

THE DEVELOPMENT OF PHOTOSYNTHESIS IN GREENING BARLEY AS
MONITORED BY CHLOROPHYLL A FLUORESCENCE

by

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Title of Thesis/Project/Extended Essay

The development of photosynthesis in greening barley

as monitored by chlorophyll-a fluorescence

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ABSTRACT

In thylakoid membranes in chloroplasts, two reaction centers, P680 and P700, use excitation energy from light-harvesting complexes to send electrons through a series of oxidation-reduction reactions. This results in the evolution of oxygen and the production of two energy-rich molecules, ATP and NADPH. The chloroplasts are underdeveloped in plants grown in the dark from seeds. When placed in the light, they complete their development undergoing changes in the light-harvesting pigment complex, and the capacity for electron transport.

If a plant, previously given light, is kept in the dark for several minutes then illuminated, the photochemistry changes over several seconds before reaching a steady-state. Fluorescence emitted from chlorophyll molecules in the light-harvesting complex competes with photochemistry for the excitation and thus can be used to monitor these changes. Fluorescence seen within milliseconds, F_0 , indicates the amount and organization of chlorophyll in the light-harvesting complex, while the peak, F_p , seen seconds later, is a measure of the oxidation-reduction change that occurred. Oxygen as an electron acceptor adds to photochemistry that competes with and quenches fluorescence.

Barley (*Hordeum vulgare* L.) was grown in the dark for 7 days and then transferred to continuous light. During 6 to 48 hours of illumination the relative peak fluorescence, $f_p = (F_p - F_0)/F_0$, correlated with the increase in

photosystem II activity measured by the oxygen evolved per unit leaf area. At 3 hours the oxygen evolving system was not fully functional and its activity was less than indicated by the fluorescence, f_p . By 48 hours, all measures matched those of greenhouse control plants grown in light for 8 days. Quenching of f by 7 atm of oxygen was 72% at 3 hours and levelled off at 50% after 12 hours of greening. Oxygen evolved per mg chlorophyll also levelled off at this point indicating that the electron transport system was complete with only chlorophyll synthesis and overall growth continuing. Oxygen quenched F_0 more at 3 hours than at 48 hours indicating that at early greening times the chlorophyll molecules were fewer in number and not as well connected to the electron transport systems.

In separate experiments on 12 day old greenhouse grown barley, fluorescence was measured at various oxygen pressures and light intensities. In air, F_0 increased linearly with light intensity while f_p increased only until the light intensity reached 8 W m^{-2} and then stayed constant. Oxygen and light intensity acted in a competitive manner with more light increasing and increased oxygen decreasing f_p .

DEDICATION

To Edward and Muriel Fraser, my parents.

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TABLE OF CONTENTS

Approval	ii
Abstract	iii
Dedication	v
Acknowledgements	vi
List of Tables	ix
List of Figures	x
I. Introduction	1
1.1 The chloroplast	1
1.2 The Z scheme	2
1.3 Fluorescence	6
Fluorescence variables	10
1.4 Oxygen quenching of fluorescence	11
1.5 Greening	13
1.6 Object of research	14
II. Materials and Methods	21
2.1 Growing barley	21
2.2 Chloroplast isolation	21
2.3 Oxygen evolution	24
2.4 Fluorescence measurements	26
2.5 Fluorescence at varying incident intensity	28
III. Results	33
3.1 Greening	33
3.2 Light intensity	36
IV. Discussion	47

4.1 Greening	47
4.2 Fluorescence at various light intensities	50
V. Conclusion	55
Appendix A: Oxygen concentration in water	56
Appendix B: Enzyme kinetics	57
Bibliography	61

LIST OF TABLES

TABLE		PAGE
3.1	Relative fluorescence in leaves in air and in 7 atm O ₂	35
3.2	Half-rise times of variable fluorescence in leaves in air and in 7 atm O ₂	35
3.3	Projected maximum relative variable fluorescence as determined from the Lineweaver-Burk plot (Figure 3.7), and light intensity for half-maximum f _p	38

LIST OF FIGURES

FIGURE	PAGE
1.1	Cross-section of a higher plant leaf. 15
1.2	Structure of a higher plant chloroplast. 16
1.3	Thylakoid model showing components involved in the light reactions. 17
1.4	The Z scheme. 18
1.5	Excitation and deexcitation processes of antenna chlorophyll. 19
1.6	The Kautsky curve: the time course of fluorescence of a higher plant. 20
2.1	Fluorescence measuring apparatus with high gas pressure. 31
2.2	Fluorescence measuring apparatus for variable light intensity. 32
3.1a	Oxygen evolution and chlorophyll synthesis during greening. 39
3.1b	Relative fluorescence, f_p , in leaves and chloroplasts during greening. 39
3.2	Oxygen evolution/disc and f_p in leaves during the accumulation of chlorophyll. 40
3.3	Oxygen quenching of relative fluorescence during greening. 41
3.4	Oxygen quenching of F_0 fluorescence during greening. 42
3.5a	The fluorescent efficiency of F_0 as a function of light intensity. 43
3.5b	Relative variable fluorescence, f_p , in air as a function of light intensity. 43
3.6	Light intensity dependence of relative variable fluorescence under various O_2 pressures. 44

3.7	Lineweaver-Burk plot of the data in figure 3.6.	45
3.8	Oxygen quenching of f_p at various light intensities.	46
4.1	Stern-Volmer plot of F_0 quenching vs. oxygen concentration.	54
B.1	Michaelis-Menten plot for enzyme substrate reaction	60
B.2	Lineweaver-Burk plot for enzyme-substrate reaction ...	60
B.3	Michaelis-Menten plot for competitive inhibition	60
B.4	Lineweaver-Burk plot for competitive inhibition	60
B.5	Michaelis-Menten plot for non competitive inhibition	60
B.6	Lineweaver-Burk plot for non competitive inhibition	60

I. Introduction

1.1 The chloroplast

Photosynthesis is the process by which plants, algae, and some bacteria use light energy to synthesize energy-rich carbohydrates. This action takes place in sub-cellular units called chloroplasts. Figure 1.1 shows a cross section of a higher plant leaf.

Figure 1.2 shows the general structure of a mature higher plant chloroplast. It is enclosed by a double membrane separating its interior from the cytoplasm of the plant cell. The outer membrane maintains structural integrity while the inner membrane controls in and out molecular traffic (Prezelin, 1981).

Extending throughout the chloroplast interior is a network of closed membranous structures termed thylakoids or lamellae. These are either flattened sacs called grana lamellae, or tubes called stroma lamellae which interconnect grana. The grana lamellae usually form into stacks (a single stack is a granum) of from 2 to 100 thylakoids each, depending on species (Kirk, 1978). All structural components of the light reactions are localized within the thylakoid membranes, while the soluble enzyme components of the dark reactions are found in the stroma

(Prezelin, 1981).

This describes the situation in higher plants. Algal chloroplasts have only unstacked lamellae and blue-green algae do not even have a chloroplast envelope; their lamellae lie exposed to the cytoplasm (Devlin, 1975). The photochemical reactions of photosynthetic bacteria take place in the highly invaginated membrane that encloses the cell (Zubay, 1983).

1.2 The Z scheme

In a typical higher plant thylakoid membrane, there are about 200 electron transport chains and their associated pigment complexes (Witt, 1979). In figure 1.3 a section of the thylakoid membrane shows the location of these components. These are arranged according to midpoint potentials of the components in figure 1.4. Photosystem II reduces the intersystem electron carriers and oxidizes water. Photosystem I oxidizes the intersystem electron carriers and reduces NADP^+ .

Photosystems II and I consist of their respective reaction centers, or "traps", surrounded by light-harvesting complexes of carotenoids and chlorophylls a and b. The number of chlorophylls per photosystem I or II ranges from 200 to 400 (Prezelin, 1981; Zubay, 1983). The chlorophylls preferentially absorb blue and red light. The carotenoids fill in the spectral gap (Salisbury and Ross, 1978). To be used for photochemistry the energy of an excited antenna must be transferred to the

reaction center. Generally, this energy reaches the trap with high efficiency, utilizing the very rapid resonance transfer to compete against other deexcitation processes mentioned in section 1.3. The transfer time between neighbouring chlorophyll molecules with parallel transition moments is about 0.5 ps requiring them to be about 15 Angstroms apart (Colbow, 1973; Colbow and Danyluk, 1976).

The photosystem II reaction center (labelled P680 because a light-minus-dark difference spectra show a bleaching maximum at 680 nm) is a specialized chlorophyll-protein complex. Upon transfer of the energy from the antenna chlorophyll P680 becomes excited. The midpoint redox potential, E_m , for the removal of an electron from the excited molecule is more negative than the midpoint potential in the ground state by approximately $h\nu/e$. For chlorophyll a ΔE_m is about 1.8 V, thus P680* is a very strong reductant. Only chlorin structures¹ are involved as transient electron acceptors in the initial charge-separation events (Crofts and Wraight, 1983). Excited P680 reduces pheophytin² (Parson and Ke, 1982) to a radical ion pair state (P680⁺Phe⁻) in less than 5 ps. Because of the involvement of a less homologous chemical structure (quinone), forward electron transfer from pheophytin a to Q_a is relatively slow (approximately 200 ps; Wraight, 1982). The even slower

¹-----
(bacterio)chlorophyll or (bacterio)pheophytin

² There is now an indication of a chlorophyll intermediate acceptor ahead of pheophytin (Wraight, 1982).

recombination with $P680^+$ (about 4 ns; Parson and Ke, 1982) allows the forward reaction to occur with high probability. Electrons are passed one at a time from Q_a to Q_b which becomes doubly reduced ($t_{1/2} = 200 - 300 \mu s$ for $Q_a^-Q_b$ to $Q_aQ_b^-$, $t_{1/2} = 600 - 800 \mu s$ for $Q_a^-Q_b^-$ to $Q_aQ_b^{2-}$; Cramer and Crofts, 1982) before doubly reducing one of the pool plastoquinone molecules ($t_{1/2} = 600 \mu s$; Witt, 1979). Positioning the electron acceptors close together and optimizing the energy drop of each step allows the rapid removal of electrons away from $P680^+$ thus minimizing the probability of wasteful back reactions.

The photooxidized reaction center is itself a powerful oxidant which causes the oxidation of H_2O to O_2 . This requires the removal of four electrons and four protons for each O_2 produced. The nature of the manganese-containing charge-accumulating complex that performs this is as yet unknown. The electrons reduce $P680^+$ and the protons freed are released to the intrathylakoid space helping to create a pH gradient with respect to the exterior of the thylakoid.

In being doubly reduced, Q_b^- also takes up two protons from outside the thylakoid. These are passed to the plastoquinone pool along with the two electrons to form plastohydroquinone, PQH_2 . This is reoxidized by passing electrons on to the Rieske iron-sulfur protein, Fe-S, and the protons to the thylakoid interior. This is the rate-limiting step on photosynthesis with $t_{1/2} = 20 ms$ (Witt, 1979). The plastoquinone pool thus acts to pump protons from the outside to the inside.

Photosystem I consists of the P700-chlorophyll a-protein complex and the surrounding antenna pigments. P700 (similarly labelled because of a bleaching maximum in the oxidized-minus-reduced spectra) may be a chlorophyll dimer (Prezelin, 1981) although there is recent evidence to the contrary (Wraight, 1982). Similarly to P680, P700 is excited by energy transferred from its antenna pigments and then reduces the intermediate acceptor, another chlorophyll a within the photosystem I protein complex, which in turn reduces a secondary acceptor labelled X, thought to be an iron-sulfur protein called ferredoxin. The subsequent two acceptors are also ferredoxins, FD_a and FD_b . These three proteins are not easily washed out of the sample preparations and are thus denoted as "bound" ferredoxins. Next, these electrons are transferred to a soluble ferredoxin FD_s , and then a flavoprotein, ferredoxin-NADP-reductase, which in turn reduces $NADP^+$ to NADPH. Another proton is taken up from the thylakoid exterior whenever NADPH is used in the carbon fixation reactions.

As a strong oxidant, $P700^+$ in turn can oxidize the intersystem carriers: plastocyanin (PC), cytochrome f, Fe-S, PQ, Q_b , and Q_a . The electron transport chain as outlined here is called non-cyclic electron transport.

Cytochrome b563 can accept electrons from the ferredoxin chain and give them to the PQ pool. As mentioned earlier, reduction of PQ requires two protons so that protons must be transported across the membrane with no net change in the redox

state of electron acceptors or donors over a complete cycle.

This pathway is termed cyclic electron transport.

In a third electron pathway molecular oxygen may be reduced back to water in a series of reactions which oxidizes ferredoxin (Allen, 1977). This reaction is termed pseudo-cyclic electron transport.

Besides the possible reduction of NADP^+ , electron transport along any pathway leads to a buildup of a transmembrane pH gradient which drives a membrane-localized ATPase ($\text{CF}_0\text{-CF}_1$, in figure 1.3). These energy-rich molecules are used in the carbon-fixation or "dark" reactions of photosynthesis.

The functioning of photosynthetic electron and energy transfer may be altered by the addition of artificial electron or proton acceptors, donors, inhibitors and uncouplers, the change of ionic or pH conditions, or by change of illumination, etc.

1.3 Fluorescence

When a photon is captured by a pigment molecule, electrons become redistributed into a set of excited singlet states (Figure 1.5). There are two main excited states available in chlorophyll a. Absorption of red light (band maxima about 680 nm) raises the molecule to the first excited singlet state. The second excited singlet state (band maxima about 630 nm) is weakly absorbing. Blue light (the Soret band, maxima about

440 nm) raises the molecule to the third and higher excited singlet states (Breton and Vermeglio, 1982; Govindjee and Govindjee, 1974). Molecules excited to higher excited singlet states return via internal conversion to the first excited singlet state in about 10^{-14} to 10^{-13} seconds, too rapidly to have any competing process occur (Govindjee and Govindjee, 1974).

From the excited singlet state, the pigment molecule may lose its energy via competing processes: reradiation of a photon as fluorescence, internal conversion (nonradiative phonon emission), cross over to the triplet state, or transferal to a reaction center for photochemistry with respective rates k_F , k_C , k_T and k_P . By resonance transfer energy very rapidly reaches a reaction center where, if it is in its ground (reduced) state, P680 can absorb the energy and become excited. The reaction center trap is said to be "open". Once excited, P680 then ejects an electron across the membrane to pheophytin. However, if the trap is already photooxidized (or "closed") the excitation may transfer to another trap or the currently excited antenna molecule will deexcite by one of the other mechanisms.

The yield of any process is the ratio of its rate to all rates. The yield of deexcitation due to photochemistry is

$$\phi_P = \frac{k_P}{k_F + k_C + k_T + k_P} \quad (1.1)$$

and is observed as the number of primary charge separations per absorbed photon. When all reaction center traps are open this yield is about 90% or more (Clayton, 1980). The fluorescence

yield is

$$\phi_F = \frac{k_F}{k_F + k_C + k_T + k_P} \quad (1.2)$$

and is observed as the fluorescence intensity

$$F = \phi_F I \quad (1.3)$$

where I is the absorbed light intensity.

Experimentally, it is necessary to distinguish between fluorescence of constant yield and that of variable yield. Constant fluorescence comes from photosystem I antenna chlorophyll which are weakly fluorescent (Kitajima and Butler, 1975), any photochemically inactive chlorophylls, and that emitted from photosystem II when all the traps are open allowing the maximum deexcitation via photochemistry and is therefore the minimum amount of fluorescence. The variable fluorescence is emitted from photosystem II antenna chlorophyll when some or all reaction centers are closed and experimentally is the total fluorescence minus the constant fluorescence.

