating the NADPH dependent pathway for formation of water from $H_2O_2$ as described by Nakano and Asada.

Abbreviations: Asc, ascorbate; DHA, dehydroascorbate; GS, glutathione; Fd, ferredoxin; PQ, plastoquinone; PC, plastocyanin; cyt-f, cytochrome f.


Asc
GSSG
$H_2O_2$
DHA
$O_2$
$2H_2O$
2GSH
$H^+$
$2H^+$
$2O_2^-$
$NADP^+$
NADPH
$4H^+$
$2O_2$
Fd
$H^+$
P430
Q
PQ
cyt-f
$PQH_2$
PC
P700
P680
$4H^+$
$O_2$
$2H_2O$
$4H^+$

The objective of this thesis was to determine the influence of molecular $O_2$ on the primary processes of photosynthesis *in vivo*, in an attempt to localize sites of action and possible regulatory mechanisms involving $O_2$. *In vivo* studies are hampered by the complexity and inaccessibility of the photosynthetic apparatus. When possible, effects studied *in vivo* were also studied in partial photosynthetic systems and whole chloroplast preparations which are less complex and more accessible to selective electron transport donors, acceptors and inhibitors and phosphorylation inhibitors and uncouplers.

Results presented in this thesis indicate at least two sites of $O_2$ influence.

1. $O_2$ acts as a strong oxidant of electron transport carriers *in vivo*, indicative of a role in both an energy dissipation pathway and regulation of ATP/NADPH ratios via pseudocyclic electron transport. The oxidation of electron carriers by $O_2$ also appears to influence the electron transport dependent regulation of $CF_O$-$CF_1$ ATPase activity *in vivo*.

2. The presence of $O_2$ appears necessary for photosystem II activity when water is the electron donor to photosystem II, suggesting a requirement for $O_2$ by the watersplitting system.

# Chapter 2

## Materials and Methods

### 2.1 Selection of Plant Species

#### 2.1.1 Higher Plants

The choice of plant species for a particular study was contingent on both availability and experimental convenience. Locally grown lettuce was used extensively for both *in vivo* and *in vitro* experiments. As experimental reproducibility is dependent on the growth conditions and history of the plant material studied, market lettuce from one local source was used which proved to give consistent results.

Studies with whole chloroplasts capable of high rates of $O_2$ evolution (> 75μM $O_2$ $mgchl^{-1}$ $hr^{-1}$) using $CO_2$ as substrate could not be done easily with lettuce. Whole chloroplasts have only been successfully isolated using mechanical methods from a limited number of higher plant species, spinach giving the highest yields of whole chloroplasts (Walker, 1980). No commercial supply of consistently fresh spinach was available which made it necessary to construct a facility for growing spinach. The spinach was grown according to the method of Walker (1980) with some minor modifications. The plants were kept individually in 1.5 l pots in a 3:1:6 mixture of perlite, sand and peat. Fifty pots were constantly supplied with a nutrient growth solution (Table 2-1) via spaghetti

tubing from a 60 l. container filled weekly. The pots were enclosed in a ventilated dark room with artificial lighting, 40% cool-white fluorescent and 60% incandescent. Day length was 8 hr and the light intensity was 50 W $m^{-2}$; plants were harvested after 60 days.

For some comparative in vivo studies, bush bean (Phaeseolus vulgaris) was greenhouse grown in soil under fluorescent augmented natural illumination. The primary leaves were harvested after 2-4 weeks.

### 2.1.2 Marine Algae

The use of marine algae was strictly regulated by the availability of local species. Fucus vesiculosis, Ulva species, Macrocystis integrifolia, Nereocystis luetkeana and Laminaria saccharina were harvested weekly and kept in an aerated sea water tank at 15°C under artificial illumination (cool-white fluorescent tubes, intensity 10 W $m^{-2}$) with a 12 hr day. In an effort to control experimental variability, algae were consistently collected from well defined sites at Barnet Marine Park in Port Moody and at Barkley Sound on the west coast of Vancouver Island.

## 2.2 Chloroplast Isolation Techniques

### 2.2.1 Whole Chloroplast Isolation

Intact Type A chloroplasts (Hall, 1972) were isolated by the method of Walker (1980). Fifty grams of spinach leaves

were chopped into 2-5mm squares and then homogenized for 1-2s by a razor blade equipped blender (Kannangara et al., 1977) in 500ml of semifrozen grinding media containing 0.36M sorbitol, 15mM $Na_4P_2O_7$ and 5mM $MgCl_2$, adjusted to pH 6.5 with HCl. The homogenate was filtered through 8 layers of cheesecloth soaked in ice cold grinding media and centrifuged at 0°C in eight 50ml clear plastic tubes by the following procedure. The centrifuge was accelerated quickly to 5000 g, held for 10s then decelerated within 30s. The supernatant was discarded and the pellet washed with ice cold resuspending medium consisting of 0.4M sorbitol, 50mM Hepes, 2mM EDTA, 1mM $MgCl_2$, 1mM $MnCl_2$, 5mM pyrophosphate and 0.5mM $KH_2PO_4$ adjusted to pH 7.6 with KOH. The pellet was then resuspended at 0°C to approximately 5ml total volume in this medium typically resulting in a total chlorophyll concentration (chl-a and chl-b) of 1-2mg chl $ml^{-1}$ as determined by the method of Arnon (1949). The chloroplasts were assayed for $O_2$ evolution with a Clarke-type $O_2$ electrode at 22°C in resuspending media, chlorophyll concentration 100μg chl $ml^{-1}$, in the presence of 10mM $HCO_3$ and catalase with an activity of 1000 units $ml^{-1}$. After an initial lag phase $O_2$ evolution rates were typically 60-80μM $O_2$(mg chl)$^{-1}$ $hr^{-1}$. The chloroplasts were judged 60-75% intact by the ferricyanide (FeCN) method (Avron and Gibbs, 1974). These chloroplasts retained their activity for 3-4 hr at 0°C.

### 2.2.2 Broken Chloroplast Isolation

Broken Type C chloroplasts (Hall, 1972) were sometimes prepared by the osmotic rupture of isolated whole chloroplasts. Whole chloroplasts were resuspended in 50ml of media without sorbitol and stirred for 2 min, centrifuged at 5000 g for 30s and resuspended in media containing 0.4M sorbitol. Broken chloroplasts were also prepared by the following technique. The lettuce or spinach leaves were chopped and homogenized in grinding media as for whole chloroplast isolation except they were homogenized for 6-8s. The filtration and centrifugation steps were similar, however the centrifuge was held at 5000 g for 30s. The pellet was then resuspended in 50ml of 0.2M sorbitol, 2mM $MgCl_2$, 20mM Tricine and 10mM KCl adjusted to pH 8 with NaOH. The suspension was again centrifuged at 5000 g for 30s and the pellet resuspended in approximately 5ml of the same media yielding a chlorophyll concentration of 1mg chl $ml^{-1}$. These chloroplasts were judged 100% broken by the FeCN test and were stable for 5-6 hr at 0°C. In studies where hydroxylamine was used to replace watersplitting as electron donor to photosystem II the procedure for hydroxylamine inhibition was that used by Izawa and Ort (1974). During isolation of broken chloroplasts the pellet was resuspended in 50ml of resuspending medium with 5mM hydroxylamine adjusted to pH 7. This suspension was stirred

at 0°C in the dark for 20 min then centrifuged and the pellet resuspended with 5ml of medium containing 5mM hydroxylamine at pH 7.6. This procedure completely inhibited FeCN dependent $O_2$ evolution (Figure 2.1).

## 2.3 In Vivo studies, sample preparation

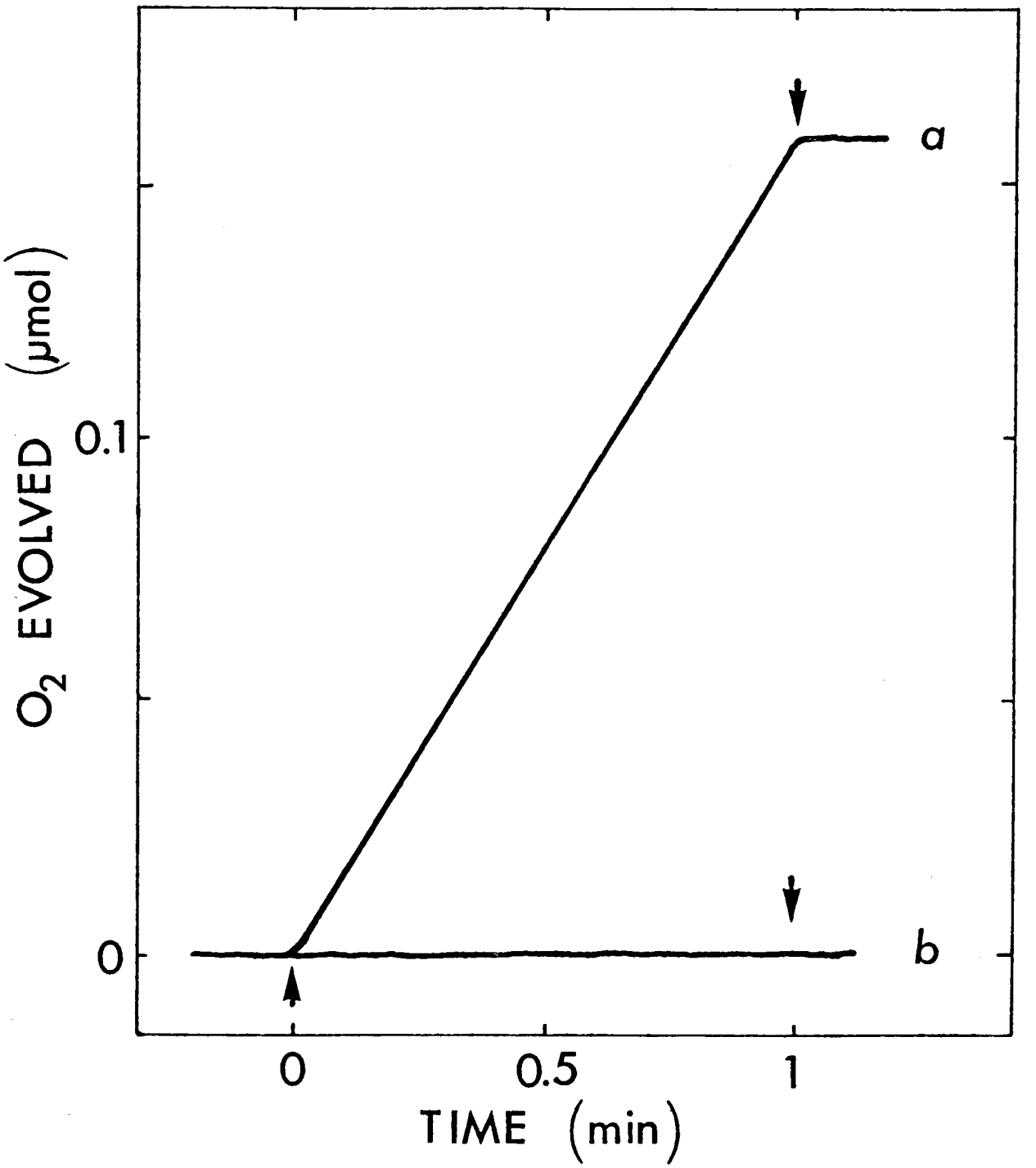
All in vivo studies of chlorophyll-a fluorescence and 518nm absorbance changes were done using 8mm diameter discs cut from leaves or thallus with a cork borer. In studies using electron transport and phosphorylation inhibitors, leaf discs were floated on aqueous solutions of either 15μM 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 1mM KCN (in 30mM tricine adjusted to pH 7.8 with NaOH) or 1mM N,N'-dicyclohexylcarbodiimide (DCCD) for 1 h in the dark prior to measurement. Algal thallus discs were inhibited with DCMU by suspending them in seawater solutions of 1μM DCMU for 1 hr in the dark prior to measurement. Discs from the brown algae (Fucus vesiculosis, Macrocystis integrifolia) were first suspended in seawater for 15 min before the DCMU treatment to partially separate the mucopolysaccarides which are exuded by the disc and interfere with the DCMU inhibition.

## 2.4 In Vitro studies, sample preparation

### 2.4.1 $\Delta A_{518}$ and FeCN reduction experiments

Chloroplasts were kept at a concentration of 2mg chl $ml^{-1}$ on ice in the dark until an aliquot was taken to make up

Figure 2.1 $O_2$ evolved versus time measured with a Clark electrode system. a) chloroplasts (50 μg chl) with 2 mM FeCN in resuspending media without sorbitol. b) hydroxylamine treated chloroplasts, conditions as in a). Light on (↑), 150 W tungsten-iodide lamp covered by Corning 2-62 red cutoff filter adjusted to 300 W $m^{-2}$ at sample. Light off (↓).


O2 EVOLVED (μmol)
0.1
0
a
b
0
0.5
1
TIME (min)

a final assay solution of 1mg chl $ml^{-1}$ for $\Delta A_{518}$ or 250μg chl $ml^{-1}$ for FeCN reduction determinations. In some $\Delta A_{518}$ experiments the assay solution contained 0.5mM pyocyanine or 2mM methyl viologen, as electron acceptors at photosystem I, and when applied the ADP and phosphate concentrations were 20mM. DCMU had a concentration of 15μM when used.

In FeCN reduction rate determinations the FeCN concentration was 5mM.

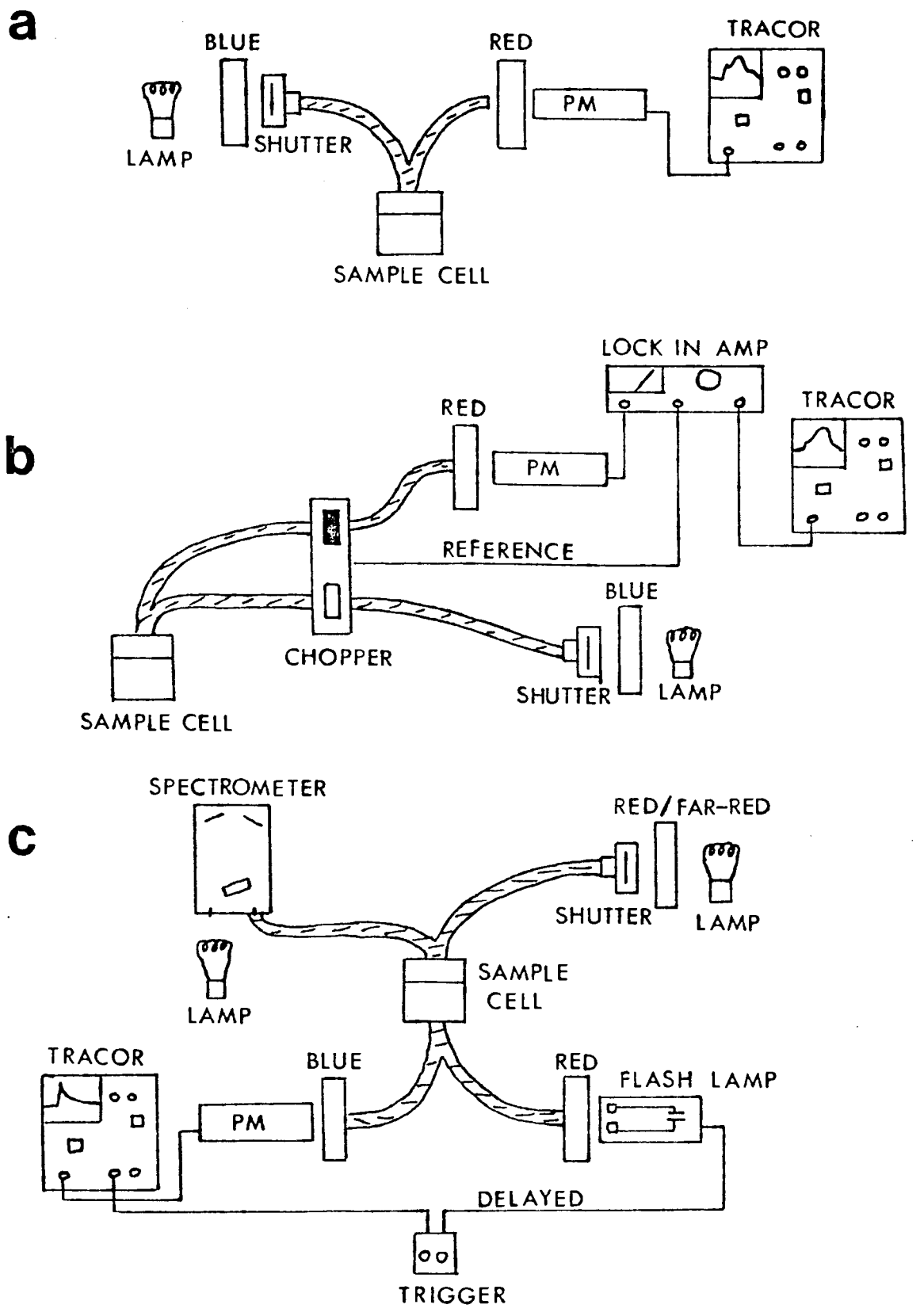
### 2.4.2 Fluorescence induction experiments

The stock chloroplast suspension contained 200μg chl $ml^{-1}$ and was kept on ice in the dark. A 50μl aliquot of stock suspension was added to 2ml of medium and then expressed through a 5μm pore size millipore filter with a hypodermic syringe. Chloroplast samples retained on the filter thus contained 10μg chl. In some cases the 2ml of medium contained 5μM DCMU, 1μM dibromothymoquinone (DBMIB), 0.5mM 2-5 dimethylquinone (DMQ) or 100μg histone (Sigma Chemical).

## 2.5 Apparatus for Fluorescence and Absorption Change Spectroscopy

A system was developed capable of measuring prompt and delayed fluorescence induction or absorbance changes induced by constant or pulsed actinic light compatible with hyperbaric $O_2$ and both *in vivo* and *in vitro* samples (Figure 2.2). The sample compartment is a smaller version of the stainless steel

Figure 2.2 Apparatus for fluorescence and absorbance change spectroscopy. a) prompt fluorescence induction apparatus. b) delayed fluorescence induction apparatus. c) $\Delta A_{518}$ apparatus. See text for additional details.


a
BLUE
RED
TRACOR
PM
LAMP
SHUTTER
SAMPLE CELL
b
LOCK IN AMP
RED
TRACOR
PM
REFERENCE
BLUE
CHOPPER
SHUTTER
LAMP
SAMPLE CELL
c
SPECTROMETER
RED/FAR-RED
SHUTTER
LAMP
LAMP
SAMPLE
CELL
TRACOR
BLUE
RED
FLASH LAMP
PM
DELAYED
TRIGGER

pressure cell described by Chandler and Vidaver (1969). The chamber has two opposing fiber optic compatible ports with parallel lucite windows. When assembled in the cell the ends of the fiber optics are separated by 8-10mm. The bottom window has a 1mm deep 9mm diameter recess into which an 8mm diameter leaf or thallus disc will fit.

For $\Delta A_{518}$ or FeCN reduction rate experiments 35µl of chloroplast suspension, 1mg chl $ml^{-1}$ or 200µg chl $ml^{-1}$, was placed on a 1mm nylon grid, 0.45mm thick and 8mm in diameter held in the recess in the bottom window. The chloroplast sample prepared in this way had a large surface area to volume ratio and equilibration to a new $O_2$ concentration was enhanced. In fluorescence studies 2ml of a chloroplast suspension (50µg chl $ml^{-1}$) was expressed with a hypodermic syringe through a 5µm pore size millipore filter, as previously described. This resulted in a thin layer of chloroplasts on the filter which could be placed in the sample chamber in the same manner as a leaf or thallus disc. This technique resulted in a very fast equilibration of the chloroplasts to different $O_2$ concentrations.

$O_2$ and/or $N_2$ were supplied to the sample cell by two high pressure Linde specialty gas mixture tanks, one containing $O_2$ with 280µl per liter $CO_2$ and the other $N_2$ with 285µl per liter $CO_2$. A 1.5 l stainless steel chamber was arranged in parallel with the sample cell to allow mixing of the $O_2$ and

the $N_2$ to any desired $O_2$ partial pressure and gas total pressure up to 150 atm. Anaerobiosis was effected *in vivo* or *in vitro* by passing $N_2$ gas with 285µl $l^{-1}$ $CO_2$ over the sample at a very slow rate for 20 min.

### 2.5.1 Fluorescence induction measurements

#### 2.5.1.1 Prompt Fluorescence

The fluorescence induction apparatus (Figure 2.2a) employed a 650W tungsten-iodide lamp shielded by a blue (Corning 4-96) filter and adjusted to 10 W $m^{-2}$ at the sample. Actinic light was led through one arm of a bifurcated fibre optics system to the top of the sample compartment while fluorescence was detected by an EMI 9558BQ photomultiplier after passing through the other fibre optics arm and 2 red filters (Corning 2-64). The bottom window of the sample cell was blocked off. Output from the photomultiplier was stored in the memory of a Tracor Northern Model TN 1710 signal averager. The fluorescence $F_p$-level (after 0.5s) and the $F_o$-level (after $10^{-9}$s, see chapter 3) were determined in digital form. Fluorescence induction time courses were recorded from the signal averager memory with a Hewlett-Packard x-y recorder. Light flashes were controlled using a Compur electronic shutter with an opening time of 2 ms, permitting reasonably precise $F_o$-level extrapolations.

