REGULATION OF CHLOROPHYLL FLUORESCENCE DURING PHOTOSYNTHESIS: A STUDY OF THE FACTORS AFFECTING CHANGES IN YIELD AND EMISSION OF CHLOROPHYLL FLUORESCENCE IN INTACT ALGAL CELLS AND ISOLATED CHLOROPLASTS

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THESIS

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I. INTRODUCTION

1. General

1.1 Foreword

Photosynthetic tissues emit fluorescence upon excitation with light. Variation in the yield of chlorophyll (Chl) fluorescence during illumination can be observed for a few seconds (fast) to several minutes (slow) (1). Fast changes in the Chl fluorescence yield have been studied extensively as they indirectly reflect the photochemical utilization of excitation energy.

Study of long term changes in the yield of Chl fluorescence has recently acquired a new impetus. From several correlative studies (see references cited in 1), it has been suggested that these slow changes, perhaps, reflect some energy-linked structural variations of the photosynthetic lamellar systems. Therefore, a study of changes in the fluorescence yield of photosynthetic pigments ought to give some insight into the primary photochemical events of photosynthesis as well as the energy metabolism associated with photosynthesis.

It is generally assumed that fluorescence is in competition with the primary photochemical events of photosynthesis. Qualitatively speaking, a high rate of electron flow reduces the yield of Chl a fluorescence, while a reduction in the electron flow enhances the fluorescence yield. This simple rule, as we shall see later, seems to be valid only within the first few seconds of illumination. Long term fluorescence yield changes do not seem to be governed by electron flow. It is postulated, rather, that these changes are controlled by the structural "state" of the pigment bed. We do not exactly know the relationship between the

structural "state" of the membranes' pigment bed and the Chl *a* fluorescence yield, although a few hypotheses have attempted to give a pictorial description of the phenomenon (see below).

Before we elaborate on the problem of this investigation, it will be instructive to discuss the current dogma of photosynthesis and to present a brief survey of the recent research that has bearing on our investigation. This survey is, however, not meant to be all-inclusive and it is only a general description of the current state of our knowledge.

1.2 Photosynthetic Unit, Two Pigment Systems and Two Light Reactions

The classical flashing light experiments of Emerson and Arnold (2) showed that brief (10 μ second) flashes of saturating light produced, on the average, one O_2 per 2400 chlorophyll molecules. Since the quantum requirements of steady state photosynthesis is 8 quanta per O_2, then 8 traps must be activated to produce one molecult of oxygen, and hence the number of chlorophyll molecules per trap is reduced from 2400 to 300. Thus, one photosynthetic unit is now conceived to be a structural unit consisting of approximately 200-300 chlorophyll bulk molecules and one trap. These bulk molecules absorb light and funnel the absorbed quanta to their specific traps where a one electron mediated photochemical oxido-reduction reaction occurs. Numerous other experiments, for example, the study of Hill reaction as a function of the size of chloroplast fragments (3) and the determination of the ratio of trap and other reaction partners to total chlorophyll molecules (4), have amply substantiated the existence of the photosynthetic unit.

A breakthrough in our understanding of photosynthesis came from the study of Emerson and Lewis (5) and Emerson *et al*. (6) who discovered a "red drop" in the quantum yield of O_2 evolution and a synergistic effect

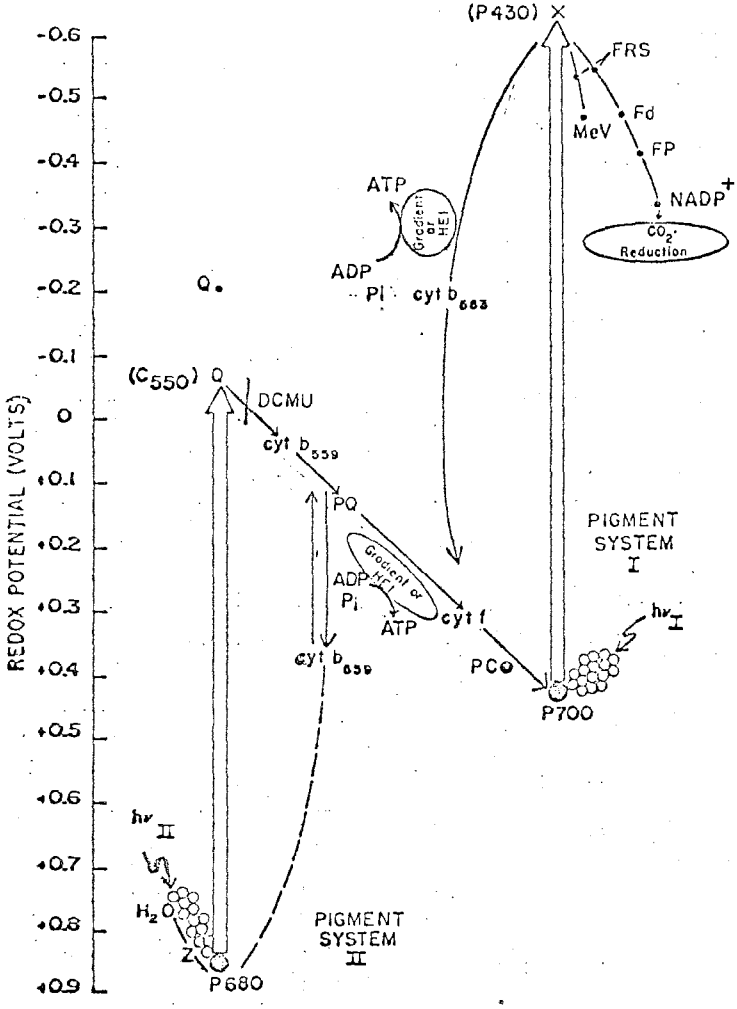
of two wavelengths of light on the rate of photosynthesis. Simultaneous excitation with a wavelength shorter than 685 nm (red light) and a wavelength longer than 685 nm (far red), yielded a higher rate of oxygen evolution than the sum of the rates when these lights are given separately (now known as the Emerson enhancement effect). Later measurements of the action spectra of the enhancement effect (7), the antagonistic effect of red and far red light on cytochrome (cyt f) (8), the quenching by far red light of fluorescence of Chl a excited with either red or blue light (9, 10) and finally, the physical separation of the two pigment systems (see review, 11) led to the proposition of two light reactions driven by two different pigment systems. Studies made with photosynthetic mutants have added additional evidence to the concept of two photosystems (see review, 12).

The bulk of experimental evidence weighs heavily in favor of two light reactions connected in series popularly known as the Z (perhaps more appropriately, "N") scheme or the modified Hill and Bendall scheme of photosynthesis. (For the original description, see ref. 13.) Figure 1 illustrates the reaction partners in this scheme together with names of a few chemicals that affect these reaction components. We describe below some of the characteristics of the two pigment systems.

1.3 The Photosystem I (PS I)

The trap of photo or pigment system I, discovered by Kok (14), is a special type of Chl a molecule called P700. P700 receives most, if not all, of its energy via a small group of long-wavelength forms of Chl a absorbing at 695-700 nm. Quanta arriving at these forms of Chl a either feed to P700 if it is "open" or escape as heat if it is "closed," which

Figure 1. A Modified Hill and Bendall Scheme of Photosynthesis. The two vertical arrows represent the two light reactions; all other dark reactions. Flow of electrons from H_2O to $NADP^+$ is designated as 'non-cyclic' electron flow and the closed pathway involving only the system I is known as 'cyclic' electron flow. A similar cyclic flow of electrons involving only system II shown as dashed line has also been suggested recently. Abbreviations used: Z. the primary donor of PS II; P680, the proposed trap of PS II; Q (C550), the primary acceptor of PS II and the quencher of fluorescence (Q. is a low potential form of the quencher Q); PQ, plastoquinone; $Cytb_{559}$ and $Cytb_{563}$, cytochrome b; Cytf, cytochrome f; PC, plastocyanin; P700, the trap for PS I; X(P430), the system I acceptor; Fd, ferredoxin; FP, flavoprotein; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ADP, ATP, adenosine di and triphosphate, pi, inorganic phosphate. DCMU and MeV shown in the diagram are not the intrinsic components but are usually added either to block or initiate non-cyclic electron flow respectively.


REDOX POTENTIAL (VOLTS)
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
+0.1
+0.2
+0.3
+0.4
+0.5
+0.6
+0.7
+0.8
+0.9
(P430) X
FRS
Fd
MeV
FP
NADP+
CO2 Reduction
ATP
Gradient H+
ADP
Pi
cyt b663
Q
(C550) Q
DCMU
cyt b559
PQ
ADP
Pi
Gradient H+
ATP
cyt f
cyt b559
PC
PIGMENT SYSTEM I
hν I
P700
hν II
H2O
Z
P680
PIGMENT SYSTEM II

perhaps partly accounts for the low fluorescence yield of system I pigments.

The excitation of P700 results in a photoreaction: the transfer of an electron from P700 to an unknown low potential acceptor X. The chemical nature of X is still unknown. (Yocum and San Pietro (15) have been able to isolate a substance called Ferredoxin Reducing Substance (FRS), which they earlier believed to be the primary electron acceptor of PS I. Hiyama and Ke (16) have observed an absorbance change around 430 nm which they claim to be due to the reduction of the primary acceptor of PS I; the chemical nature of P430 is yet unknown.) Photochemical oxidation of P700 is independent of temperature as it occurs at liquid nitrogen temperature (17) and has a quantum yield in the neighborhood of unity (18). P700 is a mild oxidant, the mid-point potential being +430 mv; and X^- is a strong reductant. The midpoint potential of X/X^- couple is estimated to be approximately -600 mv (19).

In vivo, the system I photoproduct X^- reduces $NADP^+$ (and probably other compounds). Three enzymes, FRS, ferredoxin and ferredoxin-$NADP^+$ reductase mediate the transfer of electrons from X^- to $NADP^+$ (see review, 20). The low potential viologen dyes have been shown to accept electrons from X^-, overriding the above reaction (19).

It is also known, primarily from the studies with isolated chloroplasts, that a cyclic flow of electrons occurs from X^- to $P700^+$, probably mediated by a b type cytochrome. In these reactions, no net oxidation reduction occurs, but the downhill flow of electrons releases energy, a part of which is conserved as ATP molecules (21). The occurrence of cyclic electron flow in vivo is supported by indirect and circumstantial evidence (22).

Recent studies (see literature citations in ref. 23) show that the stromal-lamellar fragments of chloroplasts only perform cyclic electron flow, while the granal-lamellar system operates in the fashion of the Z-scheme. It is very difficult to say if the above is true for all types of higher plants and algae. In view of the lack of evidence of such morphological separation of photosystems in algal cells and keeping in mind the contradictory data against such a generalization (12), we shall restrict our discussion only to the Z-scheme.

PS I can carry out partial reactions of photosynthesis if the flow of electrons from PS II is inhibited and reduced dyes that act as electron donors are added (24). PS I can then reduce $NADP^+$ or viologen dyes. A large number of such electron donors are now known, but the site specificities of these donors are not clearly understood. In the presence of some cofactors (e.g. PMS, DAD, pyocyanine and TMPD), the flow of electrons from X^- to $P700^+$ can be stimulated. Since there is no net reduction of any carrier, this system is assayed by the amount of ATP formed.

Oxygen exchange studies with a variety of algae have shown that light (particularly light absorbed by PS I) depresses O_2 uptake during "weak" illumination. This depression of O_2 uptake operates even when net feeding of electrons is blocked by poisons. This type of depression of the oxygen uptake has been suggested to be associated with PS I mediated cyclic electron flow *in vivo* (25). It is assumed that cyclic electron flow would deplete the endogenous ADP supply and thus it would depress the respiratory O_2 uptake due to limiting supply of ADP (State IV) (25). However, recent studies made with the use of uncouplers do not fully agree with the above suggestion (26).

1.4 The Photosystem II (PS II)

Photo or pigment system II evolves oxygen from water and donates (ultimately) electrons to the oxidized trap of system I, the $P700^{+}$. Unlike photosystem I, the PS II trap has not been fully characterized. However, a bleaching at 682 nm (P680-690) (27), perhaps, relates to the reaction center of photosystem II. According to Döring et al. (27) (but a view not shared by all in the field (1, 17, 28)), the trap of system II is only a sensitizer of electron transfer and does not undergo oxidation or reduction!

It is assumed that excitation of PS II trap results in the oxidation of a hypothetical electron donor Z and the reduction of an electron acceptor Q. The chemical nature of Z and Q are as yet unknown. (Recently, an absorbance change at 550 nm due to oxidation-reduction of a compound called C550 has been observed (29). It is claimed that C550 is Q (30).) The reduced Q (Q^{-}) transfers electrons through a series of intermediates to oxidized P700 ($P700^{+}$). Q in its oxidized state has been assumed to act as a physical quencher of fluorescence. Thus the ratio Q^{-}/Q is a measure of the fluorescence yield (10). Closure of the trap, and consequently a high fluorescence yield, is associated with the reduction of Q.

Transfer of electrons from Q^{-} to its immediate partner (shown as PQ in Figure 1 or sometimes represented only as a kinetic parameter A) is rapid (occurring in less than 1 msec if all Q is in the reduced state and all A in the oxidized state) (31). Fluorimetric estimation of the redox potential of Q suggests that Q is a mild oxidant; the Q/Q^{-} couple has a midpoint potential of about +180 mv (32). However, Cramer et al.'s (33) titration of Q gave two transitions at -35 and -270 mV.

During the downhill flow of electrons from Q^- to $P700^+$, some energy is conserved to generate ATP. The functional site (sites) of ATP synthesis during this non-cyclic flow is not very well elucidated and has been studied by many workers (e.g., see Neumann et al., 34).

Cheniae (35) has recently reviewed the literature pertaining to the oxygen evolving reactions and the catalysts presumed to co-operate in these reactions. The complex series of reactions leading to the light-induced accumulation of positive equivalents at the PS II reaction centers for dark oxidation of water have prompted many theoretical models (36-38). A discussion of the activation and de-activation of stored positive charges in System II donor is beyond the scope of this thesis. However, it is worth mentioning that recent studies of Joliot's group in Paris (39) and Kok's group in Baltimore (40) show that oxidation of water to molecular oxygen requires a collaboration of four oxidizing equivalents. We only imagine that a chemical species Z^{4+} is formed and that water reacts with this species to produce oxygen.

In recent years, it has also been shown that PS II can photo-oxidize some compounds other than H_2O. A variety of chemicals has been used to donate electrons to the reductant (or Z^+) side of PS II. It is, however, not clearly known which of these compounds feed electrons to oxidized Z^+, which to $Chla_2^+$, or to some immediate precursor of oxygen. It is also very difficult to say if these (donor) compounds are specific to PS II or if they can feed electrons to PS I as well, since detailed studies on the site specificity of these compounds are not available. However, it has been recently recognized (41) that hydroxylamine, a well-known inhibitor of photosynthesis, donates electrons to PS II rather specifically when

present in excess. Donation of electrons to PS II by artificial donors becomes appreciable if O_2 evolving capacity is destroyed. Yamashita and Butler (42) demonstrated photo-oxidation of a variety of chemicals in tris-washed chloroplasts. Washing of chloroplasts with a high concentration of tris at high pH (0.8 M, pH 8.) eliminates the photo-oxidation of water and promotes photo-oxidation of a good many non-physiological donors, including hydroxylamine.

In this thesis, we have studied the characteristics of the effect of hydroxylamine and other dyes in tris-washed chloroplasts. We have also analyzed the effect of hydroxylamine in the intact cells of red alga Porphyridium and also to a lesser extent in the blue-green alga Anacystis. We will elaborate on this topic later in our discussion.

2. Fluorescence Yield and Emission Characteristics of Two Pigment Systems

2.1 Fluorescence Yield

Unlike the situation in PS I where the yield of Chl fluorescence is extremely low and the incoming quanta either carry out a photochemical conversion or are lost as heat, some 3 to 10% of absorbed quanta in photosystem II are re-emitted as fluorescence. Hence, it follows that most fluorescence comes from PS II.

The decay of the excited bulk Chl of photosystem II is shared by three competitive precesses: (a) radiationless loss (this includes heat and other losses, e.g., spillover of quanta to PS I), (b) fluorescence, and (c) trapping by the reaction center of PS II. The respective first-order rate constants are represented as k_h, k_f and k_p respectively.

Thus, the absolute quantum yield of fluorescence is given as follows:

$$\phi_f = \frac{\text{quanta emitted}}{\text{quanta absorbed}} = \frac{k_f}{k_h + k_f + k_p} \qquad (1)$$

If all Q is in the oxidized state, all the traps of PS II are open, the yield of fluorescence given in equation 1 is minimum (assuming no other parameter affects the yield). If all the Q is kept reduced, the yield of fluorescence will be maximum and is represented as:

$$\phi_f^{max} = \frac{k_f}{k_h + k_f} \qquad (2)$$

Equations (1) and (2) can be rearranged to give the following relation (see 43, 44 for detailed discussions):

$$k_h = k_f \left[1/\phi^{max} - 1\right] \qquad (3)$$

If one assumes that k_f *in vivo* is the same as that of Chl *a* in solution, one can estimate k_p, k_h and k_t (rate constant of energy transfer from strongly fluorescent $Chla_2$ to weakly fluorescent $Chla_1$, included in k_h in the above equation).

Recently, Mar *et al*. (45) made estimates of the rates of the k's in three algae (*Chlorella*, *Anacystis* and *Porphyridium*). Since Q is the primary acceptor of electrons of PS II one would expect that the rate of system II reaction (V_2) (*e.g.* of O_2 evolution) will obey the following relation:

$$V_2 = \alpha_2 I \left[Q\right]/\left[Q_{total}\right] = \alpha_2 I \left[1-Q^-\right]/\left[Q_{total}\right] \qquad (4)$$

where ($\alpha_2 I$) is the absorbed quanta by PS II. It was observed that the rates are higher than to be expected from the above relation at low values of Q (46). This observation was taken to mean that the absorbed photons

of system II have the ability to seek and find an open trap if they happen to meet a closed trap. In other words, energy transfer occurs among the PS II units.

The simplest way to visualize this observation is to assume that system II reaction centers are embedded in a two dimensional common pigment bed ("lake" model) and that an absorbed photon has a choice of several traps. Fluorescence lifetime (τ) measured as a function of the yield of fluorescence gives a linear relation, which supports such a lake type makeup of the photosynthetic unit of PS II (PSU II) (47, 48). It must, however, be mentioned that other models have been proposed which assume that the system II units are isolated ("isolated puddles" model) with no transfer between them. Such a model predicts a non-linear relation between ϕ_f and τ. If, however, an inter-unit excitation energy transfer exists between the puddles, an increase of lifetime of chlorophyll fluorescence with yield (although not necessarily linear) can be observed (49). The basic problem lies in the fact that it has been difficult to resolve unambiguously if there is more than one lifetime for Chl a fluorescence in vivo (45). Sorokin (50, 51) has recently made detailed mathematical analyses of the time-course of fluorescence yield and of the fluorescence yield and lifetime relationships for both multicentral (lake) and unicentral (isolated puddles) PSU II. Interestingly enough, he suggests that these organizations of units are mutually interchangeable (50, 51).

It is assumed that the level of fluorescence will be maximum when all Q is reduced. In very bright exciting light the photochemical reduction of Q will be much faster than dark oxidation of Q and one would expect an

exponential increase of fluorescence yield from F_0 to F_{max}. Morin (52) and Joliot and Joliot (46) could not observe a first order increase of fluorescence yield in bright light. Morin (52) and Delosme (53) observed a similar non-exponential rise of fluorescence yield in the presence of orthophenanthroline or DCMU. Delosme (53) explained this non-exponential rise of yield by assuming, as discussed earlier, the occurrence of photon transfer between photosynthetic units of system II.

However, this non-first order rise of the fluorescence yield in the presence of poisons can also be explained by assuming that there are two fluorescence quenchers (e.g., Q_1 and Q_2) and/or a requirement of two photoacts for the complete reduction of Q (Q^- and $Q^=$). Recently, Joliot and Joliot (54) observed a differential reduction of the quencher Q in the dark by dithionite in the presence and absence of DCMU and they interpret this differential behavior in the chemical reduction of the acceptor as the intervention of two quenchers; and, one of the quenchers is protected from chemical reduction by dithionite when DCMU is present. Similarly, Doschek and Kok (55) observed variations in the nature of the fluorescence rise curve in isolated chloroplasts in the presence of DCMU depending on the pre-treatment of the chloroplasts--an observation which does not fully agree with the suggestion that non-first order fluorescence rise curve reflects only the photon transfer ability. (But it is possible that the prehistory of chloroplasts may alter the structural organization and may change the probability of transfer.)

Doschek and Kok (55) compared the value of fluorescence yield at any given moment to the fraction of area removed from the fluorescence rise curve until that moment and observed a quadratic relationship between

these two parameters under some experimental conditions. These authors suggested that a (two) sequential reduction of Q (Q^-, $Q^=$) describes the non-exponential rise of fluorescence yield in the presence of DCMU. Thus, a sequential reduction of two quenchers or photon transfer among the photosystem II units can explain the non-exponential fluorescence rise curve. It is difficult to decide which one of the above two governs the fluorescence rise curve in the presence of DCMU. In the absence of experimental data qualifying the two quenchers, we will assume here that fluorescence yield is governed by Q, the primary acceptor of photosystem II in the same sense as originally proposed by Duysens and Sweers (10).

It has been observed, first by Homann (56) and later by Murata (57) that addition of ions (e.g., Mg^{++}) also increases the yield of Chl fluorescence in isolated chloroplasts, over and above the DCMU level (F_∞ DCMU) which suggests that accumulation of reduced Q does not give rise to the maximum yield. These data indicate that a significant fraction of absorbed photons in system II is shared by system I, at least, in isolated chloroplasts (see section 2.2 below).

In this thesis, we shall attempt to describe and discuss the nature of enhancement and/or suppression of fluorescence yield in the presence and absence of electron flow, both in intact algal cells and isolated chloroplasts; we shall also discuss the factors that govern the yield of Chl fluorescence. The following section presents a brief summary of the time course of Chl a fluorescence yield changes during illumination. Details of the kinetics of fluorescence yield changes as well as the historical development of the subject have been discussed earlier by Munday (58) and by Papageorgiou (59) of our laboratory.

2.2 Distribution of Excitation Energy Between Two Pigment Systems

As discussed earlier, the occurrence of Emerson enhancement lends support to the mechanism of photosynthetic electron transport involving two light reactions operating in series. In order to achieve maximum efficiency of electron flow, the two pigment system units need to receive equal excitation. Two alternate hypotheses have been proposed to achieve such a distribution of absorbed quanta (60). In one model (separate package), each pigment system has almost equal amounts of bulk or antenna pigments and each has its own pigment bed; there is no communication by way of energy transfer between two pigment systems. The alternate model (the spillover) assumes more bulk pigment molecules in pigment system II with the excess absorbed quanta of pigment system II, if not needed at the reaction centers, moving to pigment system I. An extreme case of the spillover model has been recently suggested by Avron (61). In this model, a general pigment bed feeds energy to both reaction centers and the fractional distribution of quanta to these centers is regulated by reaction conditions and the presence or the absence of cofactors. The mechanism of this controlled spillover is yet uncertain.

A special role of divalent cations has been proposed by Murata (57) to control the spillover. Addition of Mg^{++} or other cations increases the yield of indophenol dye reduction, and decreases the yield of system I partial reaction. Murata (57) concluded on the basis of these results that cations suppress energy spillover from PS II to PS I. Avron and Benhayyim (62) on the other hand, find an increase in yield of $NADP^{+}$ reduction with water as a donor with added Mg^{++}. Using modulated relaxation spectrophotometry Rurainski *et al*. (63) have also shown that Mg^{++} ions enhance the yield of photoreduction of $NADP^{+}$ by H_2O. Sun and

Sauer (64) could establish that the presence of $MgCl_2$ is necessary for optimal photoreduction of $NADP^+$ by H_2O and also for Emerson enhancement effect, a condition that was previously used by R. Govindjee et al. (65). There are two possibilities to visualize how ions (e.g., Mg^{++}) can control the photochemical reactions: (a) ions prevent spillover between two pigment systems as proposed by Murata (57); (b) ions may influence a back transfer of excitation from PS I to PS II and determine the efficiency with which quanta are trapped and utilized at PS II reaction centers (64). No experimental data are available to distinguish between these two possible mechanisms.

2.3 The Characteristics of Chlorophyll a Emission in vivo

The emission spectra of chloroplasts and algae at room temperature are relatively structureless. In green algae and chloroplasts from higher plants, the emission spectrum has a main peak around 685 nm (F685) with a broad shoulder around 720-740 nm. No fluorescence due to Chl b is observed suggesting nearly 100% efficiency of energy transfer (66). (For exception, see ref. 67.) In phycobilin containing algae, phycoerythrin emits fluorescence at 580 nm, phycocyanin and allophycocyanin at around 650 nm.

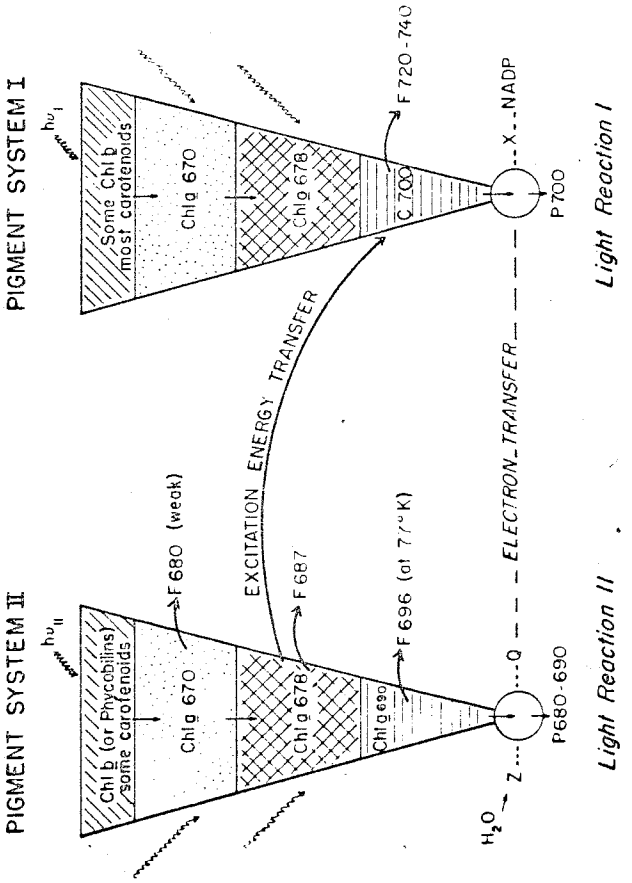
The emission spectrum of chloroplasts at low temperature, namely at $77^{\circ}K$, is three-banded with maxima at 685 nm (F685), 695 nm (F695) and 735 nm (F735). (For a detailed discussion of emission characteristics of chloroplasts and algae, see ref. 68.) The long wavelength F735 band is broad and more intense and consists of more than one component. From a comparison of emission and excitation spectra in various organisms, it has been postulated that F685 and F696 mainly originate from pigment system II and F735 originates mainly from PS I. In phycobilin containing algae, the phycobilins are mainly associated with system II and

preferentially excite 685 and 696 nm fluorescing species.

Krey and Govindjee (69, 70), working with Porphyridium attributed the increase in fluorescence intensity at 696 nm (at 77°K) or under high-illumination to be that of emission of excess of energy from the system II trap under conditions when photosynthesis is absent or saturated. These workers observed a difference maximum at 692-693 nm, at room temperature, obtained by subtracting the spectrum of normal emission from that of the emission spectrum of a DCMU poisoned sample. Further experimental support of the proposal that the F696 band corresponds to the pigment closely in "communion" with the system II trap has come from the following types of studies: (a) quenching of F696 by quinones, cytochromes, and other chemicals (71, 72), (b) the exponential build of the F696 band at 77°K, and (c) its sensitivity to Mn deficiency (73).

Recently, we have characterized the fluorescence properties of both pigment systems by analyzing the emission characteristics of system I-Chl a protein complex (obtained from a blue-green alga Phormidium luridum) and from purified system II particles from Zea mays (74). These studies indicate that F696 does not originate completely from PS II, although this system is enriched in the Chl form that fluoresces at 696 nm. Similarly, F735 band does not exclusively belong to system I, but F735 occurs in system I in larger abundance. These conclusions are in agreement with those obtained by Cho and Govindjee (75, 76) from their spectral analyses of intact algal cells. A description of various fluorescing species of Chl a in both pigment systems and energy transfer is diagrammed in Figure 2. We have not attempted to include all the information that is available in the literature. This diagram only illustrates the general compositions of two pigment systems of energy transfer from PS II to

Figure 2. A diagrammatic sketch of the possible distribution of pigment molecules between two pigment systems in green plants (modified after Govindjee *et al.*, 68). (See text for explanation.)


PIGMENT SYSTEM II
PIGMENT SYSTEM I
Chl b (or Phycobilins) some carotenoids
Chl a 670
Chl a 678
Chl a 690
F 680 (weak)
F 687
F 696 (at 77°K)
EXCITATION ENERGY TRANSFER
Some Chl b most carotenoids
Chl a 670
Chl a 678
C 700
F 720-740
ELECTRON TRANSFER
Z
Q
P680-690
P 700
X
NADP
H_2O
Light Reaction II
Light Reaction I

PS I as discussed earlier.

3. Luminescence, Oxygen Evolution and PS II Traps

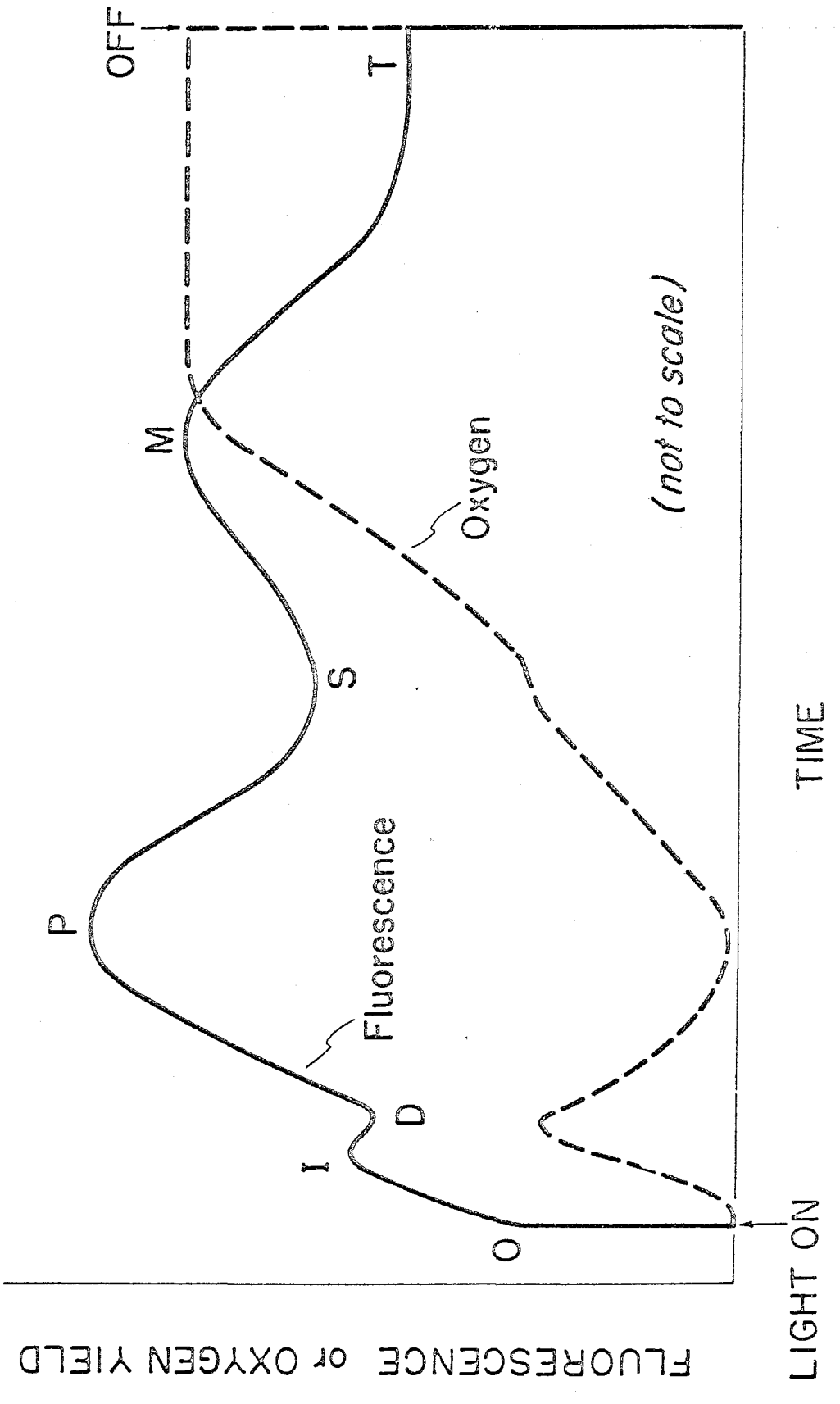
3.1 Kinetics of Oxygen Evolution and Fluorescence Changes in Algal Cells

The velocity-intensity dependence of photosynthetic reactions, the time course of oxygen evolution and fluorescence yield changes have been reviewed previously (77). Recently, Govindjee and Papageorgiou (1) have discussed quite extensively the induction of Chl *a* fluorescence and its relation to photosynthesis. Myers (78), in a recent review, has also discussed some aspects of the kinetic relation of oxygen evolution and fluorescence yield. In view of the availability of these reviews, we shall only enumerate the principal features of chlorophyll *a* transients and their relation to other photosynthetic processes.

In intact algal cells, two waves of Chl *a* fluorescence changes are observed when dark-adapted algal cells are exposed to continuous illumination. The first wave is a rapid transient lasting a few seconds (the fast fluorescence induction). The characteristic points in its time course are: O, a low initial (original) level of fluorescence yield; I, an intermediary maximum; D, a dip or minimum; P, a maximum (peak) much higher than I; and a subsequent decline from P to a broad minimum S (quasi steady state). The second wave or the slow fluorescence change (lasting up to minutes) begins with a rise from S to a maximum level M, and subsequently declines to a terminal steady state level T. The blue-green algae that have been studied here show a sluggish M to T decline, the M level being much higher than P.