For a short interval at the beginning of illumination of a dark-adapted plant both fluorescence and O_2 evolution increase substantially due to activation of their systems. Subsequent to this the quantum yields of the variable fluorescence and of the rate of oxygen evolution add up to a constant. If the rates of the other processes are proportional to that of fluorescence (they are all intra-molecular processes but depend on their environmental conditions)

$$k_F + k_C + k_T = \beta k_F \quad (1.4)$$

then ϕ_p and ϕ_f can be linked by a complementarity equation

$$\phi_p + \beta \phi_f = 1 \quad (1.5)$$

where β is a proportionality constant (Lavorel and Etienne, 1977). Thus fluorescence can give kinetic information about the photochemistry occurring at P680.

On the way to an overall steady-state, the photosynthetic apparatus passes through a set of several transitory stages, collectively known as induction. The induction of the rate of oxygen evolution reflects the rate of electron transport through photosystem II. Fluorescence induction (figure 1.6), first observed by Kautsky, hence known as a "Kautsky curve", reflects the momentary density of electronic excitation of photosystem II.

A convenient time division is made in the fluorescent induction: a fast phase ODIPS lasting a few seconds and a slow phase SMT which may take several minutes to complete. These are an initial or origin level, O, a rise to an intermediate maximum, I, a dip, D, a rise to a peak, P, and then a decline to a quasi-steady state, S. The second, slow phase begins with a rise from S to a maximum, M, and then gradually declines to a terminal, T, level (Figure 1.6).

To determine the O level fluorescence, F_0 , it is necessary to take measurements before Q_a is significantly reduced and hence k_p decreases. One can either use low light intensities or very fast illumination rise times.

The OI rise represents a partial reduction of the PQ pool while the decline to D is attributed to reoxidation of the PQ pool and Q by photosystem I (Papageorgiou, 1975). The increase in fluorescence to P occurs when photosystem I becomes saturated and unable to accept more electrons and the PQ pool becomes reduced. The fact that at moderate light intensities the maximum may be less than that observed in DCMU³ treated tissue is attributed to photosystem I not saturating which prevents the PQ pool becoming totally reduced (Baker and Bradbury, 1981).

The slow fluorescence induction phase, PSMT, is also largely due to changes in the redox state of Q but proton and other cation electrochemical gradients across the thylakoid membrane and ATP concentration in the stroma all modify the chlorophyll fluorescence emission characteristics in this phase (Baker and Bradbury, 1981).

This thesis will be involved only with the fast transient phase, OI DP, as it indicates the changes in the redox state of Q due to intersystem electron transport.

Fluorescence variables

From the complementarity equation we have

$$F_p = I \phi_{Fp} = \frac{I}{\beta} (1 - \phi_{P \text{ reduced}}) \quad (1.6)$$

³ DCMU: 3-(3,4-dichlorophenyl)-1,1'-dimethylurea blocks electron transport between Q and PQ (Izawa, 1977).

and
$$F_o = I \phi_{F_o} = \frac{I}{\beta} (1 - \phi_{P \text{ oxidized}}) \quad (1.7)$$

so
$$F_v = F_p - F_o = \frac{I}{\beta} (\phi_{P \text{ oxidized}} - \phi_{P \text{ reduced}}) \quad (1.8)$$

As discussed in sections 3.2 and 4.2, F_o is directly proportional to light intensity, so dividing by F_o gives a measure of the range of Q oxidation.

$$f_p = \frac{F_p - F_o}{F_o} \propto (\phi_{P \text{ oxidized}} - \phi_{P \text{ reduced}}) \quad (1.9)$$

Variable fluorescence, F_v , will refer to the excess at P above the constant or "dead" fluorescence, F_o . Relative variable fluorescence, f_p , is preferable to F_v in that it is more reproducible from different samples of the same sample population. The maximum fluorescence, F_{max} , is seen in the presence of electron transport inhibitors such as DCMU. Relative maximum fluorescence is defined as $f_m = (F_{max} - F_o)/F_o$.

1.4 Oxygen quenching of fluorescence

Chlorophyll a fluorescence is quenched by molecular oxygen. Vidaver et al. (1981a) have described three phases of O_2 quenching in bean leaves, spinach chloroplasts and Scenedesmus: 1) quenching of f_p ; 2) quenching of f_m ; and 3) quenching of F_o . Each phase differed in sensitivity to oxygen by approximately one order of magnitude. Half-quenching of f_p occurred with 3 to 5 atm of O_2 , f_m 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 and was tentatively related to the increased oxidation of electron transport by O_2 . Quenching of f_m and F_o were suggested to represent O_2 quenching of P680 reaction centers or Q_a and antennae chlorophyll molecules respectively.

Oxygen may act as an electron acceptor at plastocyanin (Vidaver et al., 1981b), ferredoxin (Allen, 1977), or in photorespiration (Salisbury and Ross, 1978).

The direct quenching by oxygen of the excitation of antenna pigment molecules in a bimolecular reaction adds a new deexcitation rate term $k_{O_2}[O_2]$ to the fluorescence yield equation.

$$\phi_F = \frac{k_F}{k_F + k_c + k_T + k_P + k_{O_2}[O_2]} \quad (1.10)$$

Fluorescence quenching by the action of oxygen as an electron acceptor would change the photochemistry term to include a functional dependence on O_2 concentration through the redox state of Q_a .

$$k_P \Rightarrow k_P([O_2])$$

The quenching ratio inverse,

$$\frac{F}{F(O_2)} = \frac{k_F + k_c + k_T + k_P([O_2]) + k_{O_2}[O_2]}{k_F + k_c + k_T + k_P} \quad (1.11)$$

shows the type of quenching by O_2 . A linear plot means a

 4 Oxygen concentration in water as a function of pressure is calculated in appendix A.

bimolecular process, while non-linearity shows that other pathways are involved.

1.5 Greening

In the chloroplasts of greening plants, one has the opportunity to correlate development of various aspects of photosynthetic function and structure thus getting a partial understanding of what happens when plants germinate under natural conditions.

When angiosperm seedlings are germinated and grown in the dark the relatively undifferentiated proplastids⁵ develop into etioplasts. These etioplasts characteristically contain one or more large quasi-crystalline bodies, built up of interconnected membranous tubules in regular array, termed prolamellar bodies (Kirk and Tilney-Bassett, 1978). The etioplasts contain no chlorophyll, but they contain a small amount of protochlorophyllide bound in the prolamellar bodies and some carotenoids.

Upon illumination, the prolamellar body disperses into sheets of perforated membranes, which give rise to the thylakoids. After a few hours, depending upon species and age of the seedlings, there is a fusion of the thylakoids to form

⁵ Plastids are double membrane-bound organelles found only in plants and algae and contain either chlorophyll and the photosynthetic apparatus, other pigments, or stored starch, protein, or oil (Curtis, 1979).

grana (Boardman, 1977).

The barley etioplast contains some of the electron carriers already present while others and the photosynthetic pigments are synthesized and incorporated into the forming thylakoid membranes during greening (Plesnicar and Bendall, 1973).

1.6 Object of research

The object of this thesis is to determine which photosynthetic activities could be measured by fluorescence while the composition and structure of the photosynthetic apparatus developed from immaturity caused by etiolation (growth in the dark) to maturity. The quenching of fluorescence by O_2 was to see if O_2 has different effects in the developing plant compared to the mature plant. A study was also done on the effect of high light intensity and various O_2 pressures in mature greenhouse grown barley.

The results presented will show that

1. Variable fluorescence is a good measure of photosystem II activity throughout greening.
2. Both constant and variable fluorescence are more easily quenched by O_2 during early greening although apparent at different O_2 pressure ranges.
3. High light intensity and high O_2 concentrations are counteractive in changing the redox state of Q_a .

Figure 1.1 Cross-section of a higher plant leaf.

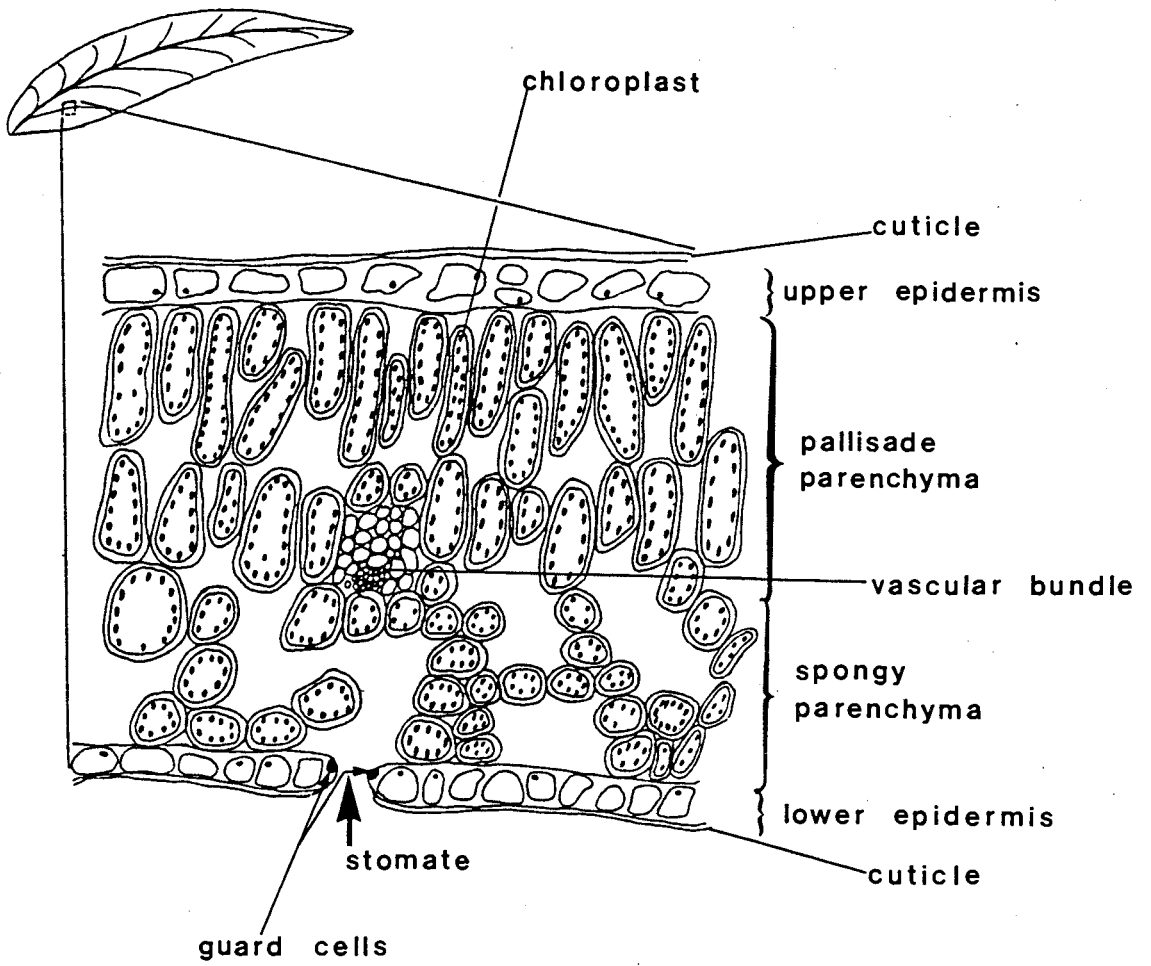


Figure 1.2 Structure of a higher plant chloroplast.

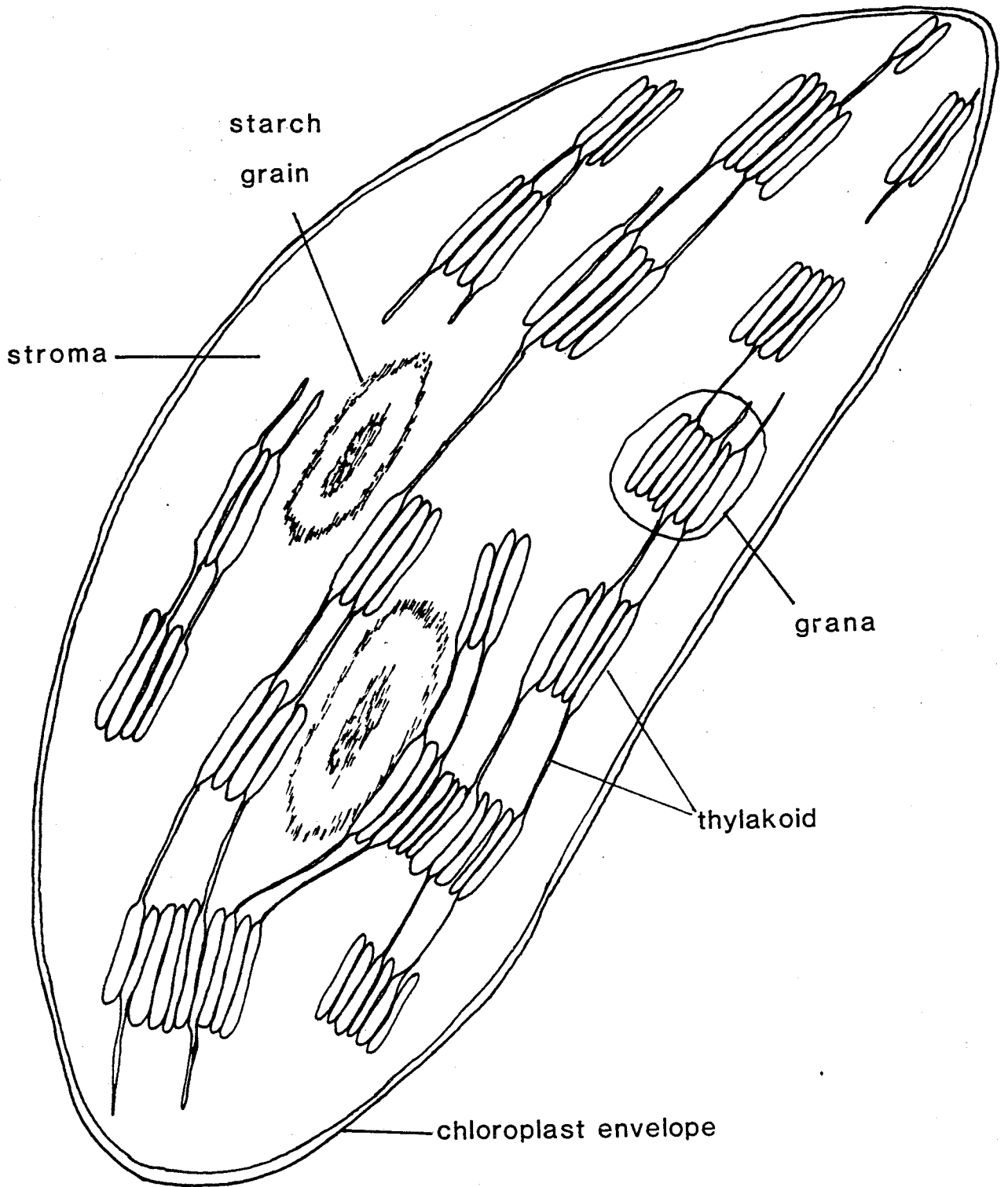


Figure 1.3 Thylakoid model showing components involved in the light reactions.