#### 2.5.1.2 Delayed Fluorescence

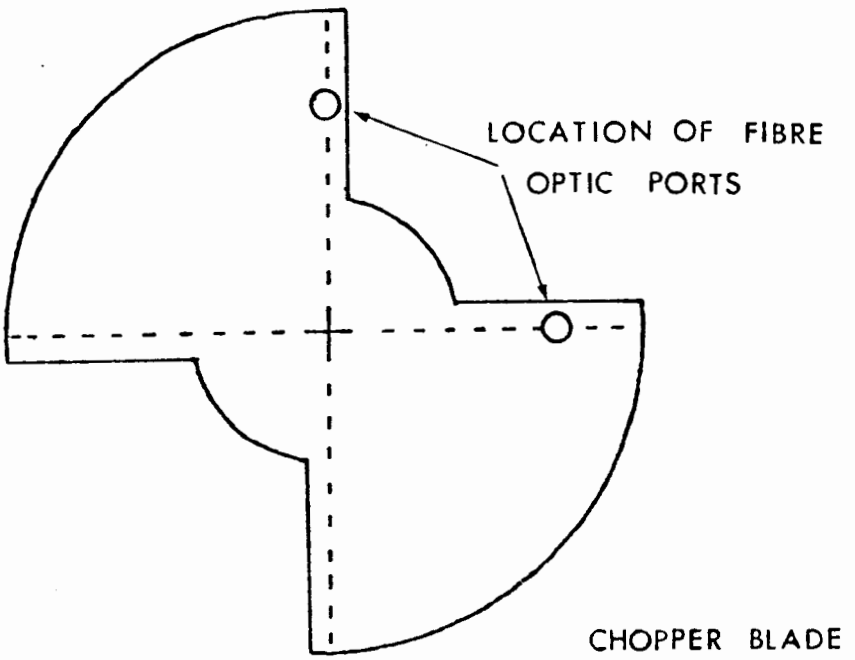
Delayed fluorescence was measured with the phosphoroscope

setup shown in Figure 2.2b. The phosphoroscope used a Princeton Applied Research optical chopper, model BZ-1, with a modified chopper blade and housing (Figure 2.3). The chopper blade had two equal opposing sectors each consisting of 1/4 of the total blade area plus an additional 0.5 cm overlap on both sides of each sector. Two fibre optic ports were mounted equidistant from the chopper blade axis 45° relative to each other. When operated at 125 Hz each fibre optic port was alternately exposed for 3.5ms and covered for 4.5 msec and at no time were both fibre optic ports exposed simultaneously.

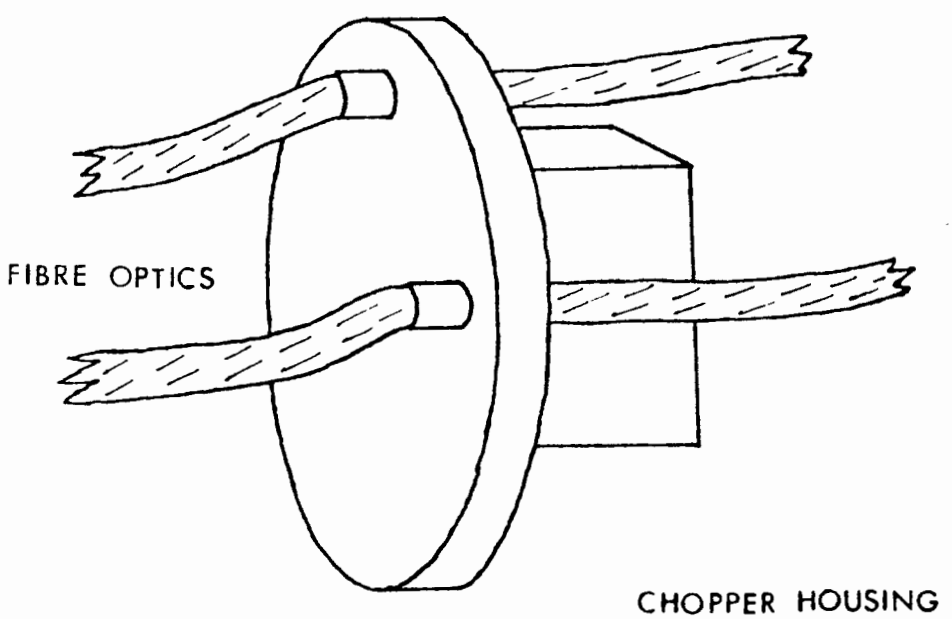
Actinic light, from a 650W tungsten-iodide lamp covered with a Corning 4-96 blue-green filter, was led to the sample chamber through one arm of a bifurcated fibre optic bundle after passing through one of the chopper's fibre optic ports. The other arm of the bifurcated fibre optic bundle carried fluoresence emitted by the sample through the second fibre optic port of the chopper and ultimately to the EMI 9558BQ photomultiplier protected by a Corning 2-64 red cutoff filter. Thus only delayed fluorescence emitted from 0.5ms to 4ms after the actinic light was blocked by the chopper blade was detected by the photomultiplier. This measurement cycle was repeated every 8 ms. The photomultiplier output was fed into a Princeton Applied Research lock-in amplifier, model HR-8. The chopper supplied a reference signal to the lock-in amplifier which was used to tune the amplifier to the modulated

Figure 2.3 Light chopper used for measuring delayed fluorescence induction transients. a) modified chopper blade showing relative location of fibre optic ports. b) chopper housing showing fibre optic ports.

a


LOCATION OF FIBRE
OPTIC PORTS
CHOPPER BLADE

b


FIBRE OPTICS
CHOPPER HOUSING

signal from the photomultiplier. The output of the lock-in amplifier was therefore a continuous measure of the average amplitude of delayed fluorescence emission from 0.5 - 4ms after the actinic light was blocked by the chopper blade each measurement cycle. This output was recorded by a Tracor Northern TN 1710 signal averager which could output data to a Hewlett-Packard X-Y recorder.

### 2.5.2 $\Delta A_{518}$ and FeCN reduction rate determinations

The 518nm or 415nm (for FeCN reduction measurements) measuring beam from a 6V 100W tungsten ribbon filament lamp, dispersed by a 3/4 meter Spex Industries Czerny-Turner spectrometer (1mm slit) was led through one arm of a bifurcated fibre optic bundle to the top of the sample chamber and adjusted to 4 mW $m^{-2}$ or 1 mW $m^{-2}$ at the sample, respectively (Figure 2.2c). The transmitted measuring beam was accumulated by a bifurcated fibre optic bundle at the bottom window, one arm leading to an EMI 9558BQ photomultiplier shielded by a blue (Corning 4-96) filter. Actinic light was led to the bottom window of the sample chamber through the other fibre optic arm, and was supplied either by a 100V 650W tungsten-iodide lamp covered by a red (Corning 2-64) filter for FeCN reduction studies, or by an EG&G FX224 metal can type flash bulb shielded by a red (Corning 2-64) filter for $\Delta A_{518}$ pulse studies. The flash bulb was used in conjunction with an EG&G FY604 litpac and a Maxwell 10$\mu$F 2000V fast discharge capacitor resulting in a pulse width at half height of 3$\mu$s.

Either red (Corning 2-64 filter) or far-red (Baltzers B-40 718nm interference filter) background light could be supplied to the sample chamber through the free arm of the top bifurcated fibre optic bundle from a 650W tungsten-iodide lamp. The photomultiplier output was signal averaged for $\Delta A_{518}$ studies by a Tracor Northern TN1710; typically 8 to 40 individual measurements were averaged depending on the time base and filter time constant used. In pulse studies, the signal averager was triggered at an adjustable time interval before the flash lamp was triggered by a two output dual pulse trigger box. The frequency of the dual pulses could be varied from 10Hz to less than $10^{-3}$Hz and the time interval between the pulses from 10ms to 100ms.

## 2.6 Experimental Protocol, Sample Pretreatment

### 2.6.1 Fluorescence induction determinations

In fluorescence induction studies the experimental measurement regime was as follows. All samples were dark-adapted for at least 1 hr prior to measurement. The sample was placed in the sample holder and the $F_o$-level determination in air was measured with a 50ms flash of actinic light. After a further dark adaption of 1 min a $F_p$-level in air was obtained with a 10s light flash. The sample was now dark adapted for 6 min after which the sample cell was flushed with the gas mixture to be used and then slowly pressurized ( 1

min) to acquire the $O_2$ concentration desired. After allowing the sample to equilibriate with the new $O_2$ concentration for 2 min in the dark the measurement pattern was repeated. A fresh sample was used for each different $O_2$ concentration.

In delayed fluorescence induction studies, the same protocol was followed except there was no $F_o$ level determination.

2.6.2 $\Delta A_{518}$ determinations

In $\Delta A_{518}$ studies leaves were dark adapted for 1 hr before measurement. In most experiments the sample was exposed to the actinic flash at the desired flash frequency for 5 min prior to data acquisition. In some experiments $\Delta A_{518}$ was determined in air and then again after a change in the $O_2$ concentration. In these experiments the actinic flashes were left running at the frequency desired during the gas change and, as in fluorescence studies, the sample was allowed to equilibrate for 2 min with the new $O_2$ concentration before data was acquired.

TABLE 2-1

Nutrient Solution for Spinach Water Culture

| Solution | Stock Concentration | Volume (ml) per 60l. nutrient solution |
|---|---|---|
| $KNO_3$ | 1 M | 360 |
| $Ca(NO_3)_2$ | 1 M | 240 |
| $MgSO_4$ | 1 M | 120 |
| $KH_2PO_4$ | 1 M | 60 |
| $MgCl_2$ | 1 M | 240 |
| Trace elements | B,Mn,Zn,Cu,Mo | 60 |
| NaFe - EDTA | 3.86 gm/250 ml | 60 |

| Trace Elements | Quantity mg in 250 ml $H_2O$ |
|---|---|
| $H_3BO_3$ | 715 |
| $MnCl_2$ | 452 |
| $ZnSO_4$ | 55 |
| $CuSO_4$ | 20 |
| $NaMoO_4$ | 7.25 |

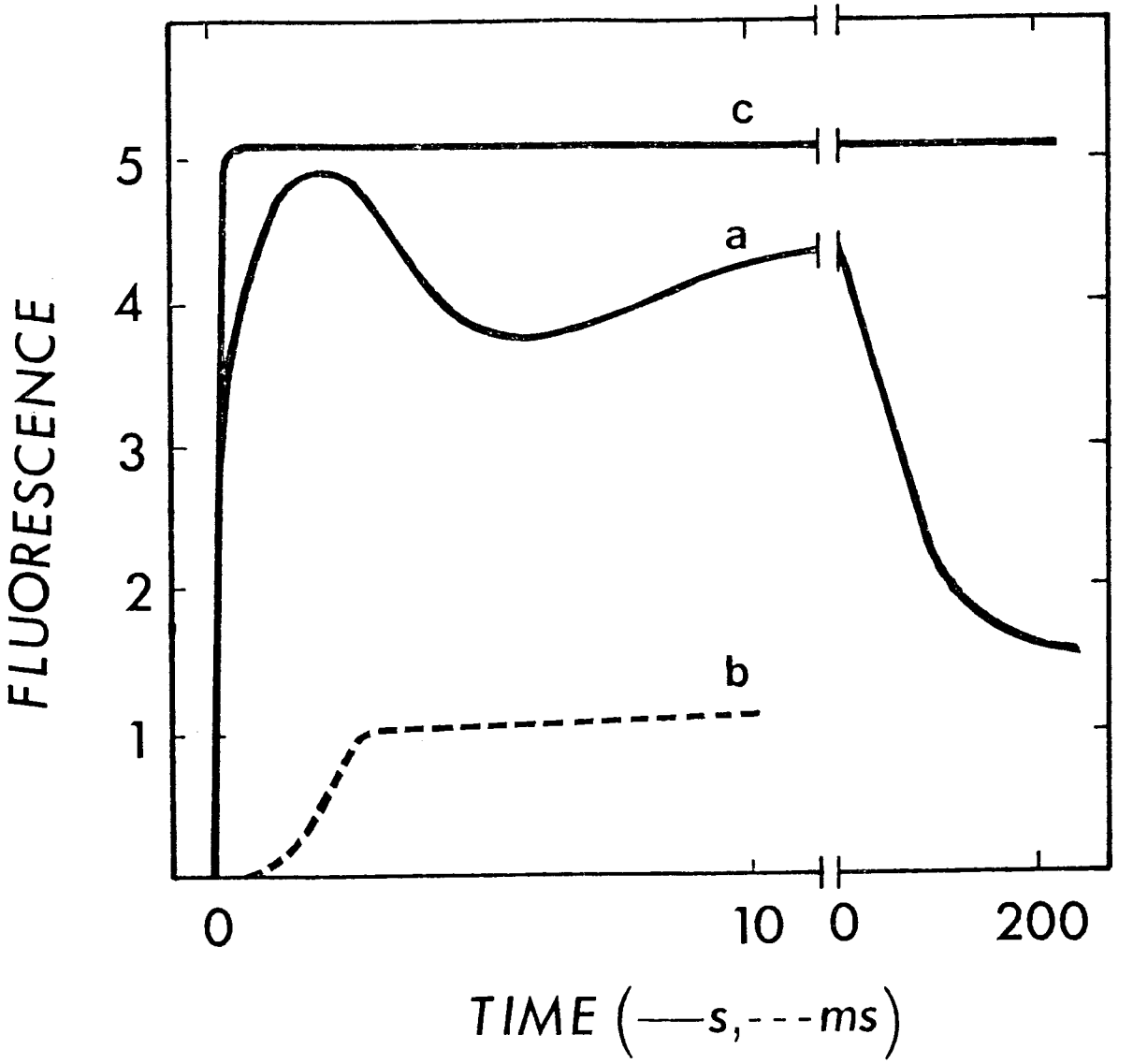
# Chapter 3

# $O_2$ quenching of variable chlorophyll-a fluorescence *in vivo* and *in vitro*

## 3.1 Introduction

Changes in chlorophyll-a fluorescence emission with time after excitation of a dark adapted leaf contain considerable information on the functioning of the photosynthetic apparatus during induction of photosynthesis. A typical fluorescence induction curve from a mature bean leaf, dark adapted for 1 hr prior to illumination is shown in Figure 3.1a. Immediately upon excitation of the leaf, fluorescence rises to an initial level, $F_o$, Figure 3.1b. This is emission from the chlorophyll-a molecules of the light harvesting complex associated with photosystem II prior to excitation energy inducing charge separation at the reaction center of photosystem II, P680. Upon excitation energy transfer to P680 and the resulting charge separation, electrons are transferred to plastoquinone via the primary electron acceptor of photosystem II, Q ($Q_A$ as in Figure 1.2). As the plastoquinone pool becomes reduced the probability of excitation energy transfer to P680 causing a further reduction of plastoquinone decreases, and the probability of energy dissipation in the chlorophyll-a pigment bed by other processes must increase. Therefore an increase in fluorescence observed as the increase in emission from $F_o$ to $F_p$ results from reduction of the plastoquinone pool (Munday and

Figure 3.1 Chlorophyll-a fluorescence induction curves for a mature bean leaf. a) typical induction curve for dark adapted leaf. b) $F_o$ level determination for dark adapted leaf (shown on expanded time scale). c) induction curve in leaf treated with 15$\mu$M DCMU.


FLUORESCENCE
5
4
3
2
1
c
a
b
0
10
0
200
TIME (—s, - - - ms)

Govindjee, 1969; Lavergne, 1974; Mohanty and Govindjee, 1974; Papageorgiou, 1975). That reduction of plastoquinone determines the increase in fluorescence from $F_o$ to $F_p$ can be demonstrated by comparing the rise time of fluorescence in untreated leaves to the rise time in leaves inhibited with 15μM 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), Figure 3.1c. Electron transport from Q to plastoquinone is blocked by DCMU treatment (Duysens and Sweers, 1963), therefore the rise from $F_o$ to $F_{max}$ in the presence of DCMU is indicative of Q reduction where Q is maximally reduced at $F_{max}$. It has been established that there are several plastoquinones, but only one Q associated with each photosystem II reaction centre (Amesz, 1977; Golbech et al., 1977) and that the initial rate of plastoquinone reduction by photosystem II is greater than the rate of oxidation by photosystem I (Witt, 1975). Thus the slow rise to $F_p$ in untreated leaves as compared to the fast rise to $F_{max}$ in the presence of DCMU indicates reduction of the plastoquinone pool as compared to reduction of Q. It is expected that $F_p$ would reach the same intensity as $F_{max}$ if the plastoquinone pool were completely reduced during dark-light induction, assuming DCMU affects the observed fluorescence emission only by blocking electron transport from Q to plastoquinone.

After the initial rise to $F_p$, fluorescence is subsequently quenched over a period of minutes to the $F_T$ level *in vivo*. Studies with *in vitro* systems have shown that quenching

of fluorescence can be induced by a number of factors. Changes in the redox state of plastoquinone (Lavergne, 1974; Vernotte et al., 1979), the magnitude of proton (Briantais et al., 1979) and other cation (Barber, 1976) electrochemical gradients across the thylakoid membrane and the level of adenosine triphosphate (ATP)(Horton and Black, 1980) can all influence chlorophyll-a fluorescence emission and may be involved in the $F_P$ to $F_T$ quenching observed *in vivo*. However, recent evidence presented by Bradbury and Baker (1981) has indicated that the bulk of *in vivo* quenching is a direct result of plastoquinone oxidation. Also, the induction kinetics of EPR signal I (an indicator of the oxidized photosystem I reaction centre, $P700^+$) and fluorescence induction are identical (Andreeva, 1982). It is apparent that the *in vivo* fluorescence induction transient is a good indicator of the redox state of the plastoquinone pool during induction of photosynthesis.

Chlorophyll-a fluorescence is quenched by molecular $O_2$. Vidaver et al. (1981) have described three phases of $O_2$ quenching *in vivo* and *in vitro*; 1) quenching of $F_P$; 2) quenching of $F_{max}$ in the presence of DCMU and; 3) quenching of the initial fluorescence $F_O$. Each phase differed in sensitivity to $O_2$ by approximately one order of magnitude. Half-quenching of $f_p = (F_P - F_O)/F_O$ occurred with 3-5 atm of $O_2$, $f_M = (F_{max} - F_O)/F_O$ with 40 atm and $F_O$

required over 400 atm of $O_2$. Only the most sensitive phase, the quenching of $f_p$, was dependent on electron transport (inhibited by DCMU treatment) and was tentatively related to oxidation of electron transport by $O_2$. Quenching of $f_m$ and $F_o$ were suggested to represent $O_2$ quenching of P680 reaction centres (or $Q^-$) and antennae chlorophyll molecules respectively.

In this chapter a comparison is made of the in vivo chlorophyll-a fluorescence emission of two brown algal species, two green algae and two higher plants in order to investigate possible differences in absorbed light energy utilization. Also, a study using various electron transport inhibitors in lettuce chloroplasts was done to localize the site of $O_2$ quenching.

There is a wide diversity of accessory pigments in algae and higher plants. For example, red, brown, and green algae are so designated because these colors result from the presence of phycoerythrin, fucoxanthin and chlorophyll-b as their respective accessory pigments. The brown algae also have chlorophyll-c. Only green algae and the terrestrial green plants share a common pigment system. Despite this diversity in pigments, the significance is presumably limited to light capture and the basic energy conversion steps are believed to be similar for all $O_2$ evolving plants (Barrett and Anderson, 1980; Mishkind and Mauzerall, 1980). Chlorophyll-a is universally present in all plants which have the capacity for water-

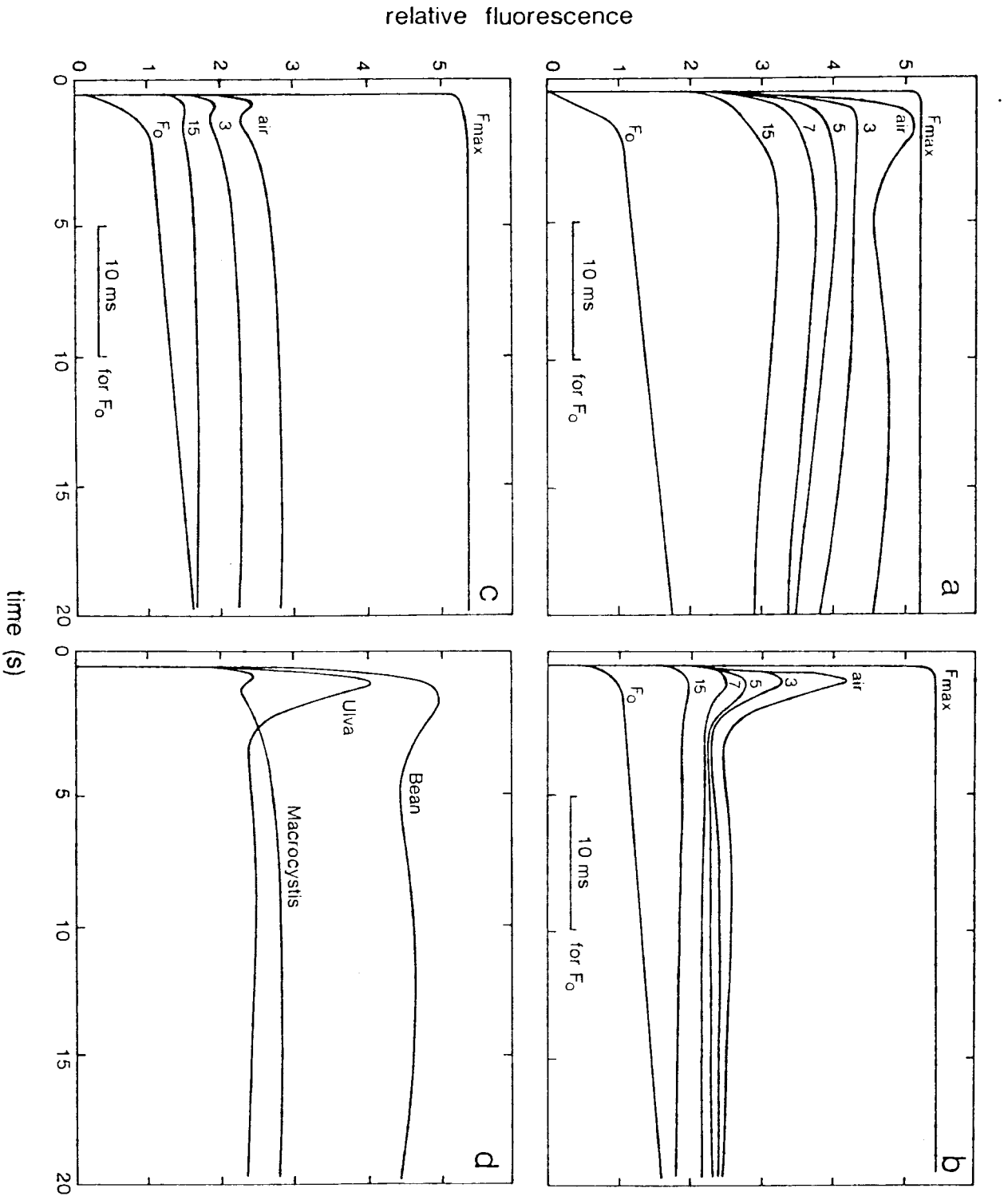
splitting and much evidence indicates that the function of accessory pigments is to transfer excitation energy to chlorophyll-a (Goedheer, 1972). Excitation energy transfer is a highly effective quencher of accessory pigment fluorescence, thus chlorophyll-a fluorescence is indicative of light harvesting by the accessory pigments as well as by chlorophyll-a.