Figure 3 diagrammatically sketches the time course of fluorescence yield fluctuations and oxygen evolution. The rate of oxygen evolution

Figure 3. A diagrammatic sketch of changes in fluorescence yield and O_2 evolution upon illumination as observed in intact green agal cells. The drawings are not to scale (after Govindjee and Papageorgiou, 1).


OFF
T
M
Oxygen
(not to scale)
S
P
Fluorescence
I
D
O
TIME
LIGHT ON
FLUORESCENCE or OXYGEN YIELD

follows a time course which is essentially antiparallel to the fast fluorescence induction with the exception of the very fast activation OI phase (79).

3.1.1 The fast Changes in Fluorescence Yield

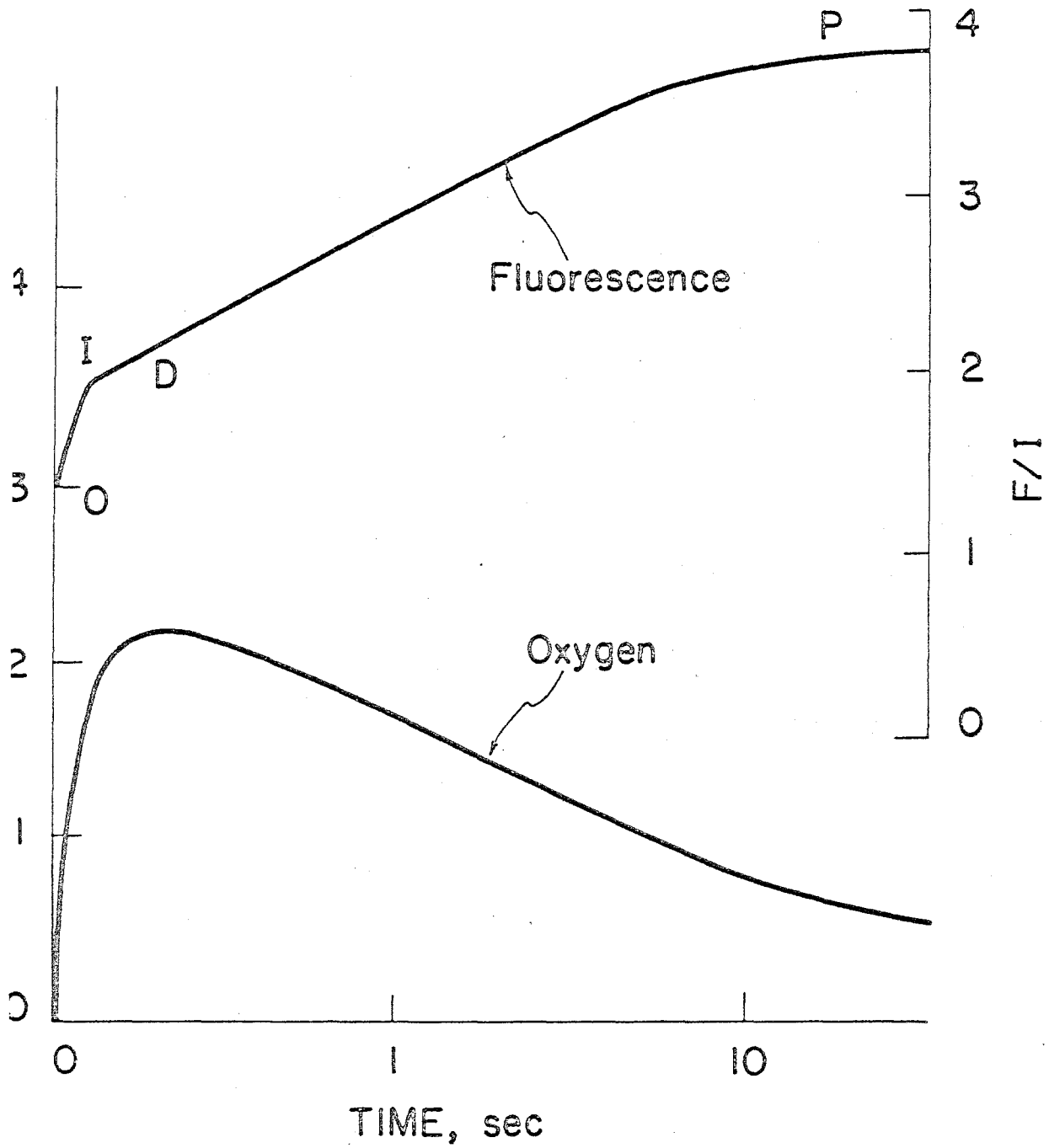
a. The OI Phase. During the activation phase, the fluorescence yield rises while the O_2 evolution remains essentially zero for a while and then increases slowly to a maximum. Thus, one observes a non-complementary rise of fluorescence and O_2 at the very beginning and then subsequent parallel rise of O_2 and fluorescence. This initial O_2 lag is pronounced in weak light and obeys the Ixt law of a constant quantum process (80), and is explained by the assumption that more than one oxidized equivalents are necessary to oxidize water while the reduction of Q requires only one quantum of light. This assumption receives support from the observation that a 200 μsec single saturating flash of light given after a long dark period will reduce all the Q (31) but a single saturating flash ($\simeq$100 μsec) will not produce any detectable oxygen (81). This explanation of the initial non-complementary relationship between fluorescence and O_2 transients is based on the assumption that the primary acceptor Q is homogenous and is reduced by one quantum of light and does not show oscillations as O_2 yield does. However, Delosme (82) recently reported oscillations in the fluorescence yield with the number of flashes and, as we have discussed earlier, there are suggestions for two quenchers (Q_1 and Q_2) or a quencher with two reduction states (Q^- and $Q^=$). It is not clear if these two forms of Q have the same quenching ability and, thus, it is difficult to predict a relationship with O_2 evolution at this time. We will only assume for our discussion that the primary acceptor Q is homogenous and requires one quantum of light for its reduction. Hence

the parallel rise of O_2 evolution observed during the major part of OI rise phase is governed by the photochemical reduction of Q and the dark reoxidation of Q^- by the A pool. The closure of traps would increase the fluorescence yield while their reopening of it would result in the O_2 evolution. The rate of O_2 evolution increases as more and more units accumulate four oxidizing equivalents but the fluorescence yield will also rise as the number of closed traps exceeds the number of traps reopened by interaction with the A pool.

According to this assumption, with very bright light, the OI rise will be maximum without any O_2 evolution and this has not been shown experimentally as of yet. But in the presence of DCMU the purely photochemical rise OI is achieved without any O_2 production. But again, even in the presence of DCMU this rise is non-logarithmic and further assumption of excitation transfer between the units of PS II is necessary to explain the OI rise curve. The ID decline has been analyzed by Munday and Govindjee (83). The dip (D) is present in aerobic cells but becomes prominent under anaerobic conditions. It appears that both systems I and II are equally effective in accelerating ID decay. Munday and Govindjee (83, 85) suggest that a fast reoxidation of Q^- by A causes the dip. (The photosynthetically produced oxygen may also quench the fluorescence and influence the acceleration of ID decay.)

b. <u>The DPS Phase</u>. The D to P rise of fluorescence is associated in time with a decline of O_2 evolution (see Figure 4). Correspondingly, the decline of fluorescence in the PS phase is accompanied by a rise in O_2 production. These kinetic patterns suggest that the sum of the Φ_p (quantum yield of photosynthesis) and Φ_f (quantum yield of fluorescence) is constant (assuming other rate processes do not alter). In spite of the close

Figure 4. The early time course of fluorescence and O_2 evolution in green alga Chlorella. Note the IP rise in fluorescence yield is accompanied by the decline in the rate of O_2 evolution. The area under the O_2 trace represents the so called 'oxygen gush' or spike. The initial rapid rise of fluorescence represents the activation reaction of Joliot. (Drawn after P. Joliot, (81).)


P
4
3
2
1
0
F/I
Fluorescence
I
D
O
Oxygen
0
1
2
3
4
0
1
10
TIME, sec

complementarity between ϕ_p and ϕ_f during this interval of illumination, there are experimental observations that do not agree with the suggestion that DPS transient can be explained on the basis of oxidation and reduction of Q.

The experiments of Munday and Govindjee (84) suggest that DP rise is due to a slow rate limiting step beyond X, the primary acceptor of PS I as the DP rise could be suppressed by eliminating this rate limiting step. Hence, at P the intermediate carriers of electron transport including X must remain reduced. However, recently Duysens (86) has reported a quenching at P by imposing a bright microsecond flash of system I light. This experiment is difficult to explain by the above hypothesis that accumulation of Q^-, A^- and X^- causes DP rise. It is possible that an intense system I pulse can cause a direct oxidation of Q^- without being oxidized by A pool. Thus the observation of Duysens cannot be taken to be incompatible with the suggestion that P results due to an accumulation of the reduced intermediates; but the problem certainly needs to be further investigated.

The kinetic pattern of the P to S decline with the simultaneous increase in O_2 evolution apparently indicates that P to S decline represents a reoxidation of reduced intermediates (Q^-, A^-). But early experiments of Duysens and Sweers (10) have shown that this is not the case. It was found that if the light was turned off at S and then immediately turned on again, the OIDPS transient can not be restored. But the fluorescence level remains low. The restoration of the OIDPS transient requires several minutes of darkness after light is turned off at S. These simple experiments suggest that PS decline is complex. Superimposed with oxidation of reduced intermediates during PS decline there

are other factors which also influence the fluorescence yield.

3.1.2 The Slow Changes in the Fluorescence Yield: The SMT Phase

Figure 3 illustrates the relationship between oxygen evolution and fluorescence yield changes during this interval of illumination. O_2 evolution increases in parallel with the S to M rise and continues to grow asymptotically to a steady state while the fluorescence yield declines after reaching a maximum M to a terminal T level. Papageorgiou and Govindjee (81, 82) have documented some of the important characteristics of SMT phase with photosynthesis. The lack of complementarity of oxygen evolution to the fluorescence change during this phase suggests that the fluorescence yield of Chl *a* is not determined by the redox state of the primary acceptor (Q) whose oxidized form is known to quench Chl *a* emission. Papageorgiou and Govindjee (87, 88) have shown that the slow changes in yield are sensitive to uncouplers but not to energy transfer inhibitors. Bannister and Rice (89) have shown that a mutant strain of *Chlamydomonas reinhardi*, deficient in a functional system I, is unable to show these characteristic SMT changes. These observations lend support to the suggestion that system I mediated reactions influence the Chl a_2 fluorescence change. In this thesis, we have attempted to further characterize, in some detail, the SMT fluorescence change and its relation to the fast OIDPS change in three representative classes of algae.

3.2 Fluorescence Transient and Oxygen Evolution in Isolated Chloroplasts

Isolated chloroplasts, in the absence of any added electron acceptor, show a biphasic OIDP type rise upon illumination (see review, 90). Unlike algal cells the fluorescence level does not undergo any significant further change in yield after attaining P (for detailed discussion see 90). These

chloroplasts, without added Hill oxidant, do not show prolonged O_2 evolution. Upon illumination, after a period of dark adaptation, one observes a gush of O_2 which declines to a low level (91). This decline of O_2 evolution is accompanied by a fluorescence rise to P and thereafter, fluorescence yield remains high and O_2 level remains low during the subsequent period of illumination.

Addition of a Hill oxidant like benzoquinone abolishes the O_2 gush but O_2 evolution increases exponentially and then asymptotically to a steady state level. Addition of substrate quantity of Hill oxidant lowers the fluorescence yield (91). In the presence of an excess of oxidant only the OI phase persists. Addition of DCMU causes a rapid but non-exponential rise of fluorescence to a maximum level (52, 53). In the absence of acceptor and in the presence of DCMU, the maximum fluorescence yield is about the same as if the exciting intensity is high.

In practice, the low constant level fluorescence is called F_o while P level is referred to as F_∞ or F_{max}. Usually the dip is not as prominent as one sees in algal cells. The difference in the yield between F_∞ and F_o is referred to as the variable fluorescence.

The fluorescence rise from F_o to F_{max} represents the gradual reduction of electron transport carriers. Malkin and Kok (92) and Malkin (93, 94) have analyzed this fluorescence rise from F_o to F_∞ in terms of quenchér Q and its immediate reaction partners which are denoted as A pool. From the rise curve of fluorescence, in the presence of DCMU, the concentration of Q was estimated to be 1/500 [Chl]. The concentration of A was estimated to be 1/30 to 1/50 [Chl], about 10 to 20 fold greater than that of Q (31, 90). As stated earlier, $Q^- + A \rightarrow Q + A^-$ is very rapid; electron transfer occurs in less than 1 millisecond if all Q is reduced and all A

is oxidized (31). The kinetic behavior of Q and A suggests that the above reaction has a low equilibrium constant. This suggests that both Q and A have a similar mid point redox potential. Of all the electron carriers participating in the electron transport between two photo acts, the plastoquinones probably represent the major fraction of the A pool. (Ogren et al. (95) have shown that about 10 percent of total plastoquinones present in the chloroplasts may be involved in the electron transport.)

3.3 Delayed Light Emission (DLE) and the System II Traps

Delayed light emission (DLE) from photosynthetic organisms has been a powerful tool to gain insight into the conversion of electronic excitation energy into chemical energy. This emission requires the reformation of excited Chl singlets from some metastable precursor species. The intensity of DLE is very weak and decays with a very complex kinetics (96). The emission spectrum of DLE matches the emission spectrum of prompt fluorescence. Lavorel (97) formulated a relationship between the intensity of DLE (L) and the yield of prompt fluorescence ϕ_f as follows: $L = \phi_f \cdot J$ where J represents the rate of transformation of excitation energy into the singlet level of Chl a.

The energy necessary to cause DLE has been discussed by many workers. Arnold and Azzi (98) invoked a mechanism in which the full span of the potential difference between ferredoxin (-550mv) and water (+810mv) was available to (metastable) electrons and holes in the Chl crystals to regenerate singlet species. Obviously, they do not recognize the Z-scheme as the possible mechanism of electron transport during photosynthesis.

Clayton (99) advocated that DLE originates by a dark recombination of an oxidized donor (Z^+) and the reduced acceptor (Q^-). Lavorel (97) pointed out that this recombination would yield an available potential energy of

about 1.0 volt and would not be sufficient to excite a Chl molecule to the singlet state, the energy of a red quantum (1.8V). Mayne (100) postulated that additional energy is made available from the high energy state of photophosphorylation which is in equilibrium with the photochemical products.

Recently Crofts, Wraight and Fleischman (101) proposed that the membrane potential component of the proton motive force (Δp) could contribute to the energy source of DLE, but the idea of recombination of Z^+ and reduced acceptors is retained. Wraight and Crofts (102) could establish conditions in which the intensity of DLE and electrochemical potential could approximate the relations: $L \propto \exp(\Delta p)$. Earlier Barber and Kraan (103) and Kraan et al. (104) observed somewhat similar relationships between chemical potential and salt stimulated DLE (also see 105, 106).

Another model, advanced by Lavorel (107) and Stacy et al. (108), assumes that singlet excitation is formed by the annihilation of two triplets. The basic postulates of the model are as follows: (a) two triplets recombine to give rise to a singlet, and (b) triplets are formed in two ways--by direct conversion of singlet to triplet at the trap and by the chemical back reaction between Z^+ and Q^-. The former contributes to the fast component of DLE while the latter contributes to the slow component. This slow component is influenced by acid-base shift, by salt, and temperature jumps. Stacy et al. (108) have shown that their experimental results fit the theoretical predictions of the triplet annihilation model. Furthermore, Mar (109) has discussed how this model could adequately explain the energetics of chemiluminescence and other types of luminescence induced by acids and salts or triggered by quick temperature jumps (TDLE). (For a recent discussion of TDLE, see Jursinic and

Govindjee, 110.)

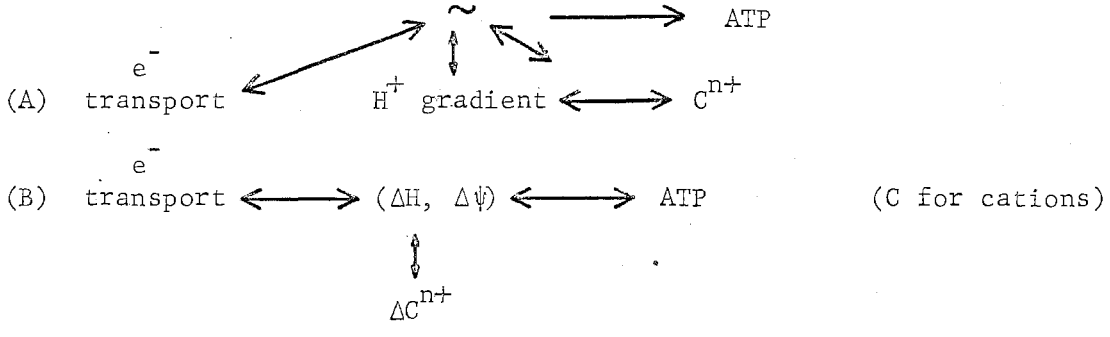
4. Energy Coupling and Related Processes During Photosynthesis

4.1 Electron Flow and Energy Coupling in Photosynthesis

Both in chloroplasts and in mitochondria electron flow is coupled to phosphorylation, that is to say that higher the rate of electron flow, greater the rate of ATP formation. If ATP synthesis stops due to some limiting condition, the electron flow stops because of a tight coupling. However, experimentally, conditions could be achieved where electron flow can continue to occur at an accelerated rate without net ATP synthesis; such a system is called an uncoupled system.

Although the phenomenology of coupling of electron transport to phosphoryation is well documented, the mechanism of coupling and energy conservation is still uncertain (see a recent review, 111). In this brief discussion we shall not elaborate on the details of the various hypotheses of energy coupling in chloroplasts and mitochondria. We shall limit ourselves to the simple enunciation of these hypotheses. This limited discussion, hopefully, may help to interpret our results more meaningfully.

Scheme A and Scheme B are the two contestant schemes for energy coupling:



Scheme A represents the modified chemical hypothesis where the as yet unidentified high energy intermediate, the squiggle (105), is involved in

ATP synthesis. (Phosphorylation of ADP during nonenzymatic oxidation of a ferroheme-imidazole complex has been described by Wang (113). A three-component Wang-reaction exemplifies a model for energy coupling through formation of energy-rich phosphorylated imidazole.)

As shown in the scheme A, the H^+ gradient may directly (or indirectly, i.e., via a cation gradient) be in equilibrium with the squiggle intermediate. The classical chemical hypothesis has been modified to imply that ΔH^+ (H^+ gradient) can make ATP via the 'squiggle' intermediate to accomodate the experimental finding that chloroplasts in dark can make ATP by changes from an acid to base (acid-base transition). The major weakness in this hypothesis is the failure to identify the "squiggle intermediate", although indirect evidence of its existence has been offered from time to time by its proponents (see reference, 113).

In 1961, Peter Mitchell (114) offered an ingenious alternate hypothesis (scheme B) known as the "Chemiosmotic hypothesis." Electron flow across a thermogradient, according to this hypothesis, does not make a chemically "squiggeled" intermediate bond, but rather an electrochemical state, a 'proton motive gradient force' across the vectorial membrane. This gradient is established due to the redox properties of the electron carriers embedded on the membrane (i.e., whether they accept an electron or a hydrogen atom upon reduction) and also due to the asymmetric localization of these carriers in the membrane matrix. If the thylakoid membranes of the chloroplasts are impermeable to ions or protons, then a gradient will be formed due to electron flow. Such a gradient, an electrochemical gradient consisting of both electrical and chemical components, constitutes a form of potential free energy and, by definition, can do useful work.

A reversible enzyme, "ATPase", located on the anisotropic membrane of the organelle harnesses the potential energy into the synthesis of ATP from ADP and Pi($H_2PO_4^-$), as follows:

$$ADP^{3-} + H_2PO_4^- \rightleftarrows ATP^{4-} + H_2O \text{ (pH 6, dehydration), and}$$

$$ADP^{3-} + HPO_4^{2-} + H^+ \rightleftarrows ATP^{4-} + H_2O \text{ (at pH 8, dehydroxylation).}$$

The existence of a membrane-bound closed space both in chloroplasts and mitochondria, of both electron accepting and hydrogen accepting redox carriers in the electron transport chain, of the vectorial ATPase on the membrane matrix, and of a link between proton translocation to cation "antiport" or anion "symport" (co-transport), lends support to Mitchell's chemiosmotic hypothesis. Moreover, the light induced pH changes in chloroplasts (108) were proven to be due to ongoing electron transport and the pH changes were related to the formation of ATP.

The post illumination ATP formation, discovered by Hind and Jagendorf (116), suggests that the ability to synthesize ATP in dark (XE) is accumulated in light. When XE and light-induced proton uptake were compared, they resembled each other (e.g., kinetics, stoichiometry), indicating that XE might represent a proton gradient. The relative abundance of XE was taken as evidence that it cannot be the "squiggle" intermediate. The idea that a pH gradient could form ATP was further tested in Jagendorf's laboratory by acid-base transition experiments mentioned earlier (110). All these observations strongly favor the chemiosmotic hypothesis but do not necessarily prove that a proton gradient is the primary energy conserving event.

There have been also many experimental observations which cannot be

explained by the chemiosmotic coupling mechanism in a straight forward way (see discussion in 118). These kinds of observations have prompted many to propose many new hypotheses of phosphorylation. One such model is designated as the fixed charge model or conformational model (118, 119). This hypothesis assumes that a proton gradient is not the source of energy for phosphorylation but may be intimately related to the energy conservation process. It further assumes that protonation of specific charges is necessary to bring about a change in the membrane conformation which activates coupling. The energy that is stored in ATP may be visualized to come from the mechano chemical change of the membrane, in a manner similar to the elastic stretching of a catapult. A hypothetical molecular mechanism has been recently proposed (Green and Young, 119). According to Dilley (118), one of the proponents of this hypothesis, the hypothesis of conformational coupling does not have the elegance of the chemiosmotic hypothesis or the simplicity of the chemical hypothesis. It resides on speculative mechanistic idea, but qualitatively it can explain many experimental observations that are difficult, if not impossible, to explain by other contesting hypotheses!

Whatever is the basic mechanism of phosphorylation, it is clear that ion flow across the chloroplast lamellae is intimately related to energy conservation. It is a difficult question as to whether the ion gradient, a chemical intermediate, or a mechanochemical elastic extension of the lamellae is the primary source of energy conservation. Ion flow and structural changes are intertwined with phosphorylation. We shall review below certain aspects of ion transport and associated structural alterations in chloroplasts.

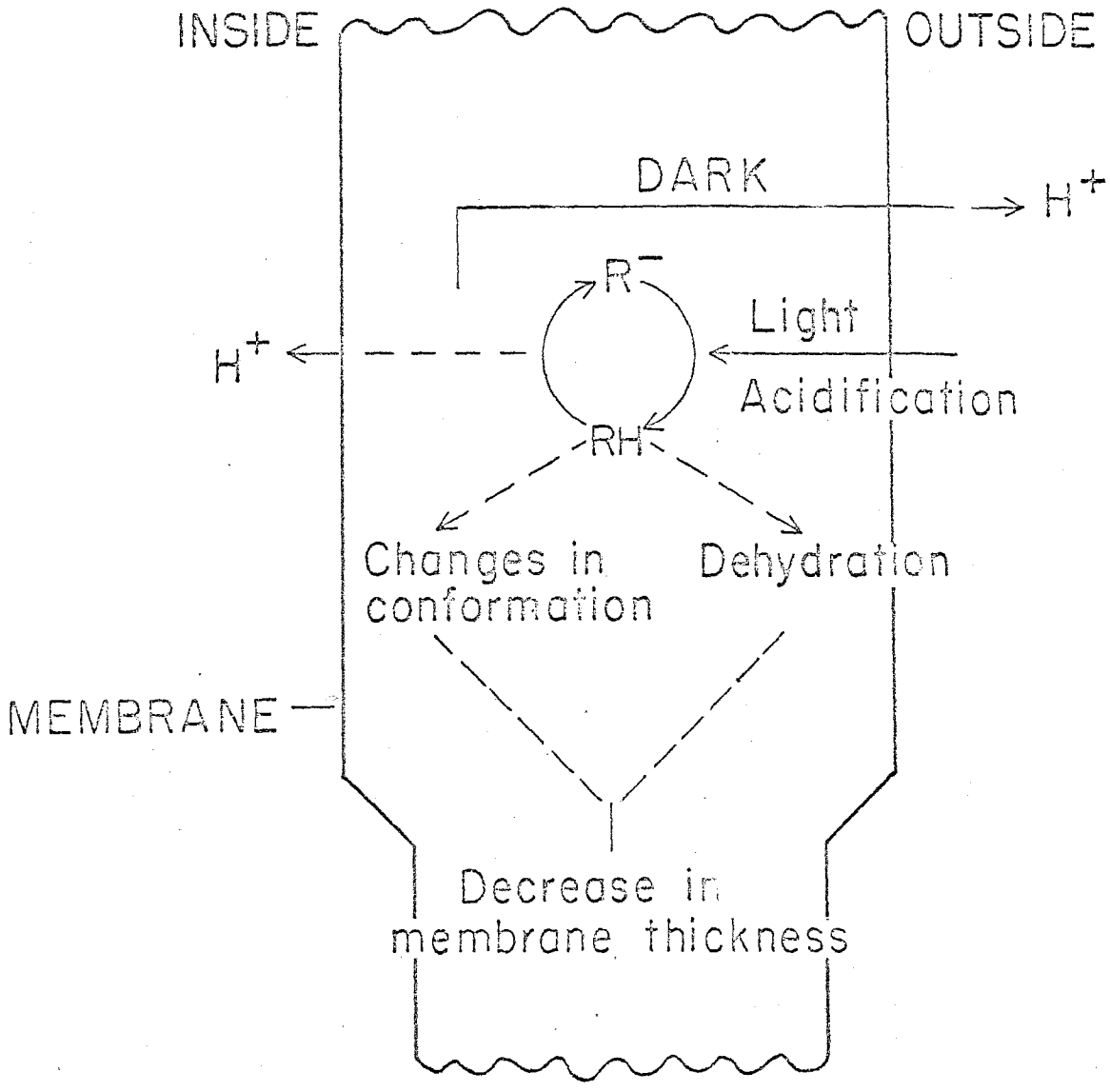
4.2 Structural States of Chloroplasts and Energy Coupling During Photosynthesis

It is now firmly established that light alters the structure of chloroplasts both *in vitro* and *in vivo*. Two kinds of gross alterations in chloroplast morphology, shrinking and swelling, occur upon illumination. The shrinking (photoshrinkage) seems to depend on a high energy intermediate or state, while swelling is mainly observed in a non-phosphorylating medium (see review, 120).

Flattening out of thylakoid membranes has been recognized as the ultrastructural basis of photo shrinkage. Murakami and Packer (121) have shown that a decrease in spacing between grana membranes and a decrease in membrane thickness cause the flattening, resulting in change of shape, ordering of the thylakoid membranes, and macroscopic volume change (121). Murakami and Packer (122) postulate that this change in membrane thickness represents the alteration in the macromolecular organization of the membrane (conformation) which causes a change in refractive index and hence the scattering changes. Claims have also been made that scattering change, the change in percent transmission, could be used to distinguish between alteration in thickness and spacing (120, 122). Figure 5 illustrates the mechanism of alteration of membrane structure as proposed by Packer and his associates.

Packer and his associates (113) recently reviewed the conditions under which light induces reversible changes in the chloroplasts' volume and organization. It has been postulated that light induced proton uptake plays a key role in governing the morphology of chloroplasts (120, 121). It is well established, both in chloroplasts and in mitochondra, that electron transport leads to proton transport. This proton uptake in

Figure 5. A diagrammatic sketch of the change in membrane conformation and configuration leading to change in the membrane thickness. Protonation of membrane charges due to light induced proton uptake has been recognized as the key event leading to structural changes of the thylakoid membrane. (Drawn after Murakami and Packer, 122.)



A MODEL FOR STRUCTURAL CHANGES

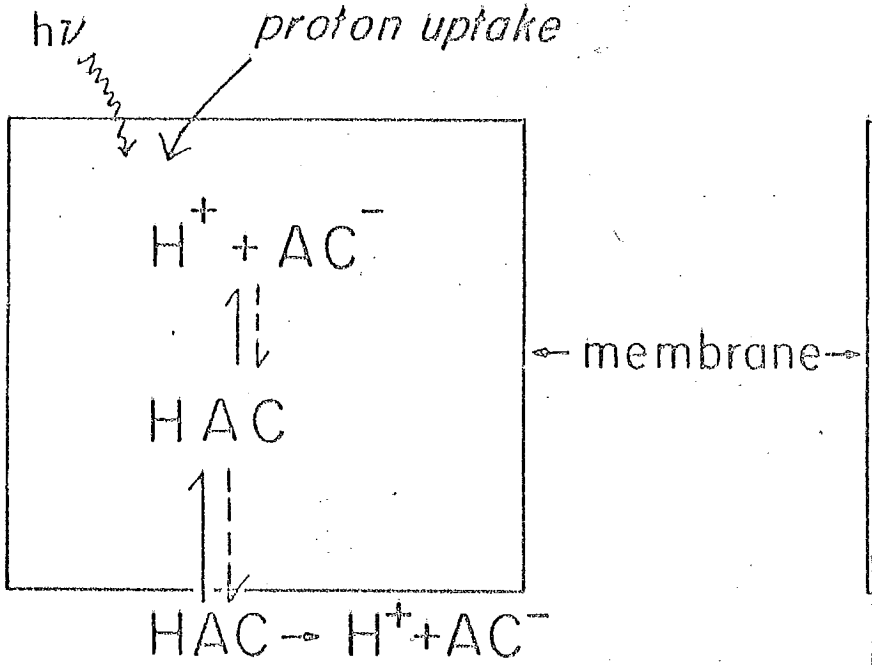
chloroplasts is associated with ion transport (cation efflux or anion influx). Obviously, massive fluxes of these ions will shrink or swell the chloroplasts.

Crofts et al. (123) proposed a hypothesis to explain the dramatic light induced shrinkage observed in isolated chloroplasts suspended in dissociable salts of weak acids. The mechanism of shrinkage is illustrated in Figure 6 (after Crofts et al., 123). Deamer and Packer (124) generalized this hypothesis to explain all structural changes induced by ionic environments. According to them, a shrinkage occurs if protons interact with ionic species to form uncharged species, otherwise a swelling will be observed due to co-transport of permeant anions to conserve the electrical neutrality. However, it must be mentioned here that a light induced swelling of the chloroplasts is not always observed in chloride media as predicted by this hypothesis. Dilley and Deamer (125) recently reinvestigated the light induced structural changes of chloroplasts suspended in chloride media and observed a shrinkage as measured by scattering changes. It is not sure what causes the swelling or shrinking in this type of ionic environment.

A second type of mechanism for the alteration of chloroplast structure has been proposed by several authors (see literature cited in ref. 118). According to this view, the chloroplast membrane is a flexible, polyanionic macromolecule with fixed charges on the surface. Membrane protonation is thought to remove loosely bound counterions and to change the membrane conformation. The idea that ions can affect the structure of chloroplast membranes and thereby regulate electron flow and phosphorylation has received some experimental support in recent years (126).

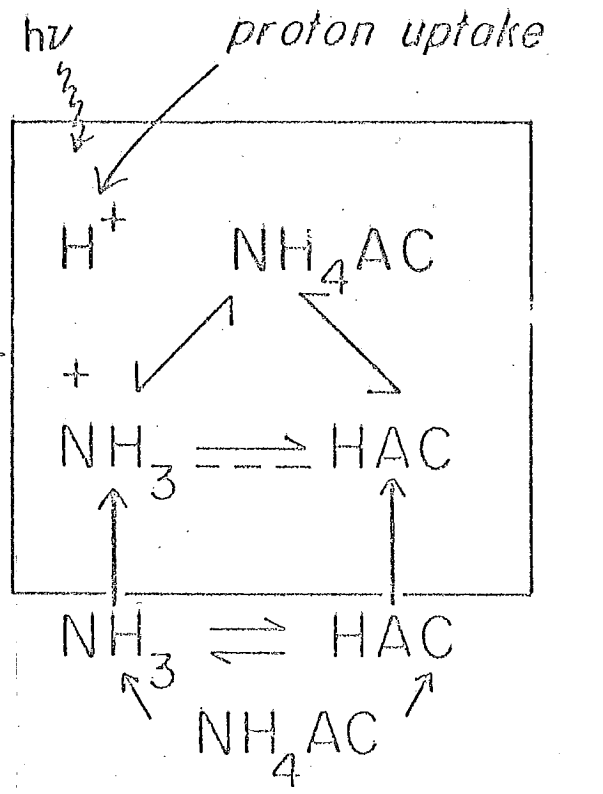
Nobel et al. (127) have pioneered a series of studies in chloroplasts

Figure 6. A model for the mechanism of light-induced shrinkage and swelling of chloroplasts in the presence of salts containing weak acid anions and weak base cations. According to the figure on the left, undissociated acids permeate freely into the membrane; the anions and the acids equilibrate between the inside and the outside. Proton uptake, during illumination of chloroplasts, decreases the internal pH, and causes an increase in the concentration of the undissociated acid. Undissociated acids come out of the chloroplasts resulting in a loss of H_2O and a decrease in volume. According to the figure on the right, uncharged molecules in the form of ammonia and acetate enter freely into the membrane. A decrease in internal pH, upon illumination, results in the formation of NH_4 acetate which rapidly dissociates into ammonium and acetate ions (not shown in the figure). This causes a decrease in the concentrations of ammonia and undissociated acetic acid in the inside space; this results in the movements of these species from the outside to the inside followed by H_2O uptake causing swelling. (After Crofts et al., 123.)