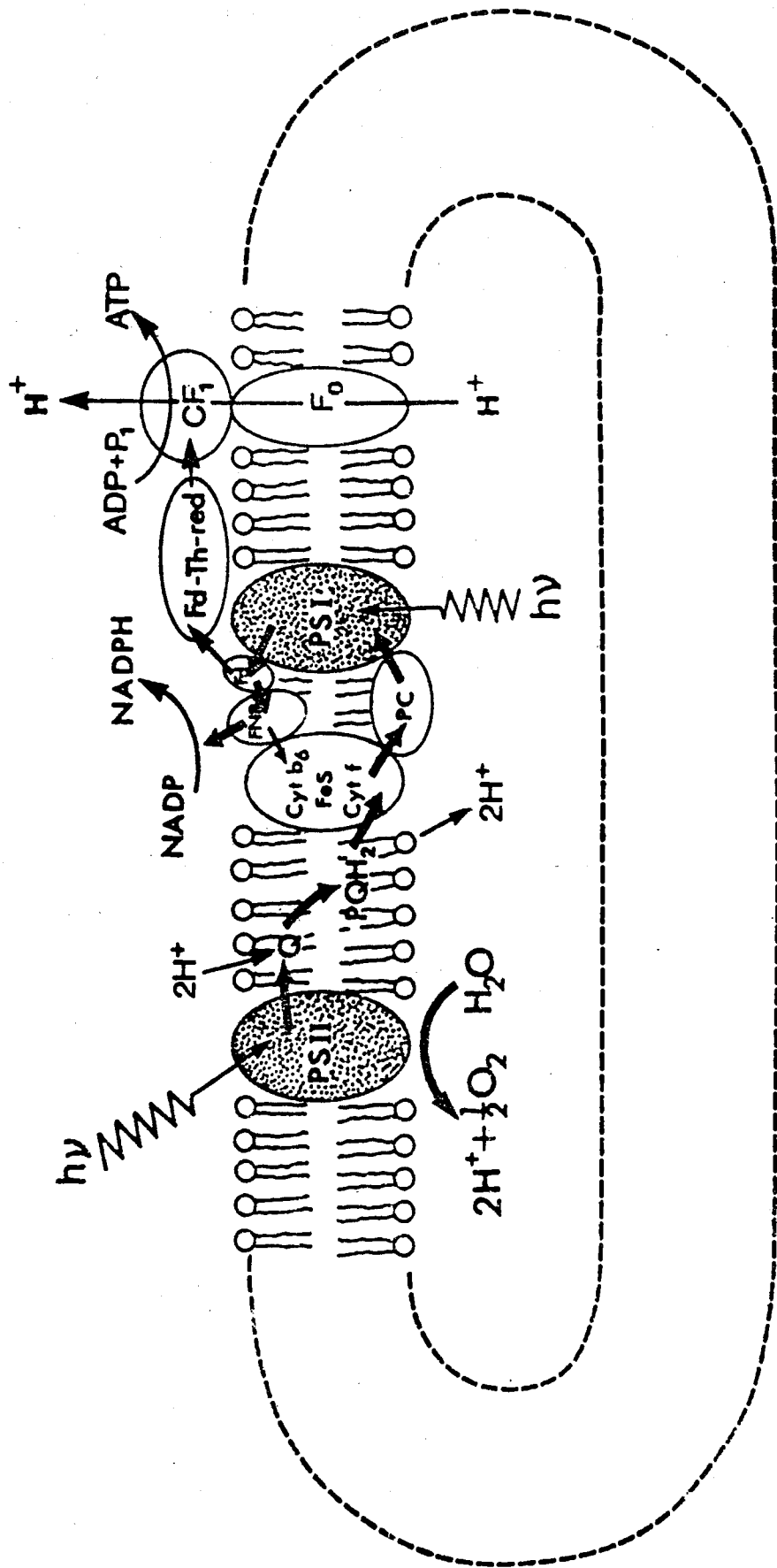
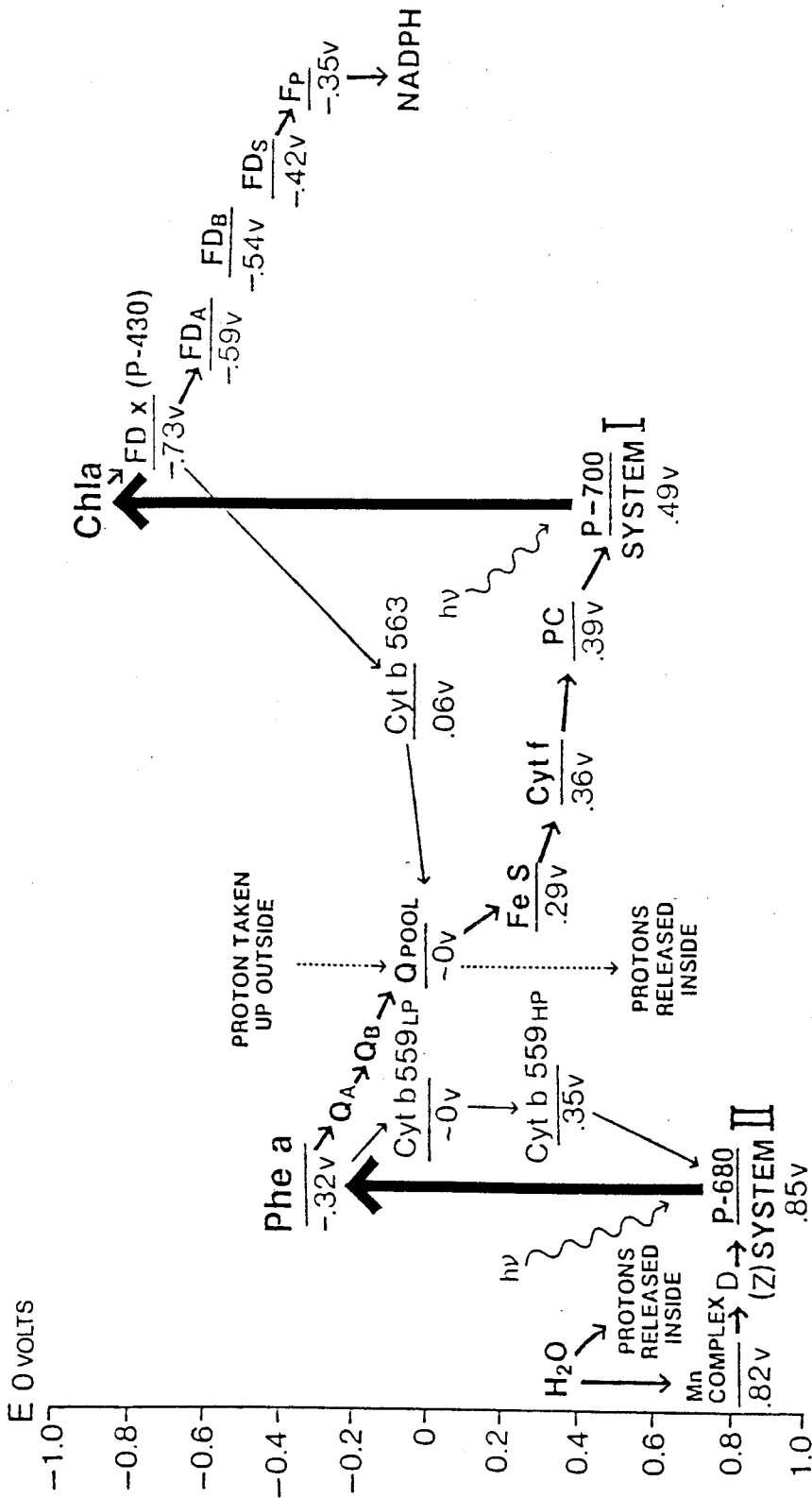


Figure 1.4 The Z scheme.

"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_{559} ; $cyt-f$ = 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.

Figure 1.5 Excitation and deexcitation processes of antenna chlorophyll.

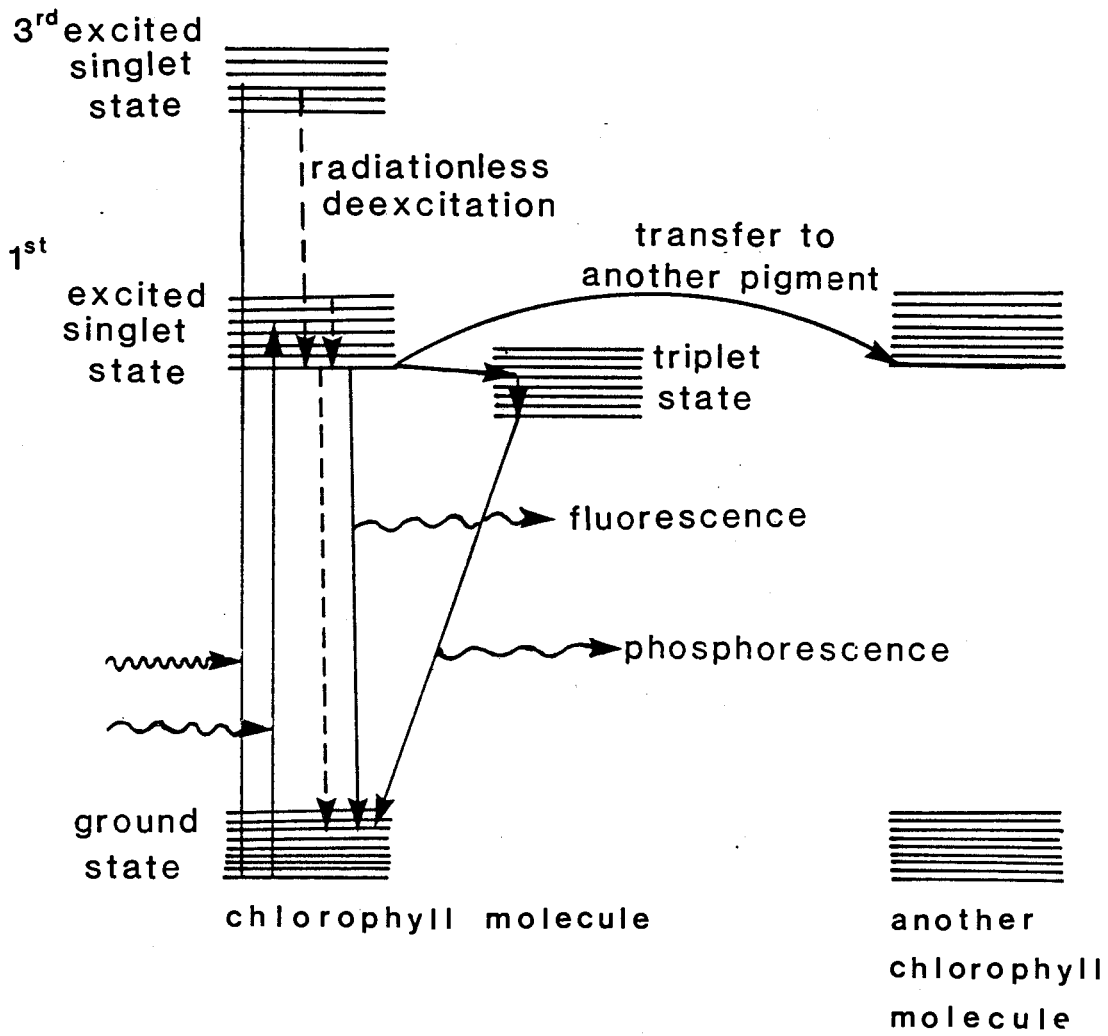
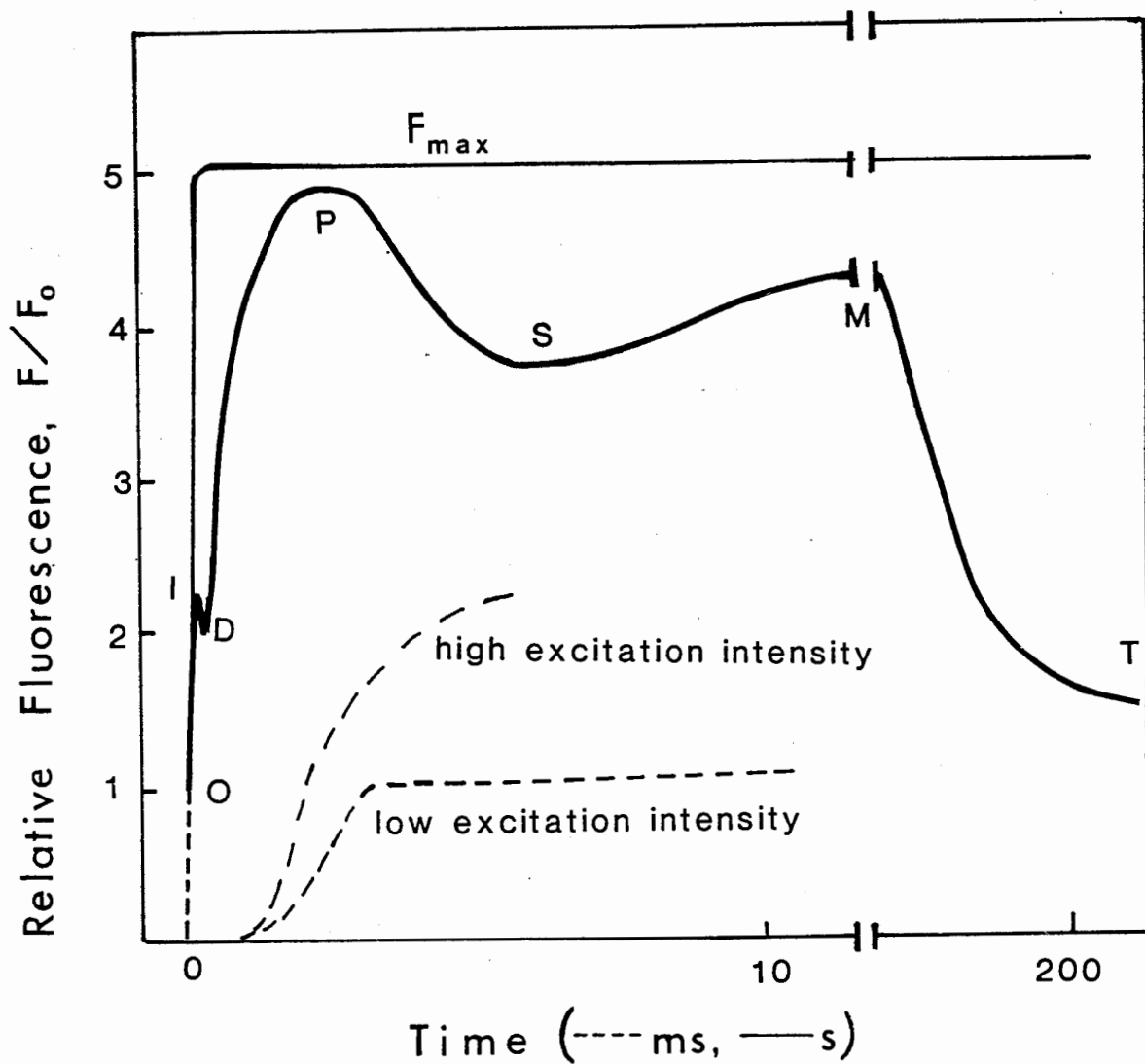


Figure 1.6 The Kautsky curve: the time course of fluorescence of a higher plant.



II. Materials and Methods

2.1 Growing barley

Barley (Hordeum vulgare L. cv. Conquest) seed was soaked in running tap water for six to ten hours and then planted on a two inch bed of vermiculite which allowed for adequate moisture and drainage. Two growing procedures were followed to produce etiolated and fully greened plants. One set of seeds were placed in the dark, at 18° to 20°C, for seven days, by which time shoots developed to about twelve centimeters tall and had started to unfurl. These were then placed under incandescent lighting of 20 W m⁻² to green. The other set of seeds were placed in a greenhouse. After eight days these shoots were about twelve centimeters tall and the leaves were fully unfurled.