A study was made of both endogenous quenching and $O_2$ quenching of $f_p$ in these diverse photosynthetic organisms. Due to the observed constancy of $f_m$ in all plants studied, relative comparisons of $f_p$ and thus of the plastoquinone redox state were possible. $O_2$ quenched both $f_m$ and $f_p$ in the marine algae in a manner similar to that observed in higher plants. Results from $O_2$ uptake experiments with chloroplasts isolated from brown algae indicated that the higher endogenous quenching of $f_p$ observed in the brown algae may result from increased electron transport to $O_2$. In chloroplasts isolated from lettuce various electron transport inhibitors were used to localize the site of $f_p$ quenching by $O_2$. The results of these inhibitor studies revealed that electron transport through plastoquinone and plastocyanin was required to observe $f_p$ quenching by $O_2$.

## 3.2 Results

Typical fluorescence induction curves for bean are seen in Figure 3.2a. The upper trace shows $F_{max}$ in the presence

Figure 3.2 Fluorescence induction curves. a) bean. b) Ulva sp.. c) M. integrifolia. d) a comparison of the bean, Ulva sp., and M. integrifolia fluorescence induction curves in air. For a, b and c $F_{max}$ was measured in the presence of DCMU, the control curve is labelled air, and in the lower traces the numbers refer to $O_2$ pressure in atmospheres. The $F_o$ trace in air is shown on an expanded time scale.


relative fluorescence
time (s)
a
b
c
d
Fmax
air
3
5
7
15
Fo
10 ms
for Fo
Ulva
Bean
Macrocystis

of DCMU; lower traces are for untreated leaves under increasing $O_2$ pressures (atm). The bottom trace is for $F_o$ in air, normalized to 1 relative fluorescent unit. Figures 3.2b and 3.2c show the same set of measurements for Ulva and M. integrifolia respectively. It is readily apparent that although $f_m \equiv (F_{max} - F_o)/F_o$ is similar in these plants, $f_p \equiv (F_p - F_o)/F_o$ is lower in the seaweeds, especially so in M. integrifolia (Figure 3.2d).

Oxygen reversibly quenched $f_p$ in all plants studied (Figures 3.2a,b,c). The $O_2$ quenching curves (Figure 3.3) show similar half-quenching $O_2$ pressures of 7 atm for bean, 6 atm for Ulva, and 5 atm for M. integrifolia, suggestive of similar mechanisms for $O_2$ quenching. $f_p$ is lower in Ulva than bean and lowest in M. integrifolia at all $O_2$ pressures.

The $O_2$ pressure required for half-quenching of fluorescence in the presence of DCMU was more than 40 atm $O_2$ in all three plants and remarkably, $O_2$ quenching of $f_m$ fits the same curve for all three (Figure 3.3).

In Table 3-1 values of $f_m$ and $f_p$ for various higher plants, unicellular algae and seaweeds are compared. $f_m$ is similar for all of the plants, yet $f_p$ is distinctly lower in the algae than in corn or bean.

The strong endogenous quenching of fluorescence observed in the marine algae and the sensitivity of fluorescence to $O_2$

Figure 3.3 $O_2$ quenching of $f_m$, dashed line, and $f_p$, solid lines, in bean (O), Ulva sp. (□) and M. integrifolia (Δ).

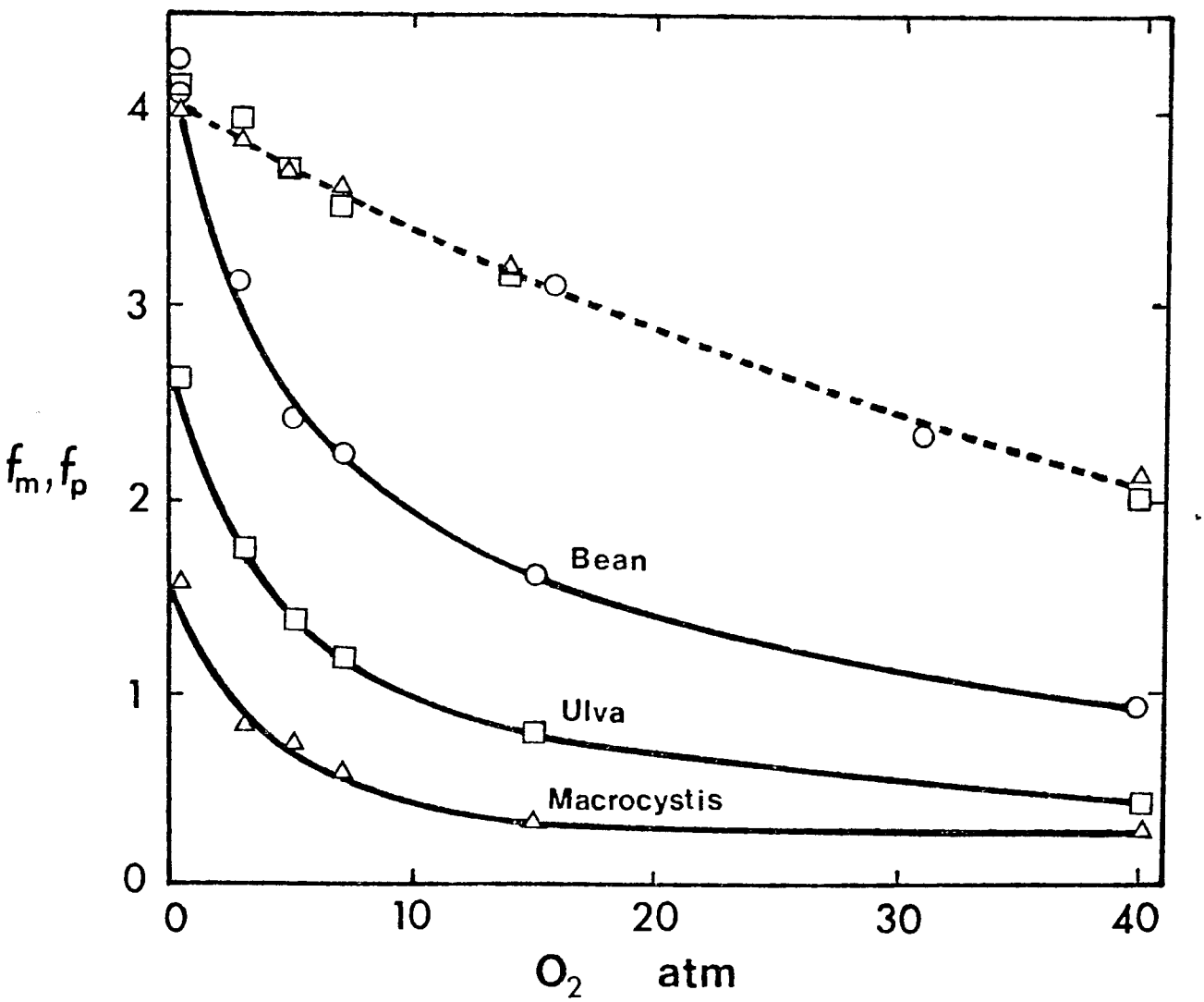

$f_m, f_p$
4
3
2
1
0
0
10
20
30
40
$O_2$ atm
Bean
Ulva
Macrocystis

TABLE 3-1

Relative variable fluorescence, $f_m = (F_m - F_o)/F_o$

$f_p = (F_p - F_o)/F_o$ for higher plants and algae

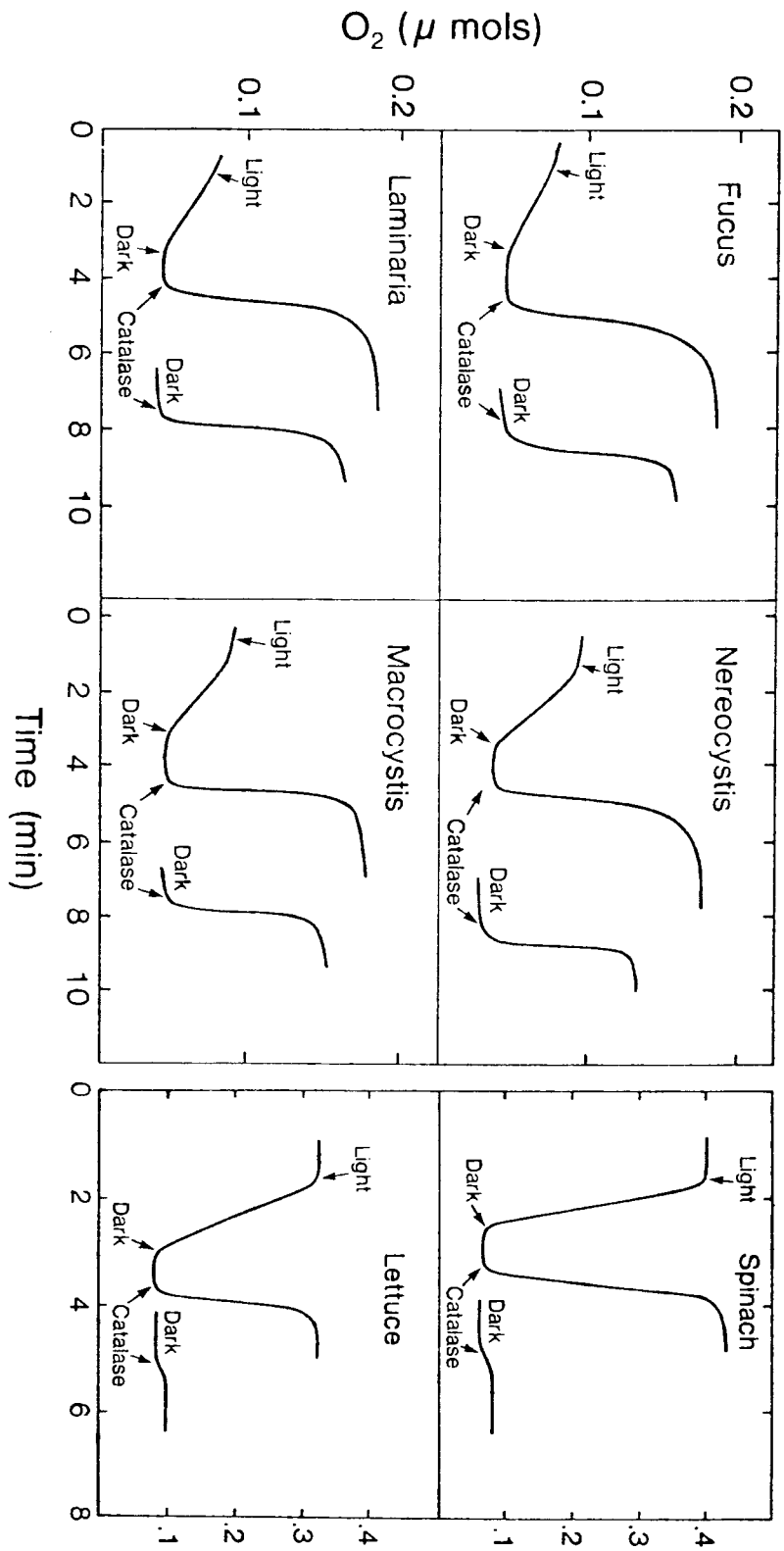
| Plant | $f_p$ | $f_m$ | $f_p/f_m$ |
|---|---|---|---|
| bean | 4.2 | 4.2 | 1.0 |
| corn | 3.7 | 3.9 | .95 |
| *Ulva* species | 2.6 | 4.2 | .62 |
| *Macrocystis integrifolia* | 1.5 | 4.1 | .37 |
| *Fucus vesiculosis* | 1.4 | 3.8 | .37 |
| *Scenedesmus obliquus* | 1.1 | 3.7 | .30 |

suggested that intersystem electron transport carriers are oxidized directly or indirectly by $O_2$. Chloroplasts were isolated from four species of brown algae (Popovic et al., 1983). It was found that well-washed, broken, brown algal chloroplasts released more $O_2$ upon addition of catalase than was taken up in the light in the presence of methyl viologen. When catalase was added to non-illuminated chloroplasts, $O_2$ was released and this $O_2$ was sufficient to account for the excess (Figure 3.4). In comparison, chloroplasts from spinach or lettuce showed little $O_2$ evolution on addition of catalase in the dark. Chloroplasts from Fucus vesiculosis isolated under a dim green safelight did not release $O_2$ on addition of catalase in the dark but would take up $O_2$ in the light in the absence of methyl viologen and ascorbate (data not shown).

In lettuce chloroplasts $O_2$ quenching of $f_p$ and $f_m$ was similar to that observed in vivo. As previously reported (Vidaver et al., 1981), inhibition of Q oxidation via intersystem electron transport by DCMU resulted in a decrease in the sensitivity of $O_2$ quenching of fluorescence by a factor of 10 in vivo or in vitro.

The site of $f_p$ quenching by $O_2$ must therefore be on the reducing side of Q. Inhibition of plastoquinone oxidation with dibromothymoquinone (DBMIB), (Trebst, 1980), retarded $f_p$ quenching by $O_2$ to the same degree as DCMU

Figure 3.4 Light dependent $O_2$ reduction in the presence of methyl viologen and subsequent $O_2$ release after the addition of catalase compared to $O_2$ release in non-illuminated samples for Fucus, Nereocystis, Laminaria, Macrocystis, spinach, and lettuce. Reaction media at 1ml contained: 50mM Hepes (pH 7.6), 1mM $MnCl_2$, 1mM $MgCl_2$, 10-15μg Chl, 1mM $NH_4Cl$, 0.2mM methyl-viologen, 6mM isoascorbate and 300 units of commercial catalase where indicated.


$O_2$ ($\mu$ mols)
Fucus
Nereocystis
Laminaria
Macrocystis
Spinach
Lettuce
Light
Dark
Catalase
Dark
Time (min)

treatment and histone, a reported inhibitor of plastocyanin function in both cyclic and non-cyclic electron flow (Brand et al., 1971; 1972), also affects $f_p$ quenching in the same manner. Quenching in the presence of 2-5 dimethylquinone (DMQ) was not inhibited and showed the same sensitivity to $O_2$ as control chloroplasts (not shown). Although DBMIB or histone treatment did not greatly influence the fluorescence induction transient the addition of DMQ both slowed the rise to $f_p$ and decreased the extent of $f_p$ indicating an increase in the effective pool size of oxidized photosystem II electron acceptors (Figure 3.6). If DCMU was added to chloroplasts with DMQ, the $O_2$ quenching of fluorescence was the same as if DMQ was not present (not shown). To determine the influence of photosystem I on $O_2$ quenching of $f_p$ a selective photosystem I inhibitor was required. Photosystem I driven $O_2$ uptake is inhibited by hydrostatic pressure in green algae although $O_2$ evolution is not greatly affected (Vidaver and Chandler, 1969). Effects of $O_2$ partial pressure with a total pressure of 135 atm supplied by $N_2$ gas are shown for bean leaves in Figure 3.7. At constant total pressure, $O_2$ quenching is even more sensitive than control quenching suggesting photosystem I is not involved and that pressure of this magnitude makes the quenching site more accessible to $O_2$.

Figure 3.5 $O_2$ quenching of fluorescence in chloroplasts in the presence of various inhibitors of electron transport. Relative fluorescence values are $f_m$ or $f_p$.

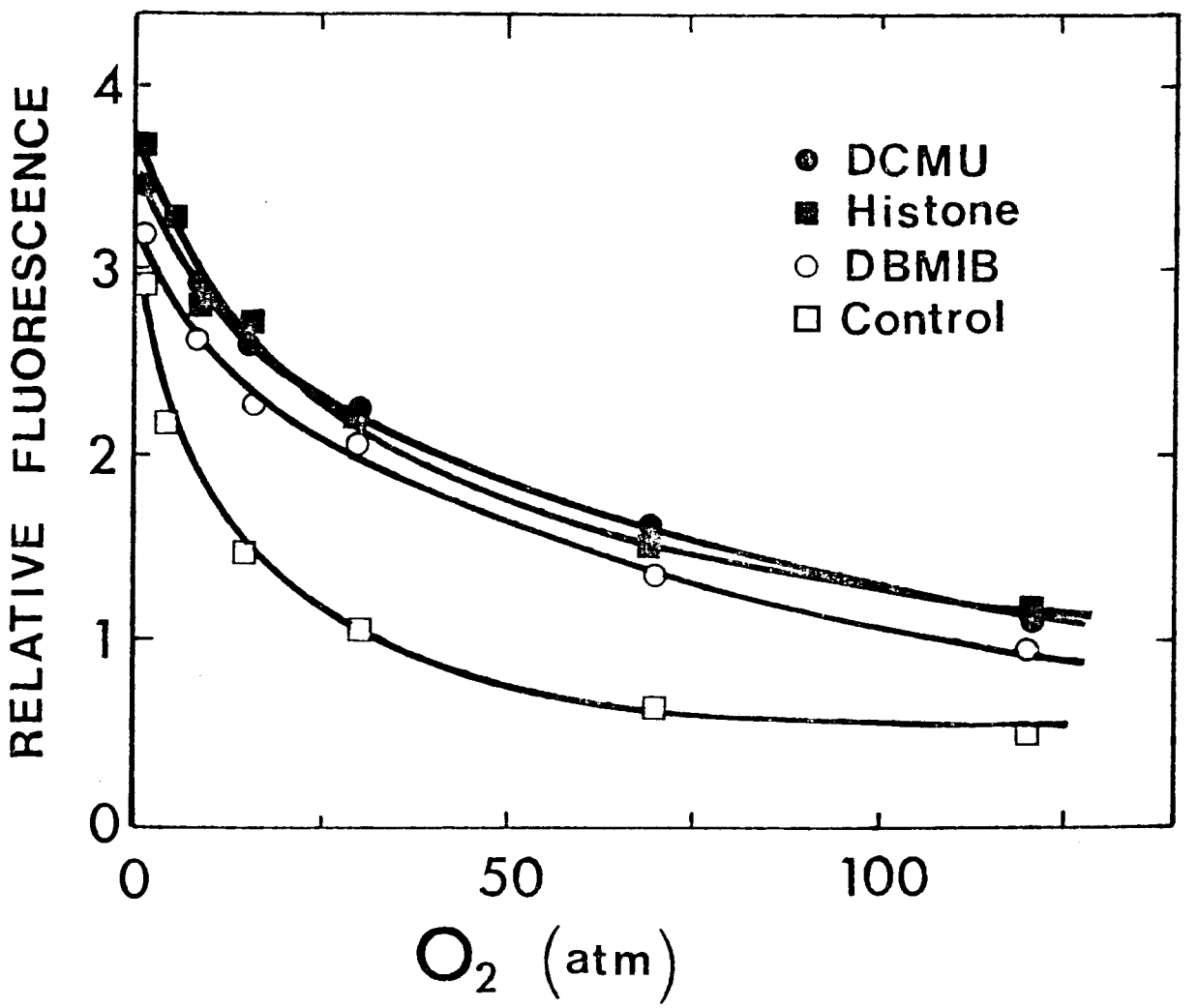

RELATIVE FLUORESCENCE
4
3
2
1
0
0
50
100
$O_2$ (atm)
DCMU
Histone
DBMIB
Control

Figure 3.6 Time courses of fluorescence induction in chloroplasts and in chloroplasts treated with 1μM DBMIB, 0.5mM DMQ, or 10μg histone per μg chlorophyll. Broken curves are $F_o$ determinations shown on an expanded time scale.