Shrinking in light in the presence of anions of weak acids

← membrane →



Swelling in the presence of ammonium salts of weak acids
(After Crofts *et al.*)

with intact outer envelopes, the so-called class I type of Spencer and Unt (128). These chloroplasts possess a high rate of CO_2 fixation and show photosynthetic control. Nobel et al. (129) have shown a light induced efflux of K^+ from these chloroplasts which is very similar to the H^+/K^+ exchange in illuminated class II chloroplasts. Whether a light induced proton uptake mediated by electron transport also occurs in intact cells is not yet clear. Claims have been made of light dependent proton uptake in intact cells of algae (130), but it has been difficult to separate the pH changes associated with the CO_2 uptake from light driven uptake of protons. Recent measurements of Atkin and Grahams (131) and also studies with mutants of Chlamydomonas by Neumann and Levin (132) seem to suggest that most of the light induced pH changes observed are due to a CO_2 pump. (However, one could think that it should be possible to separate the two effects in future research.)

Although it is not certain that H^+ ions move from the medium to the inside of intact cells, it has been established, in a series of recent investigations using microelectrodes that illumination of green intact plants and algal cells causes a change in electrical potential between the contents of the cell and external medium and these changes in electrical potential have been shown to be related to photosynthesis (133-135). Vredenberg (134) proposed that flow of H^+ from the cytoplasm to the chloroplasts is the cause of the change in the membrane potential of the chloroplasts. It is known that location of the tip of microelectrode would cause different types of changes in the membrane potential; and, because of uncertainty in resolving accurate position of the microelectrode, it is difficult to identify the observed signal with a particular membrane (135). Nevertheless, there are suggestions from the study of uncouplers

and other inhibitors that these changes in potential may be related to photophosphorylation (134). However, the elucidation of the relationship of photophosphorylation and photoinduced changes of electrical potential on the cell membrane and membranes of chloroplast requires further studies.

Although the molecular hierarchy leading to structural changes may have been somewhat worked out, as discussed earlier (120), no definite knowledge has been gained as to how it relates to energy transduction. However, the following studies reveal some of the interdependence of photophosphorylation and light induced structural changes in a general way: (a) similar kinetics of acidification of chloroplasts' interior, of the formation of post illumination XE, and of light scattering increments; (b) conditions unfavorable to XE formation suppress the scattering change; (c) optimal rate of ATP formation lowers the scattering change and vice versa, and (d) uncouplers of photophosphorylation abolish the scattering increments and ATP synthesis (see references cited in 118 and 120).

The previous discussion indicates that structural alteration of the membrane is always accompanied by energy conservation. It may or may not be a primary event in the process of energy conservation, but it is certainly associated with ATP metabolism. In this thesis, we shall relate the possible role of the structural alterations on the yield and emission of chlorophyll a fluorescence.

As sketched in schemes A and B of phosphorylation, ion transport is intertwined with phosphorylation. Various studies on the active transport of ions in intact algal cells have been made (see review, 136). These studies, in which inhibitors of various partial reactions of photosynthesis were used, were designed to learn more about the source of energy

for active transport. From such studies with intact cells of Nitella and Hydrodictyon, it is generally regarded that mostly cyclic ATP (not high energy intermediate) facilitates cation movements (K^+ uptake) in light, while chloride uptake is independent of photophosphorylation and is sensitive to DCMU. Barber (137) has observed that in Chlorella DCMU inhibits only 40% of the chloride uptake in light, and that chloride uptake is more sensitive to antimycin A than potassium uptake. Hence, although ATP is ruled out as energy source for chloride transport in Nitella, this may not be true for Chlorella (137). These types of studies prompted us to use various inhibitors to gain insight into the probable source of energy associated with structural alterations of the pigment that, in turn, affect the yield and emission of Chl a fluorescence in vivo.

5. The Thesis Problem

It has been shown that slow changes in Chl a fluorescence yield do not reflect the oxidation reduction activity of photosynthesis; they seem to be a manifestation of the interplay of a number of physiological events that are associated with photosynthesis (Papageorgiou, 59). It is difficult, at this time, to ascertain a direct cause and effect relationship between any cellular event and the slow changes in Chl fluorescence yield. Also, not fully understood is the source of energy that supports the slow fluorescence change, although a "high energy state" seems to be involved.

As stated earlier, a number of hypotheses have been proposed to account for the slow changes in fluorescence yield. Unfortunately, none of these predict straight forward experimentation to test them. They are only an illustrative description of the observations. However, most of the hypotheses imply some sort of conformational alteration of chloroplast

membranes to describe the phenomenon. Although isolated chloroplasts are knwon to undergo macroscopic structural changes, no direct correlation between these changes with the changes in fluorescence yield has been established. On the other hand, an apparent lack of the slow changes in Chl fluorescence yield has been argued to be a weakness of conformational alteration hypothesis.

Therefore, I felt that it was necessary to characterize the phenomenon of slow changes in fluorescence yield in greater detail to be able to obtain an insight into the phenomenon. To achieve this end, I attempted to study new test systems, to define conditions that modify the rate and extent of these slow changes, and to perturb these changes by the use of a wide range of known inhibitors and cofactors of partial photoreactions. We recognize the limitation of the use of inhibitors, tested in isolated systems for intact cells, but we realize that such kinds of studies have paid good dividends in elucidating many other processes (e.g., ion-transport; see references in 130). Efforts have been made to evaluate the contribution of cyclic and non-cyclic photophosphorylation as the source of energy for structural changes, and also to assess the role of such changes in slow changes in Chl fluorescence yield. We have also attempted to analyze the fast changes in fluorescence yield (OIDPS) in association with slow changes (SMT) and to evaluate if the fast and slow changes in the fluorescence are interrelated.

It has been argued that the absence of slow changes in the fluorescence yield (PS decline) in isolated class II type chloroplasts is a weakness of the hypothesis that structural modification of the thylakoid membrane controls the Chl fluorescence yield. We believe correlative studies on fluorescence yield and structure have not been made to

substantiate the above argument. Therefore, I tried to imitate conditions which are known to cause gross structural modifications of thylakoid membranes and to measure fluorescence characteristics in such systems. I did not make simultaneous measurements of scattering changes and fluorescence yield changes; but I believe that the conditions inducing structural changes have been rigorously defined and could be used safely for meaningful interpretations.

In summary, the object of this work was to gain more knowledge of the factors that regulate the Chl *a* fluorescence during photosynthesis. I cherish the hope that information gained from the present studies will provide a better understanding of the role of the structural "states" of the thylakoid membrane in influencing the yield of Chl *a* fluorescence and introduce some certainty to the concept that Chl *a* behaves as an intrinsic probe of membrane conformation. (We shall also discuss our results and those observed by other investigators in terms of existing hypotheses of slow changes in chlorophyll fluorescence.)

For the sake of convenience, we shall present our results on different organisms (*Porphyridium*, *Chlorella* and *Anacystis*) in different chapters. This will facilitate a full description of the parameters studied on a particular organism. At the end, we shall, however, compare and correlate the results obtained with different test systems and attempt to discuss them on the basis of a general hypothesis.

II. MATERIALS AND METHODS

1. Organisms Used

Both unicellular algae and isolated chloroplasts from higher plants have been used in this study. The three algae used were: (1) the green alga Chlorella pyrenoidosa, (2) the red alga Porphyridium cruentum and (3) the blue-green alga Anacystis nidulans. Occasionally, we have used other algae, namely, Cyanidium caldarium, Plectonema boryanum, and Microcystis aeruginosa.

All the algae were grown autotrophically according to Govindjee and Rabinowitch (138) in continuous light ($\sim$ 90 foot candles). A continuous flow of 5% CO_2 mixed with air was bubbled through cultures of Chlorella and Porphyridium, while only air was bubbled through the Anacystis culture. The temperature was held at $18\pm1^{\circ}C$ for Porphyridium, $20\pm1^{\circ}C$ for Chlorella and Anacystis was grown at room temperature ($23^{\circ}C$). For some experiments, Anacystis was grown for a day at $35^{\circ}C$ (and illuminated with red light ($\sim$ 60 foot candles)).

For experimentation, 3-4 day old cells of Chlorella and 5-7 day old Porphyridium and Anacystis were used. Cells were centrifuged at 500 x g for 5 to 10 minutes and resuspended with the aid of a Vortex mixer, washed and then resuspended in a buffer or in the growth medium. Usually a carbonate-bicarbonate buffer (Warburg buffer #9, 0.1 M, pH 9.2) was used for Chlorella and Anacystis, while buffer #8 with 15.2 gm of NaCl per liter of buffer (pH 8.5) was used for Porphyridium. Other buffers, when used, are mentioned in the text.

2. Preparation of Chloroplasts*

Chloroplasts of oat, maize or spinach leaves were used. Maize and oats were grown in vermiculite in continuous light. Hoagland's nutrient solution, half strength, was sprinkled twice in a week. Spinach, when used, was obtained from the local market. Chloroplasts were isolated according to one of the established procedures. In general, the leaves were washed and then homogenized for 30 seconds at high speed in a blender with phosphate buffer 0.05 M, pH 7.8, sucrose 0.4 M, and .01 M sodium chloride. The homogenate was filtered through 8 layers of cheesecloth, centrifuged for 2 minutes at 200 x g and then the resulting supernatant was centrifuged at 1500 x g for 10 minutes. The chloroplasts were finally suspended in a small volume of reaction mixture by using a rubber "policeman."

This preparation contains mostly broken grana (class II) chloroplasts. In some cases, the chloroplasts were quickly frozen with dry ice and acetone and kept in the freezer for use. Other variations in the protocol of preparation of chloroplasts, when made, are mentioned in the text.

For some experiments we have used whole intact class I chloroplasts. These chloroplasts were made essentially according to Nobel et al. (127).

3. Absorption Measurements

Absorbances were measured with a Bausch and Lomb, Inc. (Rochester, New York) recording spectrophotometer (Spectronic 505) equipped with an integrating sphere to reduce errors due to light scattering. The half-maximum width of measuring light was 5 nm. Absorbances were not corrected for "sieve" and "detour" effects. Despite the integrating sphere, all

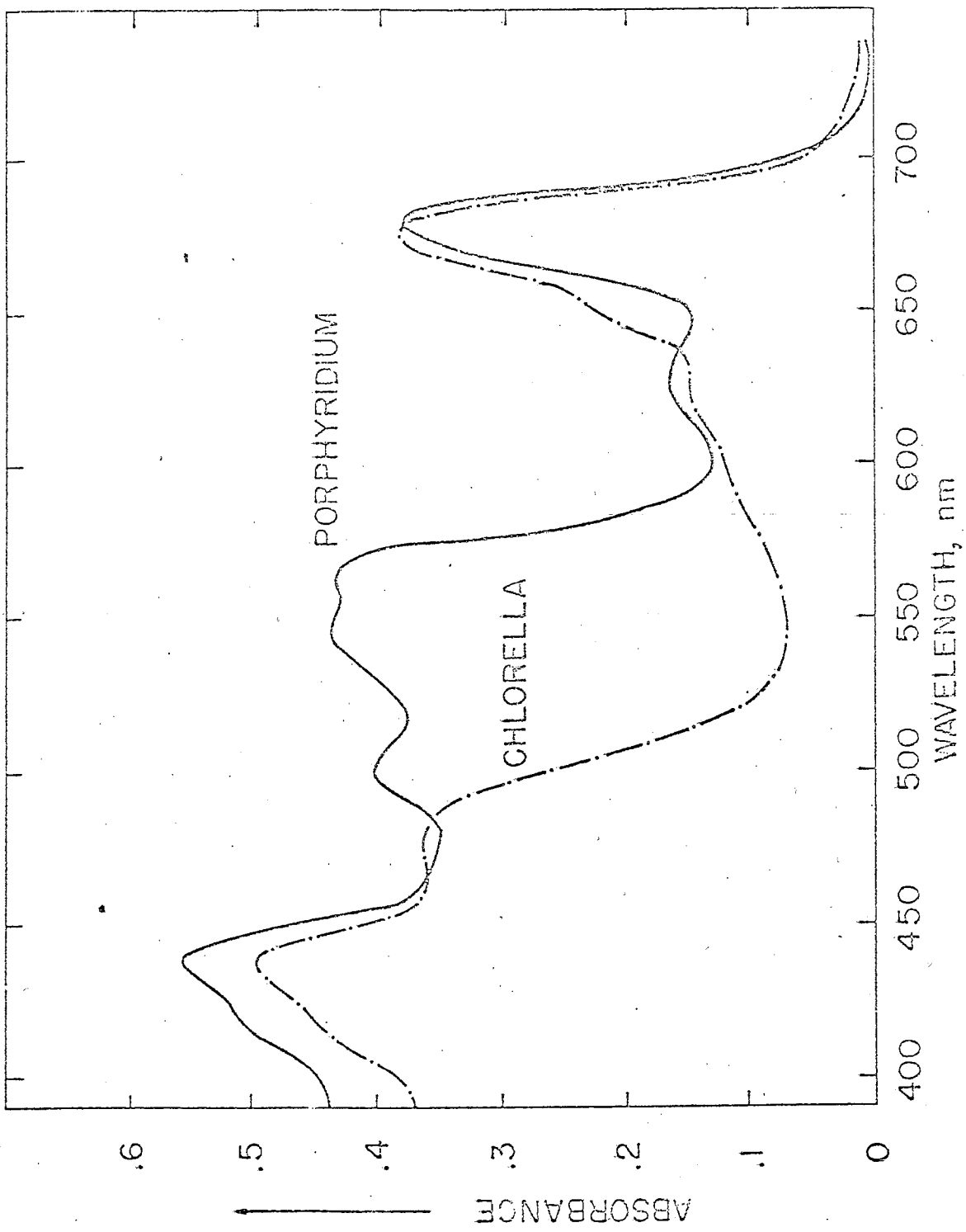
*Chloroplasts refer to chloroplasts from higher plants and not to algal plastids.

suspensions show some absorption beyond 740 nm. Since Chl *a* does not absorb beyond 740 nm, the base line is moved upwards by an amount equal to that of the apparent absorption at 740 nm. Figure 7 shows the absorption spectra of suspensions of *Chlorella* and *Porphyridium*.

For the determination of the chlorophyll concentration in algal cells, the cells were centrifuged and then extracted either with 100% methanol or 80% acetone in water. Chlorophyll could be easily extracted with 80% acetone from the cells of *Anacystis* and *Porphyridium* but not so well from *Chlorella*, and thus methanol was used for *Chlorella*. Concentration of chlorophyll was determined by using an extinetion coefficient (E^{1cm}_{663}) of 82 cm^2/millimole in the case of *Porphyridium* and *Anacystis*. In the case of the methanol extract from *Chlorella*, the following equation was used: Chl (a + b) μg/ml = 25.5 A650 + 4.0 A665, where A represents the absorbance at the wavelength indicated. In the case of isolated chloroplasts, the chlorophyll concentration was determined according to Bruinsma (139), using the equation: C (a + b) μg/ml = 27.8 A652 (in 80% acetone), where A652 refers to the absorbance at 652 nm. Absorption spectra of solutions were measured in the Cary Model 14 Spectrophotometer.

It must be mentioned here that we experimentally observed a linear relationship between the absorbance of the cell suspension at the red maximum and the amount of chlorophyll per ml of cell suspension. This linear relation could be fairly well established for absorbance of suspensions as high as 0.5 to 0.6 (an absorbance of 0.1 for 1 cm path equals ~15 μg Chl ml^{-1} of cell suspension).

Figure 7. Absorption spectra of the green alga Chlorella pyrenoidosa and the red alga Porphyridium cruentum (absorbance ($\log_{10} I_o/I$) versus wavelength); path-length, 1 cm.


PORPHYRIDIUM
CHLORELLA
ABSORBANCE
WAVELENGTH, nm
0
.1
.2
.3
.4
.5
.6
400
450
500
550
600
650
700

4. Fluorescence Measurements

Fluorescence measurements were carried out with a spectrofluoremeter designed and built by Govindjee and Spencer (140). Figure 8 shows the various parts of the apparatus. The procedure for measuring the fluorescence transient was essentially the same as that of Munday (58) and Papageorgiou (59). For measurements of emission and excitation spectra, the procedure of Cho (141) was followed. The spectra were corrected for the spectral variation of the apparatus. The spectral response of the instrument was determined according to Parker and Rees (142). Figure 9 shows the spectral response curve of the exciting monochromator and the light source (A) and of the analyzing monochromator and the photomultiplier (EMI 9558B) (B).

Light intensity was measured with a Bi/Ag Eppley Thermopile (#6161) and a 150A Keithley microvolt ammeter. The factor, 1 V = 172.4 ergs $cm^{-2} sec^{-1}$, has been used to convert the voltage registered into the intensity of light. We have also used, in the later part of our work, a Yellow Spring radiometer Model 63 for intensity measurements. We observed that the Yellow Spring radiometer read approximately 20% higher than the calibrated Eppley thermopile. We believe that this uncertainty arises due to the difference in focusing the light on the window of the thermopile and the radiometer. As we have not measured the absolute yields, this difference does not affect our results. The excitation intensity was varied by the use of calibrated neutral density Balzers filters. These neutral density filters were calibrated by measuring the transmittance in a spectrophotometer or by the use of a thermopile. Both sets of measurements agreed fairly well.

Figure 8. The block diagram of spectrofluorometer. The oscilloscope picture on the bottom left of the diagram show the phototube signal as the shutter is opened; abscissa 1 msec/division (from ref. 85).

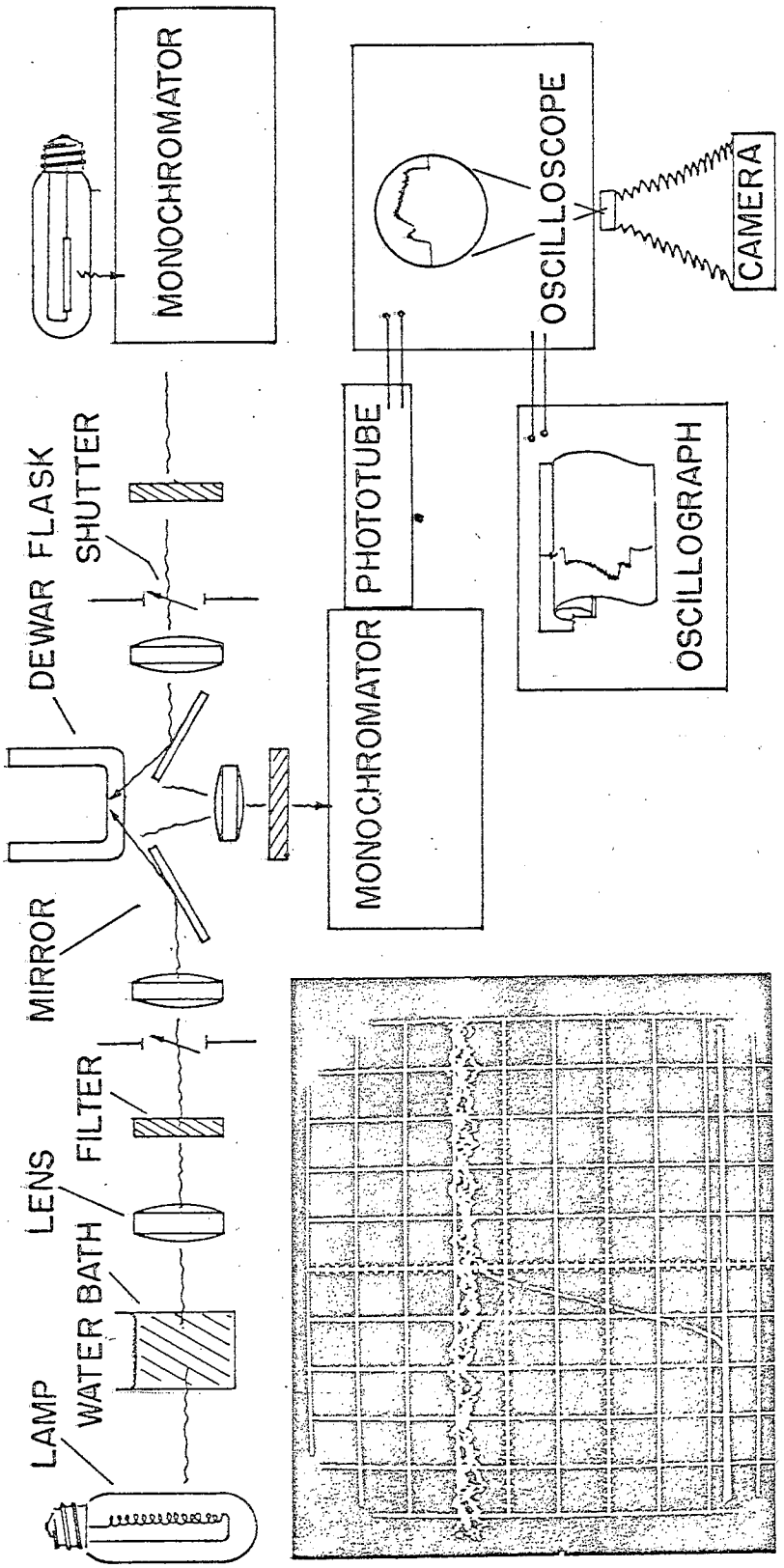
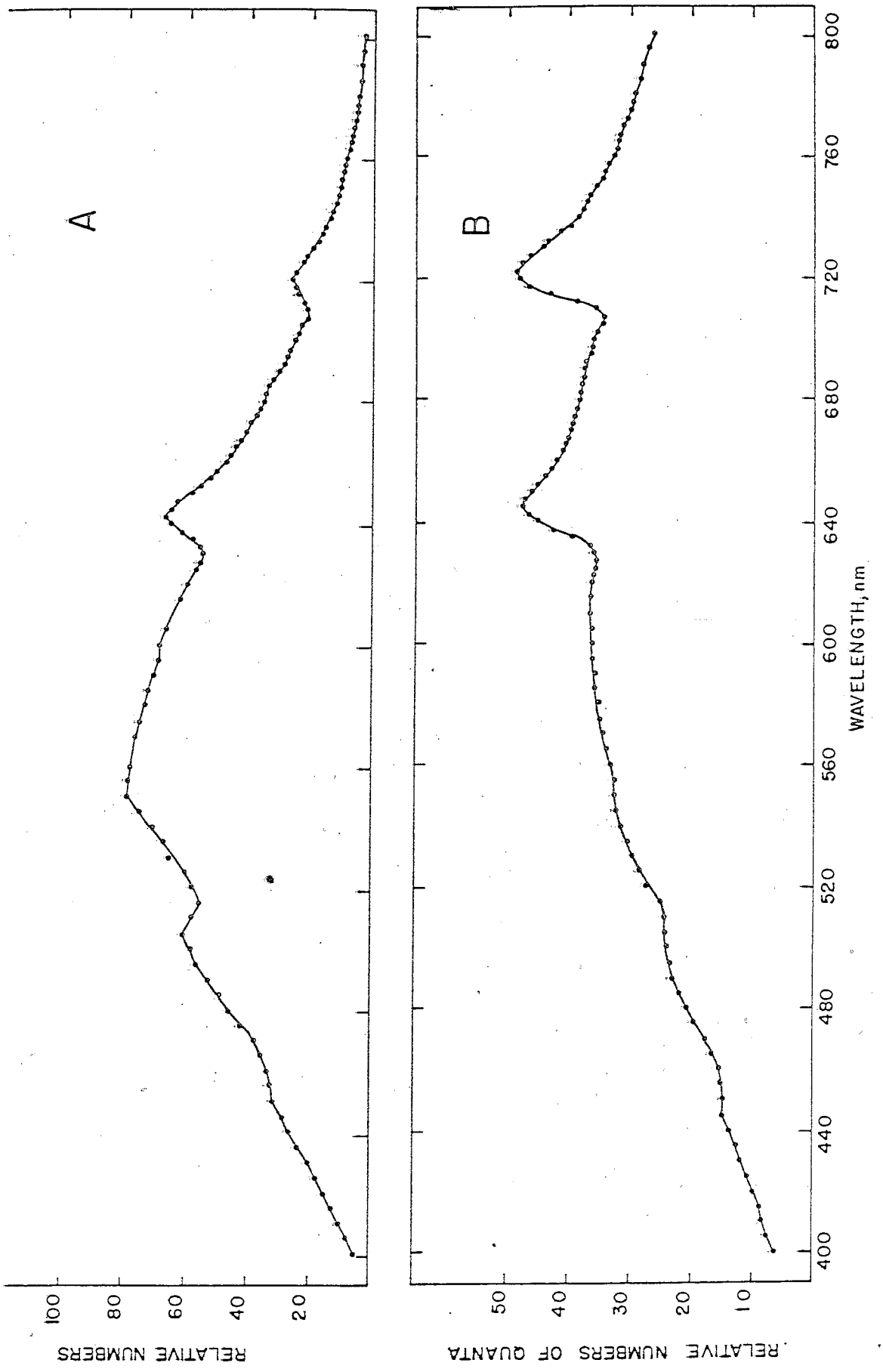

LAMP
WATER BATH
LENS
FILTER
MIRROR
DEWAR FLASK
SHUTTER
MONOCHROMATOR
MONOCHROMATOR
PHOTOTUBE
OSCILLOSCOPE
CAMERA
OSCILLOGRAPH

Figure 9. The spectral response of the spectrofluorometer. Top (A): the emission characteristics of the excitation monochromator. The light source is a 6 volt 18 amp. tungsten ribbon filament lamp operated at 21 amps. The slit width of the monochromotor (blazed at 300 nm) was 2 mm (half band width 6.6nm). The light intensity was measured by a Bi/Ag Eppley thermopile and was recorded by Keithley microvoltmeter and Brown recorder. The ordinate is presented as relative number of quanta $cm^{-2}sec^{-1}$ and abscissa as wavelength in nanometers. Bottom (B) spectral response of the analyzing monochromator (blazed at 750 nm) and photomultiplier (EMI 9558B). Details for this calibration procedure as outlined in reference (142). This calibration factor was mostly used for the correction of emission spectra.


A
B
RELATIVE NUMBERS
RELATIVE NUMBERS OF QUANTA
WAVELENGTH, nm
100
80
60
40
20
50
40
30
20
10
400
440
480
520
560
600
640
680
720
760
800

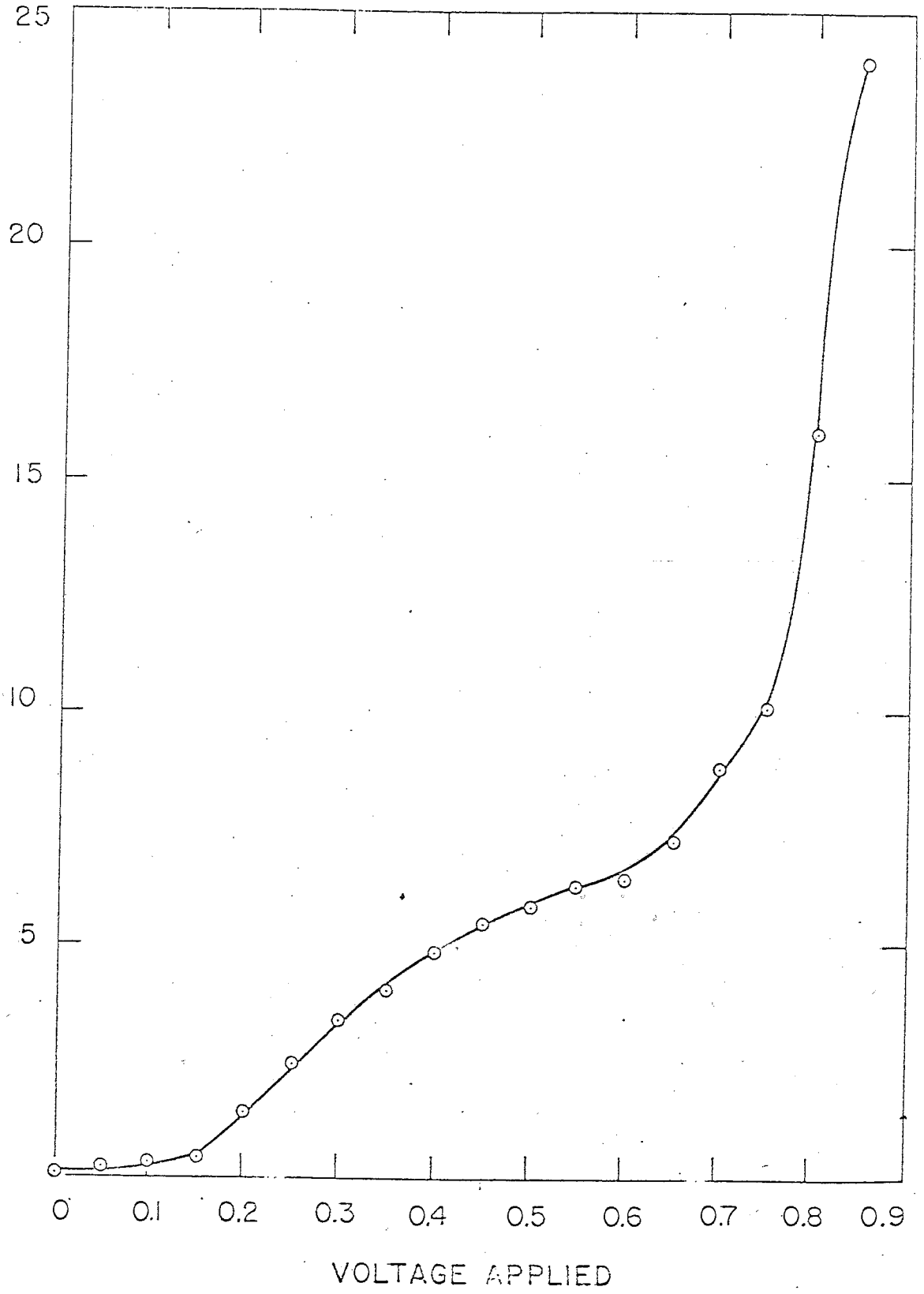
5. Measurements of Oxygen Exchange

Oxygen exchange measurements were made by the use of a stationary platinum rate electrode. This polarograph is essentially similar to that of Bannister and Vrooman (143). To mount the cell sample, a drop of algal suspension was placed on the platinum electrode and a piece of dialysis tubing was stretched tightly across the upper surface of the electrode by a gasket. In our system, 4.8 mm^3 (12 x 2 x 0.2) cells remain confined to the electrode chamber. The electrolyte solution consisted usually of a 0.05M phosphate buffer (pH 7.0) with 0.1M KCl. The platinum electrode was polarized -0.55 to -0.60 volt with reference to the Ag/AgCl (freshly coated) reference electrode. A current-voltage curve was determined according to Vrooman (144). Figure 10 shows the I-V curve and the limited plateau region between -0.5 to 0.65 volt measured without the algal cells. The voltage corresponding to the plateau region was used for O_2 exchange measurements.

The algal sample was illuminated from the top by a tungsten ribbon filament lamp regulated by a N.J.E. model 18-30 D.C. voltage regulator. Two percent CO_2 in argon or air was bubbled uniformly. Although it is possible to convert the amount of current registered into equivalents of O_2 reduced at the platinum electrode, we have presented our results in terms of relative units.

For monitoring oxygen concentration, a teflon covered Clark type oxygen electrode (Yellow Springs Instrument) with appropriate polarizing circuitry was used. The signal from the biological monitor (Model 53) was either amplified by a microvoltmeter or fed directly to an Esterline-Angus Recorder. The electrode was separated from the magnetically stirred medium which was regulated at $18 \pm 1^{\circ}C$ by circulating water. The sample

Figure 10. The current-voltage response curve (I-V curve) of the oxygen polarograph. Details for the measurement of I-V curve was essentially as outlined in reference 144.


25
20
15
10
5
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
VOLTAGE APPLIED

was illuminated by an Unitron Lamp (Frank E. Fryer Co., Illinois) with appropriate heat and Corning or Schott glass filters. The instrument was calibrated with air saturated water; at 23°C, O_2 dissolved in water was taken to be 0.25 μmoles/ml. (Occasionally we have used other electrode assemblies available in the laboratories of Dr. Boyer and Dr. Arntzen.)

6. Measurements of Delayed Light Emission (DLE)

The protocol of measurement of DLE is the same as that of Jursinic and Govindjee (110). (For details of the instrument, see ref. 110.) Two ml of the sample were illuminated for 10-15 seconds from a lamp with a regulated power supply operated at 15 amps. A blue Corning C.S 4-96 filter together with a water filter was used to filter the exciting light. After illumination, the excitation source was turned off and the photomultiplier was opened by pressing a mechanical shutter. The signal was fed to a Tektronix oscilloscope and then recorded on a Esterline-Angus recorder.

7. Other Measurements

Mr. G. Bedell helped me in measuring the amounts of ATP by an apparatus designed and built by him. The details of the procedure are outlined in his thesis (145). Briefly, the method consists of total extraction of ATP from cells by boiling them in water and measuring ATP with a luciferin-luciferase assay (146).

Hill reaction was measured spectrophotometrically using DCPIP as an oxidant. The cuvette was illuminated with saturating white light filtered through a water bath. Methyl viologen (Mev) reduction was measured polarographically by following the uptake of O_2 by reduced Mev (147). For the details of the above measurements, see thesis by M. D. L. Bakri (148).

8. Chemicals and Treatments

Most of the chemicals used in this investigation were obtained from commercial companies; some chemicals were generous gifts (FCCP, DCMU. S-6 and S-13). They were dissolved either in ethanol or in water. A very small volume of concentrated stock was added by the use of a microliter syringe (Hamilton Company) in the dark. This insignificant change in volume did not affect our measurements. When large additions were made, corrections were made for the change in volume. Usually 5-10 minutes were allowed for equilibration and penetration of the chemicals before measurements were made. The alcohol concentration, when used, did not exceed more than 2-3%. We found that at this concentration alcohol alone does not affect the measurements. Other specific treatments are described in the text.