2.2 Chloroplast isolation

Fluorescence was measured on chloroplasts in order to have better control over the amount of chlorophyll in a sample and thus have a control on the size of the fluorescence signal. The following method of isolating chloroplasts was taken from Walker (1980).

Barley leaves were washed and chopped into half-centimeter sections. These were put into a Waring blender and grinding medium (described below) was added in a ratio of 5:1 (volume/weight). The blender was operated at its highest speed for three one second bursts. The resulting mixture was filtered by eight layers of cheesecloth with a 25 μ m pore size nylon mesh below to remove large cellular debris. The chloroplasts were then sedimented by centrifuging at 3000 g for 6 minutes. The supernatant then contains mitochondria and other particles lighter than the chloroplasts while the latter have precipitated to form a pellet. The supernatant is discarded, and the pellet is gently resuspended in grinding medium. The centrifugation and resuspension were repeated to further purify the chloroplast extract. The method of Arnon (1949) was then used to determine the chlorophyll concentration of the extract so that we could place a known amount of chlorophyll in the oxygen evolution apparatus (described in section 2.3). This method measures the absorbance of the chloroplast extract in 80% acetone at 663 and 645 nm, which are the absorbance maxima of chlorophylls a and b in 80% acetone respectively. This was correlated to the amount of chlorophyll per leaf area by also measuring the total chlorophyll of an extract of 10 to 50 leaf discs, each 4 mm in diameter.

Grinding medium

The grinding medium consists of

0.33 M Sorbitol

50 mM HEPES

1 mM $MgCl_2$

1 mM $MnCl_2$

2 mM EDTA

adjusted to pH 7.0 with HCl.

Sorbitol is a nonmetabolizable sugar used to hold the grinding and resuspension media isoosmotic with the chloroplasts (Reeves and Hall, 1980). This keeps membrane proteins and lipids from losing their function and activity.

HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid), pK_a 7.55, is used as the buffer as it does not affect the water splitting system and thus does not affect the electron transport system. Also it does not bind Mg^{2+} or Mn^{2+} (Good and Izawa, 1972).

$MgCl_2$ and $MnCl_2$ are used to prevent deterioration of the chlorophyll and the oxygen-evolving mechanism in the chloroplasts during preparation and after the chloroplast envelopes are broken.

EDTA (ethylenediaminetetraacetic acid) a chelate, is used to keep magnesium and manganese in solution and not precipitate in the form of any complexes.

The pH is usually kept between pH 6.3 and 8.5, observed to give chloroplasts with maximum O_2 evolution under light (Walker, 1971).

It is essential to do the isolation fast and at cold temperatures. As the plant cells are ruptured, many enzymes are released which can harm the chloroplasts, thus washing these away as fast as possible gives the best chloroplasts. The low temperature slows enzyme activities, thus decreasing chloroplast damage. To this end, the grinding medium was cooled beforehand to a consistency of melting snow, the centrifuge was operated at 4°C, and the glassware and resuspension media were stored in a refrigerator at 4°C.

2.3 Oxygen evolution

Oxygen evolution measurements were performed on broken chloroplasts so that ferricyanide and ammonium ions could penetrate to the thylakoid membranes; an intact inner chloroplast envelope would prevent this. The ferricyanide cation can be reduced to ferrocyanide by oxidizing a part of the electron transport chain at a site which is not exactly known but is after photosystem II on the Z scheme. This site also seems to depend on the preparation procedure (Avron, 1981; Hauska, 1977). Ammonium ions are added to uncouple electron transport from phosphorylation so that the maximum linear electron transport is observed (Avron, 1981; Lilley et al, 1975; Izawa and Good, 1972).

Chloroplasts were measured for O₂ evolving ability as a measure of photosystem II activity during greening and to

compare the level of activity with those published elsewhere. The latter indicates the quality of our chloroplast isolation procedure.

A quantity of chloroplast suspension equivalent to 200 μg of chlorophyll was added to distilled water in the cavity of a Clark electrode apparatus. This consists of a platinum cathode which is held at a constant potential of -0.7 V with respect to a silver/silver chloride anode and measures the electron current produced when oxygen is reduced at the cathode (Delieu and Walker, 1972). The distilled water causes the chloroplast envelopes to break by osmotic shock. One minute later (to ensure all envelopes were broken) potassium ferricyanide and ammonium chloride were added to prepare a 2 mM solution of each and a total volume of 1 ml. Oxygen uptake was observed on the strip chart recorder at this point. The light was then turned on and the oxygen evolution recorded. The difference of the two traces gave the total oxygen evolution. The response of the Clark electrode was calibrated before and after each series of experiments by measuring the concentration of O_2 in distilled water before and after sodium dithionite was added. This is a very strong reductant and takes up all oxygen in solution.

2.4 Fluorescence measurements

To excite the chlorophyll molecules in the leaf sections and chloroplasts, they were illuminated by blue light. The resulting fluorescence was directed to a photomultiplier and the signal fed to a signal averaging device as shown in figure 2.1. A 650 W General Electric DWY Quartzline Projection Lamp was powered by a regulated power supply. The signal from a piece of dried leaf (ie. a constant fluorescence signal) showed random noise of less than one percent of its full signal.

The light passed through an infrared absorbing water filter and various lenses to be focussed onto one branch of a bifurcated random fiber optics light pipe and directed to the sample. The other branch of the light pipe passed the sample's fluorescent and any reflected light to the photomultiplier. A Corning 4-96 filter (BF) transmitted mainly blue light to excite fluorescence in the sample, while a Corning 2-64 filter (RF) covering the light pipe end inside the photomultiplier housing transmitted mainly the red fluorescence emission. The two filters were chosen to minimize excitation light reaching the photomultiplier. A contribution of about 5% of F_p to the signal due to red fluorescence of the blue filter was measured before each experiment and subtracted from the readings.

The photomultiplier, an EMI 9558QB (S20 response), was operated at 700 V with a load resistance of 200 k Ω , giving an anode to dynode current ratio of less than 0.1, so to not

deviate significantly from linearity. The photomultiplier signal was transmitted to a Tracor Northern 1710 multichannel analyzer with a signal averager module where a time trace was displayed. All fluorescence intensity readings were taken from the display (arbitrary units).

The sample cell is of a type described by Morita (1970). It is a stainless steel chamber with stainless steel tubing connecting it to a gas supply. Chamber and tubing are rated for work of over 700 atm. The plexiglas window is sealed in place by epoxy and the bottom is sealed with an O-ring when screwed tight by hand. The light pipe is separated from the sample by a clear plexiglas window. To determine the light intensity incident upon the sample, the light pipe was held up to a clear plexiglas window of the same thickness as in the pressure cell. The intensity through this plexiglas was measured by a Tektronic J16 Digital Photometer with a J6502 radiometer probe. The lamp power supply was adjusted to give a reading of 10 W m^{-2} on the photometer.

The oxygen was supplied from Union Carbide: USP grade (minimum 99.5% oxygen, maximum moisture 15ppm, balance nitrogen and halocarbons). By a system of valves and gauges this was let into the pressure cell to the desired pressure.

I had found earlier that letting in nitrogen to 100 atm as quickly as possible raised the gas temperature in the cell to at least 45°C from 24°C (room temperature) and returned to within 0.3°C of room temperature in about three minutes (measured by a

thermistor). Fluorescence of chlorophyll in phospholipid vesicles under the same conditions increased about four times, and decreased to within 1% of ambient fluorescence in about 30 seconds. For this reason the gas was let into the sample cell slowly (15 atm per minute) to the desired pressure. A minimum of another two minutes for sample equilibration was allowed and the total dark adaptation time between illuminations was a minimum of 5 minutes.

For each sample the procedure was: 1) Dark-adaption for 15 min; 2) a fast flash (about 80 ms) to determine F_0 ; 3) 4 s illumination to determine F_p ; 4) increase of O_2 pressure; 5) equilibration time 2 min; 6) dark-adaption for 5 min, and repeat from step 2.'

2.5 Fluorescence at varying incident intensity

Barley was grown in the greenhouse and used after 12 days. Plants were dark-adapted for at least 30 min and then the leaf section from the 2nd to 3rd cm from the tip of the largest leaf was placed in the sample cell.

At high light intensities, the slow shutter opening time of the previous setup did not allow accurate 0 levels (figure 1.6). For more precise 0 level measurements in air we used the following setup with a Spectra-Physics 125 He-Ne laser (Figure 2.2): a beam splitter gave a reference intensity to one photomultiplier while the remainder of the beam was focussed on

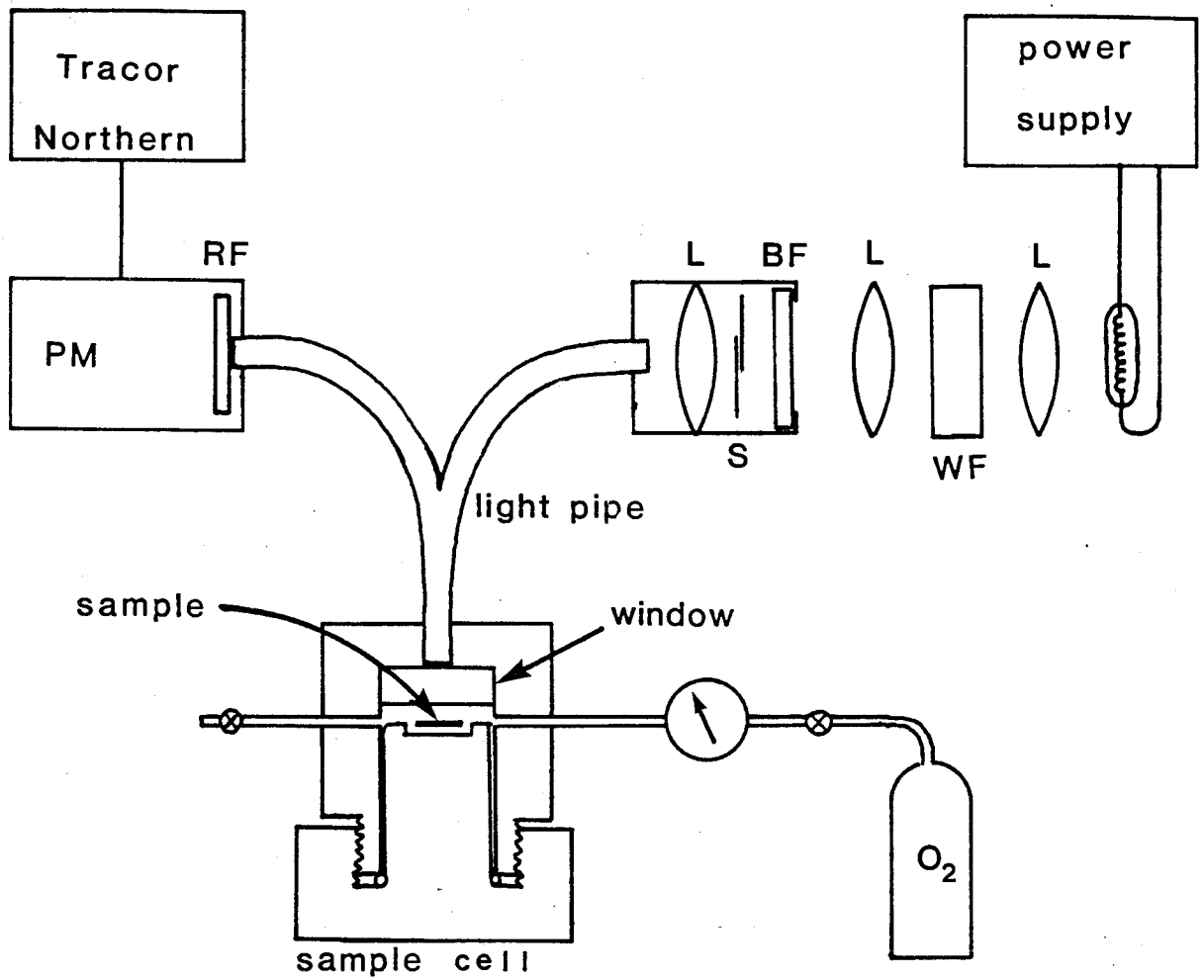
an electronic shutter blade. An aperture was placed immediately behind the shutter blade so only the focussed beam was seen. Rise times of 10-50 μ s were achieved. Some distance away one arm of a bifurcated light pipe intercepted the diverged incident beam and delivered to the cell a uniform intensity profile. The other arm carried fluorescent and reflected light to a second photomultiplier. Here a Corning 2-64 filter eliminated the reflected laser light. Both photomultipliers were EMI 9558QB operated at 800-900 V and the anode to dynode current ratios were kept below 0.08. An Ithaco 3512 ratiometer fed to the Tracor Northern signal averager the ratio of the sample to reference photomultiplier signals. Light intensity at the sample was changed by placing calibrated neutral density filters between the beam splitter and the focussing lens. In order not to saturate the sample photomultiplier over the range of fluorescence intensity a variable aperture covered the end of the light pipe inside the photomultiplier housing and measurements were repeated when this aperture was changed. There were ususally six samples at each light intensity. The error bars on the figures represent one standard deviation.

The linearity of these F_0 values in air was then used to correct the F_0 values measured with the apparatus described in section 2.4: at 10 W m⁻², F_0 was assumed to be accurate as the rise to the I level was relatively slow (figure 1.6); F_0 values at higher light intensity were extrapolated from these values. The F_0 values obtained under the various O₂ pressures were

changed proportionately to the change of those in air.