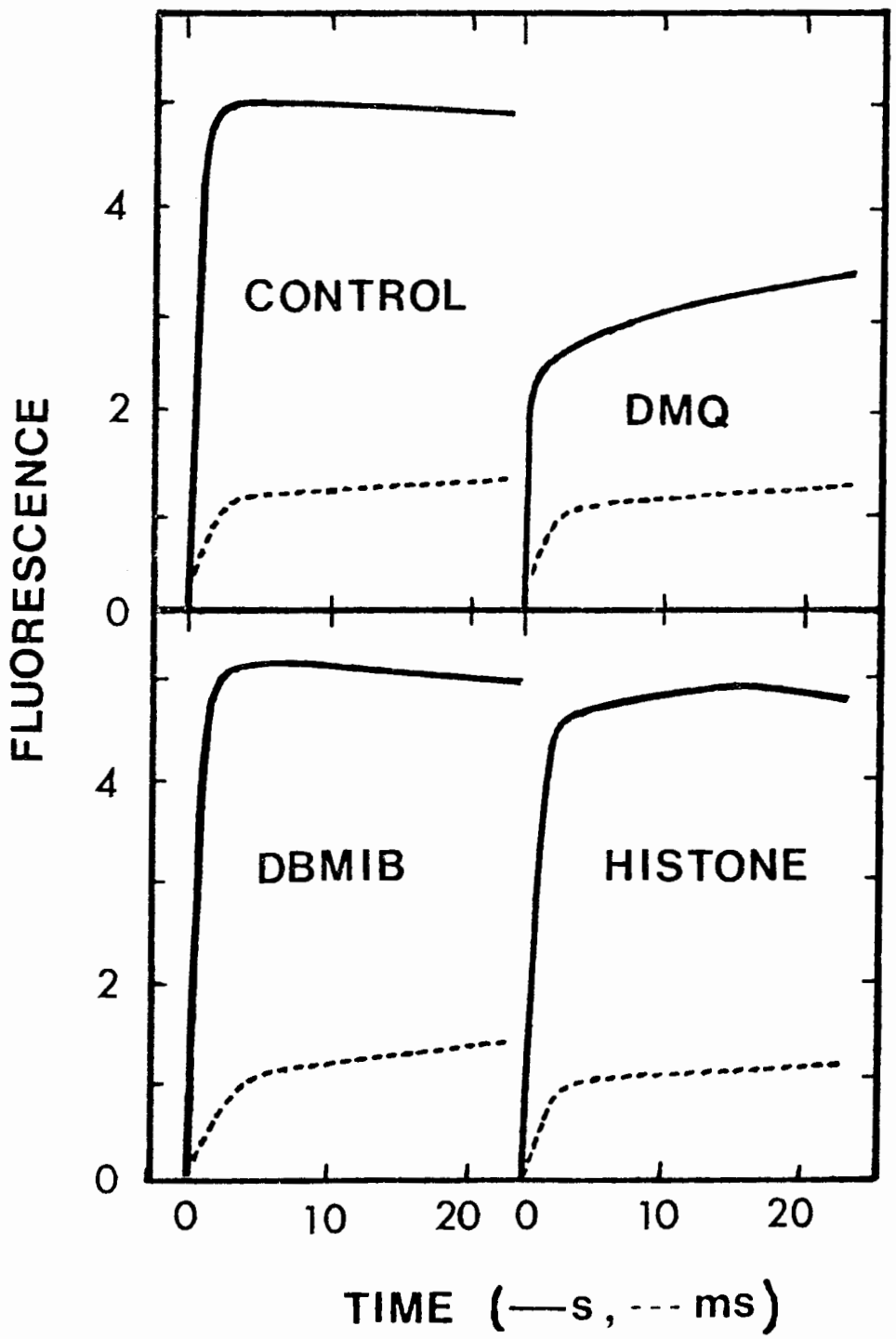
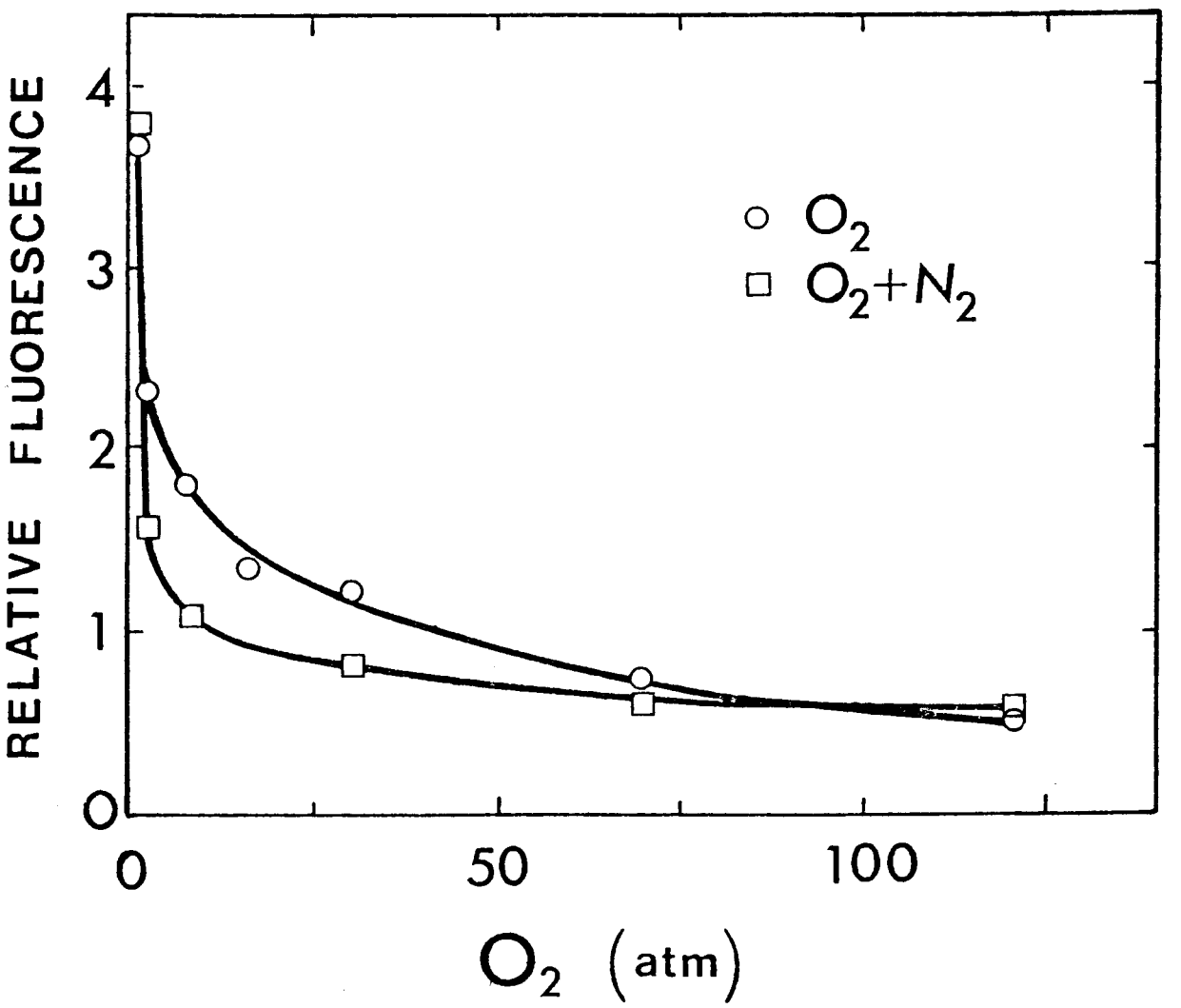

FLUORESCENCE
CONTROL
DMQ
DBMIB
HISTONE
4
2
0
4
2
0
0
10
20
0
10
20
TIME (—s, --- ms)

Figure 3.7 $O_2$ quenching of $f_p$ in bean leaves with $O_2$ present alone and with $N_2$ plus $O_2$ at a total pressure of 135 atm. The upper curve represents fluorescence yield in 100% $O_2$ at the pressure shown; the lower shows the yield at a constant pressure of 135 atm with partial $O_2$ pressure as shown on the scale.


RELATIVE FLUORESCENCE
4
3
2
1
0
0
50
100
$O_2$ (atm)
$O_2$
$O_2+N_2$

## 3.3 Discussion

### 3.3.1 A comparison of chlorophyll-a fluorescence induction kinetics and their $O_2$ sensitivity in algae and higher plants

In the interpretation of fluorescence, one usually assumes that:

1) The measured $F_o$ is the minimum fluorescence yield corresponding to the maximum possible rate of energy transfer from antennae pigments to photosystem II (Papageorgiou, 1975);

2) DCMU changes the absolute fluorescence yield by blocking electron transport oxidation of Q. (Papageorgiou, 1975), and;

3) During the initial induction of photosynthesis (5-10s) the variable fluorescence yield is determined mainly by changes in the redox state of Q (Bradbury and Baker, 1981).

With the above assumptions, the constancy of $f_m$ observed in the diverse plants studied is indicative of a functional similarity in photosystem II and associated antennae pigments which permits in vivo comparisons of the relative redox state of Q. However, as $f_m$ is a relative measure, similarities in absolute energy transfer efficiencies from antennae to photosystem II are not implied. In algae not inhibited with DCMU, variable fluorescence was seen to be highly quenched. As $f_m$ was similar in all plants, the low $f_p$ in the algae suggests that during induction Q is relatively more

oxidized in the algae than in the higher plants. Either less efficient water-splitting, more efficient electron transport to photosystem I, or higher oxidation losses from electron transport would result in a lower $f_p$. In lettuce chloroplasts $f_p$ was decreased by increasing the pool of oxidized photosystem II electron acceptors with DMQ. A major electron transport loss involves reduction of $O_2$ and the subsequent production of $H_2O_2$ (Behrens et al., 1982; Egneus et al., 1975; Marsho et al., 1979; Radmer and Kok, 1976). It has been reported that Scenedesmus obliquus shows higher rates of $O_2$ uptake ($O_2$ reduction) than spinach or soybean (Behrens et al., 1982; Marsho et al., 1979). The low $f_p$ of S. obliquus (Table 3-1) supports these results if $O_2$ is assumed to be the quencher of $f_p$. The endogenous quenching of $f_p$ is greater in the brown algae than in higher land plants. As $O_2$ is a potent quencher of $f_p$ in all the plants studied it is conceivable that the endogenous quencher is $O_2$. The higher rates of $O_2$ reduction with concomitant $H_2O_2$ production observed in the brown algae support this hypothesis.

Although electron transport may be more sensitive to $O_2$ in algae than higher plants, it is also possible that internal concentrations of $O_2$ are higher in algae. Dissipating endogenously evolved $O_2$ may be more difficult for aquatic algae than for higher land plants as boundary-layer resistances to gas exchange are much higher in an aqueous environment. For example, $CO_2$ uptake has been shown to be limited by boundary-

layer thickness in Macrocystis pyrifera (Wheeler, 1980). Increased electron transport to endogenous $O_2$ in seaweeds and other algae may partially account for the low light saturation of photosynthesis observed in the brown algae (Smith et al., 1983; Willenbrink et al., 1979).

### 3.3.2 Localization of the site of $O_2$ quenching of $f_p$

The inhibition of $O_2$ quenching of $f_p$ by treatment with DCMU in both algae and higher plants indicates the involvement of electron transport carriers on the reducing side of Q in the mechanism of $O_2$ quenching. As DBMIB, an inhibitor of PQ oxidation and pseudocyclic electron transport (Trebst, 1980), also blocked $f_p$ quenching by $O_2$, it is apparent that the $O_2$ quenching site is on the reducing side of PQ. Histone, a reported inhibitor of plastocyanin (Brand et al., 1971; 1972) also blocks $f_p$ quenching to the same degree as DCMU or DBMIB indicating that in fact electron transport all the way from photosystem II to plastocyanin is required to observe $f_p$ quenching by $O_2$.

Clearly oxidation of intersystem electron carriers by $O_2$ would quench $f_p$, and the electron transport inhibitor study showed that this site would have to involve electron transport through plastocyanin. Plastocyanin is not auto-oxidizable by atmospheric $O_2$ and after isolation of plastocyanin from spinach, it was determined that $O_2$ would not oxidize isolated plastocyanin under increased $O_2$ pressure (data not

shown). However, recent work by Haehnel (1982) has indicated that it is plastocyanin complexed to the cytochrome $b_6/f$ complex that is functional in electron transport to P700, and the $O_2$ sensitivity of this system is unknown.

The reducing side of photosystem I is not likely the site of $f_p$ quenching by $O_2$ as the addition of $O_2$ to leaves or chloroplasts in the dark decreases the $f_p$ level (usually reached within 500ms of illumination). Also, the inhibition of photosystem I driven $O_2$ uptake by increased pressure did not retard $f_p$ quenching by $O_2$ but rather made $f_p$ more sensitive to $O_2$. Ferredoxin reduces intersystem electron carriers in the dark (Mills et al., 1979; Arnon and Chain, 1979), and $O_2$ is known to oxidize ferredoxin (Hosein and Palmer, 1983; Furbank and Badger, 1983), conceivably increasing the $O_2$ concentration may drive the oxidation of intersystem electron carriers by ferredoxin. Recently, Arnon et al. (1981) have proposed a new concept for electron and proton transport in photosynthesis. The basis of this concept is their reported reduction of ferredoxin directly via photosystem II. If ferredoxin can accept electrons from photosystem II without photosystem I activity, oxidation of ferredoxin by $O_2$ would explain $f_p$ quenching by $O_2$. However the transport pathway from photosystem II to ferredoxin would have to involve plastoquinone and plastocyanin to accommodate the inhibition of $O_2$ quenching of $f_p$ by DBMIB and histone.

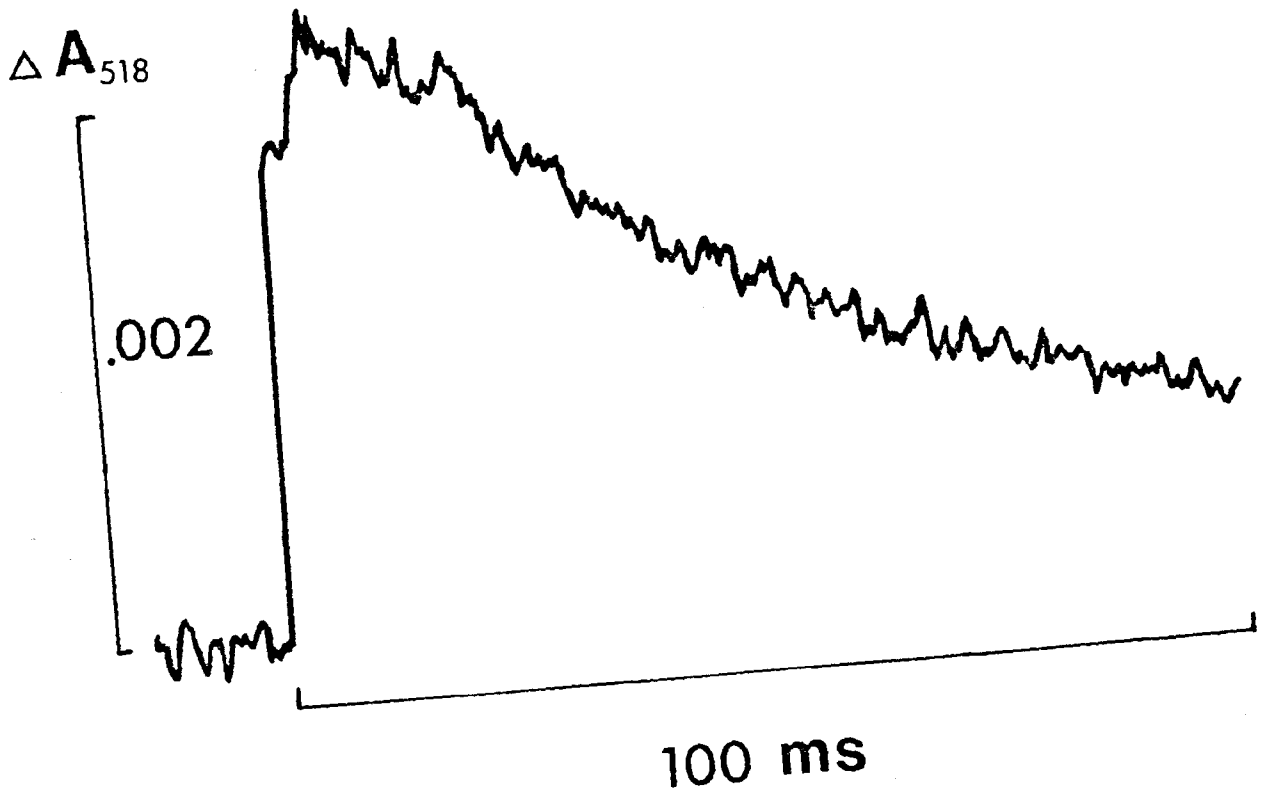
# Chapter 4

## Effect of $O_2$ on the Kinetics of $\Delta A_{518}$ in vivo

### 4.1 Introduction

Charge separation across the thylakoid membrane induced by excitation of the reaction centers P680 and P700 results in the formation of an electric field across the membrane. The magnitude of this field is large ($10^5$ V $cm^{-1}$) as the potential is held across the thin (≈ 4nm) non-conducting part of the thylakoid membrane (Witt et al., 1976). When a pigment with a different dipole moment or polarizability in the ground state than in the excited state is exposed to an electric field, it will have an energy difference between the ground and excited states that is dependent on the electric field strength. Thus the absorbance band of such a membrane bound pigment is shifted by the electric field formed across the thylakoid membrane. When measured as a light-dark absorption difference spectra, a field indicating absorbance increase peaking at 518nm ($\Delta A_{518}$) is observed (see Witt, 1979, for a review). When excited by short single turnover saturating light flashes the initial rise of $\Delta A_{518}$ is followed by a decay reflecting the slow dissipation of the electric field in the dark over a period of milliseconds. In Figure 4.1, $\Delta A_{518}$ is shown for a dark adapted lettuce leaf. This trace is the average of 40 measurements taken at a frequency of 0.25 Hz. The fast rise [< 3ns, Witt (1979] results from

Figure 4.1 Time course of $\Delta A_{518}$ in a dark-adapted lettuce leaf. The trace is an average of 40 measurements taken with a flash frequency of 0.25 Hz.


Δ A518
.002
100 ms

charge separation at both P680 and P700; the slow rise (5-10ms) is dependent on the redox poise of intersystem electron carriers and may be indicative of cyclic electron transport (Crowther and Hind, 1980) or the operation of a Q cycle (as originally proposed by Mitchell, 1976) during non-cyclic electron transport (Selak and Whitmarsh, 1982). The subsequent decay of $\Delta A_{518}$ ($t_{1/2} \approx 500$ms) is determined by the slow decay of the electric field in the dark. Addition of an ionophore will accelerate the decay by a factor of 20 or more (Witt, 1979), demonstrating that dissipation of the electric field by ion flux through the membrane is possible. In light adapted leaves the $\Delta A_{518}$ decay is faster by a factor of 20 or more over the dark adapted decay indicating new pathways of electric field dissipation in the light. The decay of $\Delta A_{518}$ in dark adapted leaves is complex (Shapendonk et al., 1979) and recently the predominance of the fast decay component after preillumination was correlated to high energy state formation (strong millisecond delayed fluorescence) and increased ATP hydrolase activity of coupling factor 1 ($CF_1$) (Morita et al, 1981; 1982). The involvement of ATP hydrolase activity of $CF_1$ is supported by <u>in vitro</u> studies where predominance of the fast decay component is observed after addition of ATP to chloroplasts with dithiol activated $CF_1$ ATP hydrolase (Schreiber and Rienits, 1982; Shuurmans and Kraayenhof, 1983). These conditions also effect an increase

in fluorescence via reverse electron flow (Schreiber and Avron, 1979).

As described in chapter 3, there is an electron transport dependent quenching of variable fluorescence by $O_2$ *in vivo* and under the same conditions $O_2$ was found to inhibit $\Delta A_{518}$ decay, thus supporting the hypothesis that the accelerated $\Delta A_{518}$ decay is dependent on high energy state formation. This chapter describes the $O_2$ influence on $\Delta A_{518}$ decay *in vivo* and evidence is presented indicating the effect is related to reduction of photosystem I acceptors via electron transport. A possible mechanism is discussed in connection with the $O_2$ inhibited ferredoxin-thioredoxin-reductase activation of $CF_1$ ATP hydrolase observed *in vitro* (Mills et al., 1980).

## 4.2 Results

The dependence of the $\Delta A_{518}$ kinetics in lettuce leaves on $O_2$ concentration and background red light are seen in Figure 4.2. Lowered $O_2$ concentrations (b) and weak red background illumination (c) decreased the magnitude and decay halftime of the $\Delta A_{518}$ compared to the control in air with no background light (a). These effects were reversible and complete within approximately 1 min. Far-red background light induced a transient acceleration of $\Delta A_{518}$ decay, Figure 4.3 shows the average of 16 flash responses given at 0.1 Hz for (a) the dark adapted control; (b) the leaf in (a) signal-averaged immediately after the addition of 0.1 W $m^{-2}$ far-red

Figure 4.2 Time courses of $\Delta A_{518}$ in lettuce leaves. Each trace is the average of 16 measurements taken with a dark interval between flashes of 10s. a) dark adapted leaf flash equilibrated for 2 min before measurement under 20% $O_2$. b) the leaf in (a) in 1% $O_2$. c) leaf under the same conditions as the leaf in (a) except with the addition of 0.3 W $m^{-2}$ red light (Corning 2-64 red cut-off filter).

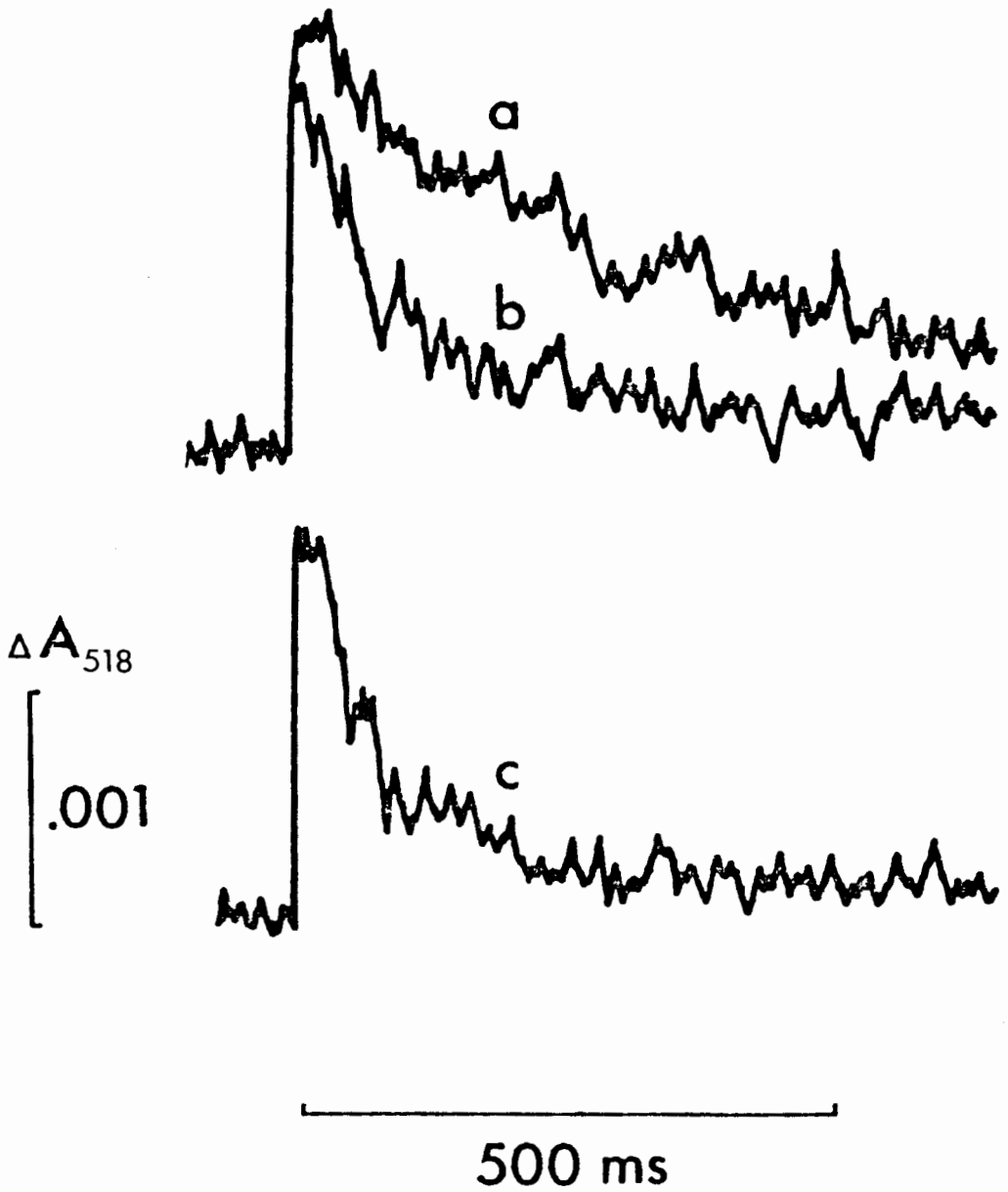
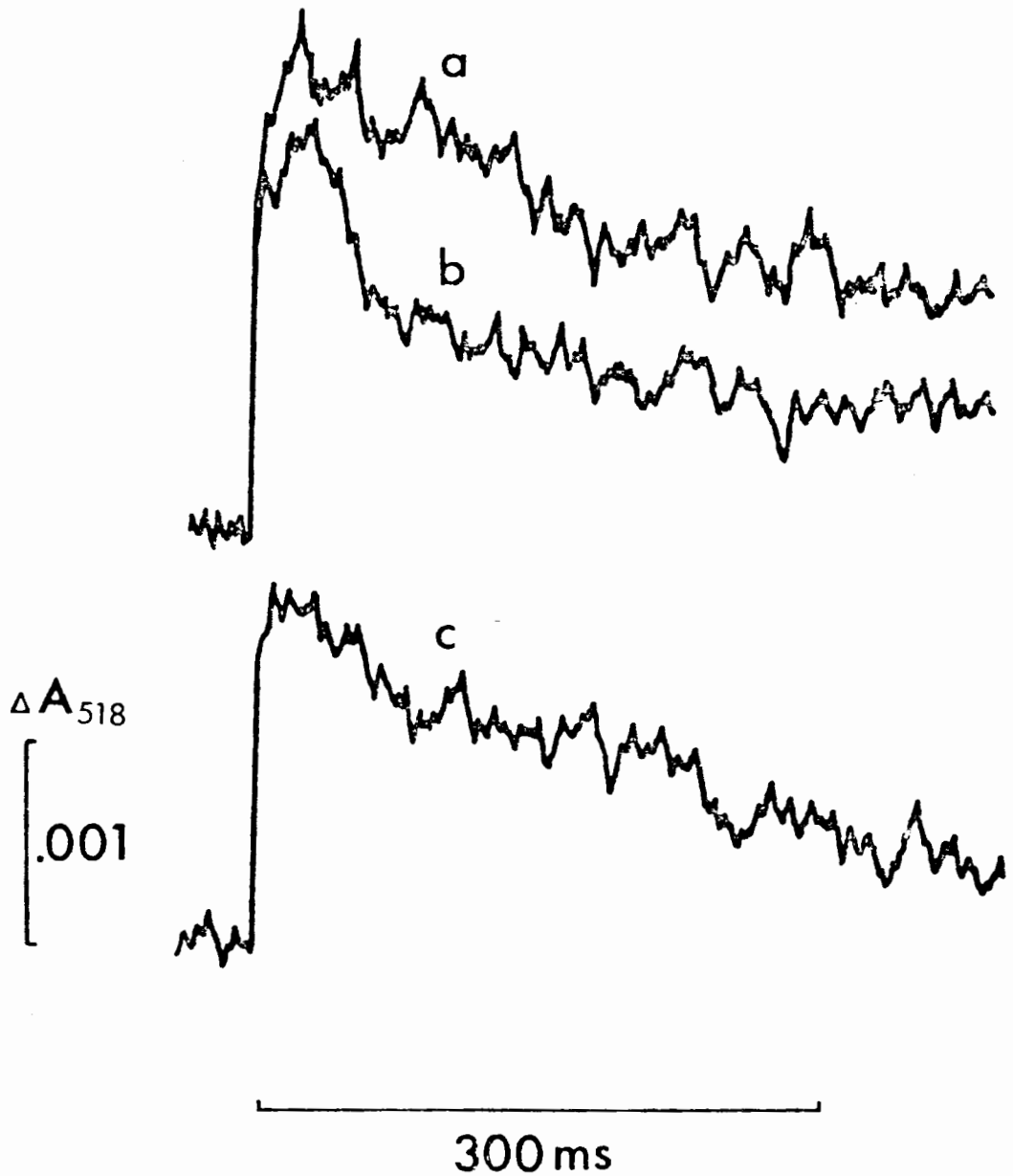