9. Presentation of Fluorescence Data

Fluorescence yield changes were represented as relative changes in the yield. We only collected a very small fraction of the total emitted fluorescence, and thus the values do not represent the absolute yields. The alteration of fluorescence amplitudes by chemicals are presented as relative changes in the yield or emission intensity. These two terms are used almost synonymously in this thesis. In some experiments the yield changes were presented as a percent change or fractional change with respect to a control condition. All time elements were measured in terms of the speed of the recorder. No special care was taken to independently calibrate the recorder speeds.

9.1 Experimental Protocol, Standardization and Sources of Error

Munday (58) has discussed in detail the methods for standardization and the sources of error that are intrinsic in fluorescence measurements.

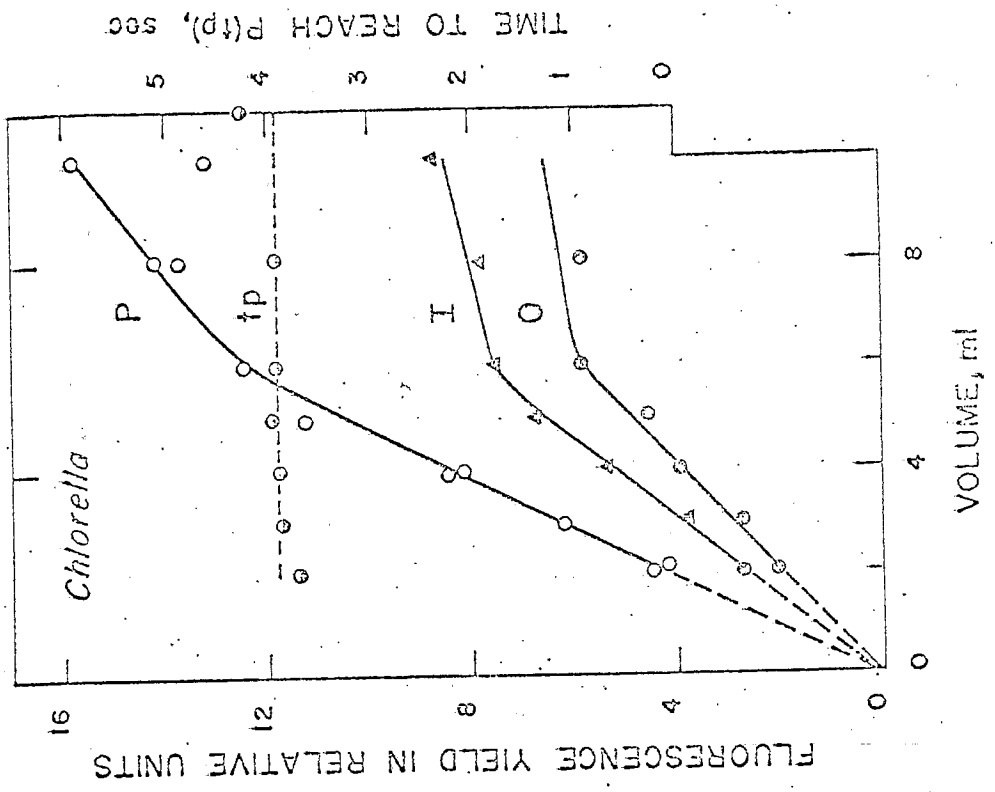
We have followed his suggestions to minimize errors. Our greatest source of variation was the differences in culture conditions. In spite of constant vigilance, fluctuation in temperature or in the rate of the bubbling of the gas or in the state of the inoculum used cause variations in the shape of the transient and we believe this does not represent the true characteristics of the organisms. No attempts were made to explore the cause and nature of unusual variations in the transient that we observed infrequently.

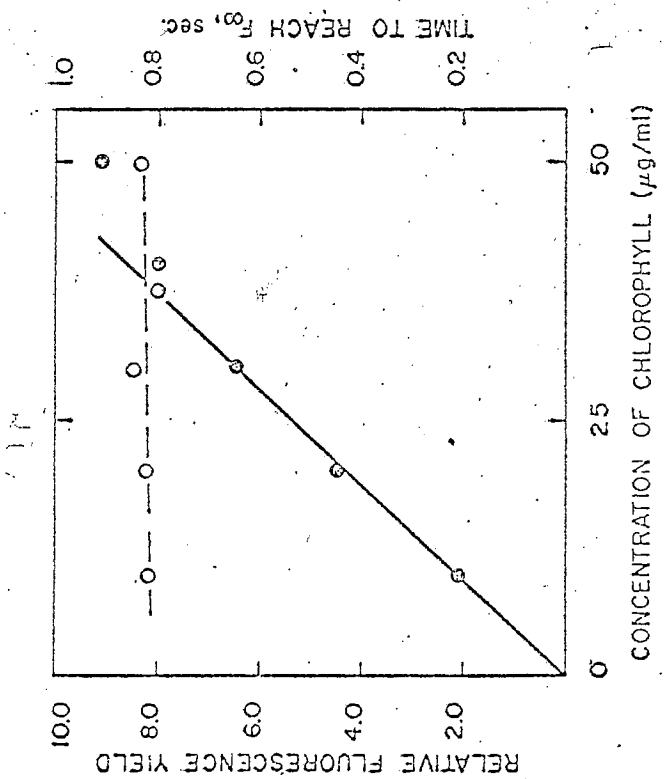
To adapt the cells to darkness, we usually keep the flask containing cells wrapped in aluminum foil in the dark for about an hour or so. Although the front surface optics used in fluorescence measurements minimizes reabsorption, it is of no great concern for the measurements of transient. However, we measured the effect of varying concentrations of chlorophyll and volume of cells on the yield of fluorescence (Figure 11). When a low volume of cells or chloroplasts were used, settling of the cells did not cause problems; however, large variations were observed with a large volume of cells due to constant settling of the cells. We therefore, routinely used 2 to 3 ml of cells in our measurements with 20 to 40 μg Chl/ml.

For most of the experiments, we had to frequently change the samples. This introduced some error due to change in geometry. We have on many occasions attempted, by repeated measurements, to estimate the extent of the variation due to variations in geometry while replacing the Dewar flask containing the sample. We estimate a maximum of about 4-6% error. We have always attempted to minimize this error by carefully removing and replacing the Dewar flask.

For the emission spectra at room temperature, we estimated the extent

Figure 11. The amplitude of fluorescence signal versus the concentration of chlorophyll and volume of the sample. Left: The fluorescence amplitude at F_∞ versus the concentration of chlorophyll in spinach chloroplasts. (Note a linear response up to as high as 40μg Chl/ml.) The top line with open circles shows the time to reach F_∞; it is independent of chlorophyll concentration. Fluorescence was measured at 685 nm (half band width, 6.6 nm); excitation light, blue (C.S. 4-96 and C.S. 3-73) with an intensity of 16 Kergs cm^{-2} sec^{-1}. Chloroplasts were suspended in Tris-Cl (0.50 mM, pH 7.8). Right: A similar experiment with different volumes of Chlorella cells suspended in Warburg buffer #9. (Note that the time to reach P (tp) (half solid circles) remains fairly invariable as the amount of cells are increased.)


FLUORESCENCE YIELD IN RELATIVE UNITS
16
12
8
4
0
Chlorella
P
tp
H
O
0
4
8
VOLUME, ml
TIME TO REACH P(tp), sec
0
1
2
3
4
5


RELATIVE FLUORESCENCE YIELD
10.0
8.0
6.0
4.0
2.0
0
25
50
CONCENTRATION OF CHLOROPHYLL (μg/ml)
TIME TO REACH F_0, sec.
0
0.2
0.4
0.6
0.8
1.0

of reabsorption of fluorescence by measuring the ratio of the intensity of fluorescence at 685 nm (F685) to the intensity at 730 nm (F730). The ratio remained fairly constant for as high as 40 μg Chl/ml. Usually, however, 10-20 μg Chl/ml was used for measuring spectra. On occasions, higher concentrations have been used to improve the signal to noise ratio. (For a discussion of problems due to reemission of reabsorbed light, see Szalay et al., 149.) For the measurement of fluorescence spectra at 77°K, we used 10 to 100 fold dilute samples than those used for room temperature spectra. Furthermore, a very small amount (0.25 ml) of cells or chloroplasts were used for 77°K measurements (74). The samples were quickly cooled. (The rate of cooling causes greater variation in the shape of the transient than the concentration of chlorophyll.)

In some experiments, we introduced a slight variation in our way of measurement of the constant level, O. The O level was estimated by giving 1/10th-1/25th second flash of weak light. These flashes had approximately 10% of the maximum excitation illumination used. Since the fluorescence yield of "O" does not vary with intensity, we believe such a procedure for estimating O level is reliable. But, we note that our measurements of the O level in this procedure gave a slightly higher (5-10%) value than when it was measured with a fast oscillographic (Midwestern Instrument 810 B) recorder operated at 64"/sec.

III. FLUORESCENCE INDUCTION IN THE RED ALGA PORPHYRIDIUM CRUENTUM

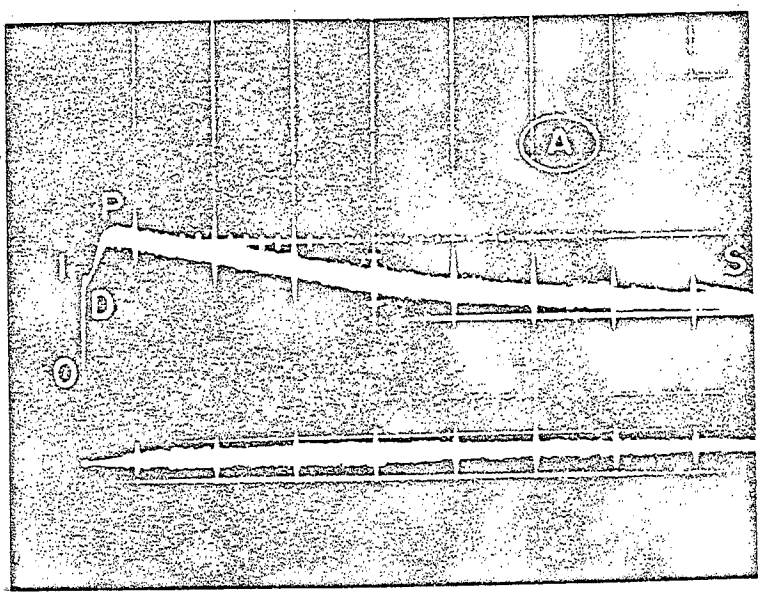
Light induced changes in the yield of chlorophyll a (Chl a) have been well-documented in intact green algae (1). In our laboratory, Munday (58) analyzed the various characteristic points in the fast (sec) induction curve (OIDPS) of the green alga Chlorella pyrenoidosa, and Papageorgiou (59) characterized the slow (min) induction curve in Chlorella and Anacystis. Several years ago, Lavorel (150) characterized the fast transient in the red alga but the slow induction has not been studied before. However, Murata (151) has described the effect of various types of illumination on the PS decay of the fast transient, and Vredenberg (152) has observed the effect of some ionophorous antibiotics on the PS decline and the restoration of the fast transient in another red alga Porphyra.

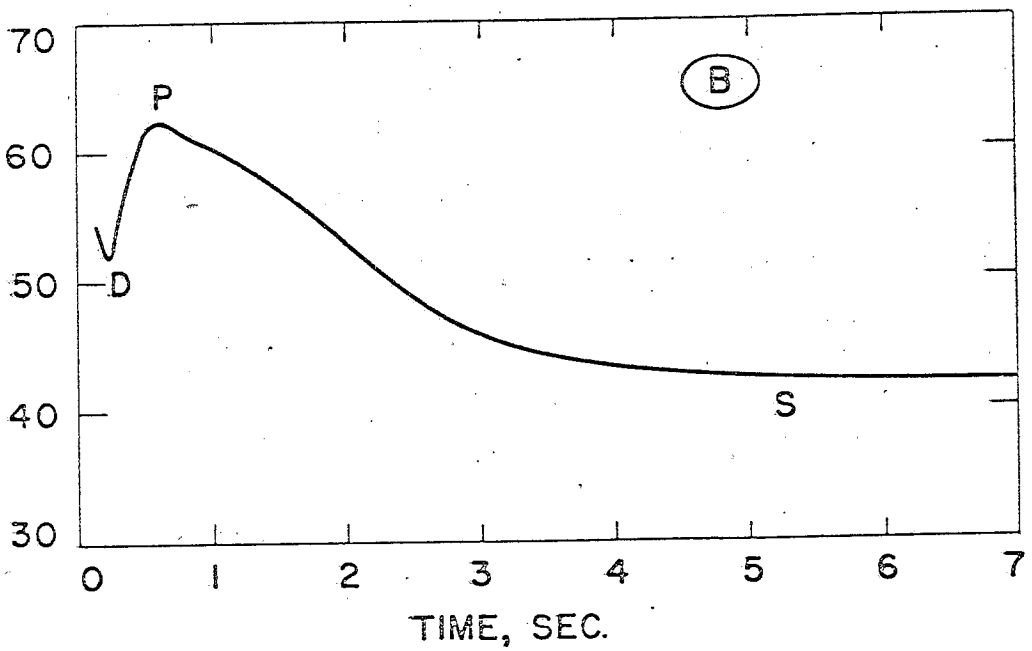
The present study extends the observations of fluorescence transients to Porphyridium cruentuma--a red alga prototype for photosynthesis research. We have analyzed the kinetics of the fast and the slow transients of Chl a fluorescence yield in the presence and in the absence of inhibitors of electron transport and uncouplers of photophosphorylation. We have also made a detailed study on the mode and site of action of hydroxylamine (NH_2OH), a potent inhibitor of oxygen evolution. The present study, made in collaboration with other members of our laboratory, has already been published (153, 154).

1. Time Course of Chl a Fluorescence and the Rate of Oxygen Evolution

Figure 12 shows the fast fluorescence transient in the intact cells of Porphyridium cruentum. After a long dark period, the fluorescence yield measured at 685 nm shows the induction phenomenon upon illumination with 540 nm green or with a broad band of blue-green light, absorbed

Figure 12. The fast transient of Chl fluorescence yield changes in Porphyridium. Top (A): oscilloscope tracing of the transient; 6 day old culture suspended in 0.1M carbonate-bicarbonate buffer, pH 8.5; λ observation, 685nm (half band width, 6.6nm); C.S. 2-58 filter before the photomultiplier; excitation blue green light (C.S. 4-96 + 3-73) (half band width, 120 nm); intensity of excitation light, 16 Kergs $cm^{-2}sec^{-1}$. Ordinate, 0.5 volt/division; abscissa 1sec/division. Bottom (B): The recorder tracing of the above transient in another (8 day old) culture of Porphyridium showing pronounced IDPS transient; 0 is not recorded on the trace. Cells were suspended in 0.05M Tris-Cl buffer, pH 8.5, plus 0.25 M NaCl. Excitation light, 540 nm with 20 nm half band width; excitation intensity, 9.2 Kergs $cm^{-2}sec^{-1}$; C.S. 2-64 filter was placed before the photomultiplier to prevent the leak of the excitation light (from Mohanty et al., 153).


A
P
D
S
C

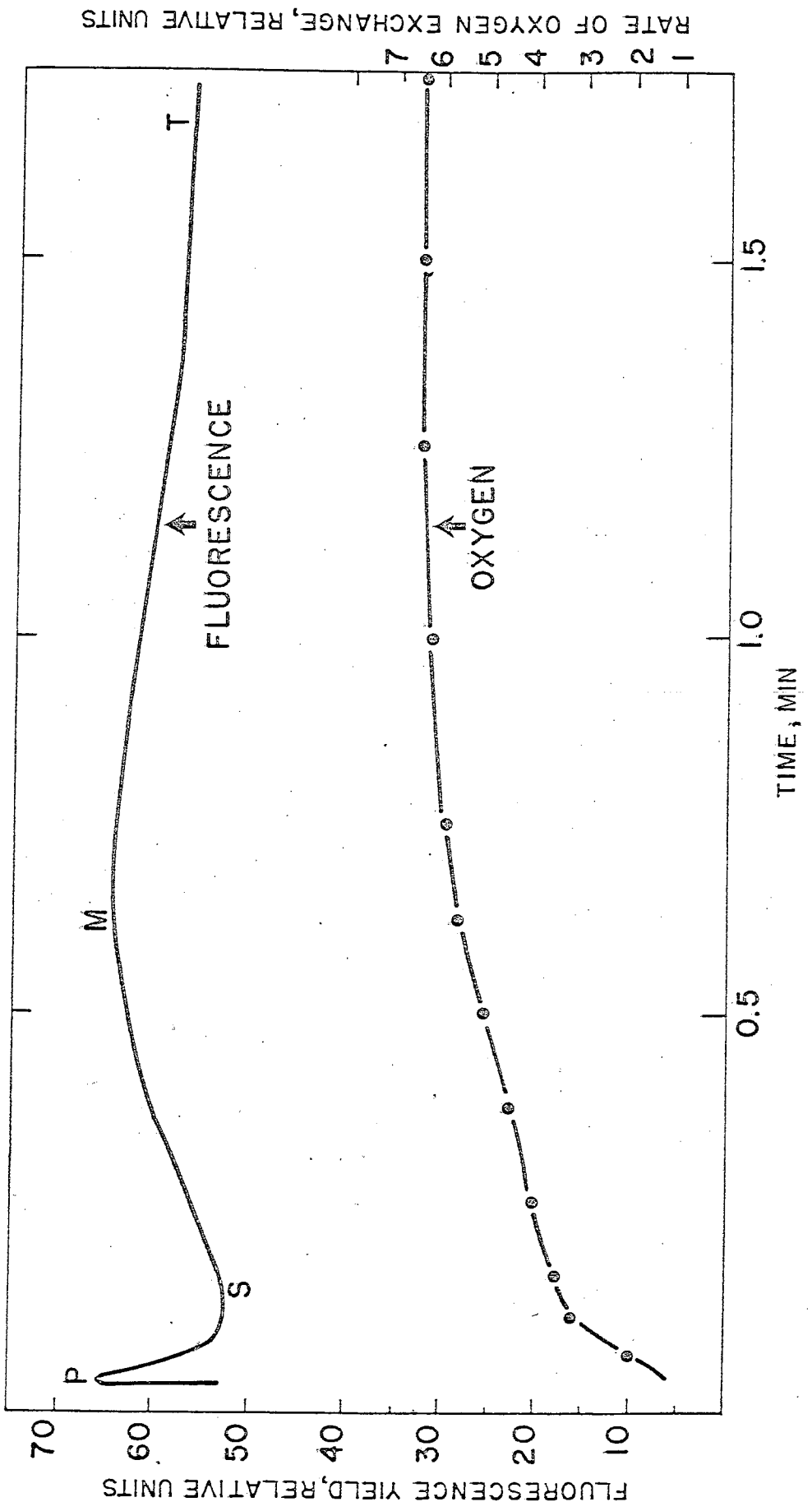

B
P
D
S
70
60
50
40
30
0
1
2
3
4
5
6
7
TIME, SEC.

mainly by phycoerythrin (System II light). Figure 12 (A) is an oscillographic record (I exc., 16 kergs $cm^{-2}sec^{-1}$) and Figure 12 (B) is a recorder trace from a different set of experiments (I exc., 9.2 kergs $cm^{-2}sec^{-1}$). These curves show clearly the various characteristic points, namely OIDPS. The ratio (P-O)/O varied, with different cultures, from 1.3 to 2.0. Blue light (440 nm) mainly absorbed by System I, does not cause this induction of Chl a fluorescence.

Figure 13 (top curve) shows the yield changes upon illumination with PS II light for a longer time period; the characteristic points SMT of the slow transient are clear. The extent of SM rise is less pronounced here than in Chlorella (59). The bottom curve of Figure 13 shows the time course of oxygen evolution in saturating green light (~12 kergs $cm^{-2}sec^{-1}$). The complementary phase of oxygen evolution and fluorescence is noticeable in the PS decline phase. Due to the slow response of the polarographic apparatus used in this experiment, the relationship between the O_2 trace to the DP phase of the fluorescence induction curve is obscure. (The initial oxygen spike is less pronounced in Porphyridium than in other algae at room temperature.) Inspite of the difference in the quality of light used for fluorescence (blue-green) and oxygen measurements (green), the results clearly indicate that the rate of O_2 evolution rises in parallel with the SM rise and increases asymptotically during the MT decline phase. These results are similar to those made in green algae (88, 89, 155). Both the oxygen and fluorescence traces could be repeated if enough dark interval is given. In these experiments, a 10 min dark period restored more than 95% fluorescence yield at P.

Thus, the time course of fluorescence yield of Porphyridium is very similar to Chlorella. The slow SMT phase shows its typical

Figure 13. The time course of the Chl *a* fluorescence yield and the rate of oxygen evolution in a 6 day old culture of *Porphyridium*. Top Curve: recorder trace of fluorescence transient exhibiting the slow PSMT phase. Experimental conditions as in Figure 12(A); the fast OID phase is not recorded. Bottom Curve: Rate of oxygen evolution as measured with a Haxo-Blinks type rate electrode with polarographic circuitry and recorded on a Heath kit servo recorder. Excitation, 545nm light; intensity, 12 Kergs $cm^{-2} sec^{-1}$; buffer, potassium-sodium phosphate plus 0.25 M NaCl, pH 7.0 (from Mohanty *et al.*; 153).


RATE OF OXYGEN EXCHANGE, RELATIVE UNITS
7
6
5
4
3
2
1
T
FLUORESCENCE
OXYGEN
M
S
P
70
60
50
40
30
20
10
FLUORESCENCE YIELD, RELATIVE UNITS
0.5
1.0
1.5
TIME, MIN

non-complementary relation to the oxygen evolution (Figure 13). The PS decline of fluorescence yield is very pronounced here. As first shown by Duysens and Sweers (10), this decline of fluorescence yield does not represent only the oxidation of the primary electron acceptor of photosystem II although an apparent complementarity with oxygen yield is observed during this phase of the induction.

2. Intensity of Excitation and the Fluorescence Induction

The time courses of the fast fluorescence induction, recorded at various intensities of illumination, are shown in Figure 14 and those of the slow fluorescence induction in Figure 15. Very low intensities fail to induce transients of measurable magnitude. As the intensities are raised the amplitudes of both the fast and the slow fluorescence changes are increased. From a set of experiments similar to the one shown in Figure 14, we plotted the time to reach P (tp) as a function of light intensity of exciting light (Figure 16). At low intensities the yield at P is proportional to the light doses, i.e., the Bunsen-Roscoe reciprocity law is obeyed. However, from Figure 15, it is clear that time to reach M did not decrease significantly on lowering the excitation intensity. This indicates that the development of the peak M is not directly related to the primary photochemical events of PS II.

Figure 17 (left) is a plot of the amplitudes of fluorescence yield at 0, P, and S, obtained from a separate set of experiments, as a function of exciting intensity. The constant level fluorescence "0" increases linearly with light intensity. This feature was first observed by Lavorel (156) and has been confirmed by Munday (58). Figure 17 (right) shows the relative variable yield at P and at S. It is clear that the

Figure 14. Fast transient of the Chl *a* fluorescence yield at different intensity of excitation, in *Porphyridium*. Left (A): Recorder tracing of the DPS transient, numbers of the curves are fractions of maximum intensity used. Intensity 1.00 = 18 Kerg cm^{-2} sec^{-1}. Other conditions as in Figure 12 (A). Right (B): The same transient reproduced from another experiment. Intensity 1.000 = 9.2 Kergs $cm^{-2}sec^{-1}$. Other details are as in Figure 12 (B) (from Mohanty *et al.*, 153).

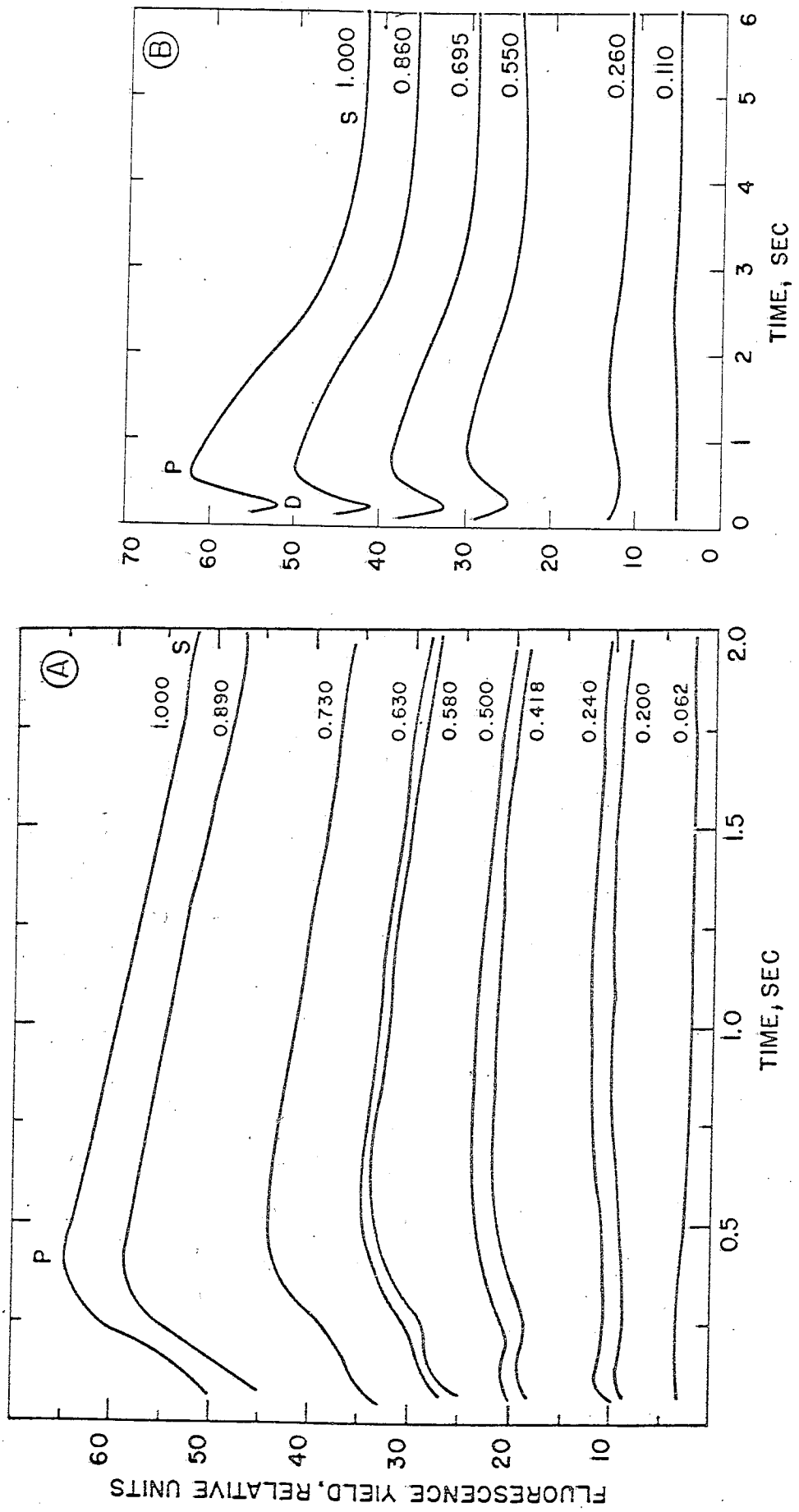

FLUORESCENCE YIELD, RELATIVE UNITS
TIME, SEC
TIME, SEC
(A)
(B)
P
S
D
1.000
0.890
0.730
0.630
0.580
0.500
0.418
0.240
0.200
0.062
1.000
0.860
0.695
0.550
0.260
0.110

Figure 15. Recorder tracing of the slow transient of the Chl *a* fluorescence yield, at various intensities of excitation, in *Porphyridium*. Intensity 1.00 = 9.2 Kergs $cm^{-2}sec^{-1}$. Other conditions as in Figure 12 (B) (from Mohanty *et al.*, 153).

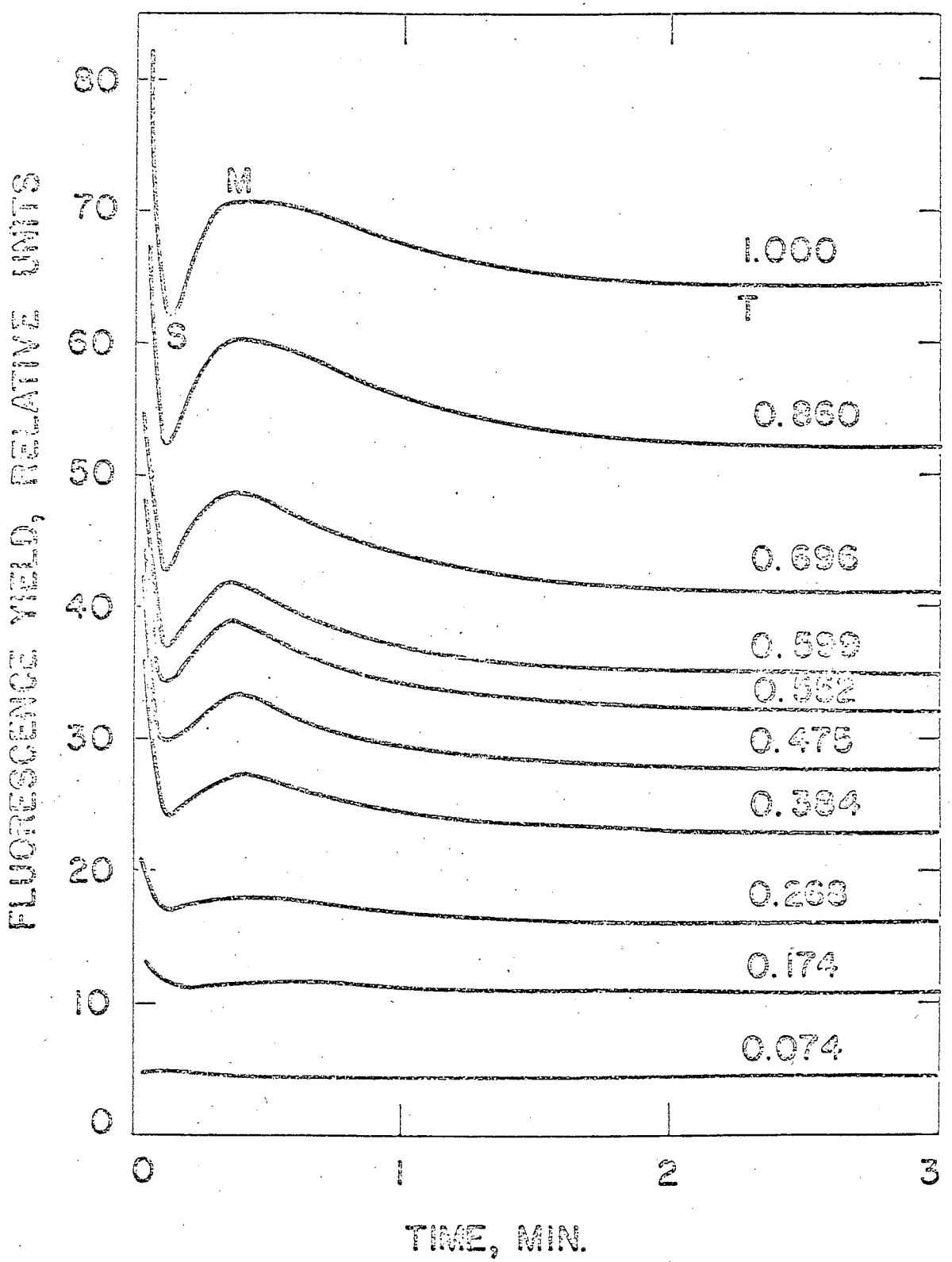

FLUORESCENCE MINUS DARK ATMS UNITS
80
70
60
50
40
30
20
10
0
M
S
T
1.000
0.860
0.696
0.599
0.552
0.475
0.384
0.268
0.174
0.074
0
1
2
3
TIME, MIN.

Figure 16. The time for the appearance of peak P as a function of exciting intensity. Highest intensity used shown as 100 on abscissa = 18 Kergs $cm^{-2}sec^{-1}$. Data for this plot was obtained from experiments similar to those in Figure 12 except that an oscillographic recorder was used to record the transient (from Mohanty *et al.*, 153).