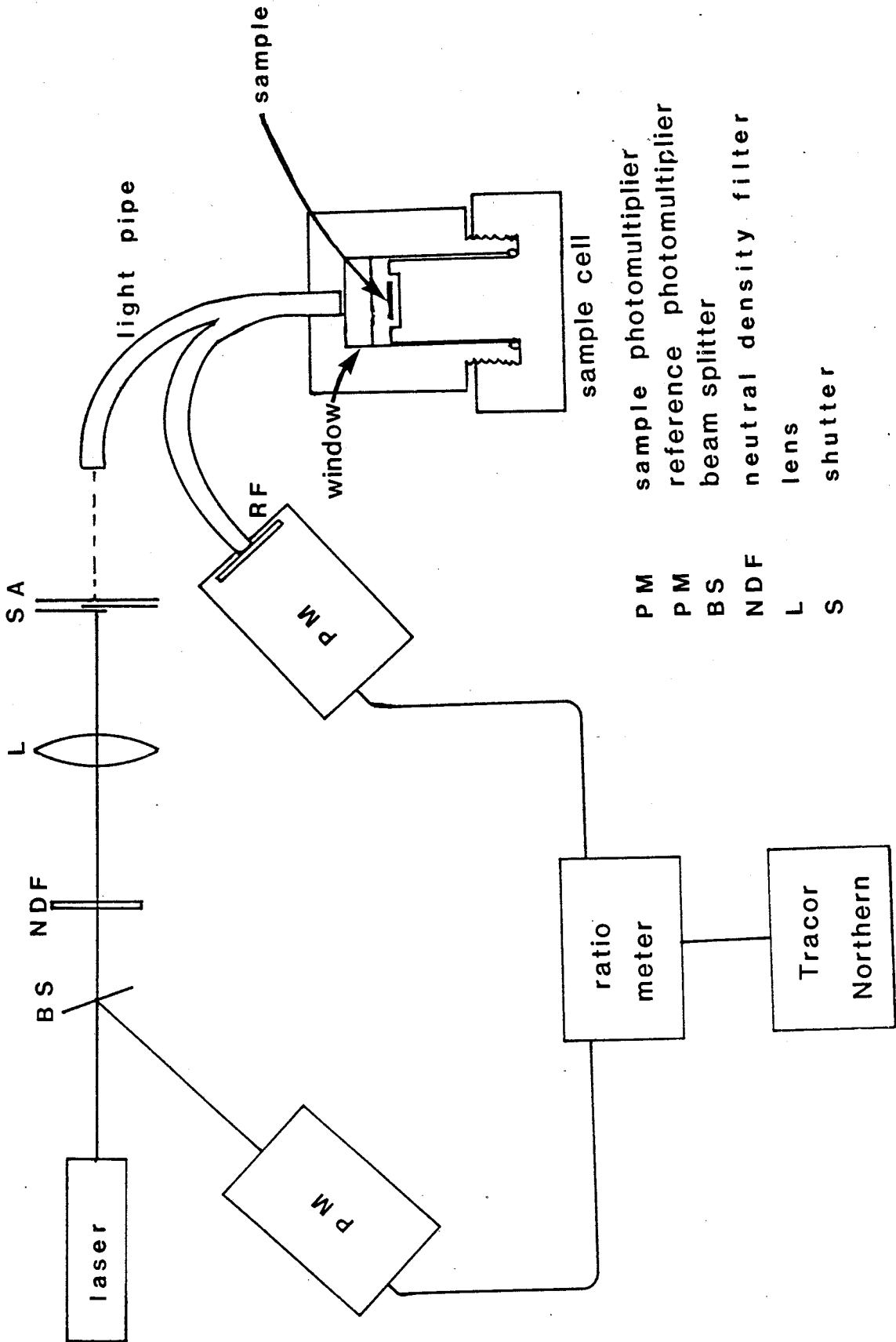
We determined that the fluorescent induction curve shapes and intensities when illuminated at 83 W m^{-2} in 58 atm O_2 and in air did not change if repeated after each of four successive five minute dark-adaptations. Therefore, a new sample was used for each O_2 pressure, increasing the light intensity after each dark-adaptation. This procedure was repeated once with a new sample for each O_2 pressure.

Figure 2.1 Fluorescence measuring apparatus with high gas pressure.



- PM Photomultiplier
- RF Red filter Corning 2-64
- BF Blue filter Corning 4-96
- WF Water filter
- L Lens
- S Shutter

Figure 2.2 Fluorescence measuring apparatus for variable light intensity.



- P M sample photomultiplier
- P M reference photomultiplier
- BS beam splitter
- NDF neutral density filter
- L lens
- S shutter

III. Results

3.1 Greening

On leaves of plants greened 3 hours only the leaf tips (approximately 1 cm) had unfurled. The rest of the leaf length was still tightly rolled. In handling and attempting to unfurl a leaf, it was often easily bruised. The tips were bright yellow for all 3 hour samples and the yellow portion extended from 2 to 6 cm down the leaf with the remainder being very light green to yellow. This wide variation in leaf pigmentation and their fragility produced the widest variation in leaf fluorescent measurements. During greening leaves became unfurled, wider, less fragile, and deeper green in colour (the yellow portion receded to the tip and disappeared by about 12 hours). Absorption measurements showed that chlorophyll content per unit leaf area increased monotonically 13-fold from 3 to 48 hours (Figure 3.1a).

Chloroplast oxygen evolution per mg chl rose steeply between 3 and 6 hours and declined to a steady rate at 12 hours. Calculated on a leaf area basis O_2 evolution followed the trend of chlorophyll accumulation, steadily increasing 30-fold from 3 to 48 hours (Figure 3.1a).

To compare O_2 evolution and fluorescence under similar conditions, we also measured fluorescence in class C (broken) chloroplasts (Reeves and Hall, 1980). Although the value of f_p was greatly reduced in chloroplasts, its change during greening paralleled that of intact leaf sections: both increased 5-fold during 3 to 48 hours greening (Figure 3.1b). Chloroplast oxygen evolution per unit leaf area and f_p showed a similar dependence on chlorophyll content (Figure 3.2).

In broken chloroplasts quenching of f_p by high oxygen pressures (figure 3.3) showed no change during greening, while quenching of F_0 was greatest during early greening and least by 48 hours and greenhouse control plants (Figure 3.4).

Later, we also determined that f_p in control leaves was half-quenched at about 7 atm of O_2 (data not shown). During greening from 3 to 48 hours the relative fluorescence range of 1 to 5 in air changed in 7 atm O_2 to 0.3 to 2.6 respectively (Table 3.1). The resultant ratios of peak relative fluorescence in O_2 to air show stronger O_2 quenching up to 12 hours greening which decreases to the expected 50% at 24 hours.

The relative rates of reduction of Q by the photosystem II reaction center were estimated by the half-rise times, $t_{1/2}$, of f_p . These decreased 4-fold from 3 to 48 hours but were not significantly changed by O_2 (Table 3.2).

Table 3.1 Relative fluorescence in leaves in air and in 7 atm O₂.

<u>Greening time (h)</u>	<u>f_p(air)</u>	<u>f_p(O₂)</u>	<u>$\frac{f_p(O_2)}{f_p(air)}$</u>
3	1.04	0.29	0.28
6	1.91	0.66	0.35
12	2.63	1.15	0.44
24	3.86	1.93	0.50
48	5.08	2.56	0.50
Control	5.59	3.24	0.58

Table 3.2 Half-rise times of variable fluorescence in leaves in air and in 7 atm O₂.

<u>Greening time (h)</u>	<u>air t_{1/2} (s)</u>	<u>O₂ t_{1/2} (s)</u>
3	0.57	0.64
6	0.35	0.32
12	0.24	0.25
24	0.22	0.22
48	0.14	0.13
Control	0.12	0.09

3.2 Light intensity

The ratio of F_o to intensity in air was nearly constant over a hundred-fold increase of incident intensity (Figure 3.5a), while over the same range f_p rose from nearly 1 to nearly 5 (Figure 3.5b). The maximum f_p was reached at about 8 W m^{-2} and did not change much with further increasing intensity.

Results of experiments similar to those of figure 3.5b using various O_2 concentrations are shown in figure 3.6. A Lineweaver-Burk plot¹ of the data is made in figure 3.7 and the projected maxima of f_p (y-intercept) are tabulated in table 3.3. The highest values of f_p were obtained in air and the lowest in 58 atm O_2 . There was a progressive quenching of all values of f_p and of its projected maximum with increasing O_2 , and likewise with decreasing O_2 partial pressures at all light used. Compared to the value in air, the light intensity, $I_{1/2}$, required to achieve one-half of the projected maximum f_p , increased as O_2 either decreased to 5% partial pressure or increased to 7 atm. At 58 atm, $I_{1/2}$ was between that for 1 atm and 7 atm O_2 .

Figure 3.8 replots data from intensity curves normalized in air for different light intensities as the ratio $f_p(\text{at given } O_2)/f_p(\text{air})$. Increased light intensity appears to partially overcome the quenching effect of O_2 ; at 7 atm O_2

¹ See appendix B.

quenching at the highest intensity was 27% and 52% at the lowest.

Table 3.3 Projected maximum relative variable fluorescence as determined from the Lineweaver-Burk plot (Figure 3.1), and light intensity for half-maximum f_p .

Amount of O_2	$f_{p, \max}$	$I_{1/2}, W m^{-2}$
Air	5.6	4.5
1 atm O_2	5.2	5.8
5% O_2	5.0	6.8
7 atm O_2	3.8	9.3
58 atm O_2	2.0	7.5

Figure 3.1a Oxygen evolution and chlorophyll synthesis during greening. Oxygen evolution per disc was calculated by multiplying O_2 evolution/chl by chl/disc. The error shown for O_2 per mg chl is about the same for all data points on that curve.

Figure 3.1b Relative fluorescence, f_p , in leaves and chloroplasts during greening.

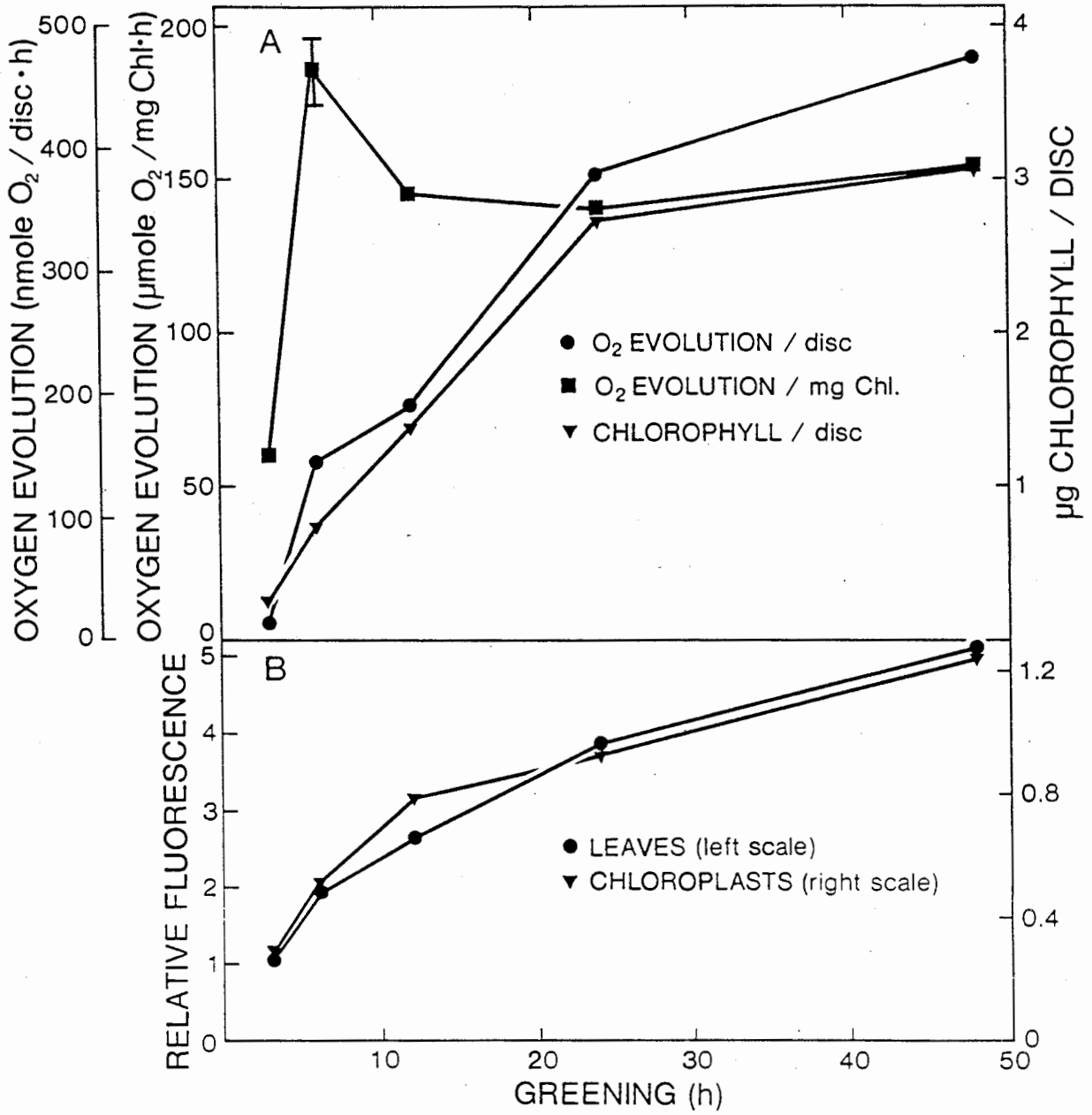


Figure 3.2 Oxygen evolution/disc and f_p in leaves during the accumulation of chlorophyll.

RELATIVE FLUORESCENCE IN LEAVES

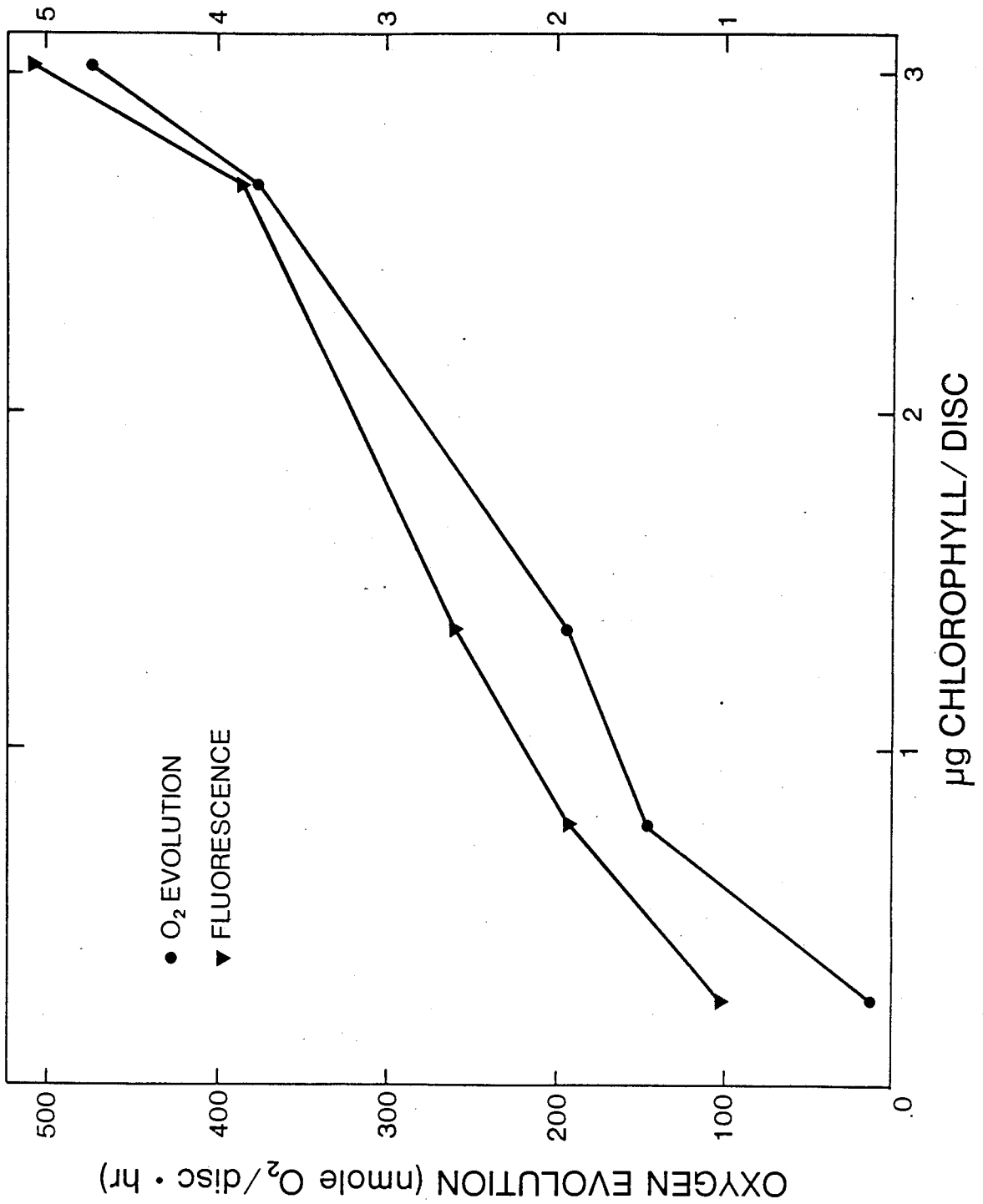


Figure 3.3 Oxygen quenching of relative fluorescence during greening. Error shown is typical of all points.