a
b
c
ΔA518
.001
500 ms

Figure 4.3 Time courses of $\Delta A_{518}$ in lettuce leaves. Each trace is the average of 16 measurements taken with a dark interval between flashes of 10s all under 20% $O_2$. a) dark adapted leaf as in Figure 4.2a. b) the leaf in (a) immediately following the addition of 0.1 W $m^{-2}$ far-red light (Balzers B-718 interference filter). c) same sample measured immediately following (b) still under the far-red light.


a
b
c
ΔA518
.001
300 ms

light; and (c) the same leaf averaged 160s later, still under the far-red light. This pattern was reversible and the transient acceleration could be observed again after approximately 2-3 min without background illumination. The transient effect of far-red light is most likely correlated to oxidation of intersystem electron carriers as sustained acceleration requires the participation of photosystem II (red light).

The acceleration of $\Delta A_{518}$ decay caused by the presence of red background illumination could be reversed by an increase in $O_2$. A linear relationship was found between the amount of added $O_2$ necessary to reverse the red light induced acceleration of decay to control levels and the intensity of the background light (Figure 4.4). This linear dependence shows a strong coupling between the $O_2$ and background light effects on $\Delta A_{518}$ decay and is most simply explained by a competitive mechanism where $O_2$ and red light affect the same site.

The $\Delta A_{518}$ decay halftime increases linearly with the dark interval between flashes ($\tau$) until a saturating dark adapted limit is reached which is independent of $\tau$ (Figure 4.5). This dark adapted limit is decreased with lowered $O_2$ demonstrating a light independent effect of $O_2$ on $\Delta A_{518}$ kinetics. However, the $\tau$ where the dark adapted limit is reached is shorter with increased $O_2$ indicating again that $O_2$ and input light energy compete in determining the $_{\Delta}A_{518}$ decay.

Figure 4.4 Plot of the $O_2$ pressure required to restore the $\Delta A_{518}$ decay halftime to the dark adapted value after the addition of background red light (Corning 2-64 red cutoff filter) versus the intensity of the background light. Data points were obtained by first adding background red light to a dark adapted sample and then adding increasing $O_2$ pressure until the decay was restored to the dark adapted level. All $\Delta A_{518}$ determinations were done with a dark interval between flashes of 10s and were the averages of 20 measurements.

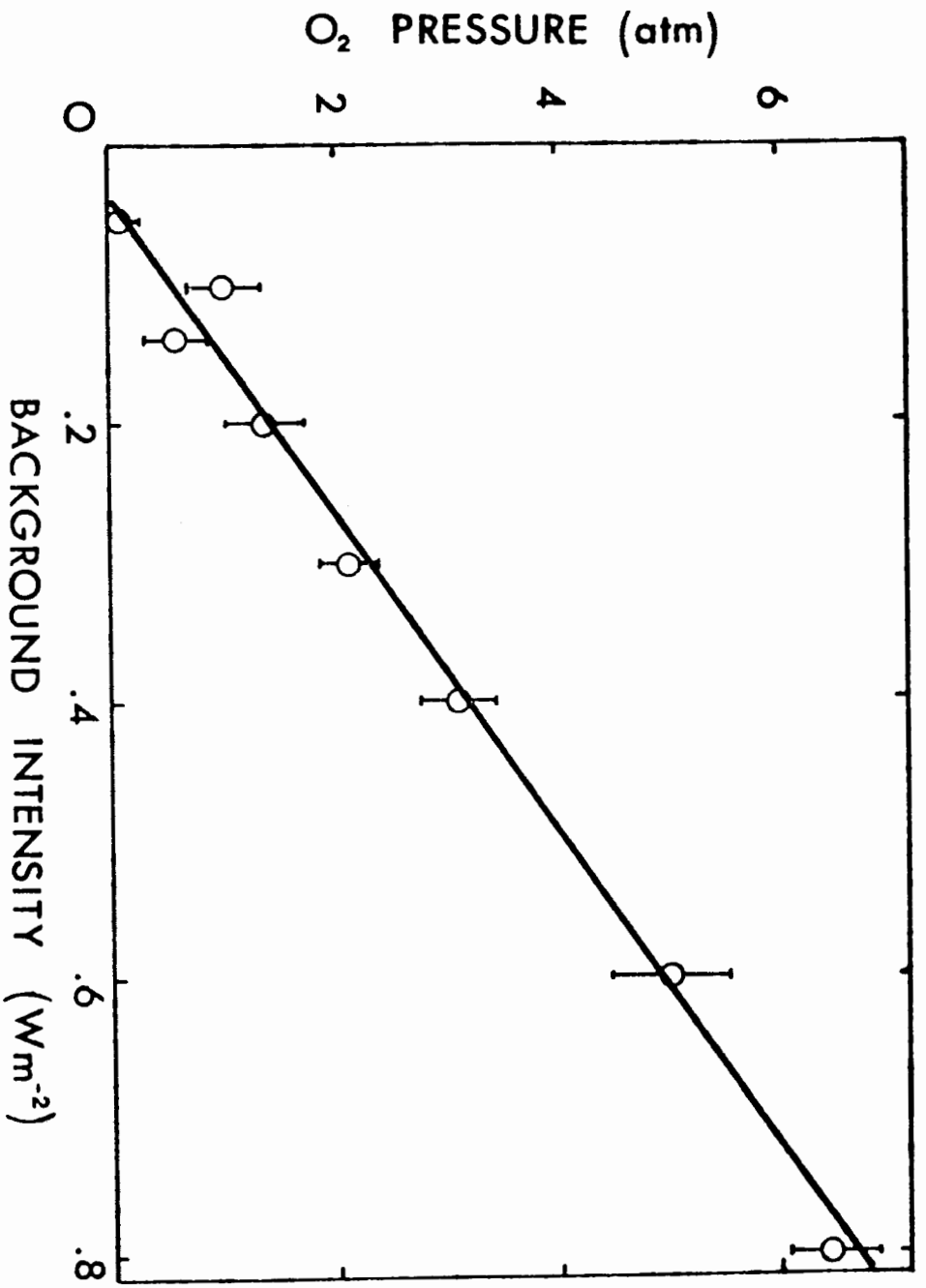
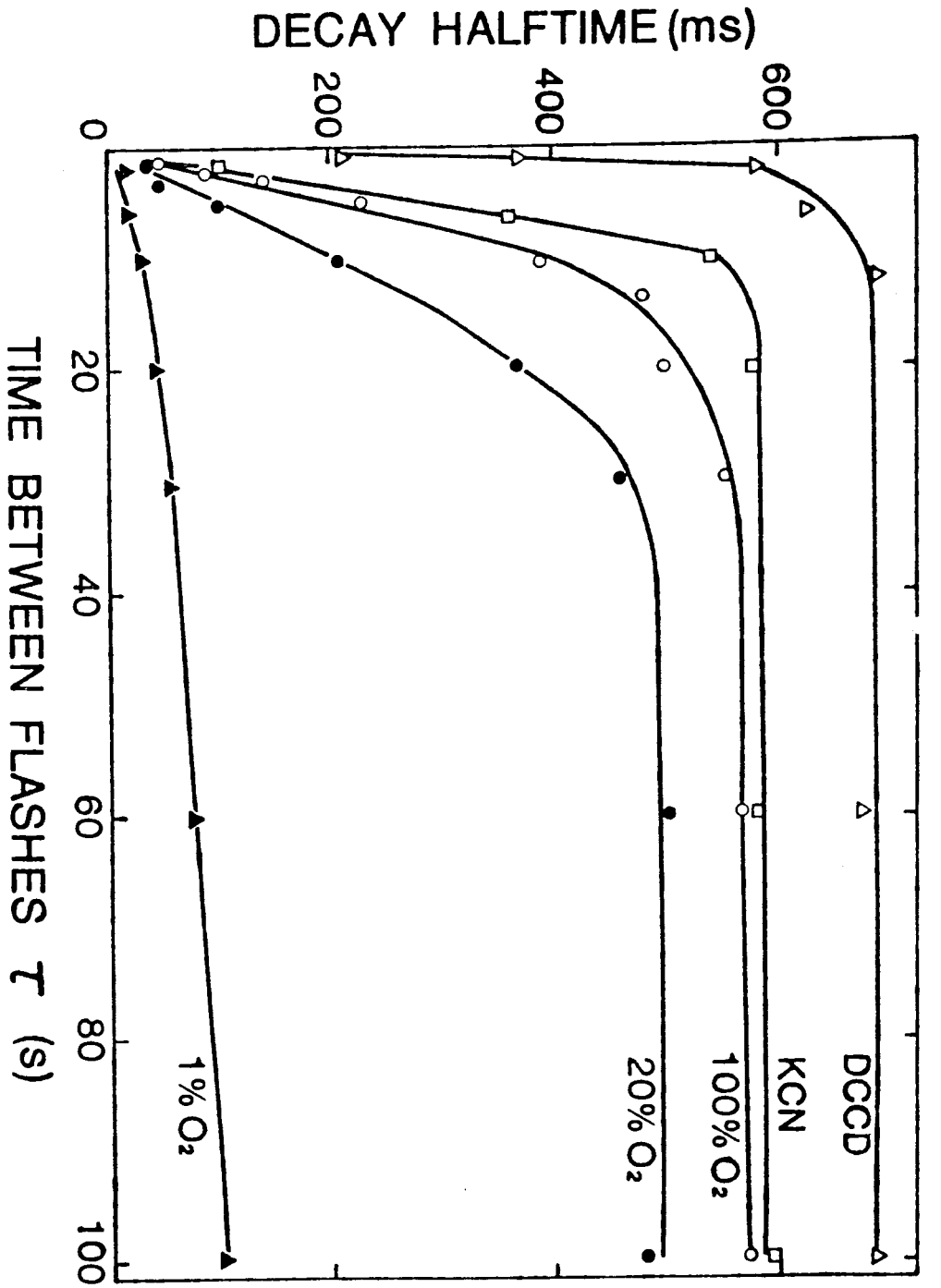

$O_2$ PRESSURE (atm)
0
2
4
6
.2
.4
.6
.8
BACKGROUND INTENSITY ($Wm^{-2}$)

Figure 4.5 $\Delta A_{518}$ decay halftime vs. the time between flashes ($\tau$) for leaves at 1% $O_2$, 20% $O_2$ and 100% $O_2$. Leaves with DCCD and KCN at 20% $O_2$. Oxygen stated as percentage of 1 atm.


DECAY HALFTIME (ms)
0
200
400
600
TIME BETWEEN FLASHES τ (s)
0
20
40
60
80
100
DCCD
KCN
100% O2
20% O2
1% O2

At $\tau$ = 10s the $\Delta A_{518}$ decay is still a function of $\tau$. Both the light dependent and independent effects of $O_2$ are manifested in the biphasic curve of decay halftime versus $O_2$ pressure (Figure 4.6). When $\tau$ is increased to 60s or at $\tau$ = 10s in the presence of DCMU (Figure 4.7) only one phase is distinguishable and the $O_2$ effect on $\Delta A_{518}$ decay saturates at a much lower $O_2$ partial pressure. Conversely, stimulation of electron transport by the addition of weak red background light or a decrease in $\tau$ to 3s causes saturation of the $O_2$ effect to occur at a higher $O_2$ pressure (> 5 atm). Although DCMU did not completely inhibit the $O_2$ effect, it did inhibit preillumination induced acceleration of $\Delta A_{518}$ decay (not shown) as previously reported (Morita et al., 1981).

DCCD, which has been reported to block proton flux through $CF_0$ (McCarty, 1980; Nelson et al., 1977) produced the longest $\Delta A_{518}$ decay halftimes at all $\tau$ (Figure 4.5) as well as inhibiting the effects of $O_2$ (Figure 4.7) and background red light (not shown) on the $\Delta A_{518}$ decay. Similarly, treatment with KCN resulted in an increase in decay halftime at all dark intervals (Figure 4.4), complete inhibition of the $O_2$ effect at long and short $\tau$ (Figure 4.7) and elimination of the acceleration by background red light (not shown).

The $\Delta A_{518}$ decay rate was faster after a 2 min preillumination with 200 W $m^{-2}$ red light in leaves treated with KCN than in leaves with DCCD and the halftime of dark recovery to

Figure 4.6 $\Delta A_{518}$ decay halftime vs. $O_2$ pressure (partial pressure below 1 atm). Time between flashes $\tau$=3s, $\tau$=10s, $\tau$=60s, and $\tau$=10s with 0.1 W $m^{-2}$ background red light (Corning filter 2-64).

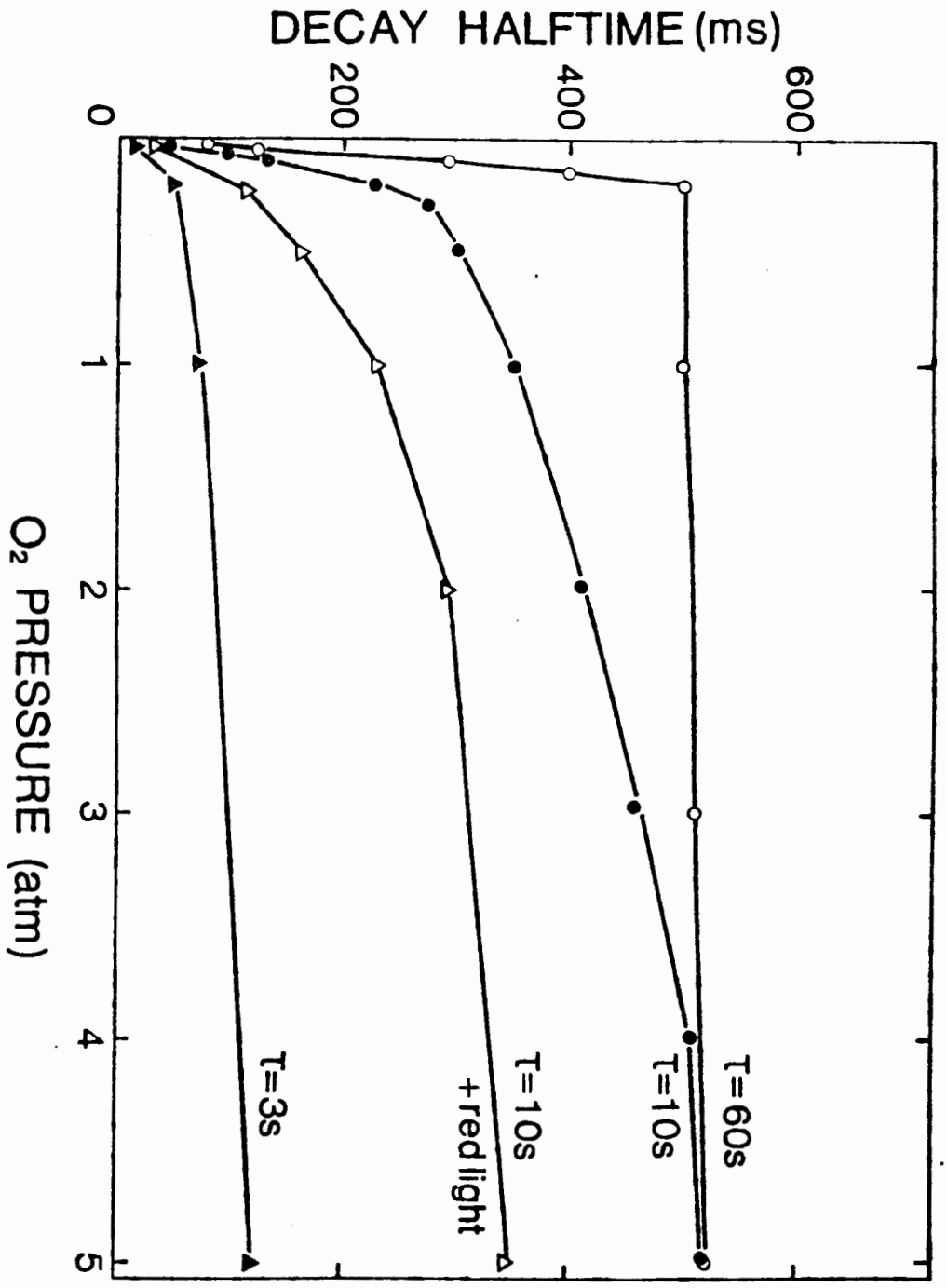
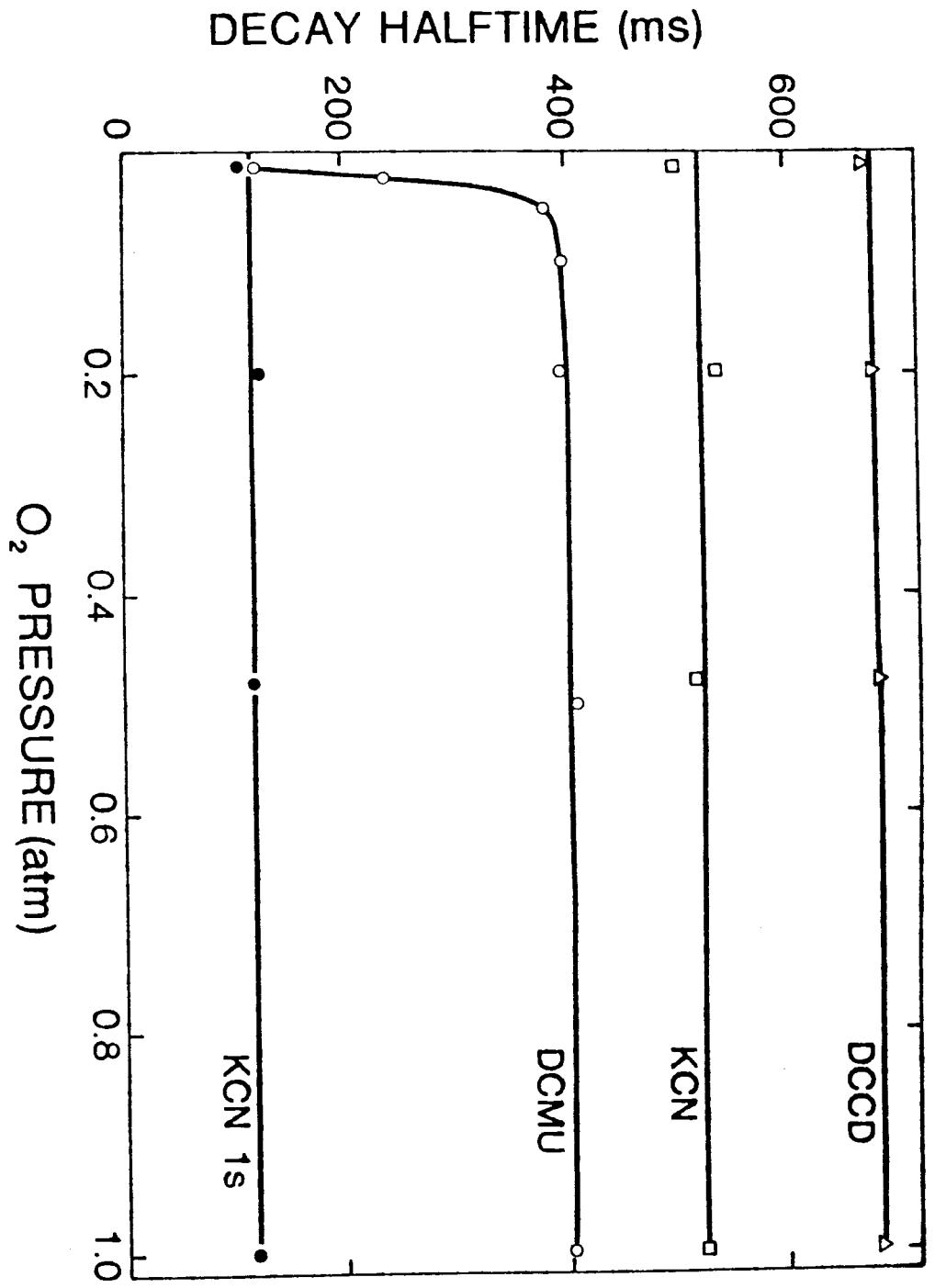

DECAY HALFTIME (ms)
200
400
600
0
1
2
3
4
5
O₂ PRESSURE (atm)
τ=60s
τ=10s
+red light
τ=10s
τ=3s

Figure 4.7 $\Delta A_{518}$ decay halftime vs. $O_2$ concentration for leaves treated with DCMU, DCCD and KCN with $\tau$=10s except for the lowest curve where $\tau$=1s.