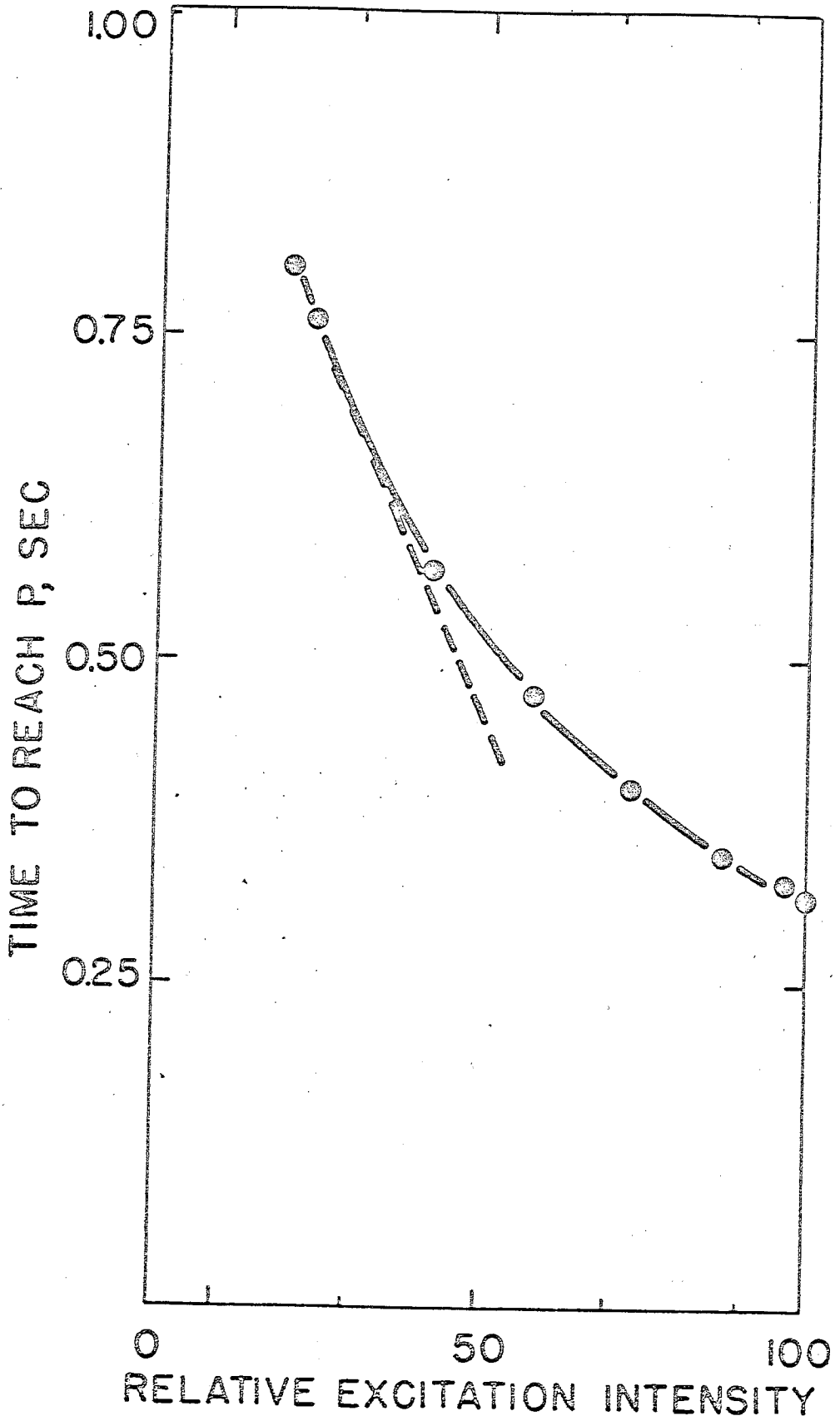
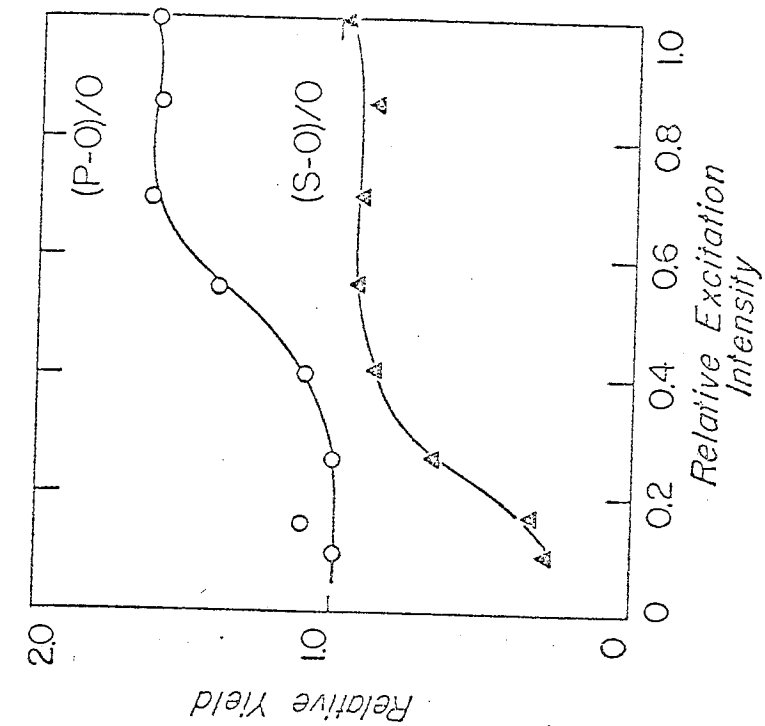
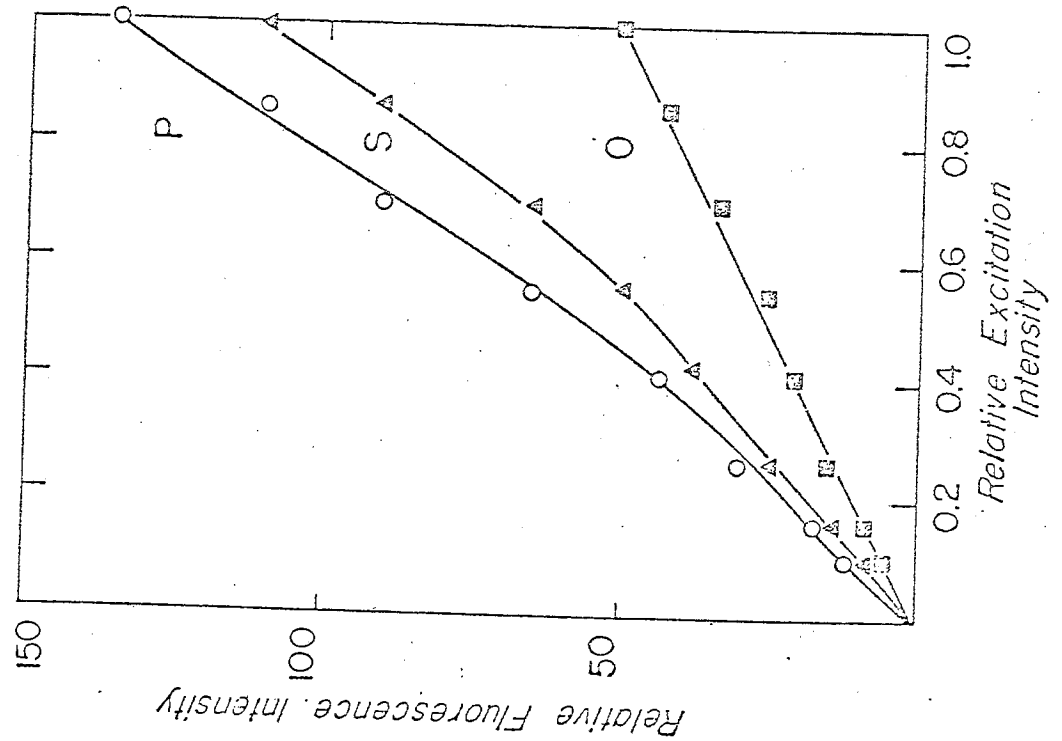

1.00
0.75
0.50
0.25
TIME TO REACH P, SEC
0
50
100
RELATIVE EXCITATION INTENSITY

Figure 17. The amplitude of O, P and S (left) and the relative variable yield (right) versus excitation intensity. The maximum intensity 1.0 = 14 Kergs $cm^{-2} sec^{-1}$. The cells were suspended in the growth medium. All other conditions of measurement as in Figure 12. Relative variable yields at P and at S were expressed as $[P-O]/O$ and $[S-O]/O$.

Turn around 90°





variable yield at S tends to saturate much earlier than at P.

Figure 18 shows our attempts to separate the different processes that are operative in both the fast and the slow induction phases. In these figures an average rate function defined as $(F_{t_1} - F_{t_2})/(t_2-t_1)$ is plotted as a function of excitation intensities. (We believe that rates of rise and decline of the yield are complex and may not be first order and thus rates defined in terms of half time may be misleading.) From Figure 18 it is clear that at the light intensities the slow changes (SM and MT) are saturated, the D-P phase still increases with increasing light intensity.

Figure 19 illustrates the constancy of fluorescence yield at T in the intensity range in which this experiment was made. On the other hand the relative yield quotient f_M and f_T which measure the ratio of fluorescence intensity at M and T with respect to that at S show characteristic dependence on intensity of excitation. It is evident that both S and M saturate at relatively lower intensities of excitation than T. From oxygen measurements (Figure 13) and the dependence of light intensities (Figures 17-19) it is clear that underlying processes that affect fluorescence SM rise and MT decline phases of the slow changes in the yield of Chl *a* are different. Furthermore, it should be mentioned here that the P to S decline phase of fast induction curve is different from its slow counterpart MT.

3. P to S Decline Phase

As noted earlier, Duysens and Sweers (10) observed that PS decline of the fast induction curve cannot be explained on the basis of redox state of the primary acceptor. They showed that, if illuminating light is

Figure 18. Average rate of changes in the fluorescence yield at various parts of the induction curve as a function of excitation intensity. (A) For DP rise and (B) for SM rise and MT decline; intensity 1.0 = 9.2 Kergs $cm^{-2}sec^{-1}$. Other conditions as in Figure 12 (B) (from Mohanty et al., 153).

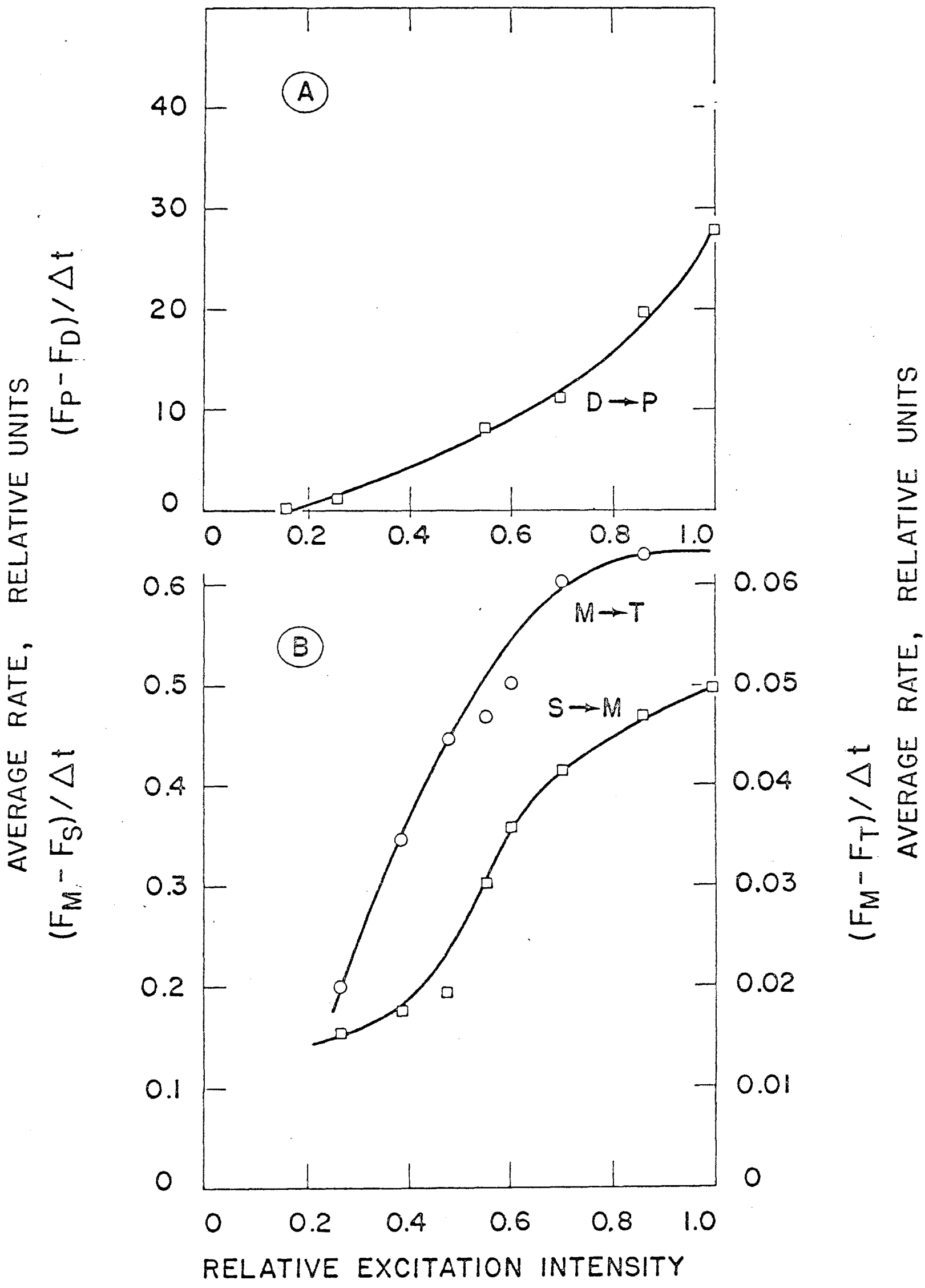

A
B
D → P
M → T
S → M
RELATIVE UNITS
(F_P - F_D) / Δt
AVERAGE RATE,
(F_M - F_S) / Δt
(F_M - F_T) / Δt
RELATIVE EXCITATION INTENSITY
0
10
20
30
40
0.1
0.2
0.3
0.4
0.5
0.6
0.01
0.02
0.03
0.04
0.05
0.06
0.2
0.4
0.6
0.8
1.0

Figure 19. Relative fluorescence yield at the stationary state T (ϕ_{FT}) and the quotients f_M ($=F_M/F_S$) and f_T ($=F_T/T_S$) versus excitation intensity. Intensity 1.0 = 9.2 Kergs $cm^{-2}sec^{-1}$. All other conditions as in Figure 12 (B) (from Mohanty *et al.*, 153).

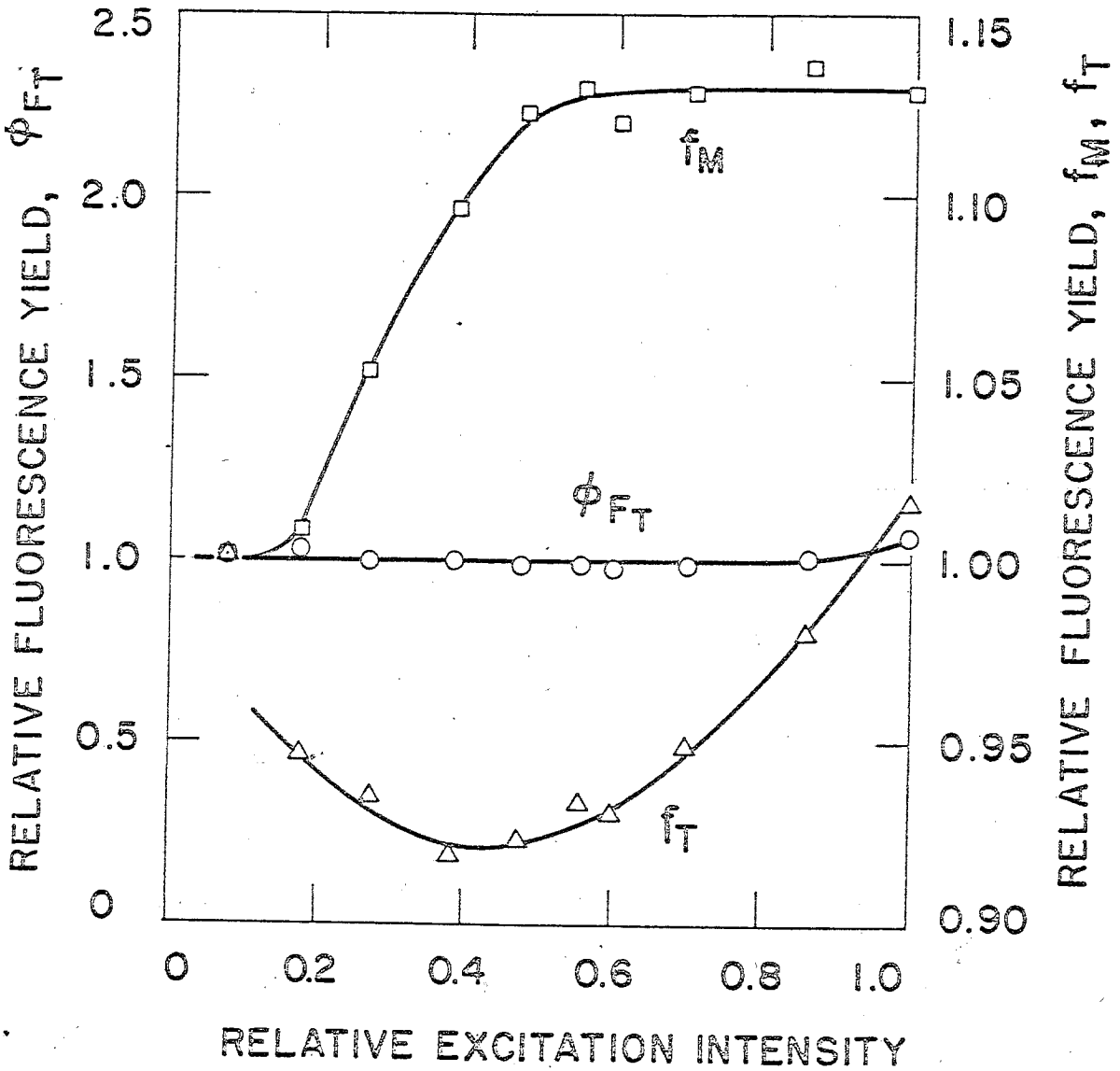
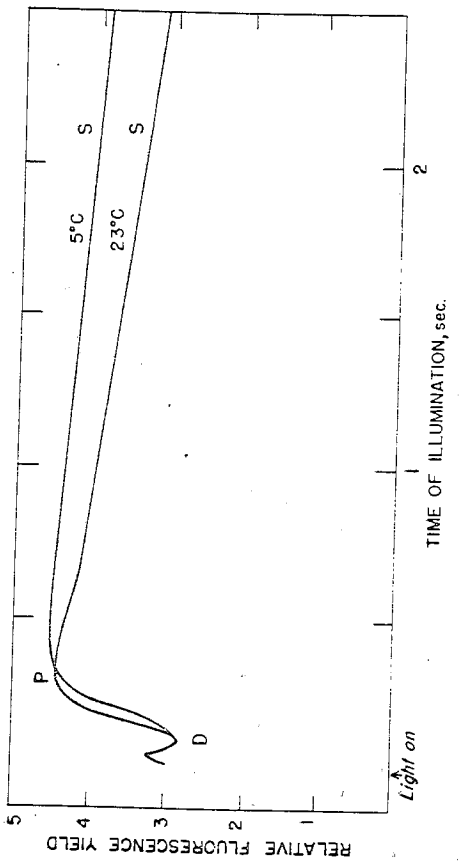

RELATIVE FLUORESCENCE YIELD, ϕ_{F_T}
RELATIVE FLUORESCENCE YIELD, f_M, f_T
RELATIVE EXCITATION INTENSITY
2.5
2.0
1.5
1.0
0.5
0
1.15
1.10
1.05
1.00
0.95
0.90
0
0.2
0.4
0.6
0.8
1.0
f_M
ϕ_{F_T}
f_T

Figure 20. The IDPS transient of Porphyridium incubated at two different temperatures during the dark adaptation period. Other details of measurement as in Figure 17.

Turn around 90° and enlarge



turned off at S and then turned on immediately again, the fluorescence yield does not rise up to the P level but remains at a low level. Duysens and Sweers (10) explained this observation by assuming that in light QH somehow transforms to a chemical, it is different from Q designated as (Q')--it can quench fluorescence like Q but it cannot be reduced by light. (In recent years Duysens (157) has rejected this simple chemical transformation hypothesis in favor of a physical state hypothesis as discussed in Chapter I.) The following experimental results indicate that PS decline is not coupled to the oxidation of QH.

Figure 20 shows the IDPS transient at two temperatures. As we did not devise any special apparatus (besides using a Dewar flask) to control the temperature during the long dark period that is necessary to restore the IDPS transient, we only made observations at room temperature and at 5^{o} C. It is clear that both DP rise and PS decline phase were slowed down at the lower temperature (5^{o} C); the PS decline seems to be more affected by change in temperature than the rise from D to P. These data indicate that perhaps Q and its immediate reaction partner in the electron transport chain are probably tightly held in the membrane matrix otherwise one would expect to observe a rapid rise of fluorescence to P at the lower temperature (5^{o} C). A slow DP rise reflects a fast electron transport from Q to A. At lower temperature, however, we would expect the rate of this electron transport to slow down. Thus, in Porphyridium it seems that the A pool remains in the more oxidized state in the dark at low temperature (5^{o} C) than at room temperature (23^{o} C).

The slower PS decline at low temperature (5^{o} C) could have been due to the slower reoxidation of QH, but we just suggested above that at 5^{o} C, this is not so otherwise we should have had a faster DP rise. In addition,

the O_2 evolution, at low temperature (5^{o} C), showed an enhanced spike as compared with the O_2 spike at room temperature suggesting a rapid reoxidation of QH by the A pool; and the subsequent increase in O_2 evolution did not alter significantly. This suggests that the PS decline does not only reflect the oxidation of QH by the A pool but is controlled by other factors as well.

Figure 21 shows the effect of a 60 sec system 1 preillumination (λ, 435 nm, intensity ~0.8 Kergs $cm^{-2}sec^{-1}$) on the PS decline. In this figure we have adjusted the fluorescence yield at P for both the preilluminated and dark adapted samples to show the effect of pre-illumination on the S level. (This normalization is justified if we assume that at P all the Q are reduced by system II light, 85.)

As observed before by other investigators in other algae (83, 84, 152, 158) S level is increased, and PS decline is slowed down by system 1 pre-illumination. The half time for the decay of the effect of pre-illumination is approximately 12-15 seconds (Figure 21, insert). Munday and Govindjee (84) reported a similar dark decay of pre-illumination effect for the suppression of P in Chlorella. The slow rate of PS decline cannot be related to the oxidation of A pool by system 1 pre-illumination. Higher oxidation level of A pool should not cause a slow reduction of the intermediates in light. Thus, P to S decline, as suggested first by Duysens and Sweers (10) does not seem to be directly related to the oxidation of QH by A in light.

Figure 22 illustrates a comparison of the decline of the yield at P in dark with that in light (PS decline). This experiment was done as follows: after a long period of darkness (10-15 minutes) the yield at P was measured and the exciting light was turned off at P and a dark

Figure 21. The effect of a 60 sec preillumination with 435 nm (half band width, 10 nm) blue light (0.8 Kergs cm^{-2} sec^{-1}) on the PS decline transient of Porphyridium. Intensity of excitation blue green light (C.S. 4-72 + 3-73) ~ 10 Kergs $cm^{-2}sec^{-1}$. Insert: Relative increase in the amplitude of S measured after 3-4 sec of blue-green light (PS II) illumination caused by the PS I preillumination over the dark adapted sample (after normalizing the yields at P) as a function of the dark time elapsed between preillumination and system II (blue-green light) illumination.

Turn around 90° and Enlarge

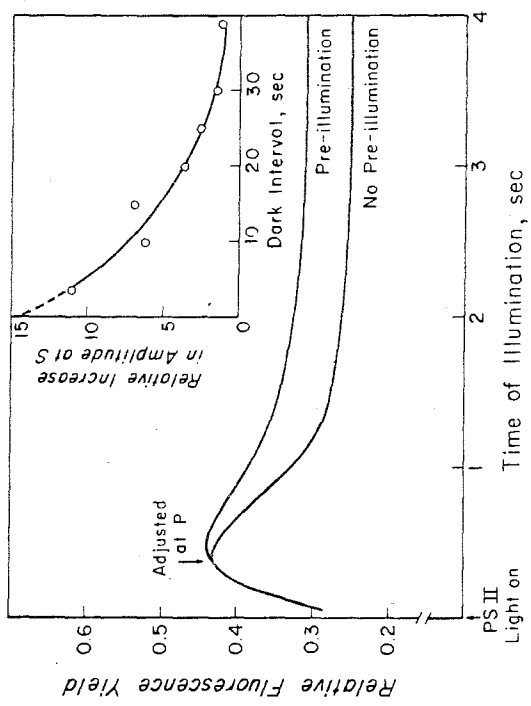
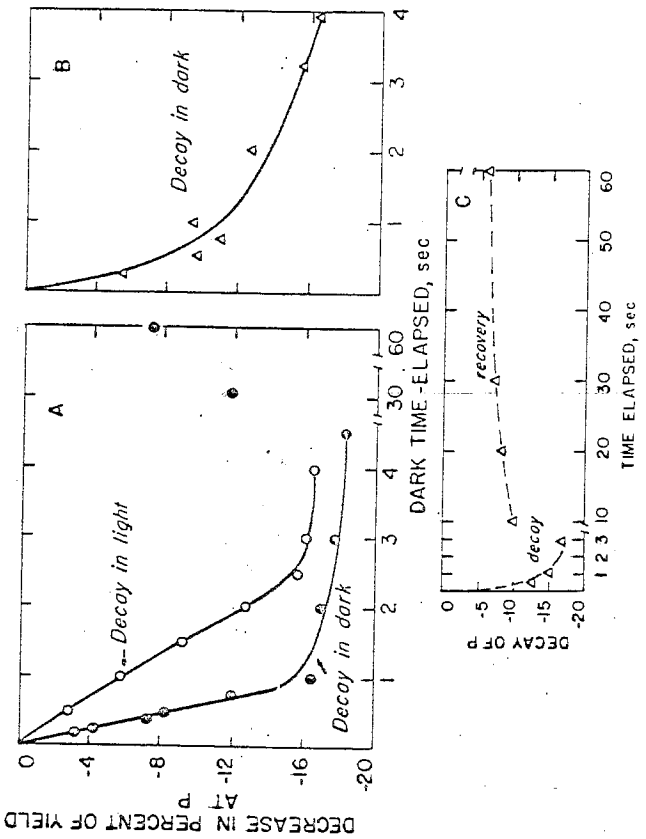


Figure 22. The PS decline in the yield of Porphyridium. Left (A): A 6 day old culture of Porphyridium suspended in growth medium was used. After 10 to 15 minutes of dark period the (System II) light was turned on until the transient reached the P level and then it was turned off. After a dark interval, as shown on the abscissa, the light was turned on again and the complete transient recorded. This cycle was repeated for each set of measurements. The decrease in the amplitude of P by the second illumination, calculated as percent decay of fluorescence at P, is plotted as a function of the dark time between two illuminations. For comparison, the decay of P (PS decline) represented as percent decrease in light is shown by the curve with open circles. Other conditions of measurement as in Figure 17. Right (B): Similar dark decay of P in a slightly older culture of Porphyridium. Bottom (C): A similar rapid decay and slow recovery of the amplitude of fluorescence at P obtained from another set of experiments.

Turn around 90° & Enlarge

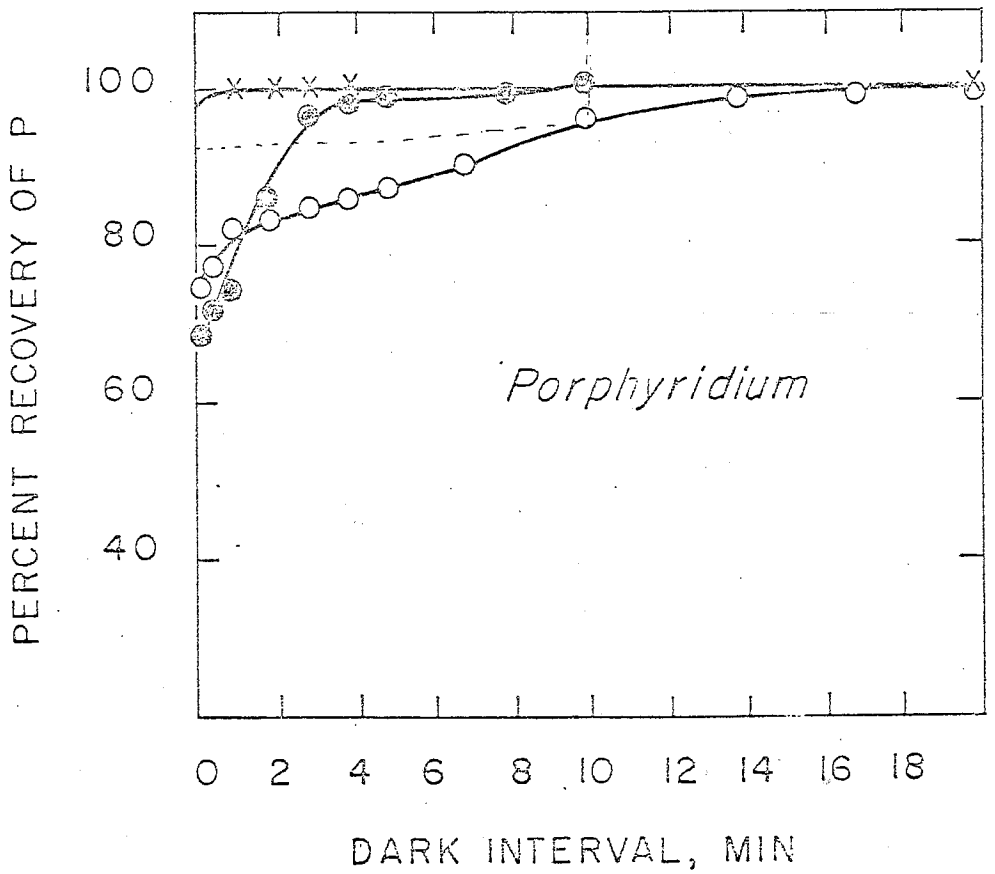


interval was interposed and then exciting light was turned on again for 3 to 4 seconds; this measurement was repeated with varying dark intervals. Figure 22 (A) shows that the yield at P declines much faster in dark than in light (with a $t_{\frac{1}{2}}$ of 0.25 - 0.5 sec in dark, and 1 - 1.5 sec in light). Figure 22 (B) is a similar experiment with an old culture of Porphyridium. This type of complex dark decay of P has been previously reported by Lavorel (159) in Chlorella in the msec region and recently by Heath (160) in chloroplasts. If, however, the dark period exceeds 10 seconds, one observes a gradual recovery of the fast transient (Figure 22 C).

4. Restoration of the Fast Transient

Figure 23 shows the amplitude at P versus the dark time in minutes. As mentioned before, a dark time of about 10 minutes (Figure 23 open circles) is necessary to restore the ability to reproduce the induction phenomenon (OIDPS). This dark time depends on the period of illumination and it also varies with organisms. System I pre-illumination recovers the transient within 1-2 minutes (not shown). Recently Vredenberg (152) reported that certain proton permeating chemicals shorten the requisite dark period to restore the induction in certain algae. We have confirmed this in Porphyridium (Figure 23, closed circles) with low concentration of FCCP (0.2μM). With 0.2μM FCCP the restoration time is shortened to 2 to 4 mins. At higher concentration (4μM FCCP), the P to S decline is suppressed, dark time to restore the transient is greatly reduced (Figure 23, crosses). (We could not, however, observe a similar enhancement of restoration in Chlorella. Because of the limited number of experiments done in Chlorella, and in chloroplasts and because of the lack of availability of nigericin which pronouncedly shortens the dark time (152), we

Figure 23. Restoration of the fast changes in Chl *a* fluorescence yield in dark. The amplitude of P after a long dark period (30 minutes) was taken as 100 percent. Untreated cells (open circles), 0.2μM FCCP (closed circles), and 4μM FCCP (crosses). Six day old *Porphyridium* cells suspended in growth medium were used. Other conditions for transient measurement are as in Figure 17.


PERCENT RECOVERY OF P
100
80
60
40
Porphyridium
0 2 4 6 8 10 12 14 16 18
DARK INTERVAL, MIN

do not speculate on the specificity of this effect.) It seems that in Porphyridium, at least, we observed a shortening of the requirement of the dark period with FCCP. It must, however, be pointed out that FCCP brings about a large number of complex changes even at a very low concentration. We will elaborate on some of these effects of FCCP in a later chapter (Chapter IV).

5. Spectral Changes During Fluorescence Induction

Lavorel (150, 161) first documented the spectral changes during fluorescence induction. He observed a relative increase in PS II fluorescence at P with respect to 0. Papageorgiou and Govindjee (87, 88) documented a large variation of spectral distribution at various points of the slow transient in Chlorella and Anacystis. Murata (162) showed that PS II pre-illumination (at room temperature) enhances the intensity of long wavelength band and decreases the intensity of F685 and F696 emission bands (as measured as) at liquid nitrogen temperature. This pre-illumination, according to Murata, alters the energy transfer from PS II to PS I.

We obtained the fluorescence spectra of Porphyridium at different stages of the induction by observing the fluorescence time course at various wavelengths. Dark periods of suitable length (10 minutes) were interposed between the recordings to restore the transient. Figure 24 shows the spectra at D, P, and S, as well as the difference spectra P minus D and P minus S. The difference bands are peaked at 687 nm for the P-D and at 684 nm for the P-S spectra while their half-band widths are about 25 nm. In Figure 25 the fluorescence spectra at S and M and the difference emission spectrum M-S are given. The maximum of the difference

Figure 24. Fluorescence emission spectra at the stage D (open circles), P (open squares) and S (solid circles) and P minus S (solid triangles) on a 5 times expanded ordinate scale. Details as in Figure 12 (B) with the exception that two C.S. 2-58 filters were used in place of one C.S. 2-64 (from Mohanty et al., 153).