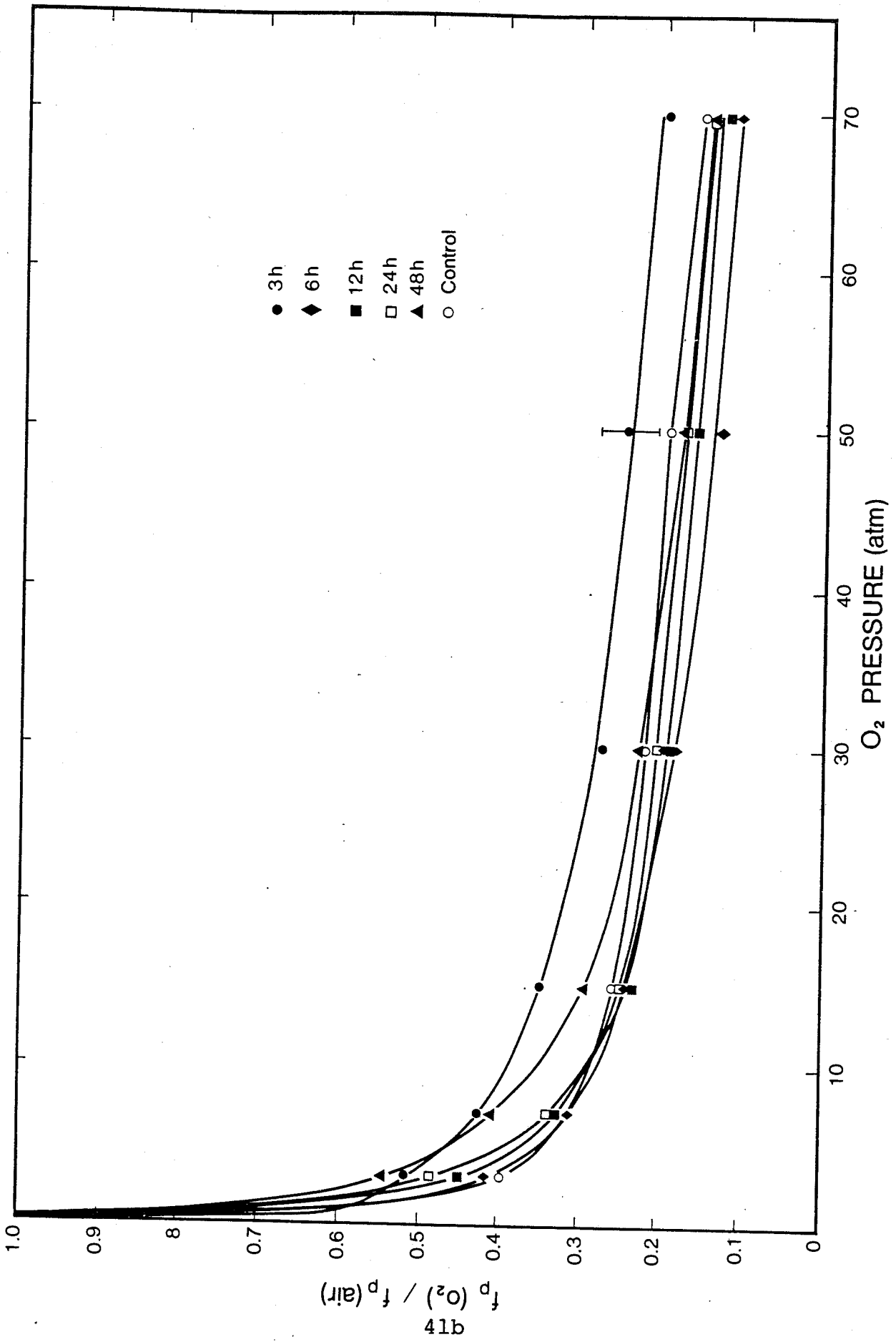


Figure 3.4 Oxygen quenching of F_0 fluorescence during greening.

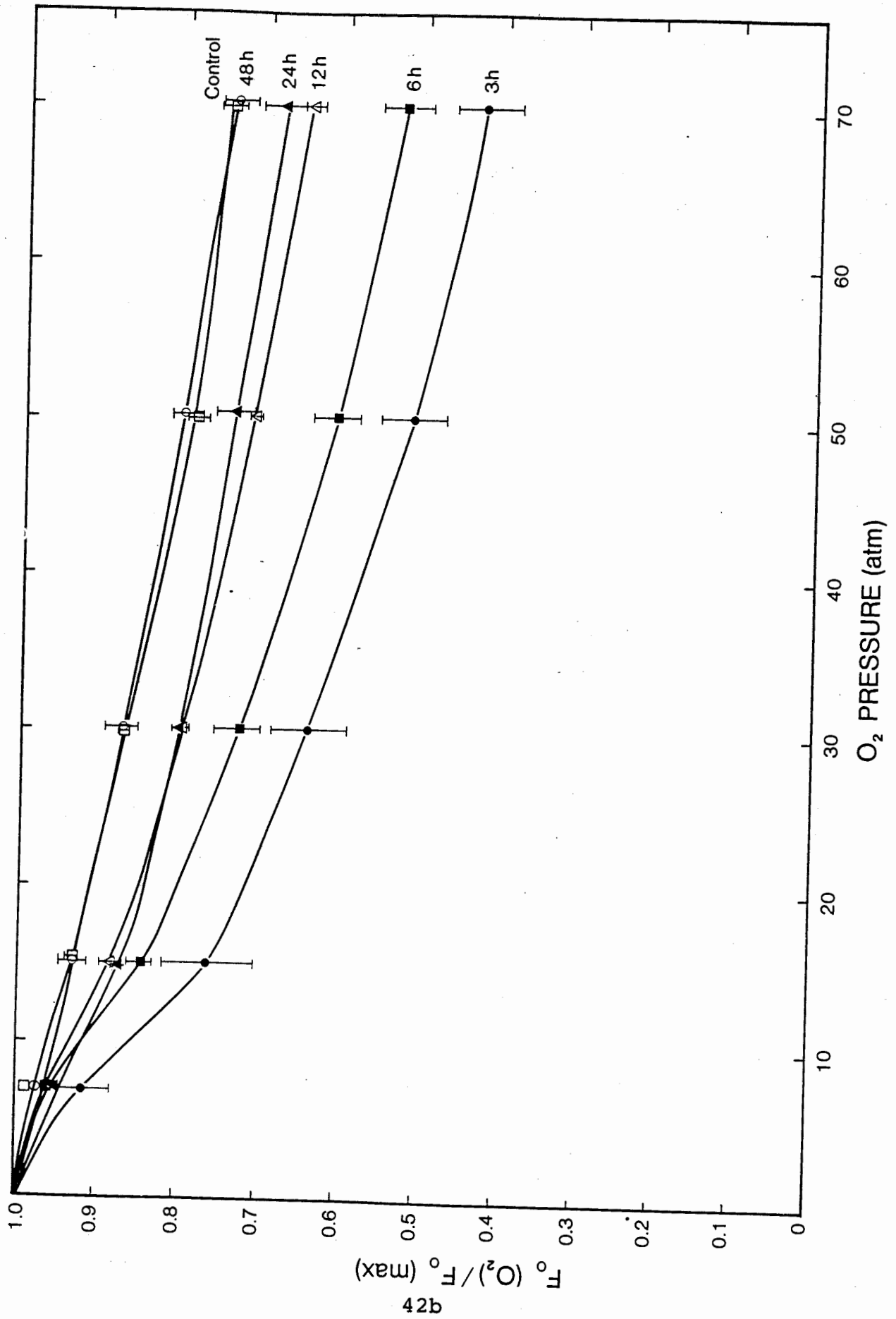


Figure 3.5a The fluorescent efficiency of F_0 as a function of light intensity.

Figure 3.5b Relative variable fluorescence, f_p , in air as a function of light intensity. Excitation at 632.8 nm. Data points are the mean of 4 to 6 samples. Error bars represent one standard deviation.

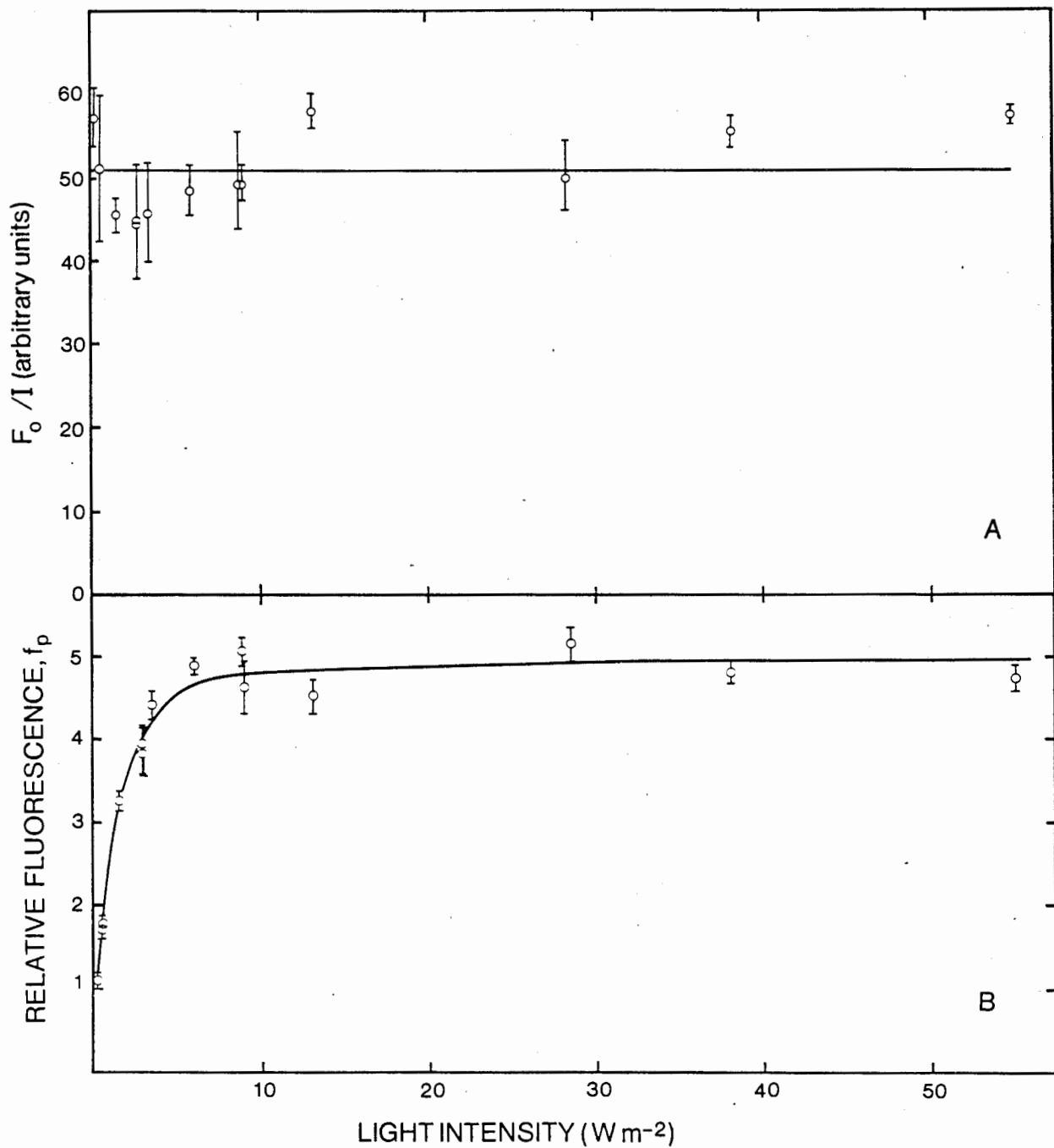


Figure 3.6 Light intensity dependence of relative variable fluorescence under various O₂ pressures. Excitation was from a tungsten lamp through a Corning 4-96 filter. Data points are the mean of two or three samples.

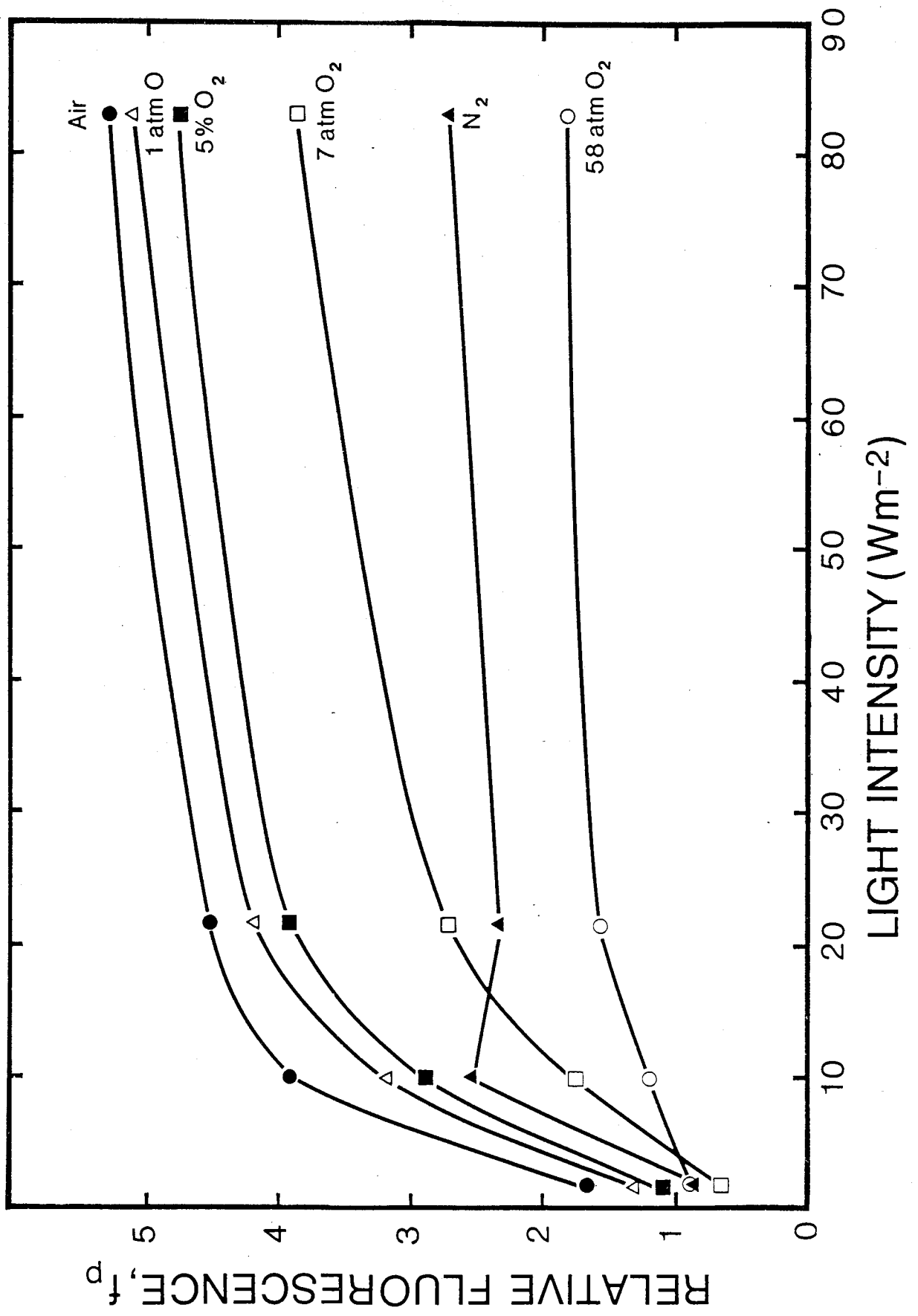


Figure 3.7 Lineweaver-Burk plot of the data in
figure 3.6.