DECAY HALFTIME (ms)
0
200
400
600
0.2
0.4
0.6
0.8
1.0
$O_2$ PRESSURE (atm)
KCN 1s
DCMU
KCN
DCCD

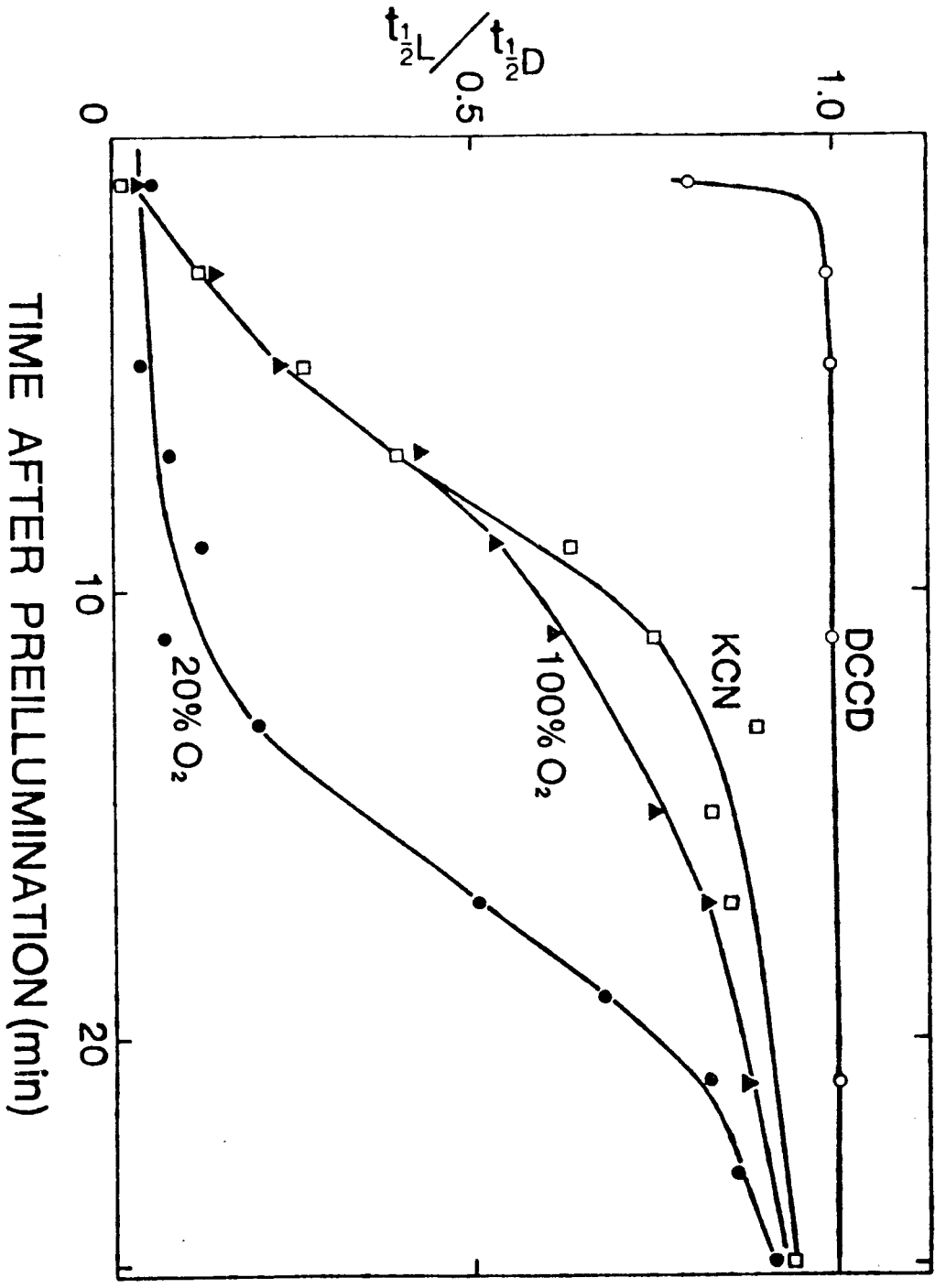
the original slow decay was much longer with KCN (8-9 min) than with DCCD (< 1 min) (Figure 4.8). The time course of dark recovery after preillumination in the presence of KCN or high $O_2$ did, however, lack the initial lag phase which increases the recovery halftime to 18 min in uninhibited leaves, correlating well with the 23 min reported by Morita et al. (1982) for spinach leaves.

## 4.3 Discussion

Factors governing the acceleration of $\Delta A_{518}$ decay by preillumination are complex. The strong correlation between ATPase activity and $\Delta A_{518}$ decay acceleration (Morita et al., 1982) has suggested that increased proton flux through activated $CF_0$-$CF_1$ ATP synthetase is responsible (Itoh and Morita, 1982). Although the mechanism is unclear, $CF_0$-$CF_1$ ATP synthetase has been shown to be activated in the light by both the transmembrane electric potential (Schlodder and Witt, 1981) and electron transport (Mills et al., 1980).

In dark adapted intact chloroplasts, ATP hydrolase activity of $CF_1$ is stimulated by repetitive flashes with a dark interval between flashes of $\tau$ = 5s (Inoue et al., 1978). This is consistent with the acceleration of $\Delta A_{518}$ decay shown to occur at $\tau$ = 5s compared to the dark adapted limit at longer $\tau$ (Figure 4.5). When the decay is measured at $\tau$ = 10s, stimulation of electron transport by addition of red background illumination accelerates the decay suggesting

Figure 4.8 The time course of dark recovery of $\Delta A_{518}$ after a 2 min preillumination with 200 W $m^{-2}$ red light, measured with $\tau$=10s for a control leaf and leaves inhibited with KCN and DCCD in air compared with leaves in the presence of 100% $O_2$. $t_{1/2D}$ is the halftime before and $t_{1/2L}$ after preillumination.


$t_{\frac{1}{2}L} / t_{\frac{1}{2}D}$
0
0.5
1.0
DCCD
KCN
100% $O_2$
20% $O_2$
10
20
TIME AFTER PREILLUMINATION (min)

that ATP-hydrolase activity is increased. When only photosystem I is activated (far-red light), the transient acceleration of $\Delta A_{518}$ decay observed is presumably limited by the reduced fraction of the plastoquinone pool. As discussed in chapter 1, plastoquinone is partially reduced in the dark (Arnon and Chain, 1979) so a transient reduction of photosystem I acceptors will result from addition of far-red light. The effect of far-red light was completely reversible after a 2-3 min dark interval which further supports this idea. This result localizes the site of background light induced decay acceleration and possibly $CF_1$ ATP-hydrolase activation to the reduction of photosystem I electron acceptors. Further support arises from the inhibition of red light induced decay acceleration by the $CF_0$-$CF_1$ inhibitor DCCD or the electron transport inhibitor DCMU. KCN, reported to inhibit plastocyanin *in vitro* by Izawa et al. (1973) also inhibited the background red light effect, suggesting that KCN inhibited electron transport *in vivo*.

The ambient $O_2$ concentration strongly affects the $\Delta A_{518}$ decay both in dark adapted and light adapted leaves. The manifestation of this effect was dependent on $CF_0$-$CF_1$ activity as all $O_2$ influence on $\Delta A_{518}$ decay was inhibited by DCCD. The involvement of electron transport is shown by the loss of $O_2$ effects on decay in leaves treated with KCN. Inhibition of electron transport from photosystem II to the plastoqui-

none pool with DCMU did not inhibit the acceleration of $\Delta A_{518}$ decay by $O_2$ concentrations of less than 5% $O_2$. These results show a similar $O_2$ dependence to that of ATP formation by cyclic electron transport in the presence of DCMU (Woo et al., 1983). Inhibition of the fast decay component of $\Delta A_{518}$ by $O_2$ occurs with a much lower concentration of $O_2$ when reduction of intersystem carriers is blocked by DCMU treatment. Oxygen appears to be competing with electron transport for control of the $\Delta A_{518}$ decay. This is shown most clearly by the linear dependence observed between the amount of added $O_2$ necessary to reverse the background light induced $\Delta A_{518}$ decay acceleration and the background light intensity. This linearity indicates a direct competition between $O_2$ and the background light induced electron transport to photosystem I acceptors. The most probable site for such a competition would be ferredoxin. Ferredoxin is reduced by photosystem I (and possibly by photosystem II; Arnon et al., 1981) and has been shown to be oxidized by $O_2$ both in the light and in the dark (Furbank and Badger, 1983; Hasein and Palmer, 1983). As $O_2$ also inhibits the $\Delta A_{518}$ decay independently of input light energy (flash frequency), any mechanism describing the effect of $O_2$ on $\Delta A_{518}$ decay must explain both light dependent and independent effects thus ferredoxin is a plausible intermediary. However, as measurements were done in vivo, the contribution of mitochondria to chloroplast adenylate levels as a function

of $O_2$ concentration must be considered. ATP has been reported to increase the contribution of the fast decay component of $\Delta A_{518}$ *in vitro* in the dark (Schreiber and Rienits, 1982; Shuurmans and Kraayenhof, 1983) just as lowered $O_2$ does *in vivo*, but it seems unlikely that lowered $O_2$ should increase the mitochondrial contribution to chloroplast ATP. Also the linear relationship between $O_2$ and background light effects on $\Delta A_{518}$ decay indicate a close relationship between $O_2$ and chloroplast electron transport.

The chloroplast enzyme ferredoxin-thioredoxin-reductase (Fd-Th-reductase) has been shown to activate $CF_1$ ATP-hydrolase activity in the presence of light induced electron transport to ferredoxin *in vitro* (Mills et al., 1980). This activation and the ferredoxin, Fd-Th-reductase activation of other chloroplast enzymes *in vitro* (Shuurmans and Jacquot, 1979) are inhibited by $O_2$. The $\delta$ subunit of $CF_1$ is a thioredoxin type protein that can activate $CF_1$ in the presence of reduced Fd-Th-reductase (McKinney et al., 1979) which supports the hypothesis that Fd-Th-reductase regulates $CF_1$ *in vivo*. Marchant (1981) has proposed that the ATP hydrolase activity observed in thylakoids, isolated after illumination *in vivo*, is a function of the chloroplast thioredoxin system.

The effect of Fd-Th-reductase on $CF_1$ is not limited to ATP-hydrolase activity, as Mills and Mitchell (1982) have

shown that ATP formation induced by an acid-base transition is increased in thiol treated chloroplasts. The $O_2$ sensitivity of the electron transport dependent ferredoxin, Fd-Th-reductase activation of $CF_1$ has been previously discussed as an anomaly, i.e. activation would only occur when the experiment was carried out under a continuous stream of $N_2$ (Mills et al., 1981). The results presented in this chapter are consistent with an electron transport dependent $O_2$ sensitive activation of $CF_1$ (giving rise to an accelerated $\Delta A_{518}$ decay) by Fd-Th-reductase *in vivo*. The activation of $CF_1$ *in vivo* is also inhibited by $NADP^+$ (Mills et al., 1981) as is ferredoxin dependent $O_2$ uptake (Furbank and Badger, 1983). It is apparent that $O_2$ may compete with $NADP^+$, Fd-Th-reductase and possibly cyclic electron transport for electrons from ferredoxin, and thus exert strong regulation of ATP and NADPH formation *in vivo*.

# Chapter 5

## The Anaerobic Inhibition of Photosynthesis

### 5.1 Introduction

The effects of $O_2$ on fluorescence induction and $\Delta A_{518}$ kinetics *in vivo* have been characterized in chapters 3 and 4. In this chapter the effects of anaerobiosis on fluorescence, $\Delta A_{518}$ and photosystem II activity are described. Evidence has been provided for the anaerobic inhibition of photosystem II activity (Vidaver, 1964; Schreiber and Vidaver, 1974; Diner and Mauzerall, 1973; Greenbaum and Mauzerall, 1976) electron transport and $CO_2$ uptake (Urbach and Fork, 1964; Slovacek and Hind, 1977; Ziem-Hanck and Heber, 1980; Satoh, 1980; Satoh, 1982) *in vivo* and in intact chloroplasts. The proposed sites of inhibition have involved photosystem II, photosystem I, cyclic and, of course, pseudocyclic electron transport. Results presented in this chapter support an anaerobic inhibition of photosystem II activity.

#### 5.1.1. Delayed fluorescence

This chapter includes both prompt and delayed fluorescence measurements. Prompt fluorescence induction (measured during illumination) has been described in detail in chapter 3.

Delayed fluorescence is emitted after illumination and persists for milliseconds in the dark. This fluorescence arises from charge recombination at photosystem II where $Q^-$ reduces $P680^+$ leaving P680 in an excited state, $P680^*$. $P680^*$ can either fluoresce, transfer its excitation energy to the light harvesting antennae chlorophylls or lose its excitation energy by non-radiative processes. Delayed fluorescence is therefore emitted by both $P680^*$ and excited antennae chlorophyll molecules (Amesz and Van Gorkham, 1978). The intensity of delayed fluorescence is much lower than that of prompt fluorescence and is effectively masked by prompt fluorescence during illumination.

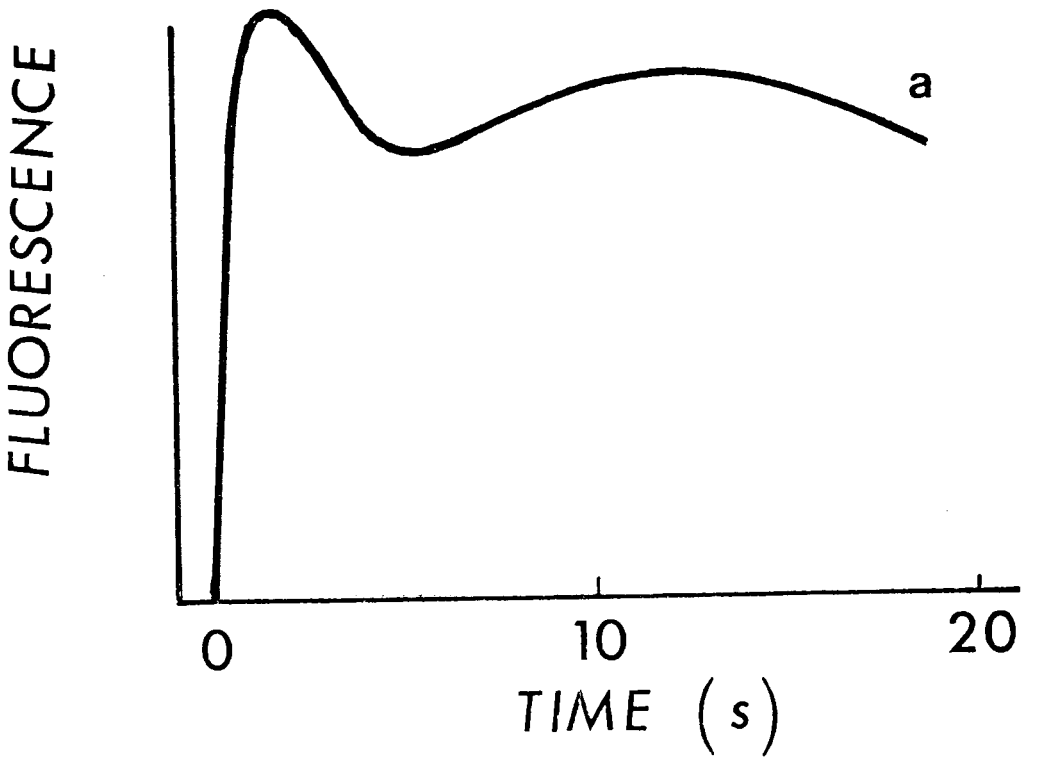
The amplitude of delayed fluorescence depends on both the concentration of $Q^-$ and $P680^+$. This recombination occurs quickly, however delayed fluorescence persists over milliseconds due to an equilibrium between the plastoquinone pool and Q. As the concentration of $Q^-$ in the dark will depend on the concentration of reduced PQ, inhibition of electron transport between Q and PQ with DCMU quenches millisecond delayed fluorescence (Morita et al., 1981). These same conditions cause an increase in prompt fluorescence. Similarly, inhibition of electron transport between the electron donor of photosystem II and P680 would also be expected to decrease delayed fluorescence but not necessarily quench prompt fluorescence.

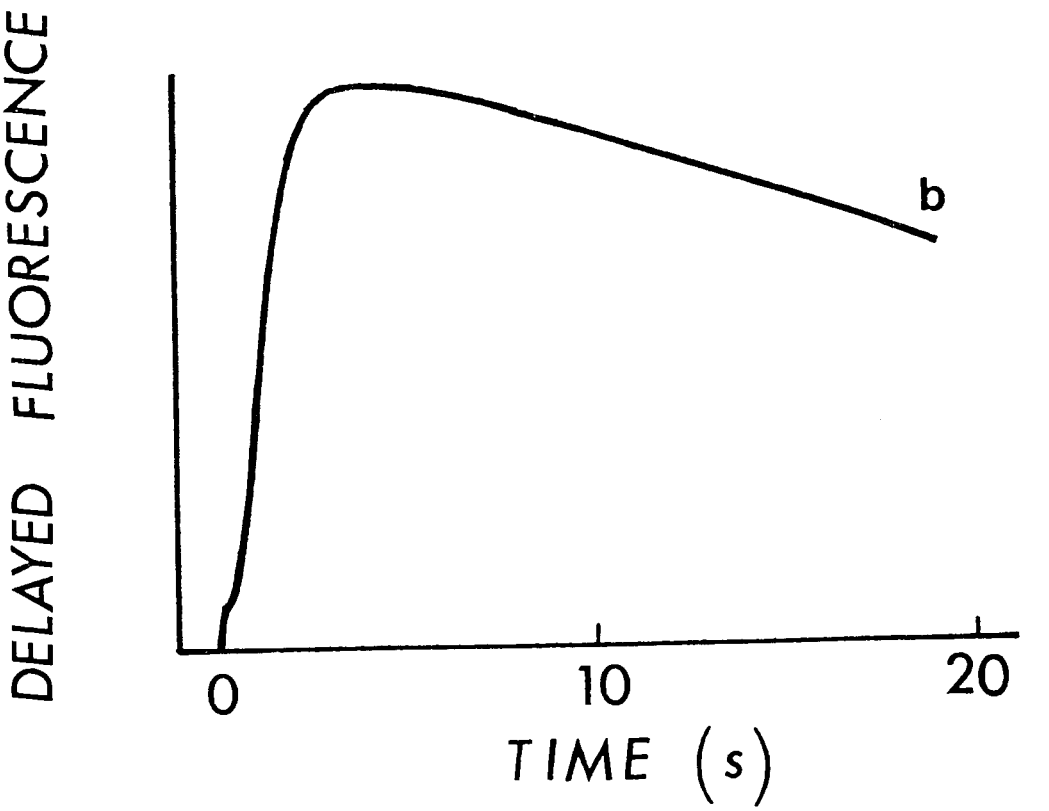
Assuming no changes in the concentration of $P680^+$ or inhibition of electron transport between Q and PQ, the induction kinetics of millisecond delayed fluorescence measured with a phosphoroscope (as described in chapter 2) should parallel the changes in the concentration of $Q^-$ with time similarly to prompt fluorescence. Large differences in the induction transients would result from inhibition of either the immediate donor or acceptor side of photosystem II. Although they differ in detail, both prompt and delayed fluorescence induction transients reach a maximum within seconds and decay over minutes in spinach leaves (Figure 5.1). Results from Morita et al. (1981) indicate that anaerobiosis quenches millisecond delayed fluorescence and increases prompt fluorescence. These results suggest the involvement of photosystem II in the anaerobic inhibition of photosynthesis. Morita et al. (1981) also reported an inhibition of preillumination induced acceleration of $\Delta A_{518}$ decay under anaerobic conditions.

In this chapter it is shown that:

1. both prompt and delayed fluorescence show similar responses to $O_2$ except under anaerobic conditions.
2. anaerobiosis inhibits light induced $\Delta A_{518}$ decay acceleration in broken chloroplasts with added ADP and phosphate.

Figure 5.1 Fluorescence induction transients for a spinach leaf in air. a) prompt fluorescence. b) millisecond delayed fluorescence. Actinic light intensity 10 W $m^{-2}$ for both curves.


FLUORESCENCE
a
0
10
20
TIME (s)


DELAYED FLUORESCENCE
b
0
10
20
TIME (s)

3. the anaerobic inhibition of light induced $\Delta A_{518}$ decay acceleration in chloroplasts is not present when hydroxylamine replaces water as electron donor to photosystem II.
4. the reduction of FeCN by photosystem II is inhibited anaerobically.

## 5.2 Results

### 5.2.1 $\Delta A_{518}$ ,prompt, and delayed fluorescence in vivo

It is immediately apparent that the lack of $O_2$ influences photosynthesis very differently than "low" $O_2$ concentrations when the effects of $O_2$ down to $10^{-5}$ atm partial pressure $O_2$ on the dark adapted $\Delta A_{518}$ decay rate in lettuce leaves are seen in Figure 5.2. The decay halftime decreases with lowered $O_2$ until approximately 0.01 atm partial pressure $O_2$. Removal of more $O_2$ now inhibits the dark decay of $\Delta A_{518}$, Curve D. Additionally, under these "anaerobic" conditions, preillumination will no longer accelerate the $\Delta A_{518}$ decay as shown by the dependence of the light adapted $\Delta A_{518}$ decay rate on $O_2$, Curve L.