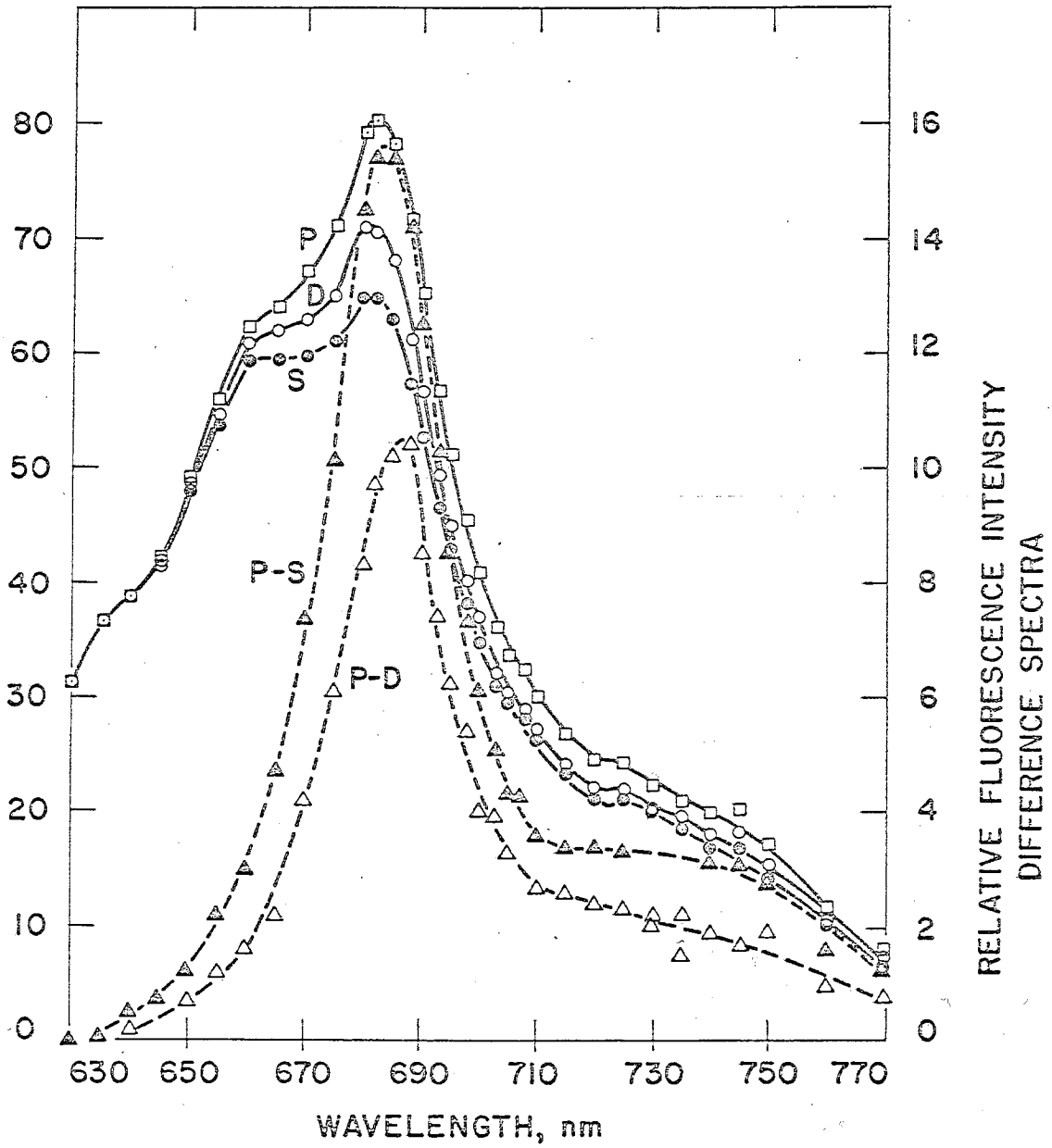
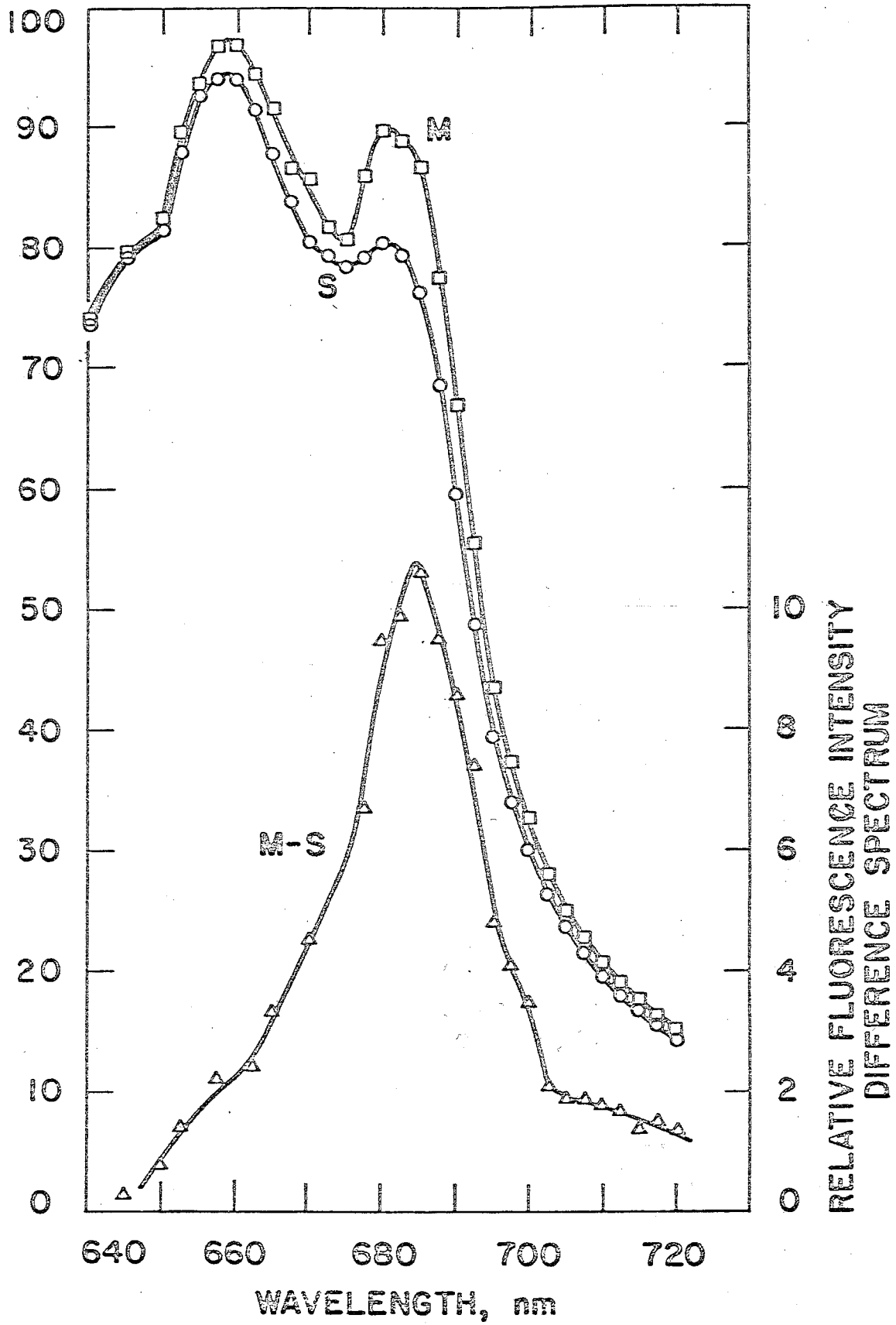

80
70
60
50
40
30
20
10
0
16
14
12
10
8
6
4
2
0
P
D
S
P-S
P-D
630
650
670
690
710
730
750
770
WAVELENGTH, nm
RELATIVE FLUORESCENCE INTENSITY
DIFFERENCE SPECTRA

Figure 25. Fluorescence emission spectra at state S (open circles) and M (open squares) of the slow fluorescence transient. Difference emission spectrum M-S is shown on a five times expanded ordinate scale. A different culture grown at much higher intensity was used in this experiment than in Figure 24; other details as in Figure 12 (B). See other details in text (from Mohanty *et al.*, 153).


100
90
80
70
60
50
40
30
20
10
0
M
S
M-S
10
8
6
4
2
0
640
660
680
700
720
WAVELENGTH, nm
RELATIVE FLUORESCENCE INTENSITY
DIFFERENCE SPECTRUM

band width is 23 nm. (Samples from different cultures were used for the spectra presented in Figure 24 and 25.)

These results indicate that throughout the fluorescence time course the fluorescence is maximal at 685-687nm (Chl a). The constancy of the allophycocyanin fluorescence yield (660nm) rules out light-induced changes in the efficiency of excitation energy transfer from allophycocyanin to Chl a. However, an increase in the yield of fluorescence in system II may be accompanied by a decrease in the fluorescence of system I because of the decrease in the efficiency of energy transfer from Chl a of system II to that of system I. Examination of Figure 24 and Table III.1 reveals that the ratio of fluorescence intensity at the peak of Chl a (mostly system II) to that at 710 nm (mostly system I) is higher at P (2.68) than at the S level (2.46), and is highest (4.31) for the P-S spectrum. This result could be interpreted to mean that at P, the fluorescence yield changes from P to S are accompanied by an increase in the efficiency of transfer from system II to system I. In the language of Duysens (157) and Bonaventura and Myers (155), the light "state 1" is converted to light "state 2." Figure 25 shows that the ratio of fluorescence peak to the fluorescence intensity at 710 nm increases when the yield changes from S to M. The exact value of this ratio at S was very different from that in the previous experiment partly because of the different culture used here. In this culture (Figure 26), as compared to the previous one (Figure 25), the efficiency of energy transfer from allophycocyanin to Chl a was lower as is evidenced by the higher allophycocyanin to Chl a fluorescence. Thus, one can suggest that the observed fluorescence yield changes from S to M has been brought about by a change in state 2 to state 1 again.

Table III.1

Ratios of Fluorescence at the Peak (Mainly System II) to that at 710 nm (System I) at Different Portions of Fluorescence Transient (From Mohanty et al., 153)

Experiment No. 1*		Experiment No. 2+		Adjusted to Experiment No. 1
D	2.63	S	4.12	2.46
P	2.68	M	4.39	2.62
S	2.46	M-S	6.12	3.66
P-S	4.31	---	----	----

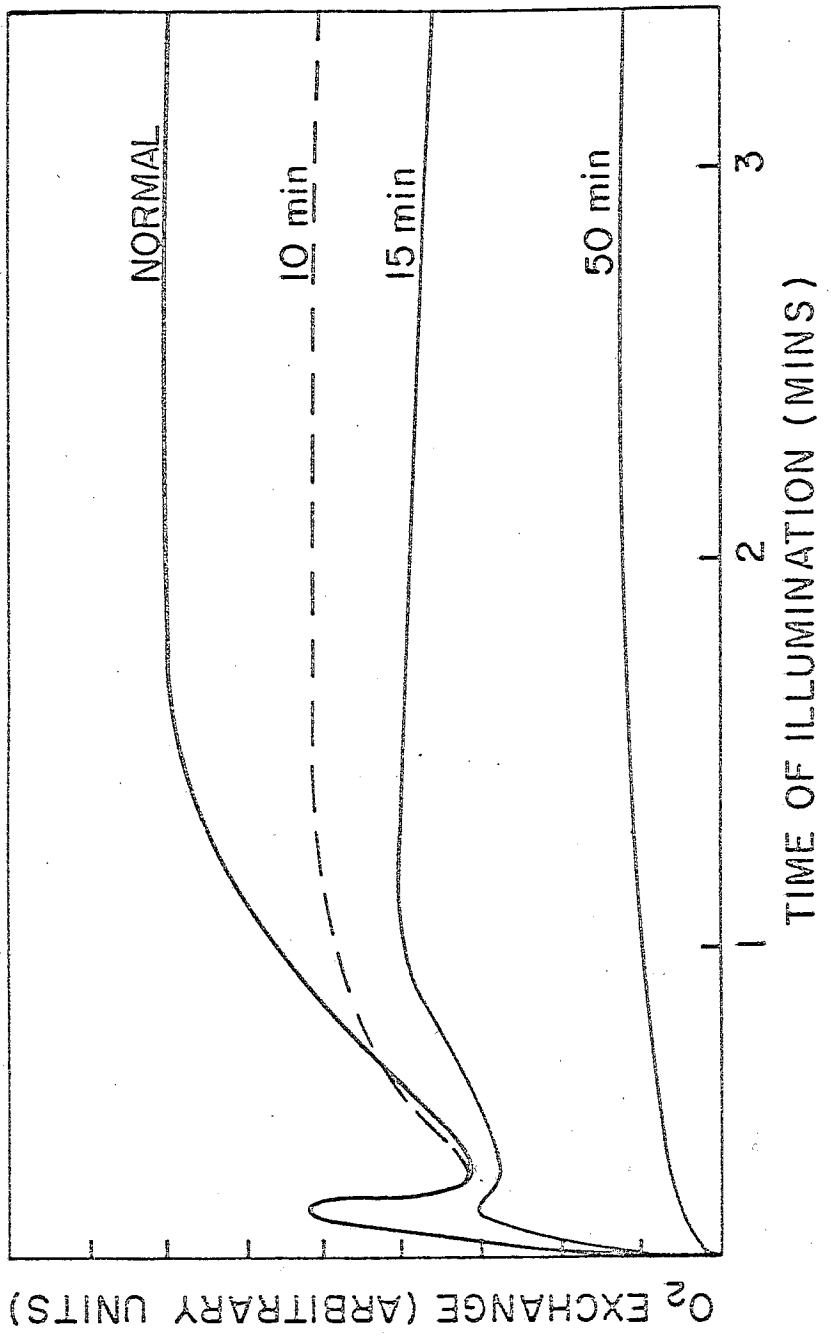
*Culture number 1, incident intensity, 1.

+Culture number 2, incident intensity, 2.

6. Effect of Electron Transport Inhibitors on the Fluorescence Yield Changes

It is well known that both substituted urea and hydroxylamine are potent inhibitors of oxygen evolution. It has been well established that substituted urea DCMU and CMU abolish the fast transient except for the very fast activation O-I rise phase (see references cited in 1). In the presence of DCMU or CMU, the fluorescence yield increases to a maximum within 2-3 seconds of illumination (~14 Kergs $cm^{-2}sec^{-1}$) and then remains constant for an extended period of illumination. Hoch and Randalls (163) recently observed a relatively slower rise of the yield of fluorescence in the presence of DCMU and FCCP in Porphyridium. From this rise curve of fluorescence they could estimate the pool size of Q to be 1Q/150 chlorophyll.

Figure 26. The time course of oxygen evolution in the presence and in the absence of NH_2OH. The number on the curves indicate the time of incubation with 1mM hydroxylamine; electrolyte, 0.05 M phosphate buffer, pH 7.0, plus 0.05 M KCl; temperature, $20^{\circ} \pm 1^{\circ}C$; 2% CO_2 in argon was uniformly bubbled. Excitation (white) light intensity = 40 Kergs $cm^{-2}sec^{-1}$ (from Mohanty et al., 154).


NORMAL
10 min
15 min
50 min
1
2
3
TIME OF ILLUMINATION (MINS)
O_2 EXCHANGE (ARBITRARY UNITS)

7. Effect of Hydroxylamine on O_2 Evolution and Fluorescence Yield Changes

Like DCMU, hydroxylamine also inhibits oxygen evolution, but Chl a fluorescence with these two poisons differ quite markedly. Delosme (164) first studied the effect of various concentrations of NH_2OH on the fast transient of Chlorella but he did not give any explanation of the changes in Chl a on fluorescence yield. Recently it has been shown that NH_2OH and many other compounds could feed electrons to PS II in isolated chloroplasts. Bennoun and Joliot (165) by the use of a rapid amperometric technique, clearly showed the photooxidation of hydroxylamine in isolated chloroplasts and in Chlorella. In view of these observations we made a detailed analysis of the action of NH_2OH on the fluorescence transient as well as on delayed light emission in Porphyridium. A part of this work, mainly on delayed light emission (DLE), was earlier reported by T. Mar (109). We discuss below the effects of NH_2OH on O_2 evolution and fluorescence induction processes.

7.1 Oxygen Evolution

First, we had to establish that in Porphyridium NH_2OH indeed inhibits oxygen evolution as it does in green plant systems. For this purpose O_2 exchange measurements were made. When Porphyridium cells were placed on a rate electrode replacement of buffer with buffer containing 1 mM hydroxylamine led to a substantial inhibition of oxygen evolution after 10 minutes of incubation (Figure 26). Longer incubation decreased the rate further. It took about an hour for the complete inhibition of O_2 evolution. At higher concentrations no O_2 evolution could be detected with our system.

Feeding of electrons by NH_2OH, at higher concentrations, was assayed by following O_2 uptake when low-potential dye methyl viologen was used (147).

It has been shown that viologen dyes accept electrons from the terminal photosystem I reduced acceptor X(19). As reduced viologens are highly autooxidizable they react with molecular oxygen yielding H_2O_2. If the catalytic splitting of H_2O_2 by endogenous catalase is prevented (by cyanide or azide), it is expected that there will be twice as much O_2 consumption in cells when hydroxylamine is a donor than when H_2O is a donor. Our results (Figure 27) indeed show that Porphyridium cells with 10 mM NH_2OH have 2-fold (0.039 μmole O_2 per ml) O_2 uptake than when it is absent. Thus, the higher rate of O_2 uptake in the NH_2OH treated than in the normal cells confirms that hydroxylamine feeds electrons, replacing water, as it does in isolated chloroplasts (166).

7.2 Fluorescence Transients

Figure 28 shows the effect of several concentrations on NH_2OH on the fast transient of Chl a fluorescence. At very low concentration (0.1 mM, lower dashed curve) only the DP phase is accelerated probably due to slower reoxidation of Q but P to S decline phase is unaffected. On increasing the concentration to 0.5 mM* the fluorescence transient undergoes a drastic change; the fluorescence increased rapidly to I phase and then abruptly declines to a low value. On increasing the concentration of hydroxylamine, the O-I rise phase is slowed (not shown here), ID decline becomes less pronounced and fluorescence yield increased slowly to a higher level with time.** If the only function of NH_2OH is to replace water as a donor, one would not expect these alterations in the time

---

*The exact concentration of NH_2OH needed to get this effect varied slightly with different cultures.

**In some cultures of Porphyridium, the constant "0" level fluorescence was found to be slightly enhanced.

Figure 27. Effect of hydroxylamine on O_2 uptake in the presence of methyl viologen in Porphyridium. Reaction mixture 50 mM phosphate buffer (pH 8.0), 250 mM NaCl, 1mM sodium azide, 0.5 mM methyl viologen and 10 mM hydroxylamine (chloride); excitation, saturating white light (from Mohanty et al., 154).

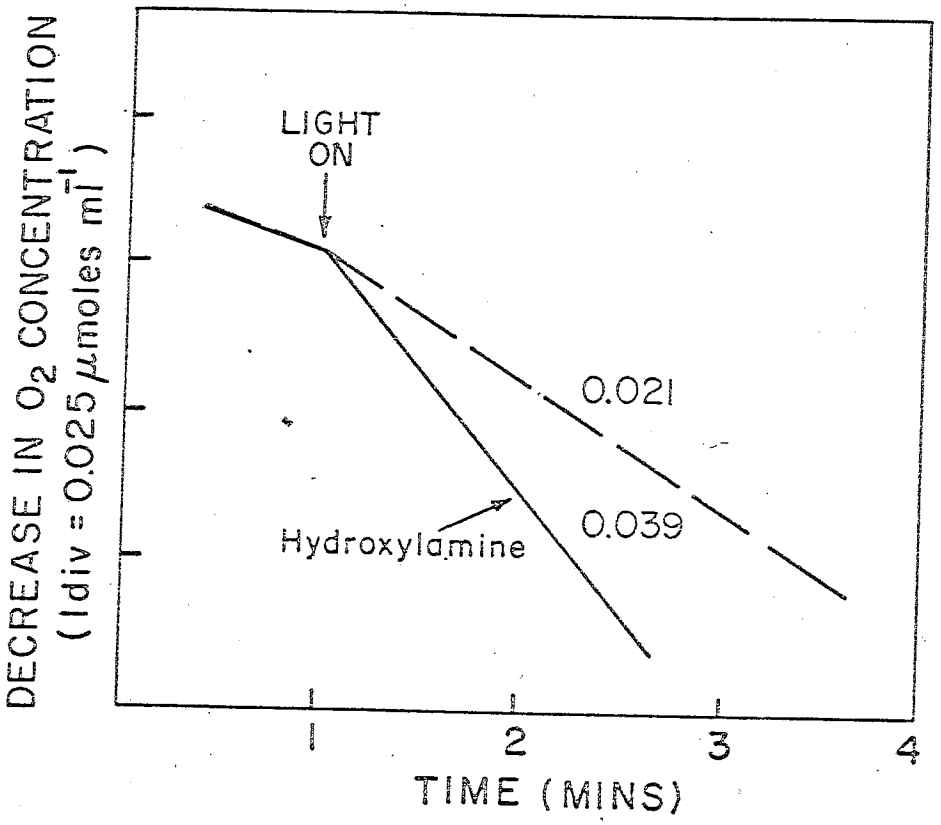
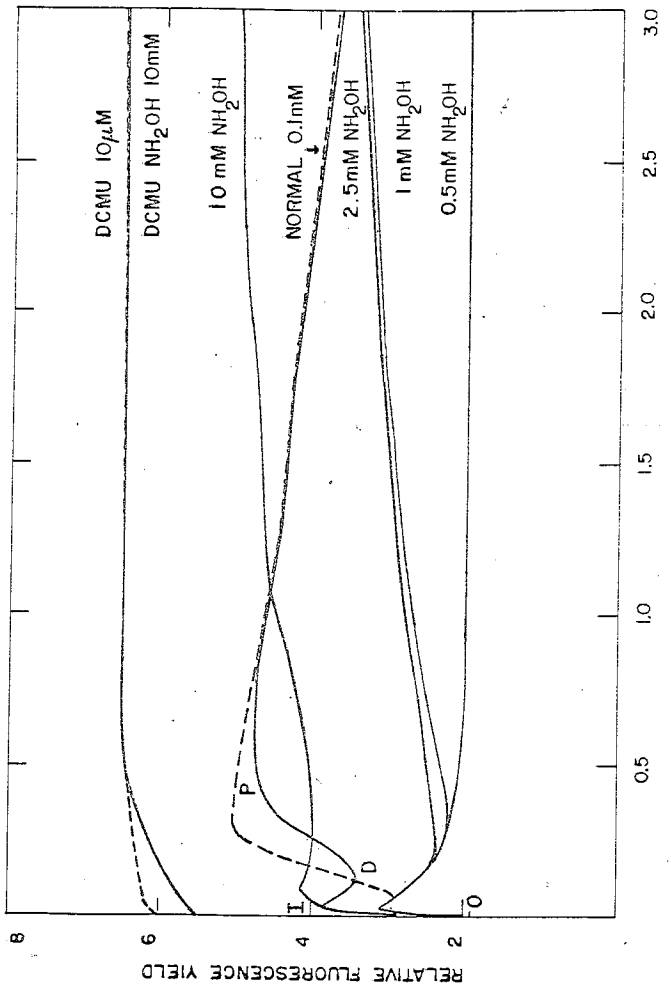

DECREASE IN O_2 CONCENTRATION
(1div = 0.025 μmoles ml^{-1})
LIGHT
ON
0.021
0.039
Hydroxylamine
1
2
3
4
TIME (MINS)

Figure 28. The effect of various concentrations of hydroxylamine (NH_2OH) on the fast fluorescence transient of Porphyridium. The fluorescence was measured at 685 nm with a 6.6 nm band width. A Corning C.S. 2-61 glass filter was used to prevent excitation light leaking into the analyzing monchromator. The cells suspended in carbonate-bicarbonate buffer (0.1 M, pH 8.5) with 15 gm/l NaCl were excited with a broad band blue light peaking at 540 nm with an intensity of 20 Kergs $cm^{-2}sec^{-1}$. The lower dashed curve represents the transient with 0.1 mM NH_2OH and the upper dashed curve the transient in the presence of 10 μM DCMU + 10 mM NH_2OH. All other concentrations of hydroxylamine are as shown. (A similar set of transient curves in the presence of various amounts of NH_2OH has been reported in ref. 154.)

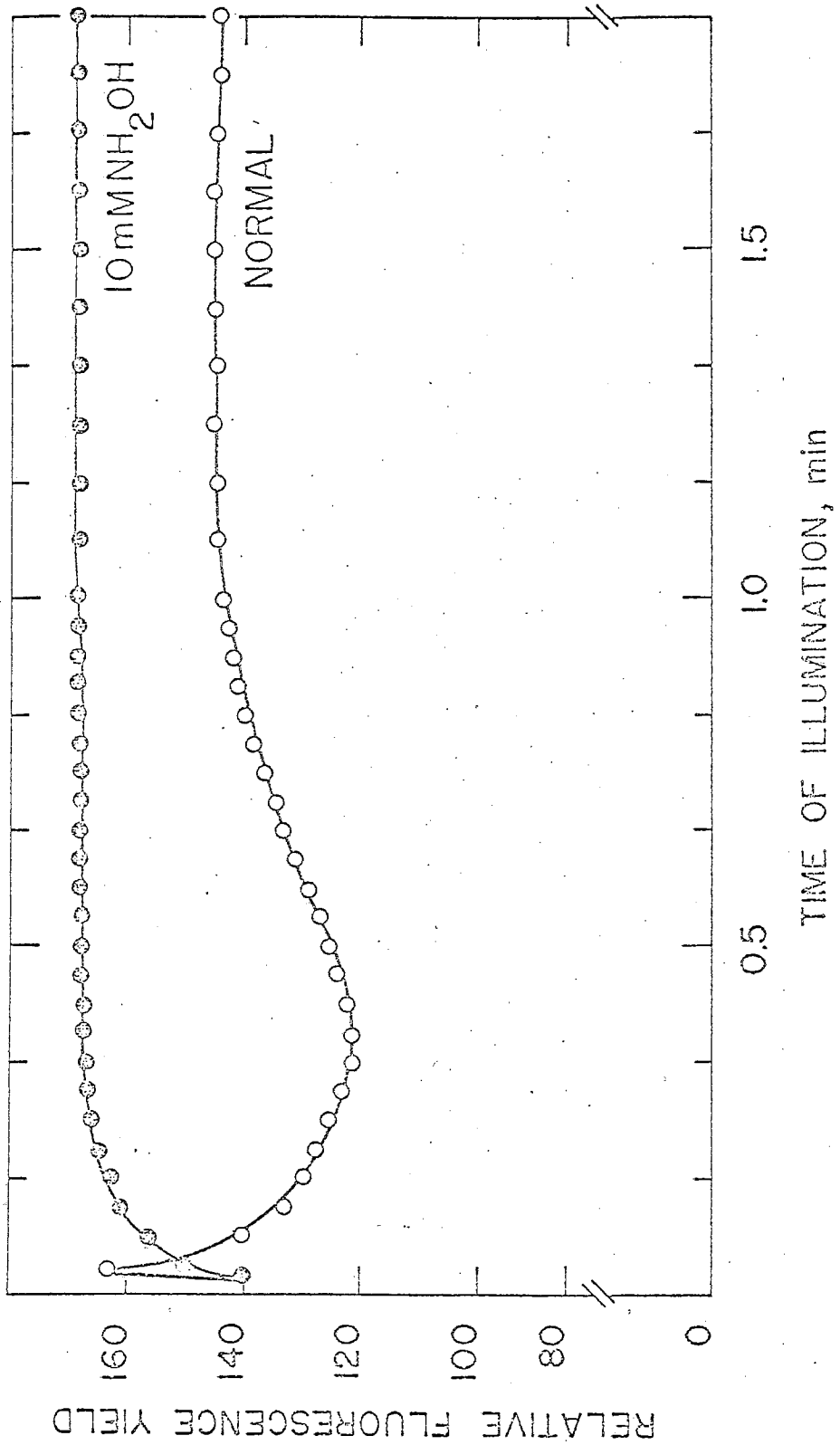

DCMU 10μM
DCMU NH2OH 10mM
10 mM NH2OH
NORMAL 0.1mM
2.5mM NH2OH
1 mM NH2OH
0.5mM NH2OH
P
D
I
O
RELATIVE FLUORESCENCE YIELD
TIME OF ILLUMINATION, sec
0.5
1.0
1.5
2.0
2.5
3.0
2
4
6
8

course of Chl a fluorescence (see also ref. 109).

We, therefore, believe that at low concentration, hydroxylamine only blocks the reaction between H_2O and oxidized donor Z, but does not feed electrons to Z^+. Thus Z^+ cannot donate electrons to Q and the fluorescence level remains low. On increasing the concentration, NH_2OH feeds electrons to PS II and consequently reduces the quencher Q and causes an increase in the yield of fluorescence. This donation of electrons by NH_2OH seems to occur at a slower rate than that by H_2O as the fluorescence transient resembles that of normal cells excited by a weak illumination.

The recovery of NH_2OH treated fluorescence transient requires a dark period and NH_2OH does not seem to have an effect on the dark restoration of Q (see below). On the other hand NH_2OH abolishes most of the PS decline in the yield of Chl a fluorescence. Figure 29 shows that 10 mM NH_2OH suppresses the SM rise also. Thus, the suppression of P in the presence of saturating amounts of NH_2OH may be due to slow donation of electrons to PS II. Figure 28, however, shows that ID decline persists even in the presence of NH_2OH. This suggests that D may be caused by the reoxidation of Q by A pool as suggested by Munday and Govindjee (83, 84). The abolition of slow changes in the yield is probably an additional secondary effect of NH_2OH. We have observed that if NH_2OH is removed from the samples by dialysis, 70-80% (measured as the yield at P) of the normal transient recovers. Cheniae and Martin (166) recently observed that they could not recover the complete oxygen evolving capacity by washing away all the NH_2OH. These results suggest that NH_2OH probably brings about some structural alterations of the chloroplast membrane--although electron transport between the two photosystems is functional in the presence of NH_2OH.

Figure 29. The slow fluorescence transient in normal and hydroxylamine treated Porphyridium. The fluorescence yield changes were monitored by a Keithley microvoltammeter and a Brown recorder. All other details of measurement as in Figure 28.

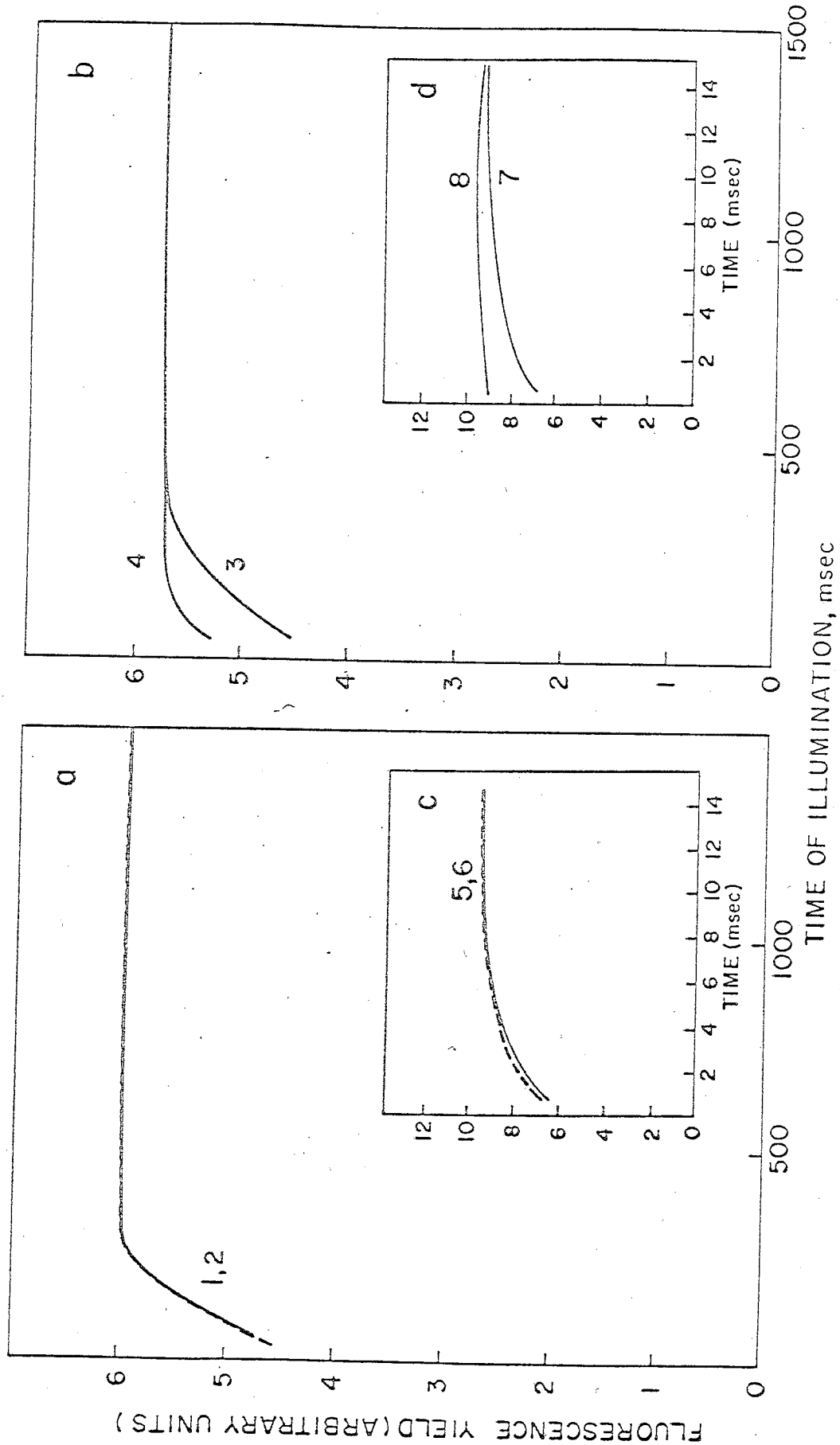

10 mM NH_2OH
NORMAL
RELATIVE FLUORESCENCE YIELD
160
140
120
100
80
0
0.5
1.0
1.5
TIME OF ILLUMINATION, min

Addition of 10 μM DCMU to normal or to NH_2OH treated samples causes rapid rise of fluorescence yield to a maximum level upon illumination (Figure 28) and then the yield remains constant without any further variation. It is well known that the high yield of Chl fluorescence in the presence of DCMU is due to the accumulation of QH (10). The OI rise in the presence of DCMU is the pure photochemical reduction of the acceptor Q by the donor Z (52, 53). Thus, in the presence of DCMU only the following reaction occurs:

$$ZP680Q \xrightarrow{h\nu_{II}} ZP680^{*}Q \longrightarrow {}^{+}ZP680Q^{-}$$

where P680 refers to the hypothetical reaction center of PS II. If the light is turned off, $Z^{+}Q^{-}$ recombine in the dark to reform reduced Z and oxidized Q. Because of this back recombination of $Z^{+}Q^{-}$, one observes the rapid rise of fluorescence yield from a low level to a maximum level upon subsequent illumination in the presence of DCMU. Figure 30 shows the result of an experiment designed to study the dark recovery of fluorescence transient in (a) the presence of DCMU alone and (b) DCMU and NH_2OH together added in the dark. Upon first illumination OI transient is clearly seen in both (curves 1 and 3). In the case of samples treated with DCMU, the transient recovers after 1-2 min of darkness (curve 2) but in the samples treated with both DCMU and hydroxylamine the initial transient did not recover (curve 4) even with a dark period of 10 minutes or more and it remained high. This experiment clearly indicates that the back recombination between $Z^{+}Q^{-}$ was prevented by hydroxylamine. Similar results were obtained in oat chloroplasts (see later) and Chlorella (insert Figure 30, c, d). Bennoun (167) independently obtained similar results

Figure 30. Recovery of the initial fluorescence transient in dark in Porphyridium (main figures: a, b) and in Chlorella (insert c, d). Curve 1, in (a) initial transient in the presence of 20 μM DCMU; the curve 2 the same transient repeated after a dark period of 10 minutes. Curve 3 in (b) initial transient in the presence of 20 μM DCMU and 10 mM NH_2OH. Curve 4, the same transient repeated after 10 minutes of darkness. Curve 5 in (c), initial transient with 10 μM DCMU in Chlorella; Curve 6, repeated after 1 minute of darkness. Curve 7 in (d), the initial transient in the presence of both 10 μM DCMU and 10 mM NH_2OH; curve 8, the same transient after 1 minute of darkness. Note the difference in time to reach the maximum fluorescence in Porphyridium and Chlorella; the intensity of illumination for the latter was higher than the former. The excitation intensity for Porphyridium was 9 Kergs $cm^{-2}sec^{-1}$ (from Mohanty et al., 154).


a
b
c
d
1,2
3
4
5,6
7
8
FLUORESCENCE YIELD (ARBITRARY UNITS)
TIME OF ILLUMINATION, msec
TIME (msec)

with spinach chloroplasts and Chlorella. (His work came to our attention after all our work was completed.) Using different concentrations of NH_2OH, Bennoun (167) observed the progressive decline of the OI rise curve in DCMU treated samples. Both our results and those of Bennoun confirmed the earlier suggestions of Izawa et al. (168) that NH_2OH might block a back reaction between Z^+Q^-.