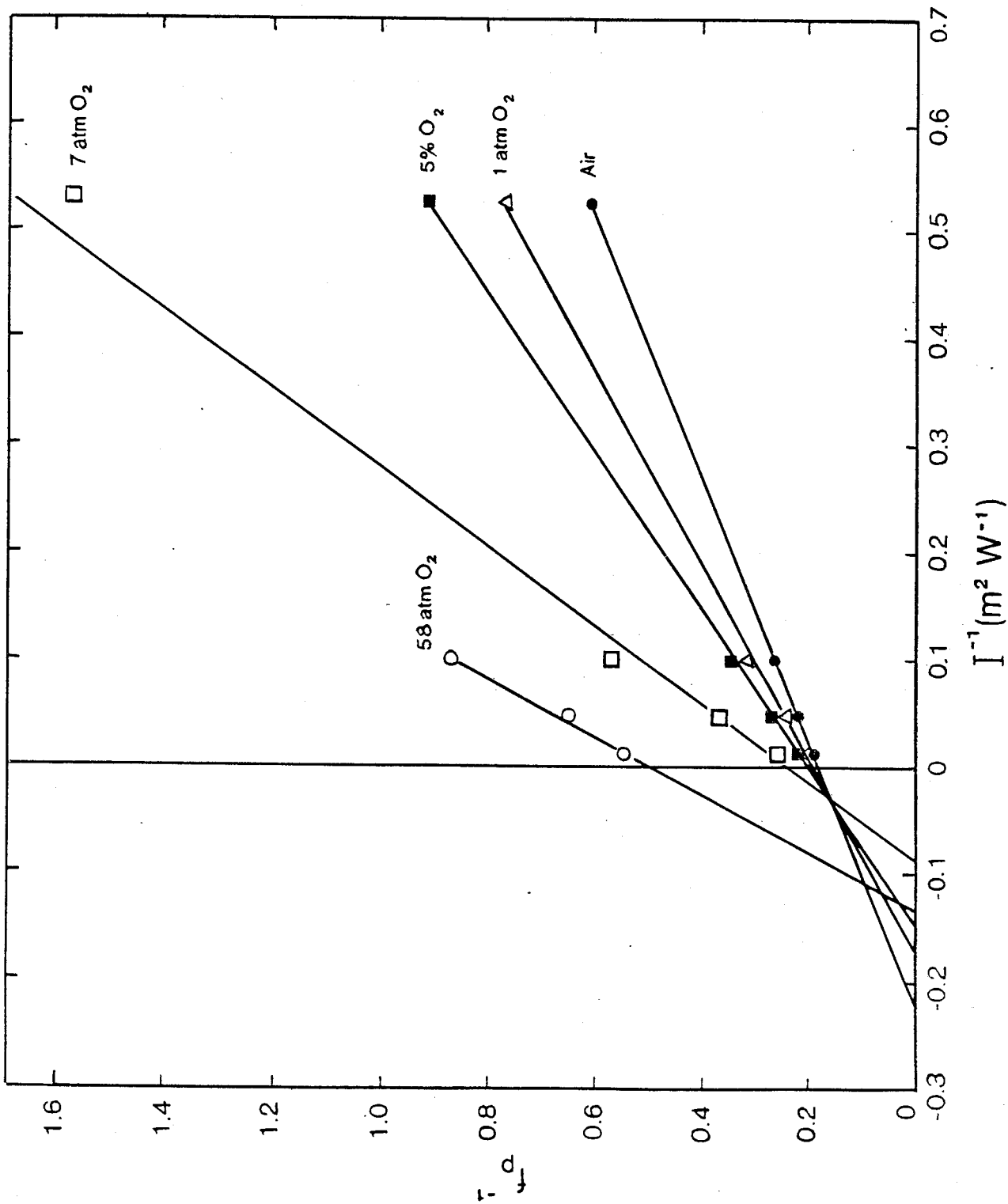
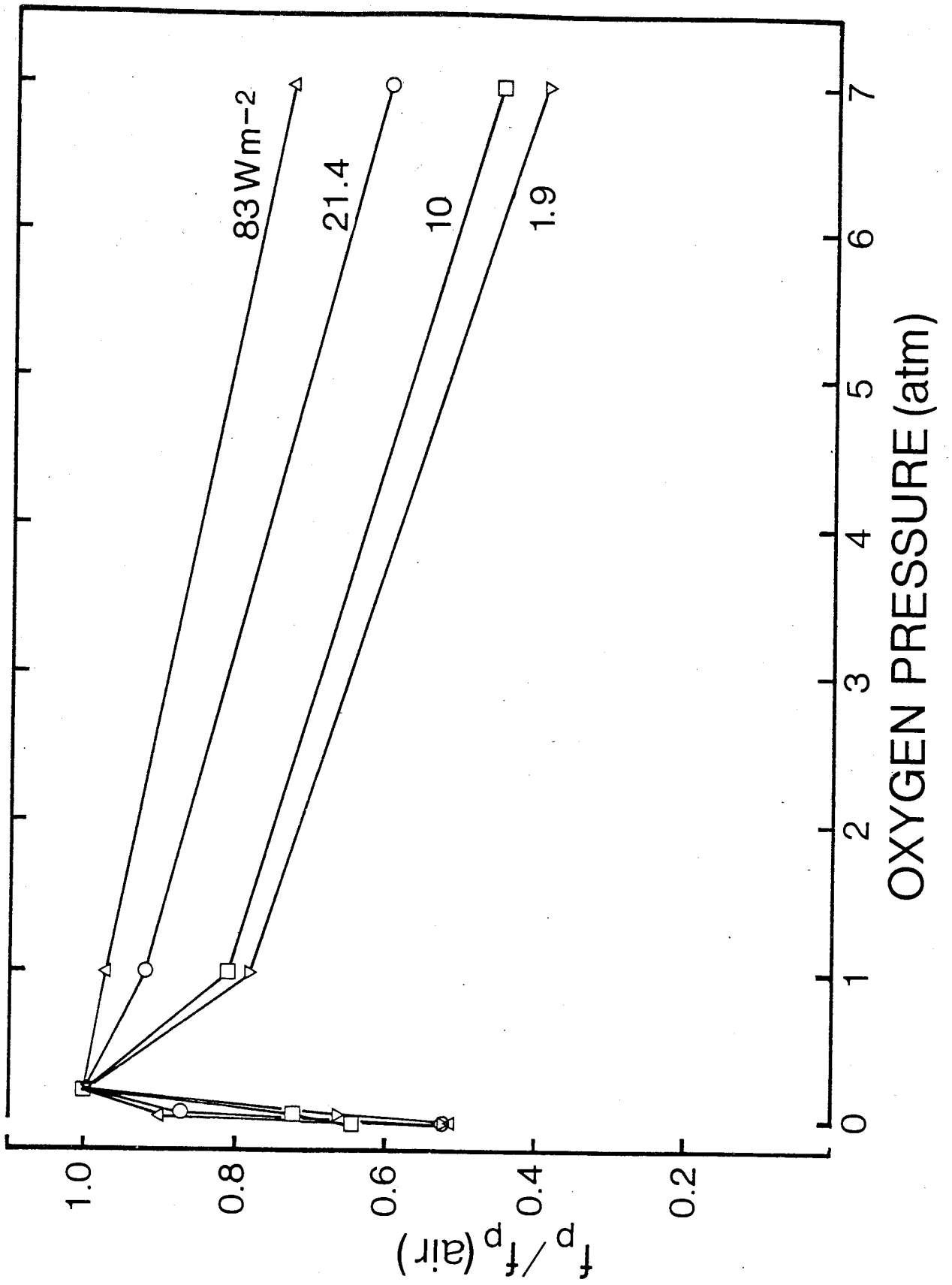


Figure 3.8 Oxygen quenching of f_p at various light intensities.



IV. Discussion

4.1 Greening

During greening there is a steady accumulation of chlorophyll per unit leaf area. However, O₂ evolution per chlorophyll increased sharply to a maximum at 6 hours and then declined to a steady level at 12 hours. On a leaf area basis, O₂ evolution also increased steadily. The maximum in O₂ evolution per chlorophyll has been observed by others (Baker and Butler, 1976; Henningsen and Boardman, 1973; Plesnicar and Bendall, 1973) and is explained by the differential development of chlorophyll and O₂ evolution. In the first minute of continuous illumination, the initial protochlorophyllide is transformed to chlorophyll (Kirk and Tilney-Bassett, 1978) and there is a lag in further chlorophyll synthesis of 2 to 4 hours. The O₂ evolving apparatus is formed or activated about 2 hours after the onset of illumination, and is not dependent on chlorophyll synthesis which can be speeded up by the addition of a precursor δ -aminolevulinic acid (ALA) (Nadler, Herron and Granick, 1972). Having been initiated, O₂ evolution per chlorophyll reaches a maximum probably when the water-splitting systems are completely formed (Kirk and Tilney-Bassett, 1978). This later chlorophyll fills in the antenna adjacent to the

reaction centers. Transfer between light harvesting pigments becomes more efficient, enhancing light energy conversion via the reaction center. As O_2 evolution per unit leaf area depends on the number and efficiency of reaction centers per unit area it increases throughout greening (Fig. 3.1).

Relative variable fluorescence is dependent on the size and efficiency of the light harvesting complex surrounding reaction centers rather than on the number of reaction centers. For each reaction center and its affiliated light-harvesting complex, as the chlorophylls become more numerous and better connected to the reaction center, there is a greater probability of an antenna excitation being trapped by this or a neighbouring reaction center. Subsequent excitation has a greater probability of not deexciting via this trap and of being emitted as fluorescence. Thus, f_p will not reflect the peak in O_2 evolution per chlorophyll.

Reduction of O_2 by electron transport is known to occur during CO_2 assimilation in chloroplasts (Egneus et al, 1975; Radmer and Kok, 1976). Exogenously added O_2 is also known to modify variable fluorescence induction and quench its yield in leaves and chloroplasts (Vidaver et al., 1981a,b). Since the ratio of the photosystem II acceptor (Q) to chlorophyll is high in the early greening stage and this ratio diminishes with further greening (Baker and Butler, 1976), the enhanced sensitivity of f_p to quenching by 7 atm O_2 in leaves during early greening is presumed a consequence of high Q/chlorophyll.

At this stage of greening there is less excitation reaching the reaction center for the same light intensity and so fewer electrons are transported from photosystem II, through Q, to photosystem I. Oxygen may then be removing a larger fraction of electrons. The small effect of 7 atm O₂ on the half-rise time from O to P, t_{1/2} (Table 3.2), indicates little influence on photosystem II reaction centers, in agreement with the observations of Vidaver et al (1981a). The rise to P occurs because the PQ pool has been reduced by photosystem II while oxidation of the PQ pool by PSI or by O₂ is after the rate-limiting step in photosynthesis: the reoxidation of PQ by the Rieske Fe-S protein (Allen, 1977; Vidaver et al., 1981b; Witt, 1979). The decrease in t_{1/2} as greening progresses occurs because of an increase in antenna size and efficiency so that more excitation can reach the reaction centers and hence electrons will reduce the PQ pool in a shorter time.

Fluorescence quenching by various oxygen pressures

At the O-level the rate of deexcitation due to photochemistry is a maximum.

$$k_p = k_{p \max} \quad (4.1)$$

From equation 1.10, if as for the C-level, O₂ acts as a direct deexcitation pathway of the chlorophyll, we have equation

$$\frac{F_0}{F_0(O_2)} = \frac{k_F + k_C + k_T + k_{p \max} + K_{O_2} [O_2]}{k_F + k_C + k_T + k_{p \max}} \quad (4.2)$$

$$= 1 + K_{O_2} [O_2] \quad (4.3)$$

If fluorescence is seen to be quenched in this linear fashion, the fluorescent molecule is said to obey Stern-Volmer quenching (Brand and Witholt, 1966). Figure 4.1 shows that the O-level quenching does obey Stern-Volmer quenching. The quenching is much more effective at early greening times because the antenna chlorophylls are not as well connected to the reaction centers as in mature plants. This decreases the rate of deexcitation going to the reaction center and increases $F_o(\text{air})/F_o(O_2)$.

Relative variable fluorescence does not obey Stern-Volmer quenching, nor does one expect it to. There is not a linear dependence of f_p on the redox state of Q (Lavorel and Etienne, 1977), and since O_2 accepts electrons near photosystem I the dependence on O_2 concentration is even less direct.

4.2 Fluorescence at various light intensities

With the apparatus described in section 2.5 our measured O-levels increased linearly with light intensity, also observed at similar light intensities in Anacystis nidulans (Mohanty and Govindjee, 1973) and swiss chard chloroplasts (Malkin, 1968). Since the deexcitation rate due to fluorescence is independent of light intensity, the maximal rate due to photochemistry and the other rates of deexcitation are also independent of light

intensity. This is expected since when all reaction center traps are open increased light intensity will not change the number of initially open traps. On the other hand, f_p in air increases with intensity up to about 8 W m^{-2} with no further increase at higher intensities. Dependence of f_p on light intensity below 8 W m^{-2} indicates that in air Q_a remains partially oxidized, perhaps because photosystem I activity exceeds that of photosystem II. Above 8 W m^{-2} , f_p is constant probably because Q_a is completely reduced so that k_p is a minimum. Additional light has no effect on f_p as prompt fluorescence now depends entirely on the number of excited antenna chlorophylls.

The intensity required for saturation is dependent on the intensity under which the plants were grown and may range from 20% to 100% (depending on species) of the light intensity during growth (Boardman, 1977; Daubenmire, 1974). The solar constant is 1360 W m^{-2} of which about 300 W m^{-2} is the photosynthetically active radiation (400 - 700 nm) at the earth's surface at 50° latitude under a clear sky. Our plants were grown at 20 W m^{-2} .

In broken pokeweed chloroplasts the electron acceptor $\text{Fe}(\text{CN})_6^{3-}$ increases the light requirement for saturation (Melis and Homann, 1975). In our experiment O_2 has a similar effect in barley leaves. Figure 3.6 shows that at any given light intensity, increased O_2 decreases the value of f_p and at any given O_2 pressure, f_p rises to higher values at higher light intensities. Increasing the light intensity overcomes the

quenching of f_p by O_2 (Figure 3.8). Table 3.3 shows that higher light intensity is required at larger O_2 pressure (up to 7 atm) to obtain one-half of its projected maximum value of f_p . At larger pressures, $I_{1/2}$ is reduced for reasons unknown.

The lowered relative variable fluorescence observed in 1 atm nitrogen may be an artifact. Schreiber and Vidaver (1974) have shown that in anaerobic conditions, the photosystem II acceptors may be reduced in the dark over several minutes to hours. This would give a high O-level and thus a low f_p . In our experiments, the sample remained in the sample cell and was flushed with nitrogen before each increase in light intensity. This may have made the sample increasingly anaerobic and thus artificially increased the O-level as the experiment progressed. We did not add any artificial electron-acceptors or preilluminate with far-red light to keep photosystem II in an oxidized state.

Another explanation is that anaerobiosis may prevent oxygen evolution and hence electron transport through Q (Slovacek and Hind, 1977). Anaerobic conditions would prevent oxidation-reduction changes of Q and hence variable fluorescence.