In Figure 5.3 a similar response to $O_2$ is found for the amplitude of millisecond delayed fluorescence in spinach leaves. Both anaerobic and high $O_2$ concentrations decrease the amplitude of delayed fluorescence. There are clearly two distinct sites of $O_2$ influence on both $\Delta A_{518}$ decay kinetics

Figure 5.2 Decay halftime of $\Delta A_{518}$ in lettuce leaves versus $O_2$ pressure (partial pressure below 1 atm) for dark adapted leaves (D) and leaves pre-illuminated for 1 min with 10 W $m^{-2}$ blue (Corning filter 4-96) light (L).

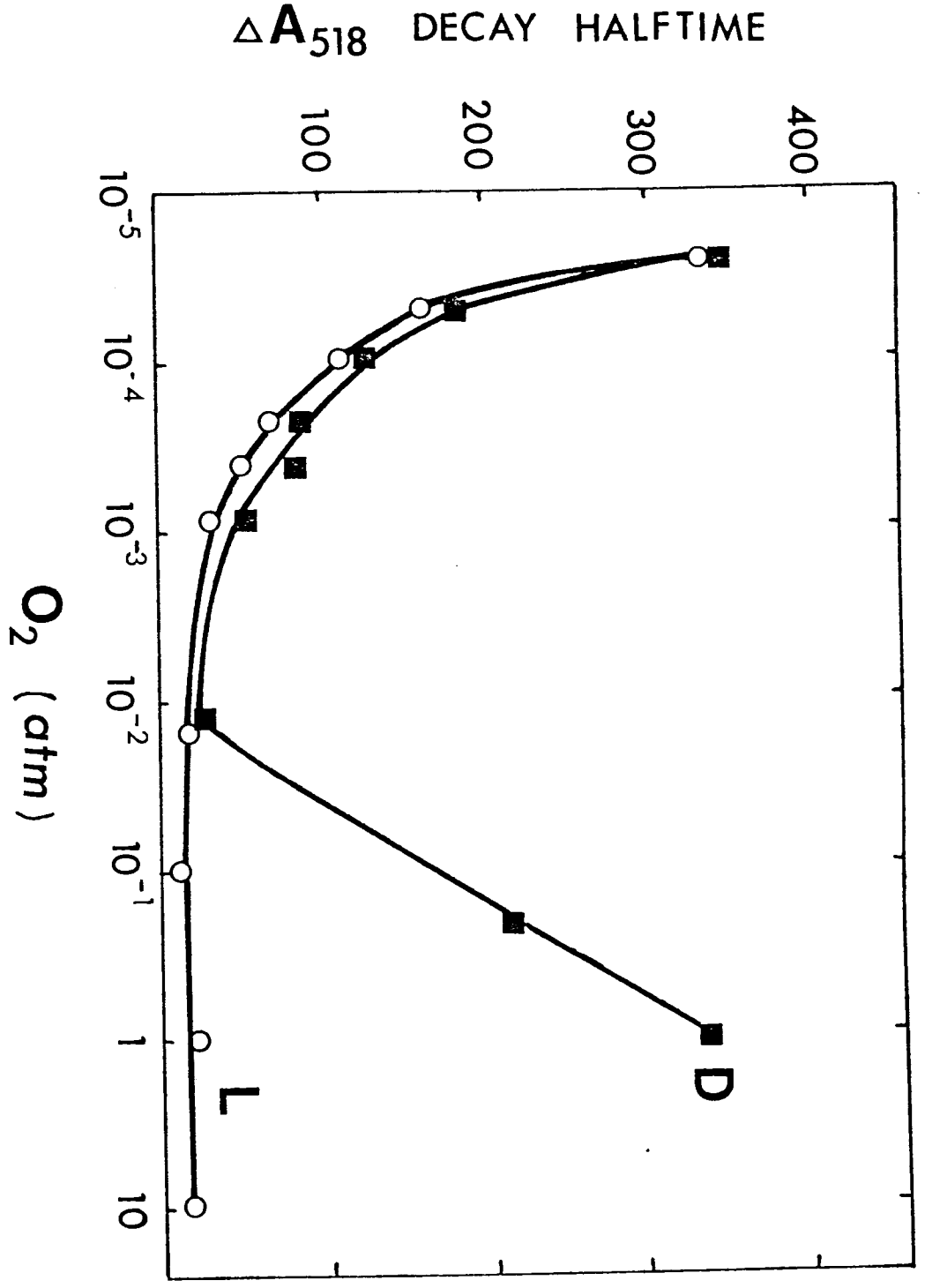
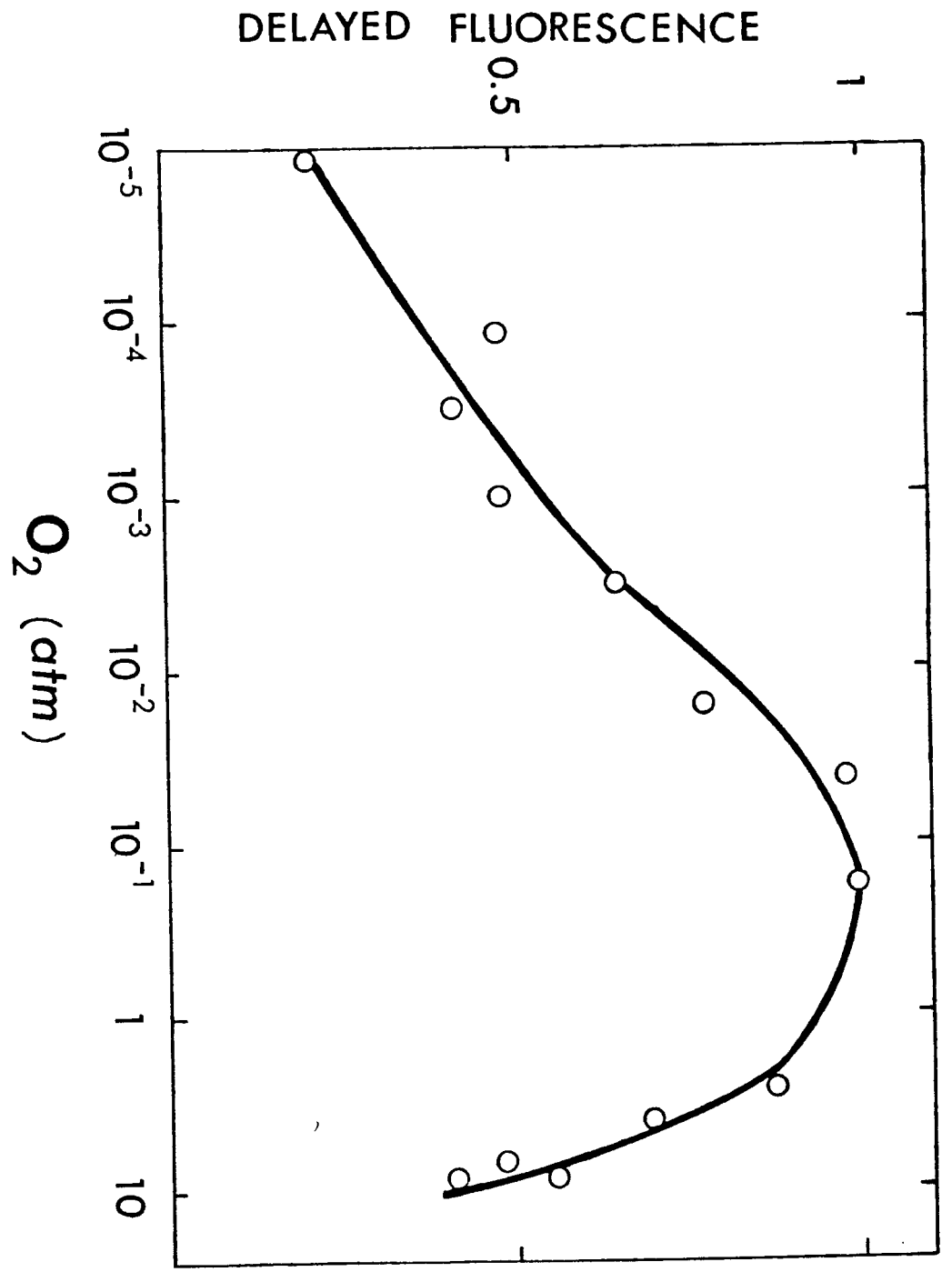

ΔA518 DECAY HALFTIME
100
200
300
400
O2 (atm)
10-5
10-4
10-3
10-2
10-1
1
10
L
D

Figure 5.3 Amplitude of peak in induction curve of millisecond delayed fluorescence in spinach leaves versus $O_2$ pressure (partial pressure below 1 atm).


DELAYED FLUORESCENCE
0.5
1
$10^{-5}$
$10^{-4}$
$10^{-3}$
$10^{-2}$
$10^{-1}$
1
10
$O_2$ (atm)

and delayed fluorescence emission. In Figure 5.4 prompt fluorescence, $f_p$, is shown as a function of $O_2$ concentration. The curve is the same as that presented in chapter 3 except concentrations of $O_2$ less than 0.2 atm are now included. No dichotomy in the $O_2$ effect is apparent.

### 5.2.2. $\Delta A_{518}$ decay kinetics under anaerobic conditions *in vitro*

Anaerobically, preillumination did not accelerate the decay of $\Delta A_{518}$ in lettuce leaves; similar results have been reported for *Zea mays* (Morita et al., 1981). The acceleration of $\Delta A_{518}$ decay is also inhibited by DCMU *in vivo* as previously reported by Morita et al. (1981) (Figure 5.5). In broken lettuce chloroplasts preillumination did not accelerate the $\Delta A_{518}$ decay appreciably (not shown) as it does in the presence of ADP and phosphate. An acceleration of decay was observed on addition of ADP and phosphate to chloroplasts in the dark which has been reported for spinach (Junge et al., 1970). The $\Delta A_{518}$ decay halftime decreased with increasing ADP and phosphate concentration in both dark adapted and light adapted chloroplasts, however, light adapted chloroplasts showed a distinctly greater decay acceleration than the dark adapted chloroplasts (Figure 5.6).

At a concentration of 20 mM ADP and phosphate, preillumination decreased the $\Delta A_{518}$ decay halftime from 120 ms to 60

Figure 5.4 Amplitude of peak in induction curve of prompt fluorescence in lettuce leaves versus $O_2$ pressure (partial pressure below 1 atm).

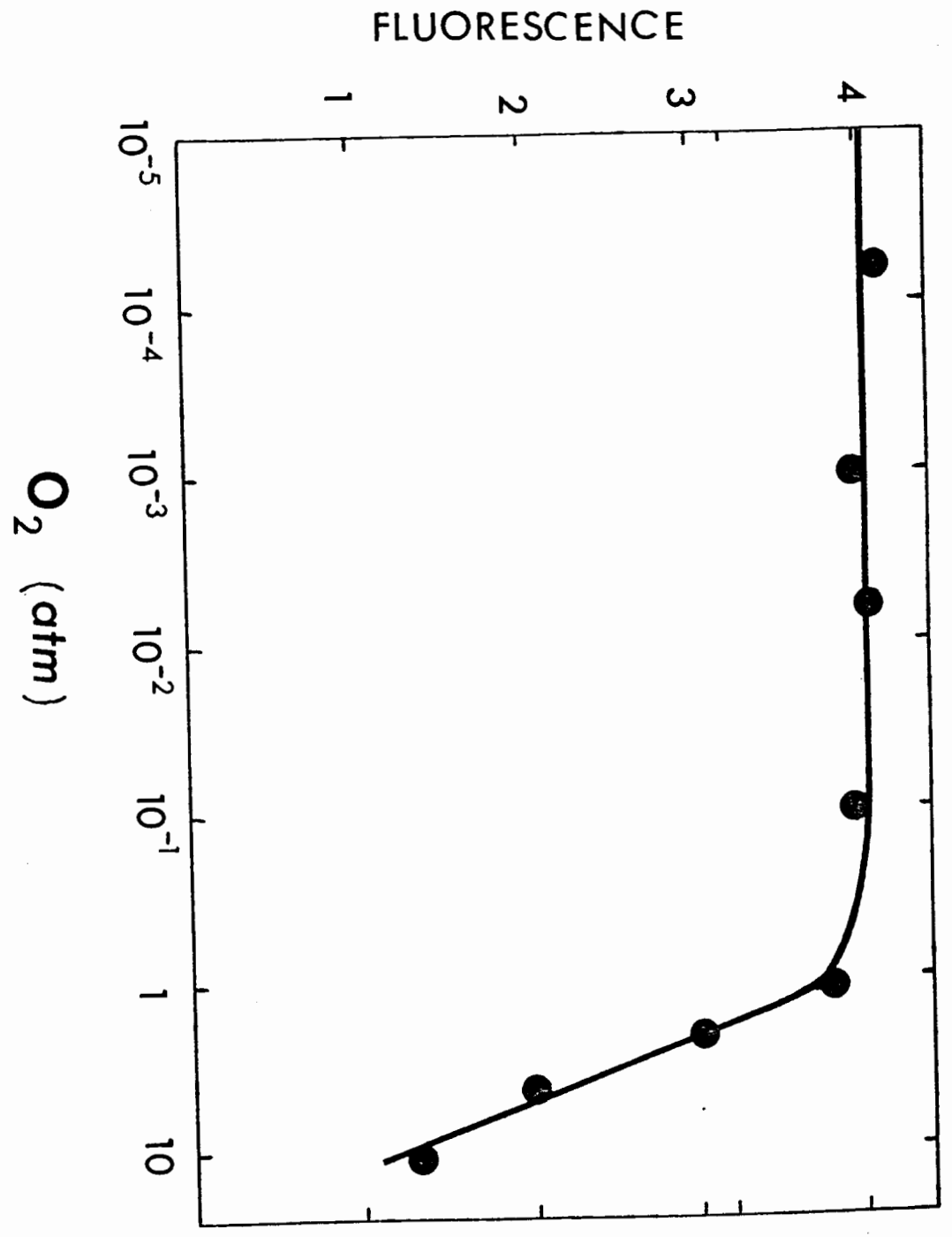

FLUORESCENCE
1
2
3
4
$10^{-5}$
$10^{-4}$
$10^{-3}$
$10^{-2}$
$10^{-1}$
1
10
$O_2$ (atm)

Figure 5.5 $\Delta A_{518}$ in lettuce leaves in air. The numbers refer to decay halftimes in ms. From top to bottom the traces are: leaf dark adapted for 1 h (D), preilluminated (L) for 1 min at 10 W $m^{-2}$, preilluminated anaerobically (L,-$O_2$), and preilluminated in the presence of DCMU. Traces are the averages of 20 measurements taken at a frequency of 0.017 Hz.

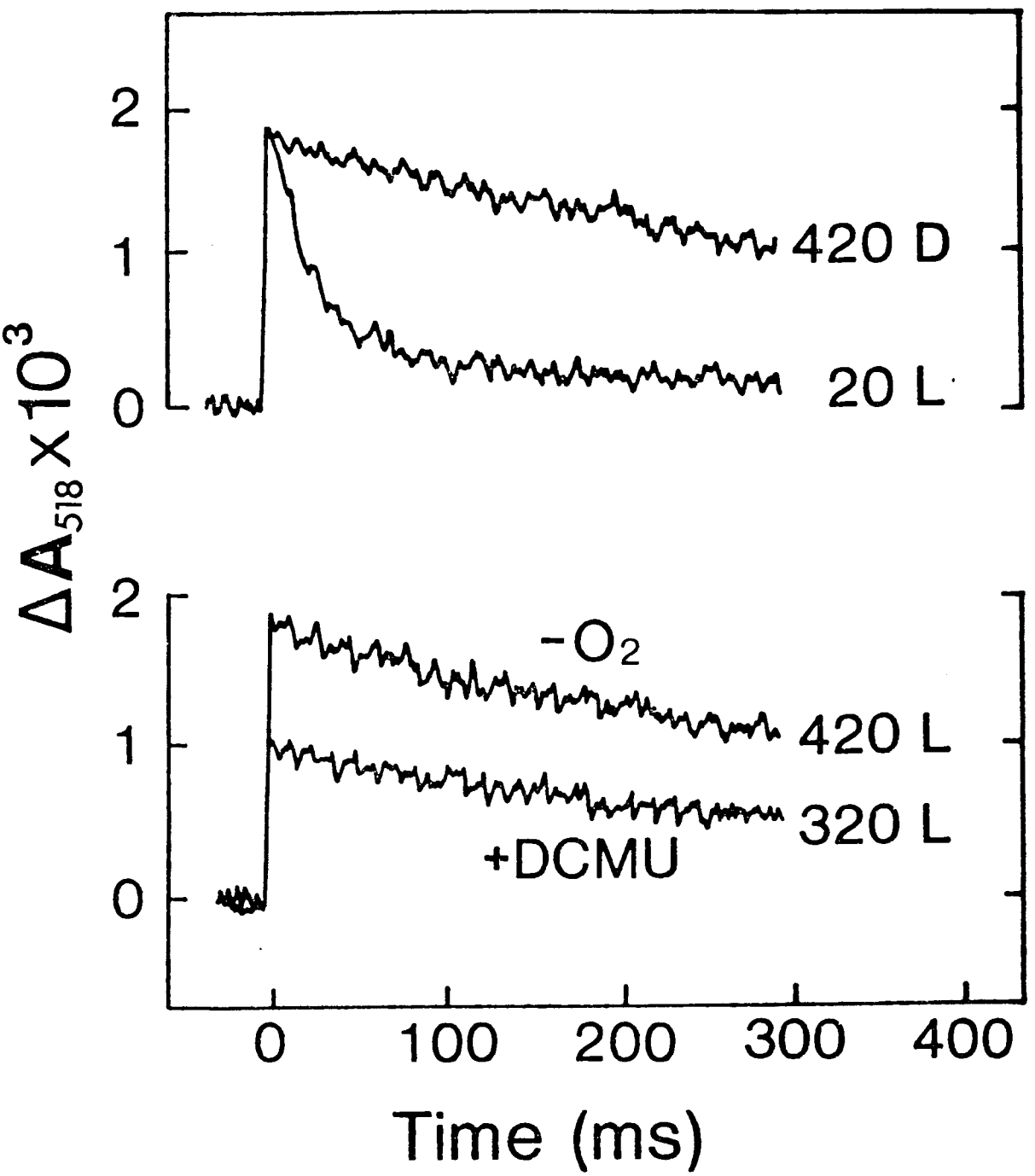
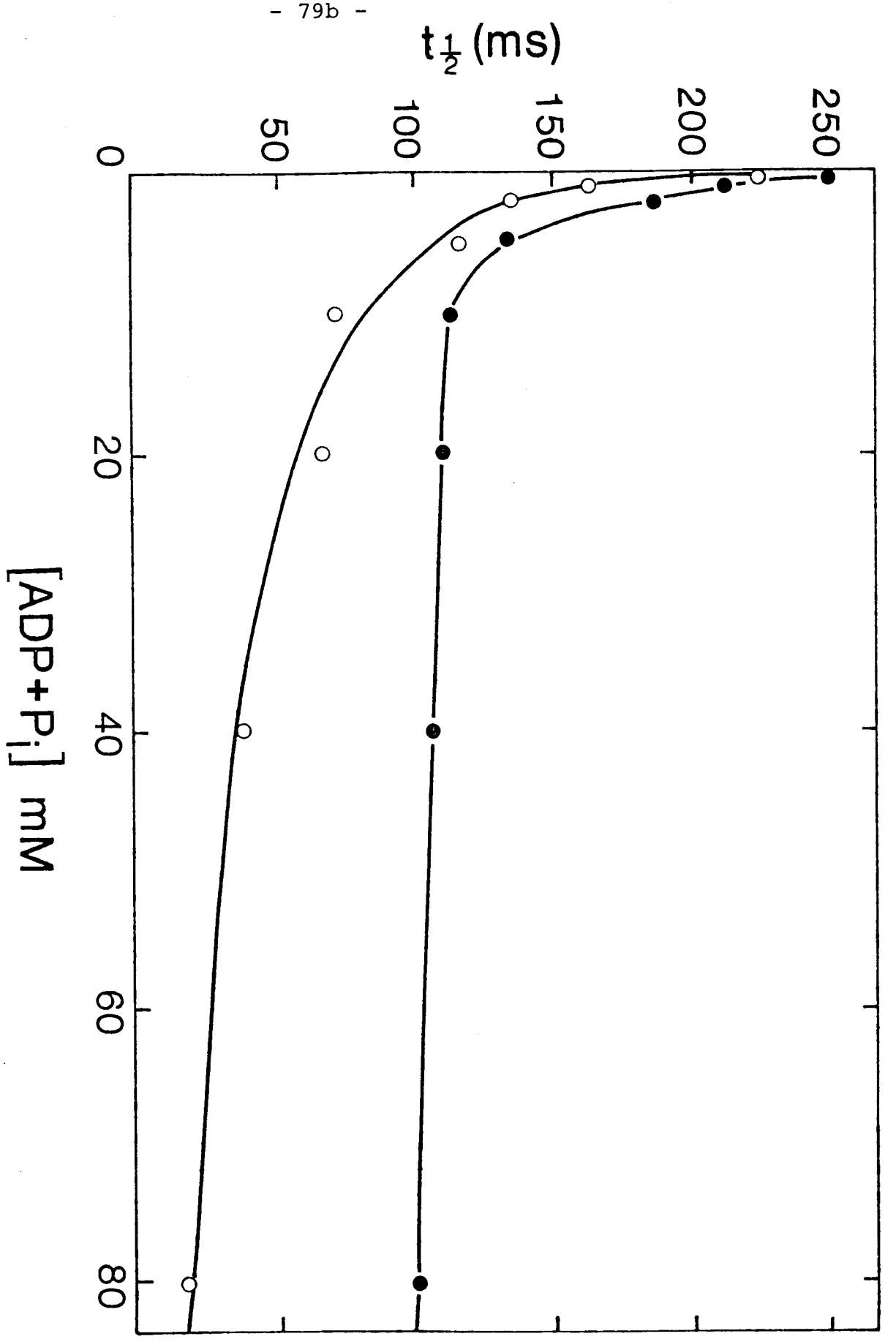

2
1
0
420 D
20 L
$\Delta A_{518} \times 10^3$
2
1
0
$-O_2$
420 L
320 L
+DCMU
0
100
200
300
400
Time (ms)

Figure 5.6 Decay halftime ($t_{1/2}$) of $\Delta A_{518}$ in lettuce chloroplasts versus concentration of added ADP and phosphate following preillumination (O) or dark adaptation (O).