This observation is in agreement with the earlier findings of Bennoun and Joliot (165) that (unlike H_2O) the photooxidation of NH_2OH does not require accumulation of more than one positive equivalent. It is clear that some residual photo-oxidation of NH_2OH occurs in the presence of DCMU. This has been experimentally shown to be the case (165).

The measurements of DLE with and without NH_2OH further confirms the suggestion that NH_2OH reduces Z^+ to Z:

$$Z^+ \; P680 \; Q^- \xrightarrow[NH_2OH]{\text{dark}} Z \; P680 \; Q^-$$

If the slow component of DLE is assumed to originate from the back recombination of Z^+ and Q^- (108, 109), one would expect a dramatic decline in the intensity of DLE in the presence of NH_2OH and DCMU. This is what was found (also see 108, 167). The details of the theory, assumptions, and kinetics of various components of DLE have been discussed by Stacy et al. (108, also see ref. 109).

The above results with fluorescence recovery transient as well as on DLE suggest that NH_2OH inhibits back reaction between Z^+ and Q^-. This inhibition of back reaction is due to reduction of Z^+ by NH_2OH and can explain the fact that at low concentrations, NH_2OH inhibits the photo-oxidation of water and at higher concentrations, it feeds electrons to

PS II. These results are in agreement with studies made earlier with spinach chloroplasts and the green alga Chlorella by other workers (165, 168).

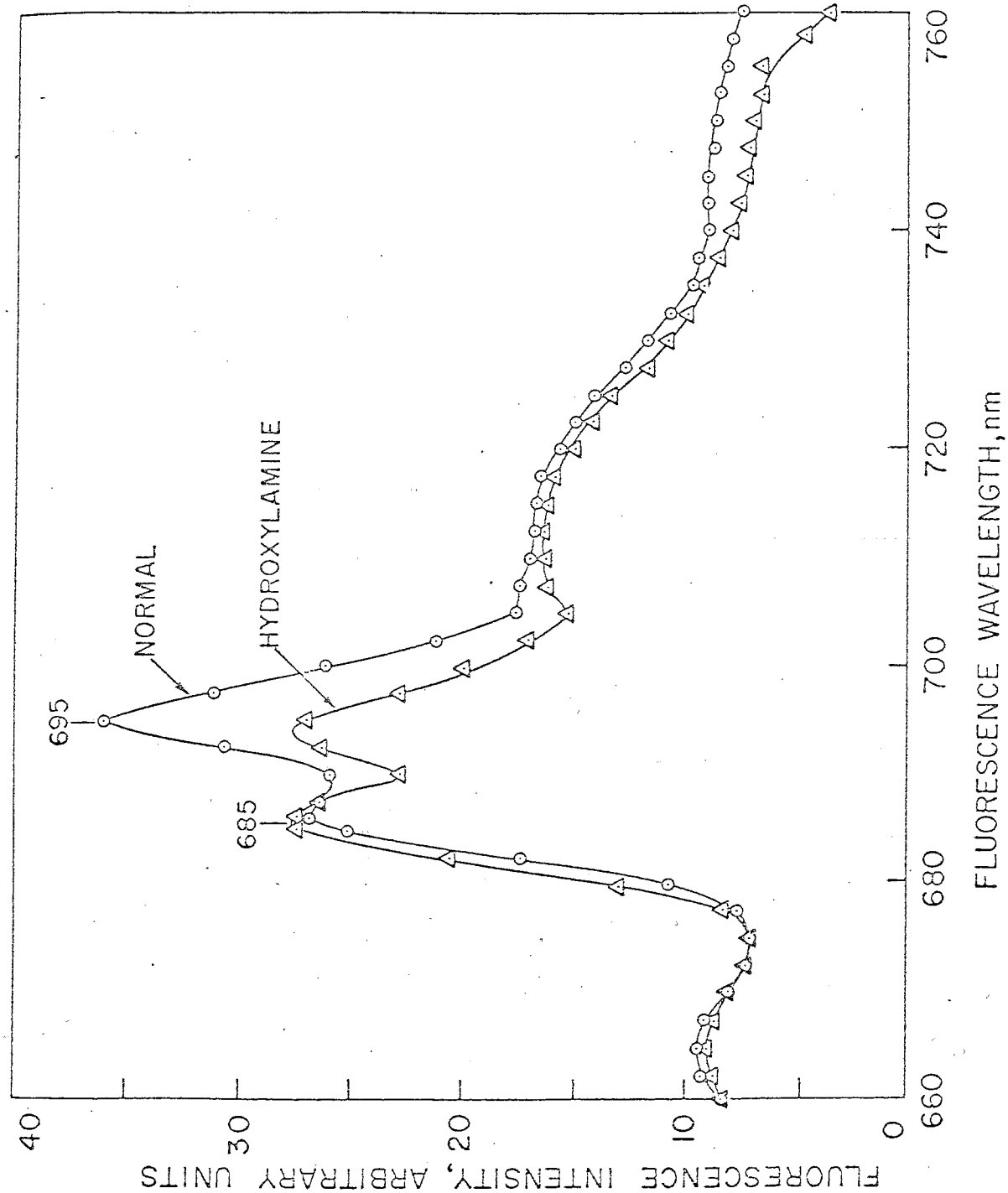
7.3 Emission Spectra at 77° K

As discussed in the introduction, there are three main emission bands of chlorophyll at 77° K, F685, F696, and F712 representing emission peaks at 685, 696, and 712 nm (see ref. 68 and literature citations therein). Earlier analyses (70, 75, 76, 169) of these emission bands in our laboratory indicate that F685 is emitted mainly from the bulk chlorophyll a of System II. F696 is associated mainly with the energy trap of photosystem II and F712 largely with chlorophyll a from the pigment system I. Figure 31 shows the emission spectra at 77° K of normal cells and cells treated with NH_2OH (in the absence of DCMU). If cells were pre-illuminated with white light from a 60-W tungsten lamp (for about 5 min) and then quickly frozen, there was a considerable lowering of the F696 band in the case of cells treated with hydroxylamine alone.* However, if the cells were not pre-illuminated, no lowering of the F696 band was observed. (The amount of lowering of this band with respect to F685 varied from culture to culture.) Normal cells with or without DCMU and cells treated with NH_2OH plus DCMU gave essentially similar emission spectra whether the cells were pre-illuminated or not.

It is known that the manganese-deficient cells have lower intensity of F696 band than the healthy cells (73). Studies of Cheniae and Martin (73, 166) indicate that NH_2OH extracts Mn^{2+} in dark. We do not believe

*At higher concentrations of NH_2OH besides the lowering of F696 band an increase in the long wave (712 nm) band was also observed.

Figure 31. Emission spectra of normal and hydroxylamine treated Porphyridium cruentum at 77° K. Curves normalized at 660 nm because we do not expect any change in the yield and emission of phycocyanin fluorescence; samples were pre-illuminated with white light (60 watt bulb placed at a distance of 20 cm.) and then cooled; hydroxylamine, 0.5 mM (from Mohanty et al., 154).


NORMAL
HYDROXYLAMINE
695
685
FLUORESCENCE INTENSITY, ARBITRARY UNITS
0
10
20
30
40
660
680
700
720
740
760
FLUORESCENCE WAVELENGTH, nm

that the lowering of F696 band in pre-illuminated NH_2OH treated cells is due to the loss of manganese as we do not see a decrease of this band in NH_2OH plus DCMU treated cells, and cells with NH_2OH that were not pre-illuminated. In the case of hydroxylamine treated cells, pre-illumination will cause a steady photooxidation in contrast to all other cases. Although in the case of cells with both hydroxylamine and DCMU, we expect a residual photooxidation of hydroxylamine (as shown by Bennoun and Joliot, 165), this small amount of photooxidation is not expected to cause a substantial lowering of the F696 band. Thus, we assume that photo-oxidation of NH_2OH leads to a quenching of fluorescence at 696 nm. It is, therefore, reasonable to assume that NH_2OH feeds, at lease in Porphyridium, very close to the System II reaction center as F696 has been suggested to originate in or near the System II trap (71).

8. Effect of Uncouplers of Phosphorylation on the Slow Fluorescence Yield Changes

Papageorgiou and Govindjee (87, 88) have shown the effect of uncouplers like atebrin and FCCP on the slow changes in the yield of Chl a fluores-cence in Chlorella and Anacystis. Figure 32 illustrates the effect of atebrin and FCCP together with CMU, DCMU on the SMT change in Porphyridium.

At low concentration (2-5 μM) of FCCP (Figure 32 (B)), the P to S decline rate becomes slower, but the O_2 evolution is not suppressed to any great extent. At higher concentrations (10 μM or more) a suppression of oxygen evolution is seen (Table III.2). Atebrin, at concentrations usually used to uncouple photophosphorylation in chloroplast reactions (10μM), inhibited SMT change to the same extent as 10μM FCCP (Figure 20 A). Phloridzin, the energy transfer inhibitor of ATPase, did not suppress SMT transient significantly.

Figure 32. Effect of electron transport inhibitors and phosophorylation uncouplers on the slow changes in fluorescence yield in Porphyridium. Left (A): Normal cells; with 10 μM DCMU; with 10 μM CMU; with 10 μM FCCP; 10 μM Atebrin and 2 mM Phloridzin. Right (B): slow transient with and without FCCP (2 μM or 5 μM). The yield was normalized at peak P. Other details are as in Figure 12 (from Mohanty et al., 153).

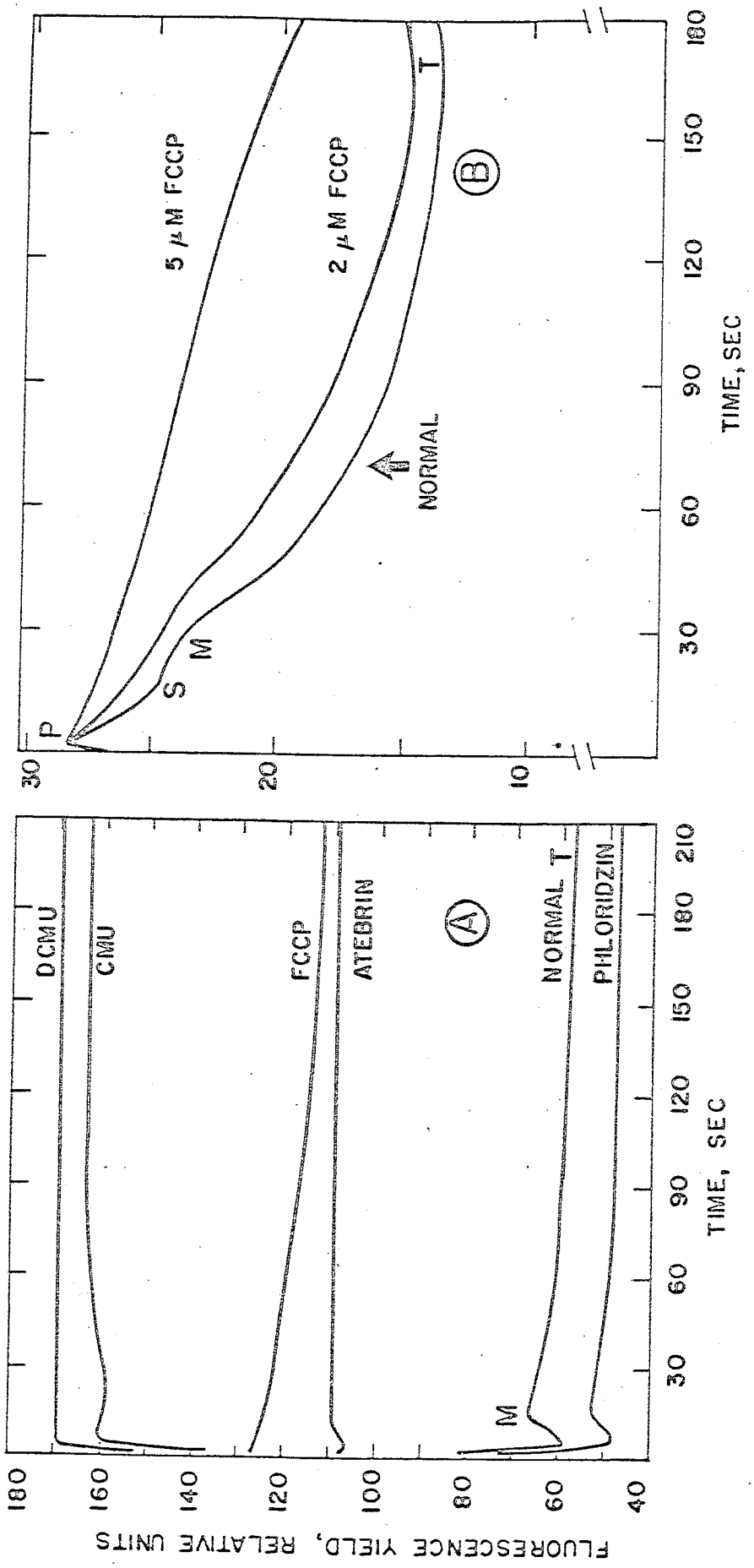

FLUORESCENCE YIELD, RELATIVE UNITS
180
160
140
120
100
80
60
40
DCMU
CMU
FCCP
ATEBRIN
NORMAL T
PHLORIDZIN
M
A
30
60
90
120
150
180
210
TIME, SEC
30
20
10
P
S
M
T
5 μM FCCP
2 μM FCCP
NORMAL
B
30
60
90
120
150
180
TIME, SEC

Table III.2

Effect of the FCCP on the Steady State (3 min.) O_2 Exchange Measured on a Rate Electrode in Saturating White Light

Concentration of FCCP*	Rate of O_2 Exchange (Evolution) (Relative Units)
1. 0	42
2. 2μM	40
3. 5μM	35
4. 10μM	19

*The other experimental details are same as described in the legend of Figure 26.

In the subsequent chapter we will report the effects of uncouplers like FCCP on the fast fluorescence transient in Chlorella.

9. Fluorescence Induction in Glutaraldehyde Fixed Cells

Intact cells of Porphyridium fixed with glutaldehyde did not show any change in the absorption spectrum of the pigments in vivo (Figure 33) (fixation of cells with aldehyde was made according to the method of Hallier and Park, 170). However, Cohen-Bazire and Lefort-Tran (171) have reported a depression in the concentration of phycoerythrin although no change in energy transfer from phycocyanin to Chl a was observed upon fixation (172). The DCPIP reduction in the fixed cells, suspended in a phosphate buffer at pH 6.8, was found to vary from 30 to 80 μmoles of dye reduced/mg/Chl/hr in saturating white light. These rates of electron transport were approximately the same as those of Hallier and Park (170). Figure 34 shows measurements of oxygen evolution with CO_2 as oxidant in

Figure 33. Absorption spectra of normal (solid) and fixed (dashed) cells adjusted at the red peak. Fixation was in 3% (v/v) glutaraldehyde in 0.05 M phosphate buffer pH 7.8 (from Mohanty et al., 153).

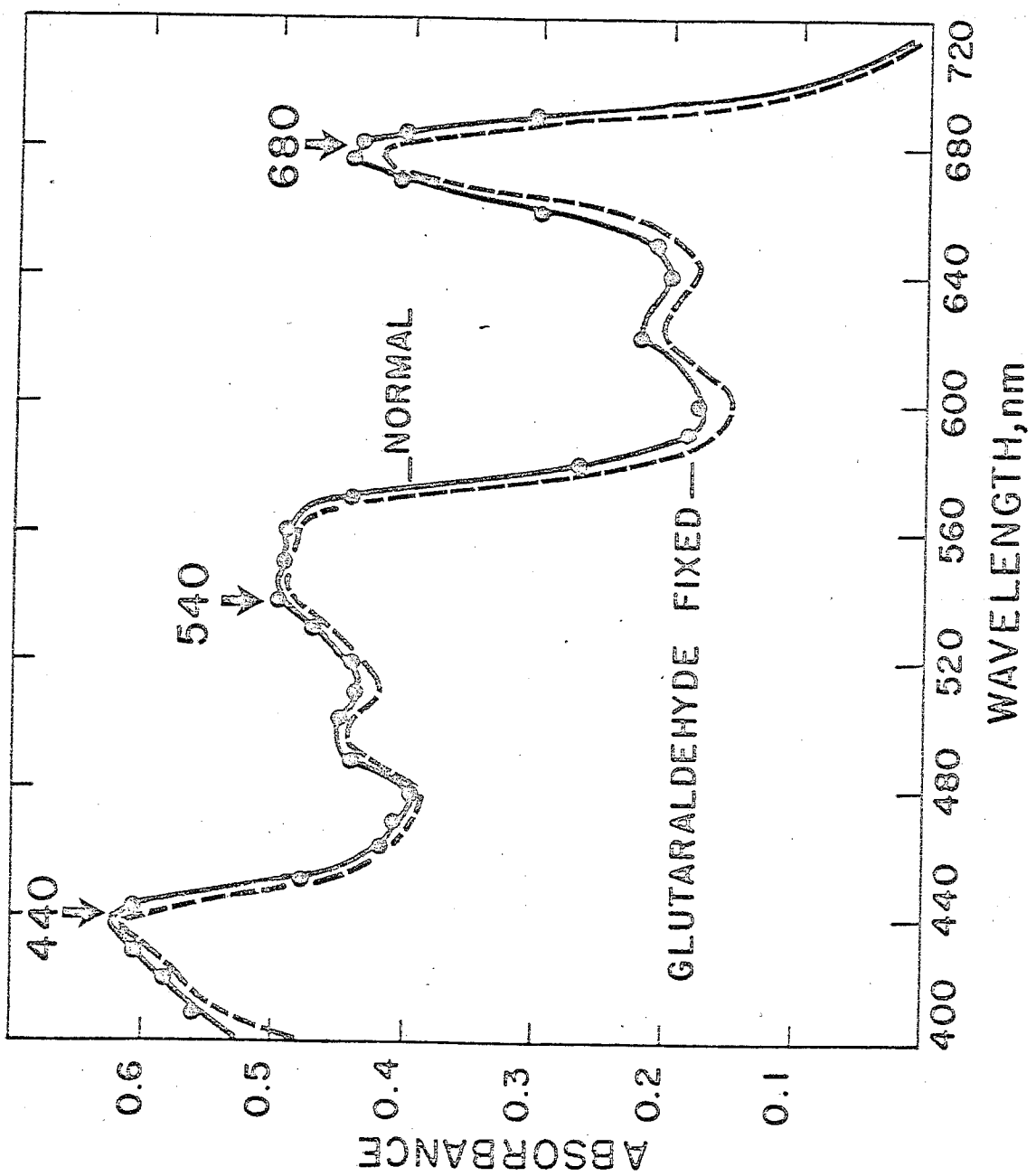
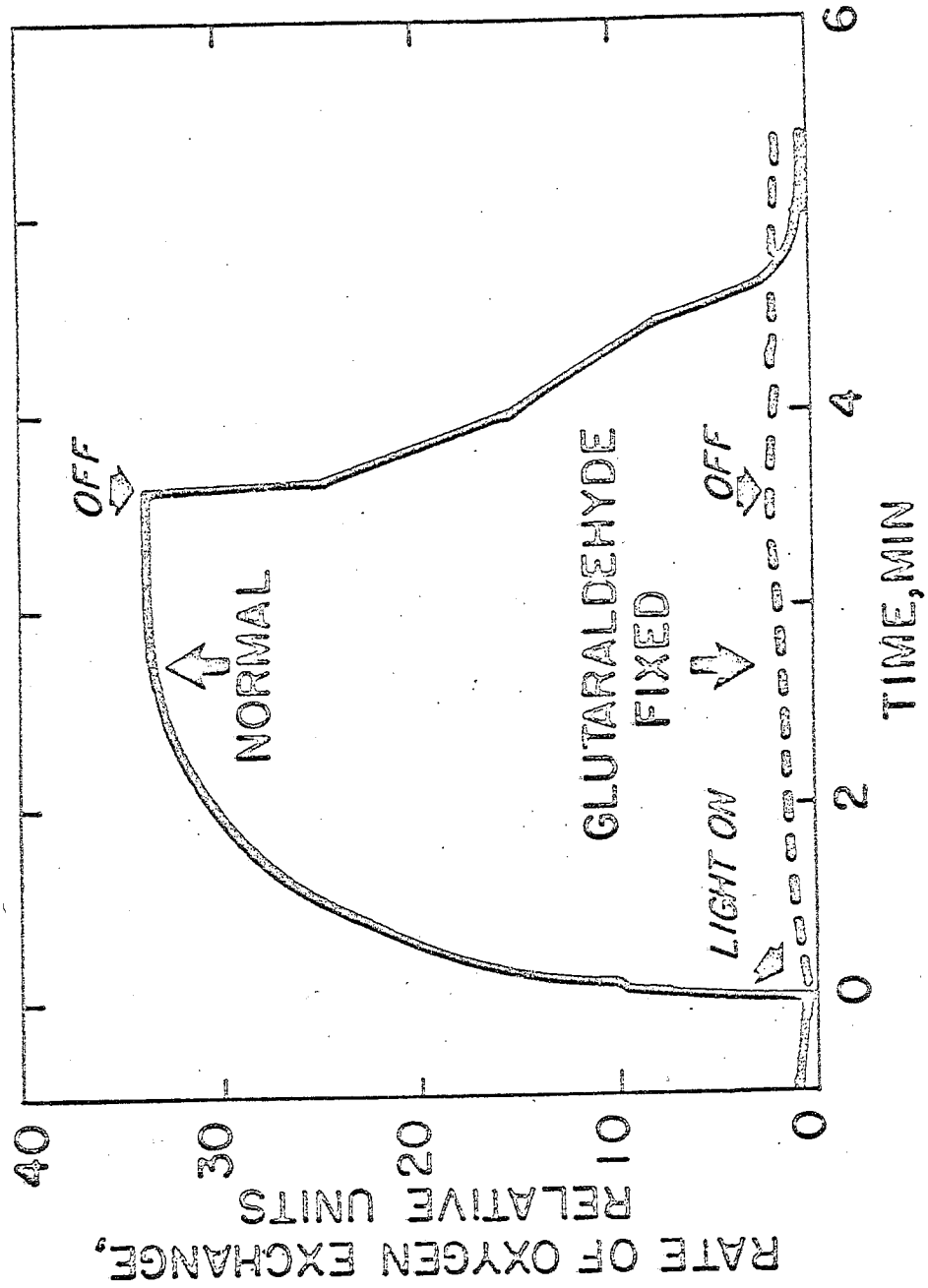

440
540
680
NORMAL
GLUTARALDEHYDE FIXED
ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
400
440
480
520
560
600
640
680
720
WAVELENGTH, nm

Figure 34. Time course of oxygen evolution in normal and glutaraldehyde fixed cells with CO_2 as an acceptor. Details of measurement as in Figure 13; (from Mohanty *et al.*, 153).


RATE OF OXYGEN EXCHANGE,
RELATIVE UNITS
40
30
20
10
0
0
2
4
6
TIME, MIN
OFF
NORMAL
GLUTARALDEHYDE
FIXED
LIGHT ON
OFF

both normal and fixed cells upon illumination with 540 nm light. No measureable oxygen evolution was observed in the fixed cells.

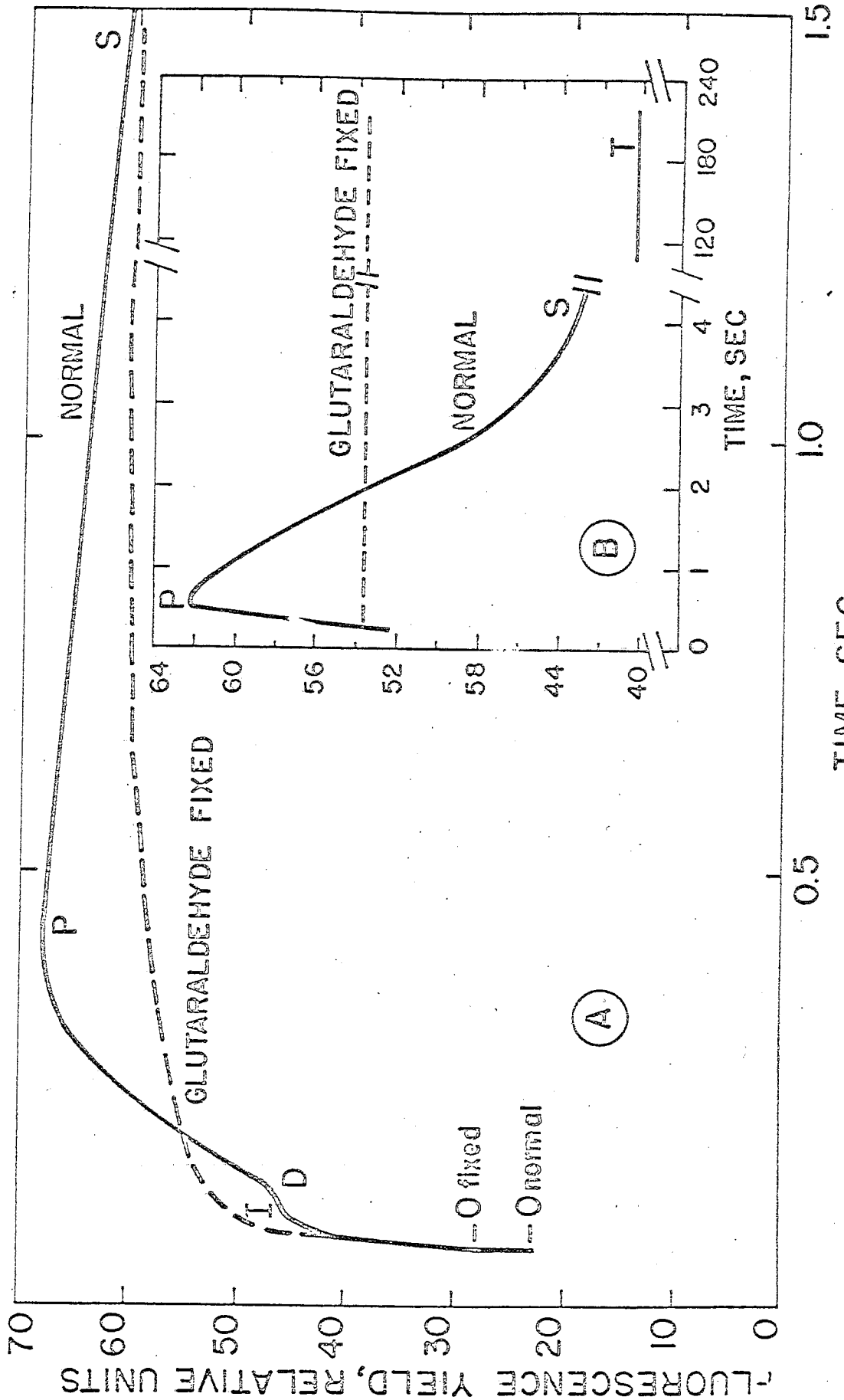
Glutaraldehyde fixed Porphyridium cells are devoid of DPSMT fluorescence change (Figure 35); the very fast OI phase is, however, present. We do not know if these fixed cells make ATP or not, although fixed chloroplasts are known not to phosphorylate (173,174). We, however, suggest that the loss of ability to fix CO_2 and thus evolve O_2 with CO_2 as oxidant, may be related to the possible unavailability of ATP needed for the Calvin-Benson cycle. The suppression of slow fluorescence changes, is, perhaps, due to the loss of ability for structural changes to occur upon fixation with aldehydes which are known to make molecular cross linkages between SH and $-NH_2$ groups of amino acids in proteins (175).

10. Summary and Conclusions

The time fourse of Chl a fluorescence yield in Porphyridium, after a period of darkness, is very similar to that of green alga - both in sec. and the min. range (Figures 12 and 15).

These changes are restricted to the fluorescence yield of Chl a, while that of phycocyanin remains constant throughout the induction period. Consequently, the emission spectrum of Porphyridium is variable (Figures 24 and 25). The room temperature emission spectrum shows a higher F685 at M than at S. We also confirmed the earlier observation of Lavorel that P has relatively more system II emission than S. Murata (162) observed a decrease in the intensity of F684 and F695 and increase of F712 at 77° K by pre-illuminating the cells of Porphyridium with system II light. From these observations Murata suggests that pre-illumination of pigment system II changes the efficiency of excitation transfer

Figure 35. The fast and the slow fluorescence transient in normal and fixed cells. Samples were adjusted to equal absorbance at the red peak (0.3). The main figure (A), the fast OIDPS transient; the O level was measured by giving short flashes as outlined in Chapter II. The dashed curve represents data for the fixed cells. Insert (B): The DPS transient and a portion of T level. Excitation as in Figure 12 A and B; (from Mohanty et al., 153).


NORMAL
GLUTARALDEHYDE FIXED
P
S
T
D
O fixed
O normal
A
B
TIME, SEC
FLUORESCENCE YIELD, RELATIVE UNITS
0
10
20
30
40
50
60
70
0.5
1.0
1.5
64
60
56
52
58
44
40
0
1
2
3
4
120
180
240

from chlorophyll a of System II to System I. Our results are in agreement with the suggestion of Murata (162); we observed an alteration in the efficiency of energy transfer from Chl a of system II to Chl a of system I without any change in the efficiency of energy transfer from phycocyanin to Chl a.

According to this hypothesis, P to S decline reflects a change from a high system II yield (State 1) to a relatively low system II yield (State 2) due to redistribution of quanta (1, 78, 155). The interconversion of these hypothetical states seem to be triggered by different colors of light (155, 162). PS II light accelerates the change from State 1 to State 2 rather rapidly (Figure 22) and PS I light prevents this interconversion (Figure 21). The high yield P (State 1) seems to decline much more rapidly in the dark to a low yield (State 2) (Figure 22) than in System II light, although a long dark period shifts it to the high yield State 1.

The experimental results obtained from Porphyridium also amplify the earlier suggestions that long term fluorescence changes are not associated with the oxidation reduction level of the primary acceptor Q and these changes may be indirectly linked to energy conserving processes. This view is supported by the following observations. (1) Absence of correlation with the rate of oxygen evolution as shown previously by other workers (87, 89) and in Figure 12. (2) Early saturation and intensity dependence of P-S and S-M phases as compared to DP phase, and the sensitivity of these changes to uncouplers (87, 88) and Figures 18, 19 and 32. (3) Furthermore, abolition of the slow changes in the fluorescence yield when a net artificial electron transport (from NH_2OH) occurs which probably inhibits the cyclic electron flow and ATP formation (Figure 29).

Nevertheless, these slow changes do not seem to be completely independent of the photosynthetic electron flow as these changes are sensitive to DCMU and CMU (Figure 32).

Results obtained with cells fixed with glutaraldehyde (Figure 35) further suggest that some kind of structural alteration is associated with these slow changes in the yield. However, the gross macroscopic alteration of structure may not be directly associated with the slow changes in the yield and the slow phase may originate from microscopic changes that precede the gross structural alterations.

Fixation of cells and chloroplasts with glutaraldehyde (or formaldehyde) immobilize the structural changes as shown by the absence of scattering and volume changes (174). Unfortunately, CO_2 reduction is also (Figure 34) abolished, perhaps, due to the lack of ATP formation. Fixed chloroplasts do not have the capability for transmembrane ion transport (174). Fixed cells have, however, normal electron transport ability in that they can reduce indophenol as well as viologen dyes although at reduced rates. Fixed cells do not exhibit slow fluorescence changes (Figure 35). These observations strongly suggest that electron transport per se contributes very little to the slow fluorescence changes in intact cells; the latter seem to be more intimately related to the energy coupling process than electron flow during photosynthesis.

It is not possible from these studies to evaluate if the slow fluorescence changes are related to proton transport across cellular membranes as Vredenberg (176) has proposed from electrical potential measurements with Nitella. However, we believe that fluorescence changes are related to the energy dependent structural changes of the thylakoid membranes. Other phenomena like the proton movement and phosphorylation affect

fluorescence via these structural changes. However, West and Packer (173) have shown that, upon illumination, glutaraldehyde fixed chloroplasts can take up protons - although at a reduced rate. (Movements of other ions into and out of chloroplasts are, however, largely eliminated.) If we assume that fixed cells behave as fixed chloroplasts, then it follows that proton uptake alone is not responsible for PSMT fluorescence yield change in whole cells.