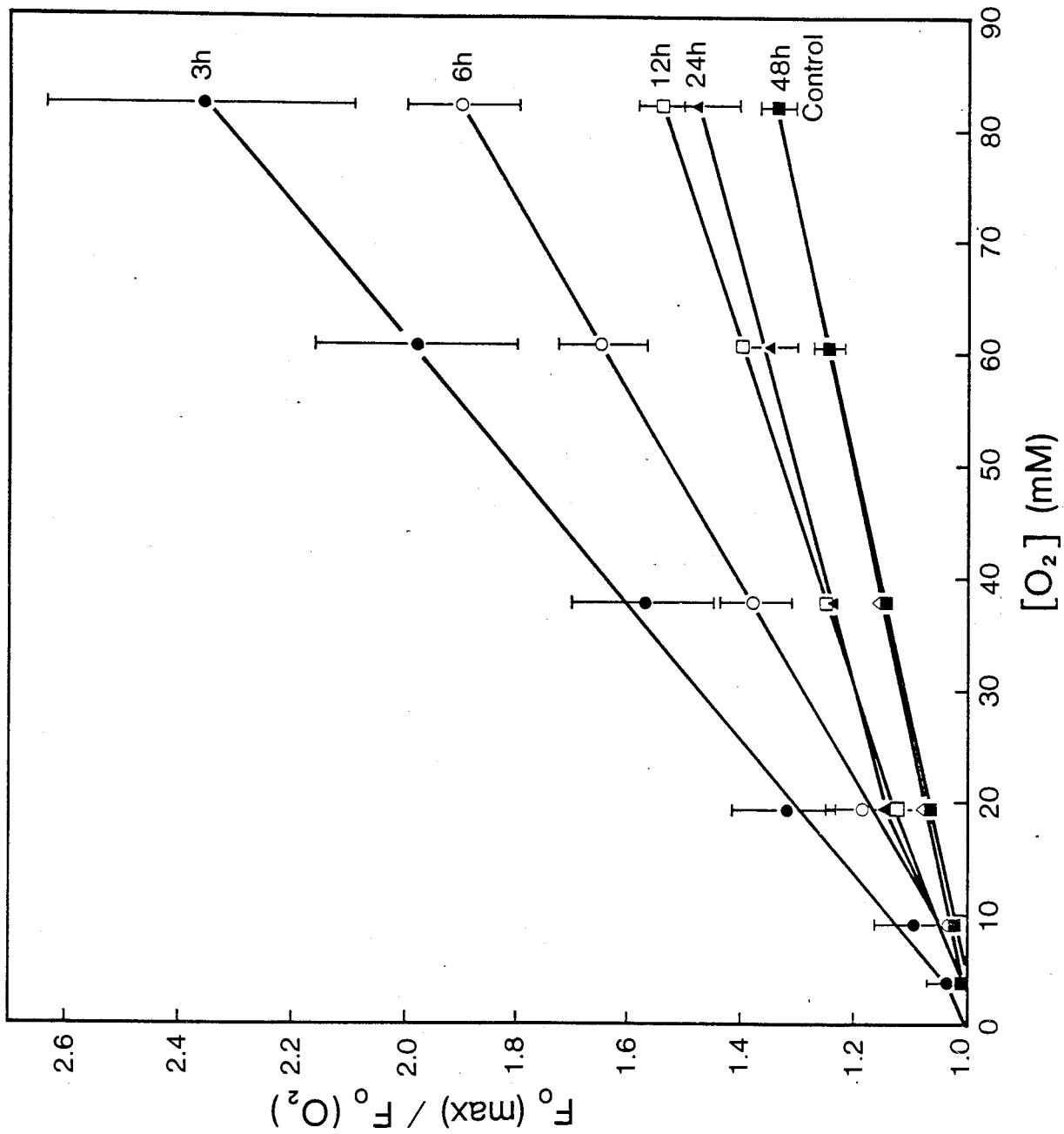
The electron transport system may be viewed as analagous to an enzyme system¹, with light as the substrate and O_2 as an inhibitor. For 1 and 7 atm of O_2 , increasing light intensity would cause more electrons to flow through the electron

¹-----
See appendix B

transport chain keeping the components (especially Q) more reduced, thus increasing f_p . Increasing O_2 increases the demand for the electron flow, keeping the intersystem electron carriers and Q less reduced and quenching f_p . Thus oxygen acts as a competitive inhibitor.

At 58 atm phase II quenching comes into effect so that the maximum f is much reduced. Also the fact that the Lineweaver-Burk plot for 58 atm is displaced to the left of the intersection of the other lines suggests that phase II is a non-competitive mode of quenching.

Figure 4.1 Stern-Volmer plot of F_0 quenching vs. oxygen concentration.



V. Conclusion

This thesis gives the results of two series of fluorescence observations of barley. In the first, the development of the photosynthetic apparatus in barley takes place in two stages. The water-splitting system shows little activity at 3 hours of greening but has been completely activated by 12 hours after the onset of continuous illumination. This was indicated by two occurrences; the oxygen quenching of f_p was greater before 12 hours and levelled off at this point, and the oxygen evolution per mg chlorophyll levelled off after 12 hours. However the synthesis and organization of chlorophyll is still going on at 48 hours as shown by the increasing chlorophyll content per unit leaf area and the decreasing oxygen quenching of F_0 .

In the second experiment, oxygen and light intensity showed counteracting effects on the relative variable fluorescence emitted from photosystem II. Increased oxygen quenched f_p while increased light intensity enhanced it.

APPENDIX A: OXYGEN CONCENTRATION IN WATER

To calculate the concentration of O₂ in the chloroplasts, atmospheres of oxygen were converted to mM by the the following equation developed by Krichevsky and Kasarnovsky (1935):

$$\log \frac{f}{N} = \log K + \frac{v P}{2.303 RT} \quad (A.1)$$

where f is the fugacity of the gas in atm (Hougen et al., 1959)

N is the mole fraction of the gas in the solvent (water)

K is Henry's coefficient

$$K_{O_2, 23^\circ C} = 3.215 \times 10^7 \text{ mm Hg} = 4.23 \times 10^4 \text{ atm (Loomis, 1928)}$$

v is the partial molal volume of the gas

$$v_{O_2} = 31 \text{ ml (Lauder, 1959)}$$

P is the total pressure of the gas in atm

R is the gas constant = 0.08205 atm l mole⁻¹K⁻¹

and T is the absolute temperature in degrees Kelvin.

At 23°C = 296.15 K

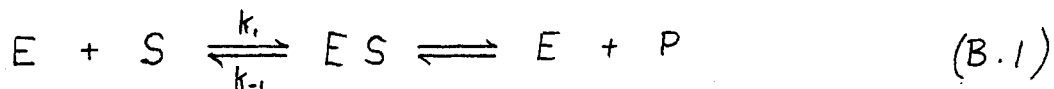
$$N = f \cdot 10^{-[4.626 + 0.000554 P]} \quad (A.2)$$

The molarity of O₂ is then calculated from

$$[O_2] = \frac{N}{1-N} [H_2O] = \frac{N}{1-N} 55.6 \text{ M} \quad (A.3)$$

APPENDIX B: ENZYME KINETICS

An enzyme acting on a single substrate species, S, forms an enzyme-substrate complex, ES, which breaks down into the enzyme and a product, P (Lehninger, 1982; Stryer, 1982). These reactions, with forward and reverse rates, may be written as



If the product is removed by further reactions, the product-enzyme back reaction is negligible. The rate of formation of the product (the "velocity" of the reaction) is then

$$V = k_2 [ES] \quad (B.2)$$

The rates of formation and breakdown of ES are

$$\text{rate of formation of } ES = k_1 [E][S] \quad (B.3)$$

$$\text{rate of breakdown of } ES = (k_{-1} + k_2)[ES] \quad (B.4)$$

In a steady-state condition these two rates are equal

$$k_1 [E][S] = (k_{-1} + k_2)[ES] \quad (B.5)$$

or

$$[ES] = \frac{k_1 [E][S]}{(k_{-1} + k_2)} = \frac{[E][S]}{K_m} \quad (B.6)$$

where K_m is the Michaelis constant $K_m = (k_{-1} + k_2)/k_1$. (B.7)

Usually the substrate concentration is much larger than the enzyme concentration, so the amount of uncombined substrate is nearly equal to the total concentration of S. The concentration of free enzyme is equal to its total concentration minus its bound concentration.

$$[E] = [E_t] - [ES] \quad (B.8)$$

So
$$[ES] = ([E_t] - [ES]) \frac{[S]}{K_m} \quad (B.9)$$

$$[ES] = \frac{[E_t][S]}{([S] + K_m)} \quad (B.10)$$

The maximal rate, V_{max} , occurs when all the enzyme is bound by the substrate $[E_t] = [ES]$ (B.11)

so that
$$V_{max} = k_2 [E_t] \quad (B.12)$$

$$V = \frac{k_2 [E_t][S]}{([S] + K_m)} = \frac{V_{max} [S]}{([S] + K_m)} \quad (B.13)$$

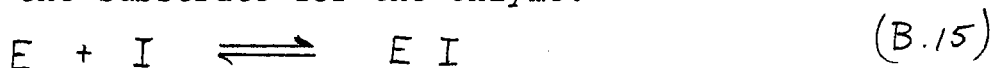
When $[S] = K_m$ then $V = V_{max}/2$ so that K_m is the substrate concentration at which the reaction rate is at one-half of its maximum value. This activity is shown in figure B.1.

Taking the reciprocal of both sides of equation B.13 gives

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max} [S]} \quad (B.14)$$

This double-reciprocal or Lineweaver-Burk plot gives a straight line as shown in figure B.2.

An inhibitor of this enzyme can act in two ways. It can compete with the substrate for the enzyme:



where EI in this form does not break down into a product nor can it react with the substrate. Secondly, the inhibitor can combine with the enzyme in such a way that it does not prevent the binding to the substrate, but does prevent the breakdown into a product.



In the first case, further increasing the substrate concentration wins the competition over the inhibitor so that K_m has been effectively increased by the factor $(1 + \frac{[I]}{K_I})$, where $[I]$ is the inhibitor concentration and K_I is the dissociation constant of the enzyme-inhibitor complex.

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m (1 + \frac{[I]}{K_I})}{V_{max} [S]} \quad (B.17)$$

See figures B.3 and B.4.

In the second case, increasing the substrate concentration cannot overcome the inhibitor's effect as it can still attack the enzyme-substrate complex after it is formed. If the inhibitor binds to E and ES with equal affinity and the substrate binds to E and EI with equal affinity then V_{max} is decreased by the factor $(1 + \frac{[I]}{K_I})$

$$\frac{1}{V} = \frac{(1 + \frac{[I]}{K_I})}{V_{max}} + \frac{K_m}{[S]} \cdot \frac{(1 + \frac{[I]}{K_I})}{V_{max}} \quad (B.18)$$

See figures B.5 and B.6.

Figure B.1 Michaelis-Menten plot for enzyme-substrate reaction.

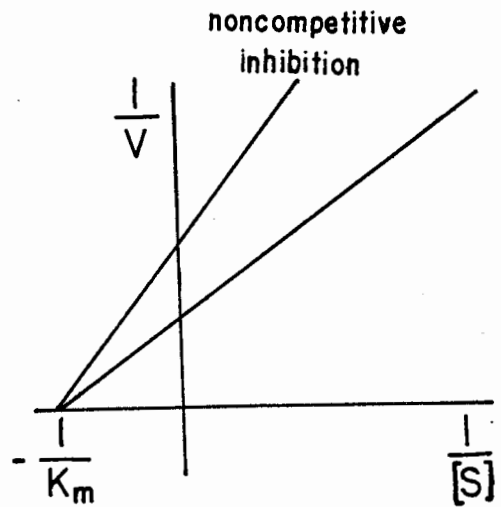
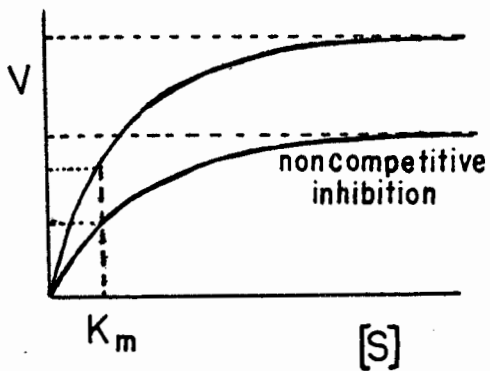
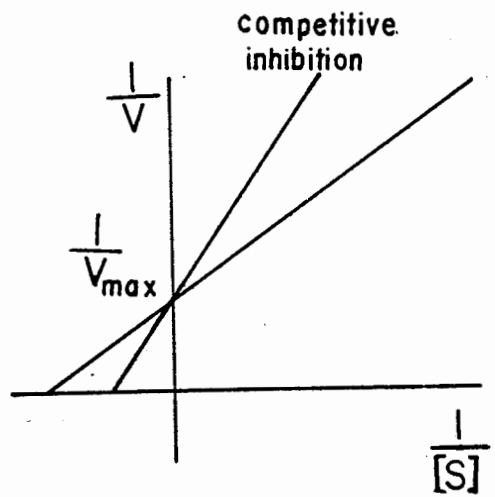
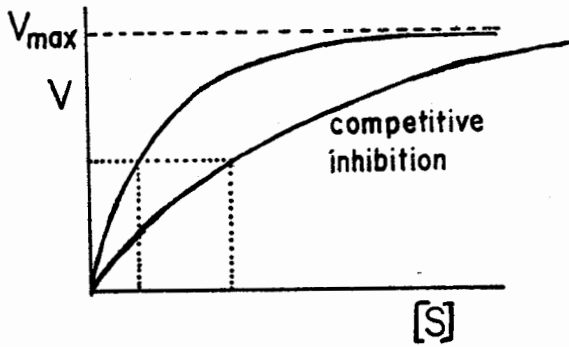
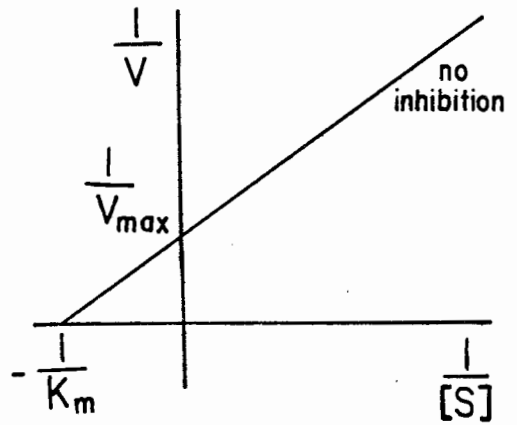
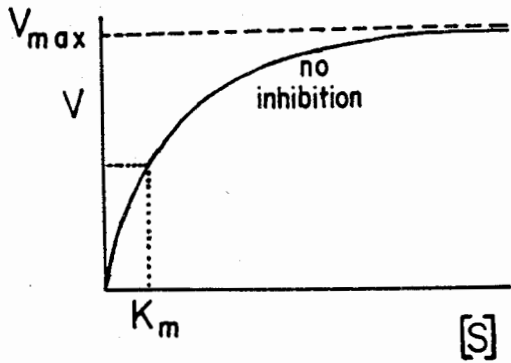
Figure B.2 Lineweaver-Burk plot for enzyme-substrate reaction.

Figure B.3 Michaelis-Menten plot for competitive inhibition.

Figure B.4 Lineweaver-Burk plot for competitive inhibition.

Figure B.5 Michaelis-Menten plot for non competitive inhibition.

Figure B.6 Lineweaver-Burk plot for non competitive inhibition.



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