$t_{\frac{1}{2}}$ (ms)
0
50
100
150
200
250
[ADP+$P_i$] mM
0
20
40
60
80

ms. A light induced decrease in decay halftime was also observed in the presence of methyl viologen from 230 ms to 120 ms or pyocyanine from 220 ms to 60 ms (Figure 5.7).

Treatment of chloroplasts with hydroxylamine did not induce a large change in the dark adapted $\Delta A_{518}$ decay rate. These chloroplasts also showed a light induced acceleration of decay in the presence of added ADP and phosphate (Figure 5.8a,b). The light induced acceleration of decay observed in chloroplasts was inhibited by the lack of $O_2$. However, the acceleration was not inhibited anaerobically in hydroxylamine treated chloroplasts (Figure 5.8c).

Table 5-I compares the relative light-induced acceleration of $\Delta A_{518}$ decay (dark adapted decay halftime, $t_D$/light adapted decay halftime, $t_L$) in hydroxylamine treated and control chloroplasts in the presence and absence of $O_2$ under various conditions. In the control chloroplasts $t_D/t_L$ approaches 1 under all conditions anaerobically, but aerobically is greater than 2 in the presence of ADP and phosphate, methyl viologen, or pyocyanin. In hydroxylamine treated chloroplasts, $t_D/t_L$ is greater than 3 in all cases with or without $O_2$ in the presence of the same additives as the control chloroplasts. Under all conditions in both control and hydroxylamine treated chloroplasts the addition of 15$\mu$M DCMU inhibited light-induced decay acceleration (not shown) as it did *in vivo*.

Figure 5.7 $\Delta A_{518}$ in lettuce chloroplasts in air. The numbers refer to decay halftimes in ms, a) from top to bottom: dark adapted (D) chloroplasts; chloroplasts with 20 mM ADP and phosphate (*), dark adapted (D) and preilluminated (L) for 1 min at 10 W $m^{-2}$. b) Chloroplasts with 2 mM methyl viologen (MV), dark adapted (D) and preilluminated (L). c) Chloroplasts with 0.5 mM pyocyanine (Py), dark adapted (D) and preilluminated (L). Traces are the averages of 20 measurements taken at a frequency of 0.33 Hz.

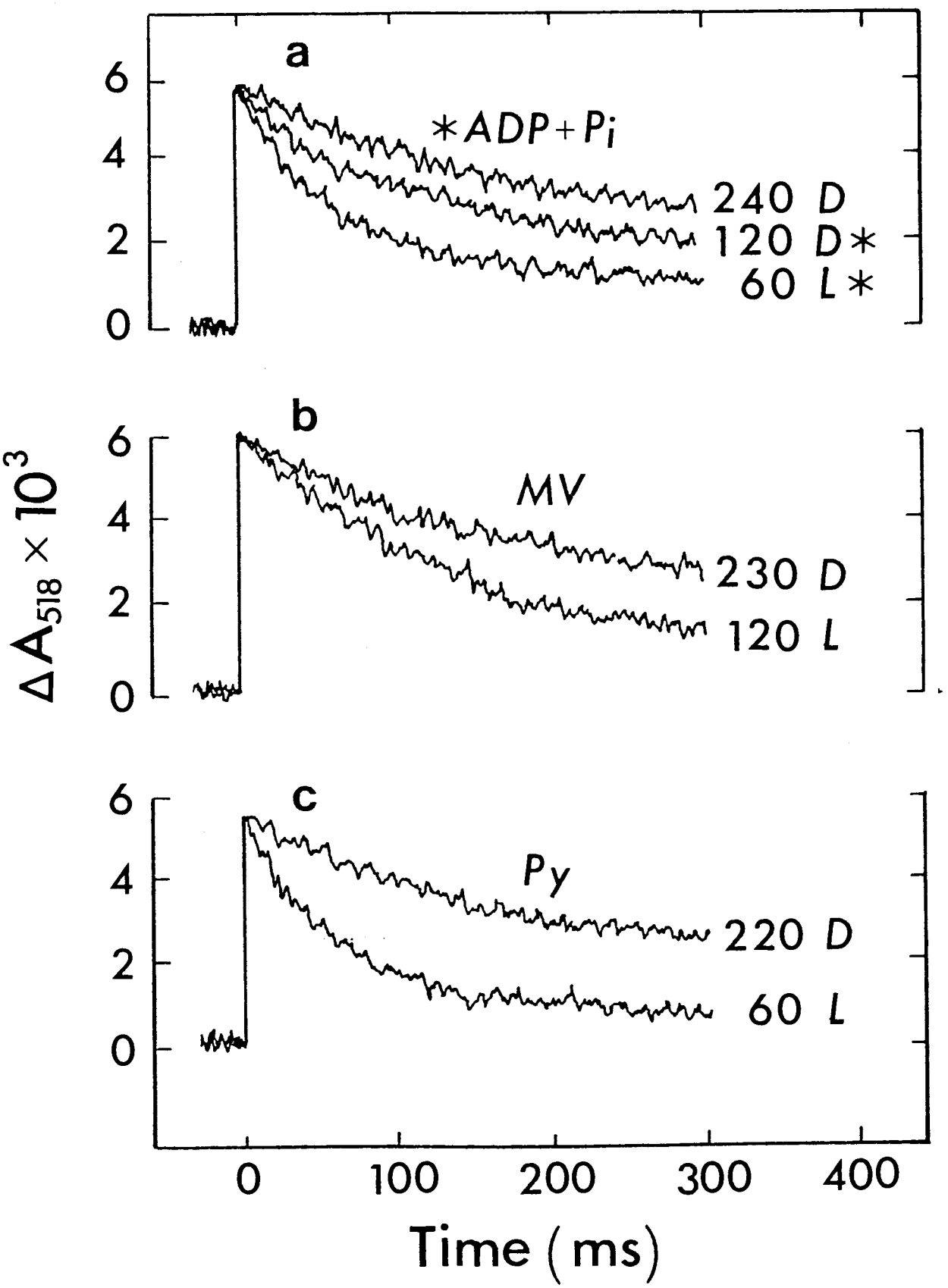

a
*ADP+Pi
240 D
120 D*
60 L*
b
MV
230 D
120 L
c
Py
220 D
60 L
6
4
2
0
ΔA518 × 10^3
0
100
200
300
400
Time (ms)

Figure 5.8 Flash induced 518 nm absorbance change in lettuce chloroplasts with 20 mM ADP and phosphate. Traces labelled (*) were treated with hydroxylamine ($NH_2OH$). a) dark adapted (D) chloroplasts, b) preilluminated (L) chloroplasts, c) anaerobic ($-O_2$) preilluminated (L) chloroplasts. See Figure 5.7 for other details.

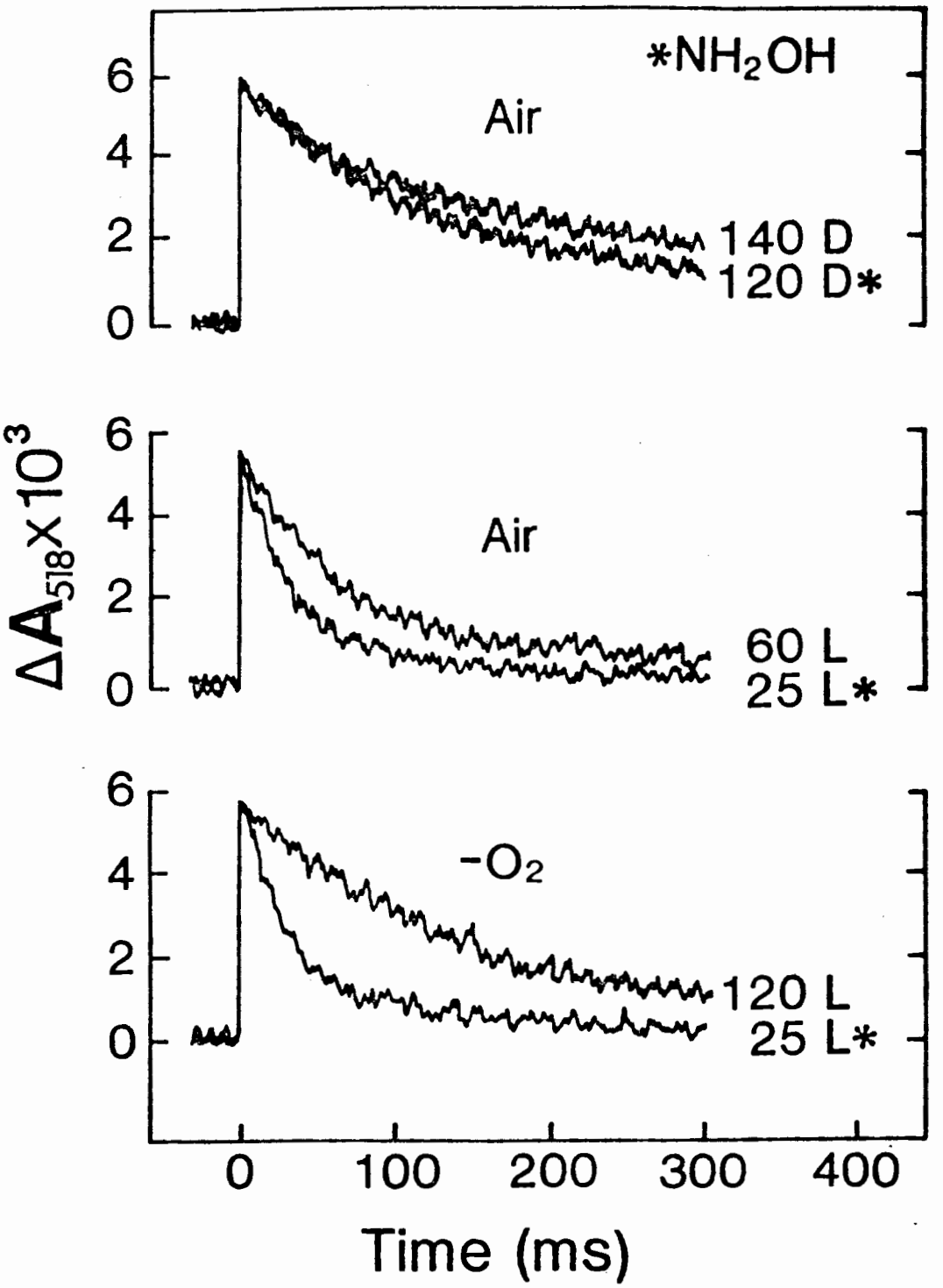

*NH2OH
Air
140 D
120 D*
Air
60 L
25 L*
-O2
120 L
25 L*
ΔA518X10^3
0
2
4
6
0
100
200
300
400
Time (ms)

Table 5-1

The relative light induced acceleration of $\Delta A_{518}$ (dark adapted decay halftime, $t_D$/light adapted decay halftime, $t_L$) in control and hydroxylamine treated chloroplasts under various conditions.

| Additions | control chloroplasts | | hydroxylamine treated chloroplasts | |
|---|---|---|---|---|
| | $(t_D/t_L)_{air}$ | $(t_D/t_L)N_2$ | $(t_D/t_L)_{air}$ | $(t_D/t_L)N_2$ |
| none | 0.97 | 1.0 | 1.1 | 1.0 |
| ADP and phosphate | 2.1 | 1.2 | 4.8 | 4.8 |
| pyocyanine | 3.7 | 1.3 | 4.4 | 4.3 |
| methyl viologen, ADP and phosphate | 3.4 | 0.92 | 3.3 | 3.3 |

$D/t_L)_{air}$ is the relative light induced acceleration of $\Delta A_{518}$ in air.

$(t_D/t_L)N_2$ is the relative light induced acceleration of $\Delta A_{518}$ under anaerobic conditions. See chapter 2 for reagent concentrations and measurement details.

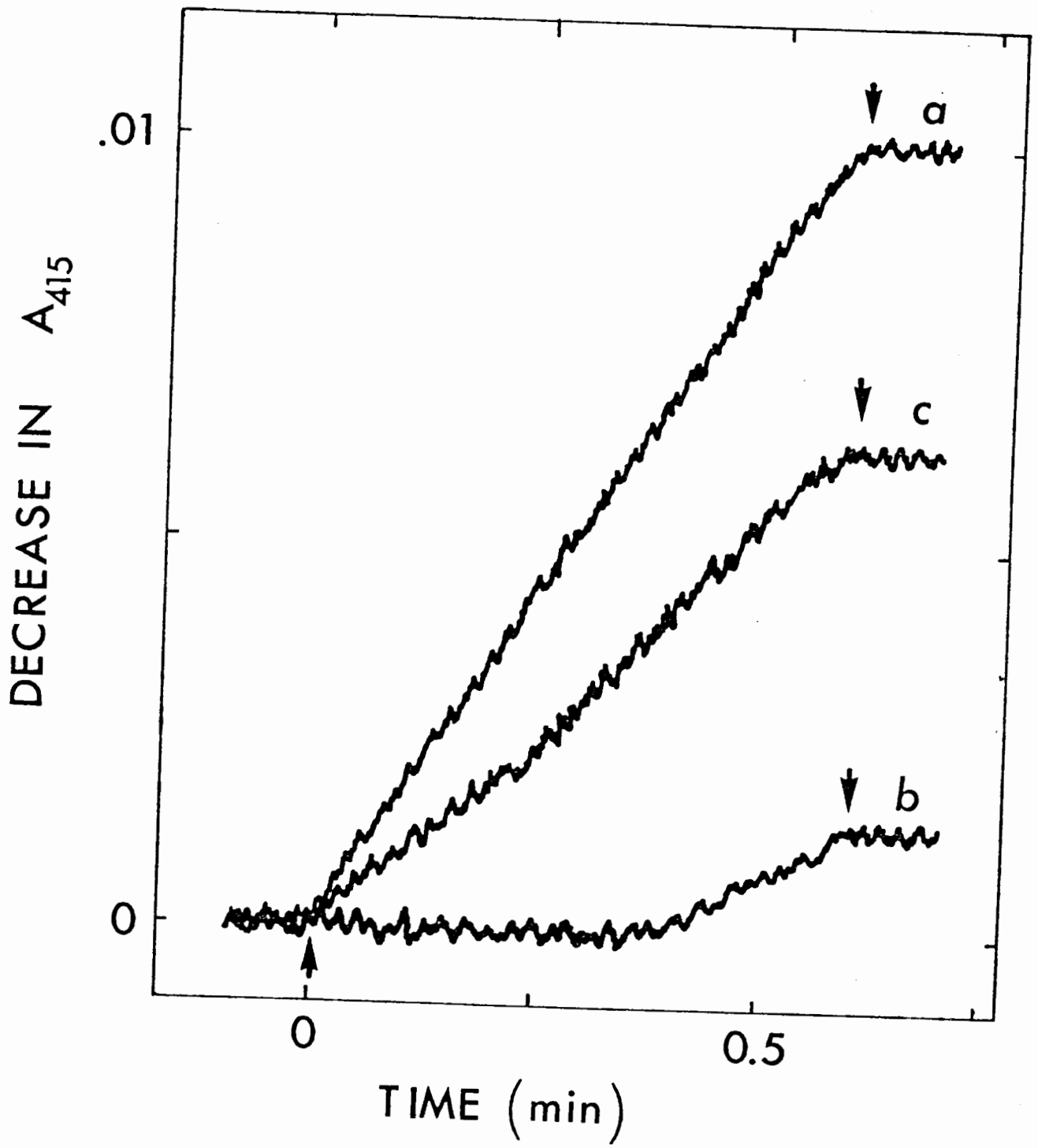
### 5.2.3 Photosystem II activity under anaerobic conditions

Photosystem II activity in chloroplasts was determined by the reduction rate of FeCN in the light as described in chapter 2. The reduction of FeCN (Figure 5.9a,b,c) was inhibited by 20 min exposure to $N_2$ (curve b). The rate achieved on return to air is shown as 5.9c. Time dependent inhibition of photosystem II (Drechsher and Neumann, 1982) was observed with 20 min exposure to FeCN in air which produced a reponse identical to curve 5.9c. The anaerobic inhibition of FeCN reduction under these conditions was fully reversible.

## 5.3 Discussion

The effects of increased oxygen concentrations on both fluorescence induction and $\Delta A_{518}$ decay kinetics have been discussed in terms of increased oxidation of electron transport carriers in chapters 3 and 4. It has been shown in this chapter that decreased $O_2$ concentrations only support the correlation between $O_2$ concentration and redox state of intersystem electron transport carriers to a certain limiting $O_2$ concentration. At $O_2$ concentrations below this limit (anaerobiosis) both delayed fluorescence and the $\Delta A_{518}$ decay are inhibited as they are at high $O_2$ concentrations. However, the anaerobic inhibition of delayed fluorescence differs from the inhibition by increased $O_2$ as anaerobic inhibition is characterized by a complete loss of the initial slow rise of delayed fluorescence while increased $O_2$ concentra-

Figure 5.9 The decrease in FeCN absorption at 415 nm versus time. a) the reduction rate in air immediately after the sample was placed in the sample holder. b) another sample measured after a 20 min exposure to $N_2$ in the dark. c) the sample in b returned to air. Light on (↑), Light off (↓). See chapter 2 for details of the measurement technique.


.01
0
DECREASE IN $A_{415}$
a
c
b
0
0.5
TIME (min)

tions only decrease the ultimate amplitude of delayed fluorescence. Similarly, both anaerobiosis and increased $O_2$ decrease the dark adapted $\Delta A_{518}$ decay rate, but only anaerobiosis decreases the light adapted $\Delta A_{518}$ decay rate. It has been reported that intact chloroplasts show an optimum in light induced $\Delta pH$ formation (Ziem-Hanck and Heber, 1980) and $CO_2$ fixation (Woo et al., 1983) at a low $O_2$ concentration (0.01 atm and 0.05 atm respectively) when photosystem I is primarily responsible for electron transport. Higher or lower $O_2$ concentrations inhibit both $\Delta pH$ formation or $CO_2$ fixation in these chloroplasts. These conditions are known to support cyclic electron transport around photosystem I and anaerobiosis or increased oxygen have been suggested to inhibit cyclic electron transport by over-reduction or over-oxidation of intersystem electron transport carriers, respectively. This idea was initially formulated by Arnon and Chain (1975) who proposed that the "redox poise" of intersystem electron carriers determined the rate of cyclic electron transport and was influenced to some extent by $O_2$.

In the previous two chapters both the quenching of variable fluorescence and inhibition of $\Delta A_{518}$ decay induced by increased $O_2$ were explained by mechanisms involving the oxidation of electron carriers by $O_2$. However, results presented in this chapter indicate that the anaerobic inhibition of light induced $\Delta A_{518}$ decay acceleration is not a mani-

festation of over-reduction of intersystem electron transport carriers. The anaerobic inhibition of light induced $\Delta A_{518}$ decay acceleration is dependent on the electron donor to photosystem II. When hydroxylamine replaced water as electron donor there was no longer an $O_2$ requirement for light induced $\Delta A_{518}$ decay acceleration. As previously described in chapter 4, $\Delta A_{518}$ decay acceleration has been correlated to ATPase activity of $CF_1$ (Morita et al., 1981; Itoh and Morita, 1982), and $CF_0$-$CF_1$ ATPase has been shown to be activated in the light by both the transmembrane electric potential (Schlodder and Witt, 1981) and electron transport (Mills et al., 1980). An $O_2$ requirement for $\Delta A_{518}$ decay acceleration in the light could stem from an anaerobic inhibition of ATPase directly or from inhibition of electron transport and concomitant high energy state formation at many sites. However, the dependence of the anaerobic inhibition on the photosystem II electron donor suggests water-splitting to be the site. The anaerobic inhibition of FeCN reduction by chloroplasts in the light supports this idea. An inhibition of photosystem II electron transport could affect $CF_1$ activation by inhibiting the reduction of ferredoxin and thus activation of $CF_1$ by ferredoxin-thioredoxin-reductase and also by the inhibition of high energy state formation induced by linear electron transport.

## Chapter 6

### Summary

This thesis presents evidence indicating at least two sites of $O_2$ influence on photosynthetic electron transport *in vivo*.

1) $O_2$ acts as an oxidant of electron transport carriers. The electron transport pathway from photosystem II to $O_2$ is blocked by DCMU, DBMIB and histone, indicating the involvement of plastoquinone and plastocyanin in this pathway. The role of $O_2$ as terminal electron acceptor suggests the operation of pseudocyclic electron transport both as an energy dissipative mechanism and a means of generating ATP. The oxidation of electron carriers by $O_2$ also appears to influence an electron transport dependent modulation of coupling factor activity as determined by $\Delta A_{518}$ decay rates.

2) Low levels of $O_2$ appear necessary for maximal photosystem II activity. This $O_2$ requirement is dependent on the electron donor to photosystem II in that $O_2$ is not required when hydroxylamine replaces water as donor to photosystem II.

As discussed in chapter 3, the site of electron trans-

port oxidation by $O_2$ is not clear. The fluorescence quenching data indicates the involvement of plastoquinone and plastocyanin in the pathway to $O_2$, however it does not distinguish whether $O_2$ acts on the reducing or oxidizing side of photosystem I. Oxygen has long been known to oxidize soluble ferredoxin and recent work has indicated that pseudocyclic electron transport via ferredoxin may regulate ATP/NADPH ratios in $C_3$ and mesophyll cells of $C_4$ species (Furbank and Badger, 1983; Furbank et al., 1983). However, the efficient fluorescence quenching by $O_2$ during induction of photosynthesis ( 500 ms) suggests that intersystem electron carriers were oxidized in the dark, indicative of a site on the oxidizing side of photosystem I. In the presence of an electron donor to plastoquinone chloroplasts generate ATP in the dark via a mechanism dependent on functional plastoquinone and the presence of $O_2$ (Bottin et al., 1981). It appears that $O_2$ may act as terminal electron acceptor for pseudocyclic electron transport on either side of photosystem I. Results presented in chapter 4 reflect the antagonistic effects of light induced electron transport and increased $O_2$ on the $\Delta A_{518}$ decay kinetics. Although the acceleration of $\Delta A_{518}$ decay induced by increased electron transport was observed transiently with photosystem I light, the antagonistic effect of increased $O_2$ could result from oxidation on either side of photosystem I

and thus does not help localize the site. The data presented in chapters 3 and 4 do however support the contention that electron transport to $O_2$ does occur *in vivo* and may help to regulate ATP/NADPH ratios as well as form an energy dissipative pathway under high light conditions.

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