In this study, we have also analyzed the effect of hydroxylamine on the fast transient (OIDPS) of Porphyridium (Figure 28). This potent inhibitor of photosynthesis blocks the flow of electrons from H_2O, and at higher concentrations, it donates electrons to PS II (Figures 27, 28). Because of the ability of NH_2OH to donate electrons without a requirement for the accumulation of oxidized equivalents, it inhibits dark recombination between Z^+ and Q^-. This function of NH_2OH was also observed independently by Bennoun (167) in Chlorella and chloroplasts.

Furthermore, the changes in emission property of the F696 band, which was previously shown to be associated with PS II reaction centers suggests that NH_2OH acts very close to the Photosystem II trap. Unlike DCMU, this inhibitor lowers the yield of F696 band. It seems that the F696 band reflects the redox state of the trap as well as other changes of the structural alteration or changes in the environment of the trap as initially observed by Cho and Govindjee (75).

IV. FLUORESCENCE INDUCTION IN THE GREEN ALGA *CHLORELLA PYRENOIDOSA*

1. Introduction

The chlorophyll *a* fluorescence transient of the green alga *Chlorella pyrenoidosa* was recently studied by Munday (58) and Papageorgiou (59). Munday (58) reviewed the literature of fast transients up to 1968. Papageorgiou (59) has analyzed the effect of light intensity, some electron transport inhibitors and uncouplers of phosphorylation on the slow changes in the fluorescence yield and on 0_2 evolution. Bannister and Rice (89) have similarly measured simultaneously 0_2 and fluorescence yield changes in the wild and a few mutant species of *Chlamydomonas*.

Both the data of Papageorgiou and Govindjee (87, 88) and that of Bannister and Rice (89) present supportive evidence that cyclic electron flow may influence the slow changes in the yield of Chl a_2 fluorescence. Bannister and Rice (89) reported a slow change in the yield of Chlorophyll *a* fluorescence in the presence of DCMU in *Chlamydomonas*, which was not observed by Papageorgiou and Govindjee (87) in *Chlorella*. Also, Papageorgiou and Govindjee (88) observed a suppression of fluorescence yield by uncouplers like FCCP and atebrin in *Chlorella*. These authors interpret this suppression of fluorescence yield to be due to an inhibition of both cyclic and non-cyclic photophosphorylation. There are also reports of quenching of fluorescence yield by FCCP in the presence of DCMU, both in intact algal cells (177) as well as in isolated chloroplasts (178, 179). The nature of this quenching is still unknown.

In this chapter, we will discuss the experiments that were designed to study the following:

(a) The effect of system I illumination during the slow SMT phase. This will enable us to assess the extent of dependence of the slow changes in the yield on the oxidation level of Q.

(b) The interdependence of slow changes in the yield, namely SMT phase, and the fast changes in the fluorescence yield (OIDP Phase).

(c) The investigation of the nature and extent of slow changes that are present when net electron transport is blocked by poisons.

(d) The effect of salts on fluorescence yield changes and its correlation with other phenomena.

We believe such types of investigations are necessary to gain insight into the cause of slow changes in fluorescence yield, and its relation to photosynthesis.

Figure 36 shows the typical characteristic points of the fast transient in Chlorella. The P/0 ratio is about 3.0. This ratio varied, depending upon the culture condition, from 2.0 to 4.0. Figure 37 (left) describes the changes in the amplitude at 0, P and S with various exciting intensities of illumination. There is no change in the slope of this F vs. I curve at 0 level, suggesting a constant yield of fluorescence at 0 (158, 161). The yields of fluorescence at S and P change with exciting intensity, but the increase in yield at P is much greater than at S (measured after 3-4 seconds after illumination).

These results are similar to that of Munday and Govindjee (83, 84) who observed a greater increase in the fluorescence yield at P than at S or at I. Figure 38 (right) is a plot of relative variable fluorescence yield at P and at S. We observe that the fluorescence yield at P does

Figure 36. The typical fast Chl a fluorescence yield changes in Chlorella pyrenoidosa exhibiting characteristic OIDPS transient. The cells were suspended in Warburg buffer #9 (0.1M, pH 9.2). The sample was illuminated with a blue green light (C.S. 4-96 plus C.S. 3-73 filter; intensity of excitation, 18 Kergs $cm^{-2}sec^{-1}$). The sample was kept in dark for 7 minutes before illumination. Fluorescence was measured at 685nm (half band width, 6.6nm). A corning C.S. 2-61 filter was used before the photomultiplier to guard the leak of excitation light. Fluorescence signal was recorded with Esterline Angus recorder. The 0 level was measured with a short duration (1/20 sec) weak intensity flash.

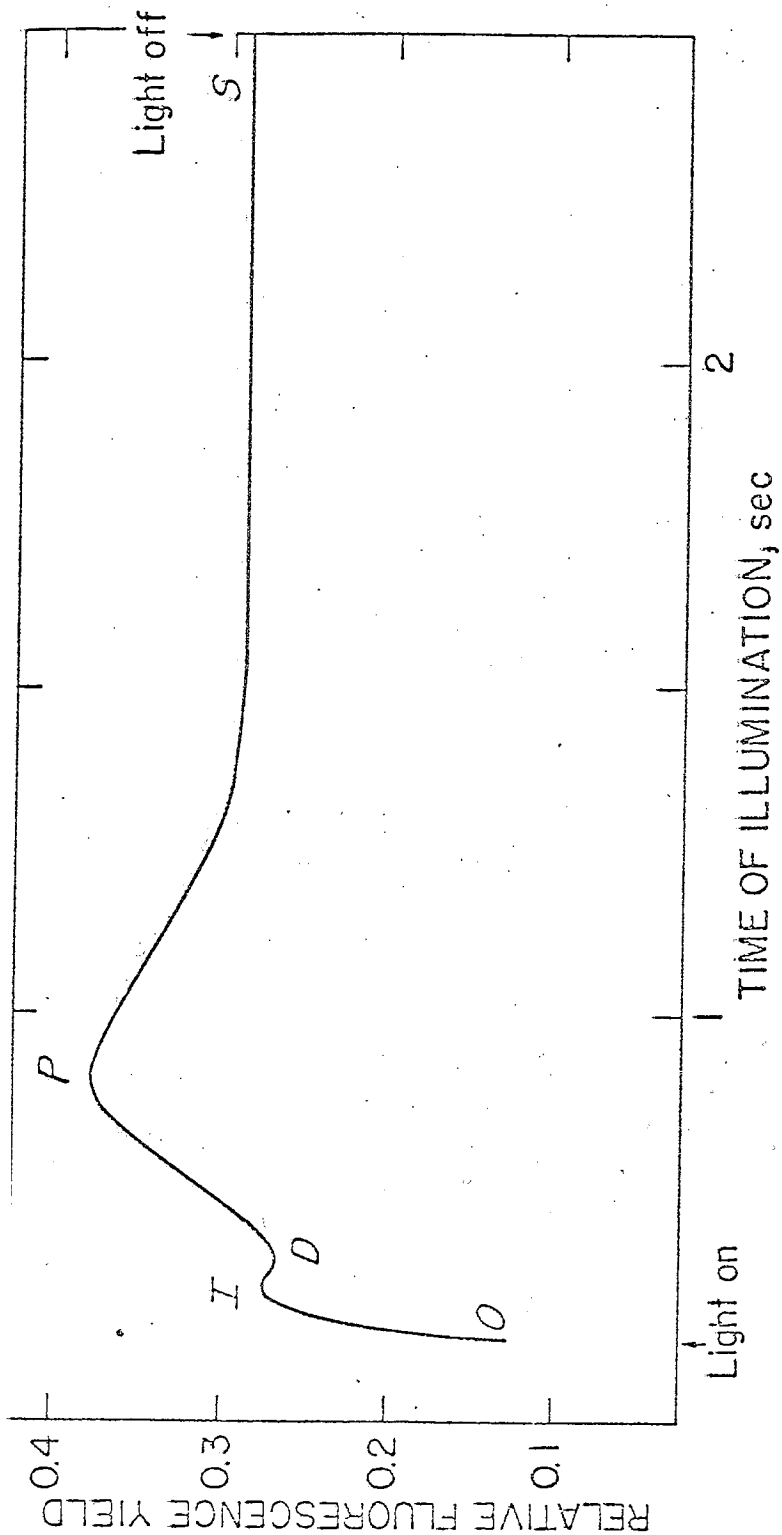
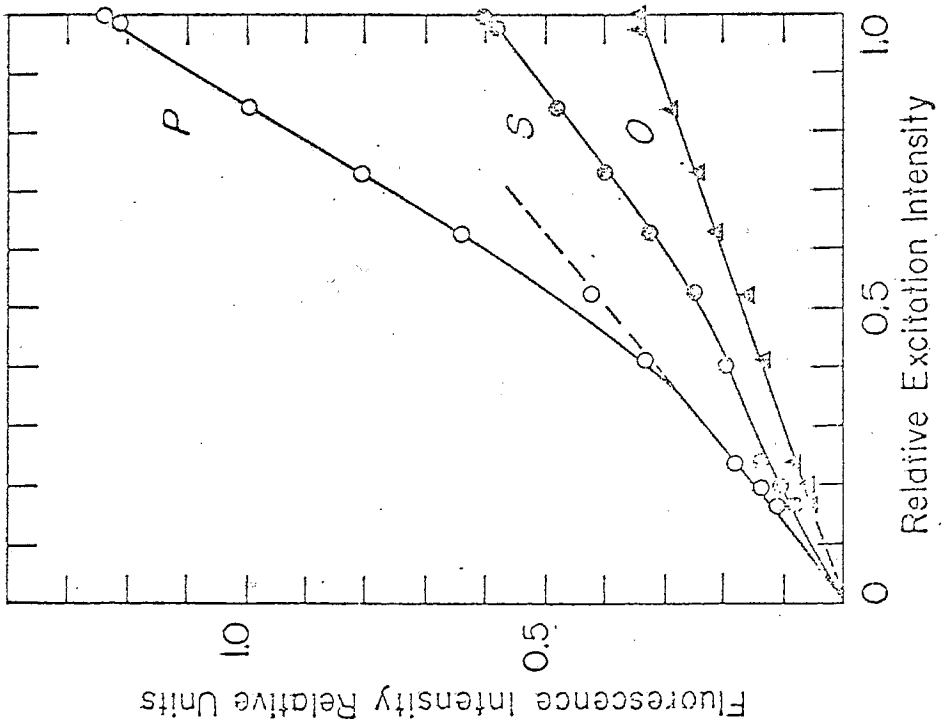
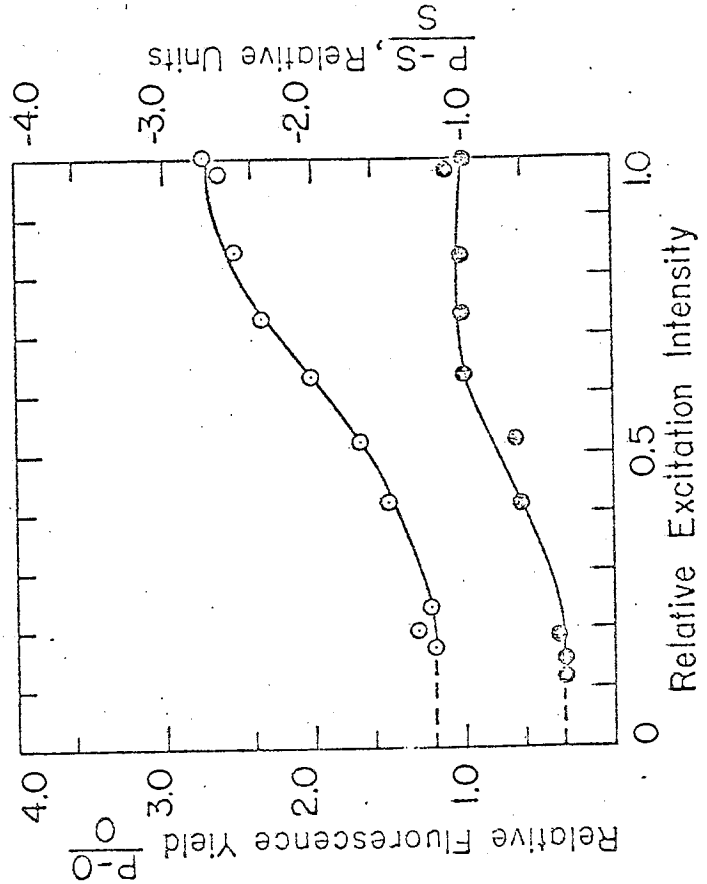

Light off
S
P
I
D
O
Light on
TIME OF ILLUMINATION, sec
2
1
0.4
0.3
0.2
0.1
RELATIVE FLUORESCENCE YIELD

Figure 37. (Left) Variations of fluorescence intensity and yield as a function of excitation intensity in Chlorella. Amplitude of fluorescence measured at 685 nm versus excitation intensity. Fluorescence transient was recorded with an oscillographic recorder. Illumination, blue light (C.S. 4-72 and C.S. 3-73; half band width, 6.6 nm); intensity 1.00 = 12 Kergs cm^{-2} sec^{-1}.

Figure 38. (Right) Relative variable fluorescence yield at P (open circles) represented as (P - 0)/0 and (P - S)/S (solid circles) versus excitation intensity.


Fluorescence Intensity Relative Units
1.0
0.5
0
0.5
1.0
Relative Excitation Intensity
P
S
O

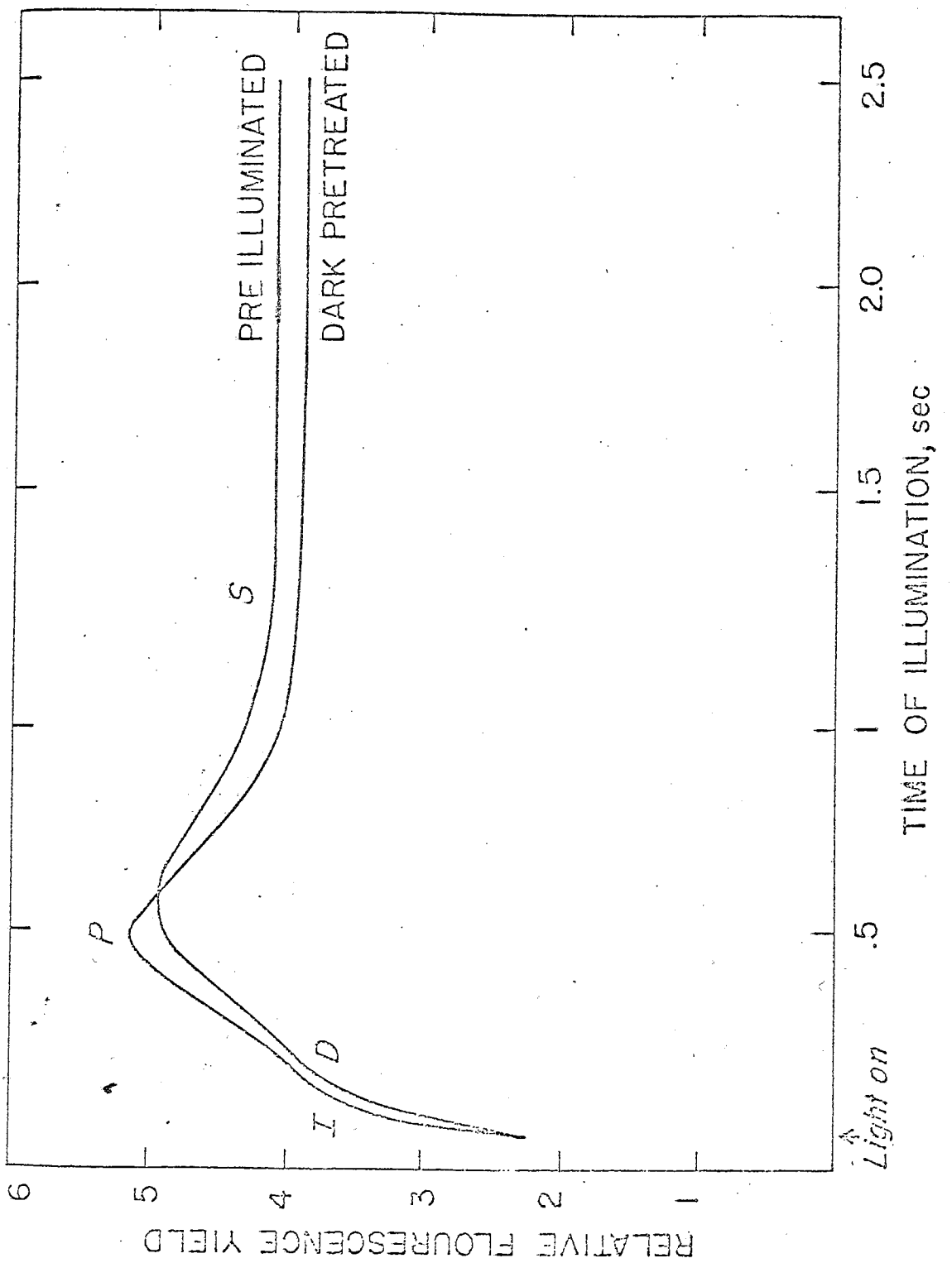

Relative Fluorescence Yield P-O / O
4.0
3.0
2.0
1.0
0
0.5
1.0
Relative Excitation Intensity
P-S / S, Relative Units
-4.0
-3.0
-2.0
-1.0

saturate at the highest intensity used. Since the fluorescence at 0 varies linearally with absorbed light (158, 161), (P-0)/0 represents the relative quantum yield of fluorescence at P. The (P-S)/S curve saturates at a lower intensity of excitation than at P.

2. Effect of Pre-Illumination on the PS Decline Phase of the Fast Fluorescence Transient

The effect of pre-illumination on oxygen evolution has been well documented (180), but, the effect of pre-illumination on the fast transient has only recently been described by Munday and Govindjee (84) in Chlorella, Bannister and Rice (89) in Chlamydomonas, Vredenberg (152) in Porphyra and Murata (151, 162, 181) in Porphyridium and in Porphyra. Figure 39 shows the typical transient of cells kept in the dark (7 minutes) and the cells pre-illuminated with 710 nm. light for 1 minute and then excited by a broad band system II light. As compared to the dark adapted sample, both DP rise and P-S decline are slower in the pre-illuminated sample as we observed in Porphyridium (Chapter III), and as Vredenberg (152) reported in Porphyra. These results also confirm the observation of Munday and Govindjee (84) in Chlorella. The decrease in P seen in Figure 39 may not be the true decrease in P as reported by Munday and Govindjee (84) as a two second dark time intervened between pre-illumination and excitation with system II light. It is, however, clear that system I pre-illumination even after a period of 2 sec dark intervention has slowed down the D to P rise and P to S decline. As discussed earlier, the slow D to P rise is explained to be due to rapid reoxidation of reduced QH by constituents of pool A which are expected to remain in a more oxidized state in system I pre-illuminated cells than in dark-adapted cells, but then the slower P to S decline cannot be explained on the basis of oxidation and

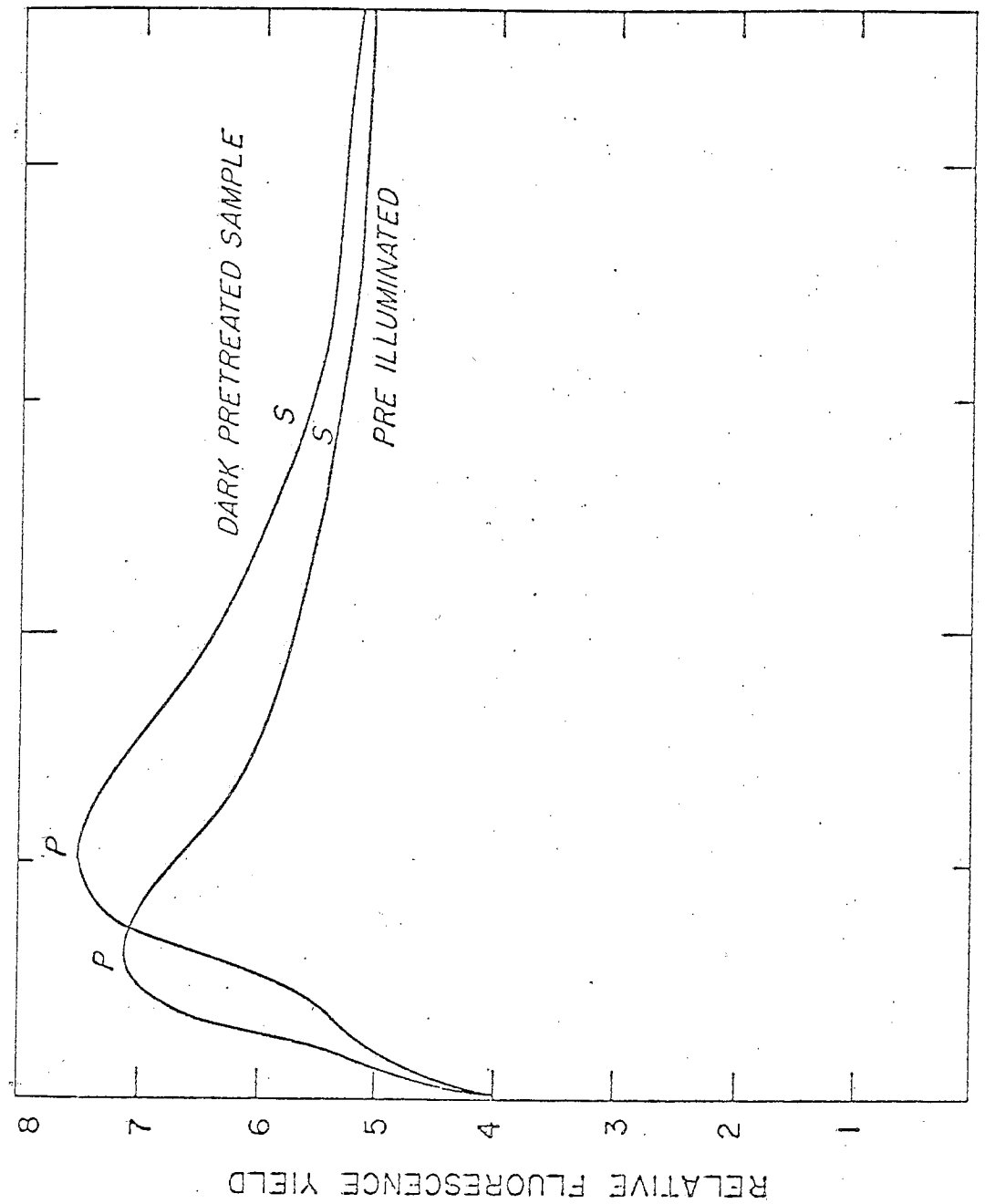
Figure 39. Enhancement of yield at S level by system I illumination in Chlorella. Dark pre-treatment, samples were kept in the dark for 7 minutes and then the fluorescence transient was measured. Pre-illumination, 710 nm light (intensity, $1.3 \times 10^{+2}$ ergs $cm^{-2} sec^{-1}$) for 60 seconds; transient was measured 2 sec after the cessation of pre-illumination; all other conditions as in Figure 36.


RELATIVE FLOURESCENCE YIELD
6
5
4
3
2
1
PRE ILLUMINATED
DARK PRETREATED
S
P
D
I
Light on
.5
1
1.5
2.0
2.5
TIME OF ILLUMINATION, sec

reduction of Q. Some other factor(s) must govern the slow P to S decline. Figure 40 shows the effect of short exposure of system II light on the fast transient of Chlorella. The upper trace is the transient of the usual 7 min dark adapted sample, and the lower (pre-illuminated) trace was measured as follows: The sample was kept in the dark for 7 minutes, and then system II broad band blue-green light was turned on until fluorescence reached P level and then it was turned off. After 2 seconds of darkness the same light was turned on again and the complete transient was recorded. This experiment may be regarded as a short term system II light pre-illumination. In the pre-illuminated sample, there is a rapid D-P increase and a faster P to S decline with respect to dark adapted sample. The rapid DP rise is due to slower dark reoxidation of QH by the constituents of the A pool which remain in a relatively more reduced state due to prior system II illumination. Again, the rapid P to S decline cannot be easily explained on the basis of oxidation reduction reactions. The results of Figures 39 and 40 suggest that system I and system II light act in an antagonistic manner on this unknown factor(s) which regulates the P to S decline as they do in redox reactions of Q: PS II light accelerates while PS I light retards it. These results confirm the findings of Munday and Govindjee (84, 85) and I report here my data to emphasize that the fluorescence change associated with P to S decline cannot be easily explained on the basis of reoxidation of the primary acceptor QH in spite of the apparent complementarity of the rate of O_2 evolution and fluorescence (89).

In the following section, we will describe some results of the interaction of system I and system II light during the SMT transient of Chlorella.

Figure 40. Effect of brief system II pre-illumination on the Chl a fluorescence transient in Chlorella. Dark pre-treated, usual transient with 7 minutes darkness; pre-illuminated with blue-green light until P level was reached and then the excitation light was turned off and again it was turned on and the transient recorded. The two transients are adjusted at 0 (2.53 scale units). The sample containing approximately 80 μg Chl/3 ml, was suspended in phosphate buffer (0.05 M, pH 8.0) plus 0.01 M NaCl. Excitation, blue-green light, peak at 480 nm (band width, 120 nm); intensity = 14 Kergs $cm^{-2}sec^{-1}$.


RELATIVE FLUORESCENCE YIELD
8
7
6
5
4
3
2
1
Light on
1
2
TIME OF ILLUMINATION, sec
DARK PRETREATED SAMPLE
PRE ILLUMINATED
P
P
S
S

3. Time Dependent Quenching of Fluorescence by System I Light During the Slow Fluorescence Transient

In 1960, Govindjee et al. (9) reported a decrease in fluorescence yield of chlorophyll a of system II when Chlorella was exposed to system I light ($\lambda_1 > 685$ nm). This observation was extended to spinach chloroplasts and several other algae by Duysens and Sweers (10). The quenching of $Chla_2$ (Chla excited by system II light) fluorescence by system I light is explained by the antagonistic effect of two lights on the primary electron acceptor of system II. We have measured the time dependence of this quenching effect on the various regions of the slow induction phase. The details of the experimental protocol have been published earlier by Mohanty et al. (182) and are briefly summarized below.

Fluorescence was excited by intense blue light (λ peak, 480 nm; half-maximum band width, 100 nm; absorbed quanta, approx. 4×10^{14}/sec $\cdot$ cm^2), and was measured at 685 nm (half-band width, 5 nm). Quenching was caused by 710 nm light (half-band width, 13 nm; absorbed quanta, approx. 5×10^{11}/sec $\cdot$ cm^2). 546 nm light of similar band width and intensity caused no change in fluorescence yield. Filters were used to transmit fluorescence and to prevent nearly all the exciting light from entering the measuring monochromator. With 710 nm light alone, the light leak was very low, less than 2% of blue-excited fluorescence; it was measured and subtracted from the blue + 710 nm excited fluorescence.

In our experiments a series of 5 minute dark-5 minute (blue) light cycles were used. Blue light preferentially excited system II. The system II fluorescence yield (observed at 685 nm) undergoes a slow induction labelled S, M and T. Interaction with system I was studied by adding 710 nm light at different times during the induction of fluorescence

mentioned above.

The 710nm light caused no fluorescence quenching in the first 5-10 seconds of the (blue) light period (Figures 41 and 42). Instead, the fluorescence yield increased slightly (1-5%). We considered the possibility of an apparent increase due to nonlinearity in the fluorescence (F) versus intensity (I) curve and the fact that about 10% of the 710 nm light quanta excite System II. However, the intensity (I) of blue light was high enough so that the fluorescence (F) was in the second linear portion of the curve F versus I. This linearity was tested by adding 546 nm light (absorbed almost equally in the two systems; the intensity of this light was adjusted to give the same signal as 710 nm light). No change in the fluorescence yield was observed with 546 nm light in contrast to results with 710 nm light. The small increase in fluorescence yield caused by 710 nm light during the first 5-10 seconds of System II light is, therefore, considered real. The absence of quenching during this period was observed in Chlorella cells suspended in their growth medium as well as in Warburg buffer No. 9.

After 10 seconds of blue light, however, 710 nm light caused quenching (Figure 41), and this quenching increased up to 0.5 to 2 minutes, and then decreased slowly until about 3-5 minutes (Figure 41). The amount of quenching varied from culture to culture but remained constant in one sample; it varied from 7 to 18%. When the maximum quenching was 7% (Figure 41 (a)) the quenching effect reached a peak at about 0.5 to 1 minute and the effect declined to 3% by 3 minutes. However, when the maximum quenching was 10 to 15%, the peak occurred at 1-2 minutes and the decline in quenching effect was still high at 5 minutes (10 to 14%) (Figure 41, b).

Figure 41. Percent change in fluorescence yield due to 710 nm light at various times during the SMT fluorescence transient. Top (a): experiments with three different cultures (thick line with open triangles, solid circles and crossed circles) are shown; the scale to the left. For comparison, a typical SMT transient is shown with thin line with small open circles; scale to the right. Bottom (b): similar experiment with two other cultures (large open circles and solid squares) exhibiting higher percentage of quenching of the fluorescence yield by 710 nm supplementary light. The thin-line curve (small open circle, with abscissa scale to the right) represents the usual SMT fluorescence transient recorded with a Keithley microvoltammeter and a Brown recorder (from Mohanty et al., 182).

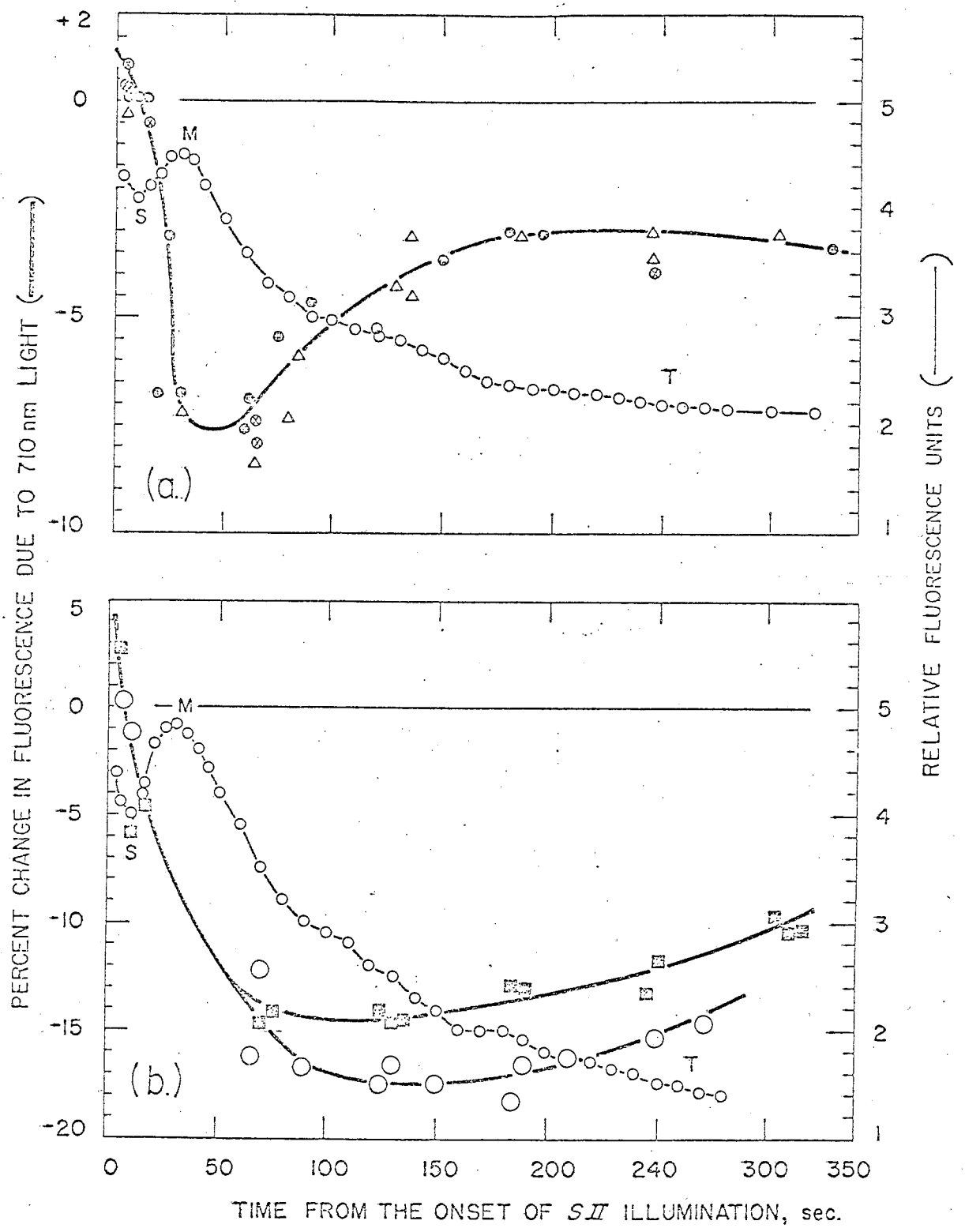

+2
0
-5
-10
M
S
T
(a.)
5
0
-5
-10
-15
-20
M
S
T
(b.)
PERCENT CHANGE IN FLUORESCENCE DUE TO 710 nm LIGHT
RELATIVE FLUORESCENCE UNITS
0
50
100
150
200
240
300
350
TIME FROM THE ONSET OF SII ILLUMINATION, sec.

Figure 42. Percent change (ΔF) in fluorescence yield in Chlorella pyrenoidosa caused by supplementary illumination of 710 nm. Fluorescence measured at 685 nm excited by blue light (absorbed quanta, approximately 4×10^4 quanta $cm^{-2} sec^{-1}$). (A): 710 nm far red light given after 5 sec. of blue (mainly PS II) illumination, (B,C): Same given after successive periods of 40 sec. The level of fluorescence prior to 710 nm illumination was adjusted to read zero; the light leak due to 710 nm light alone has been subtracted (from Mohanty et al., 182).

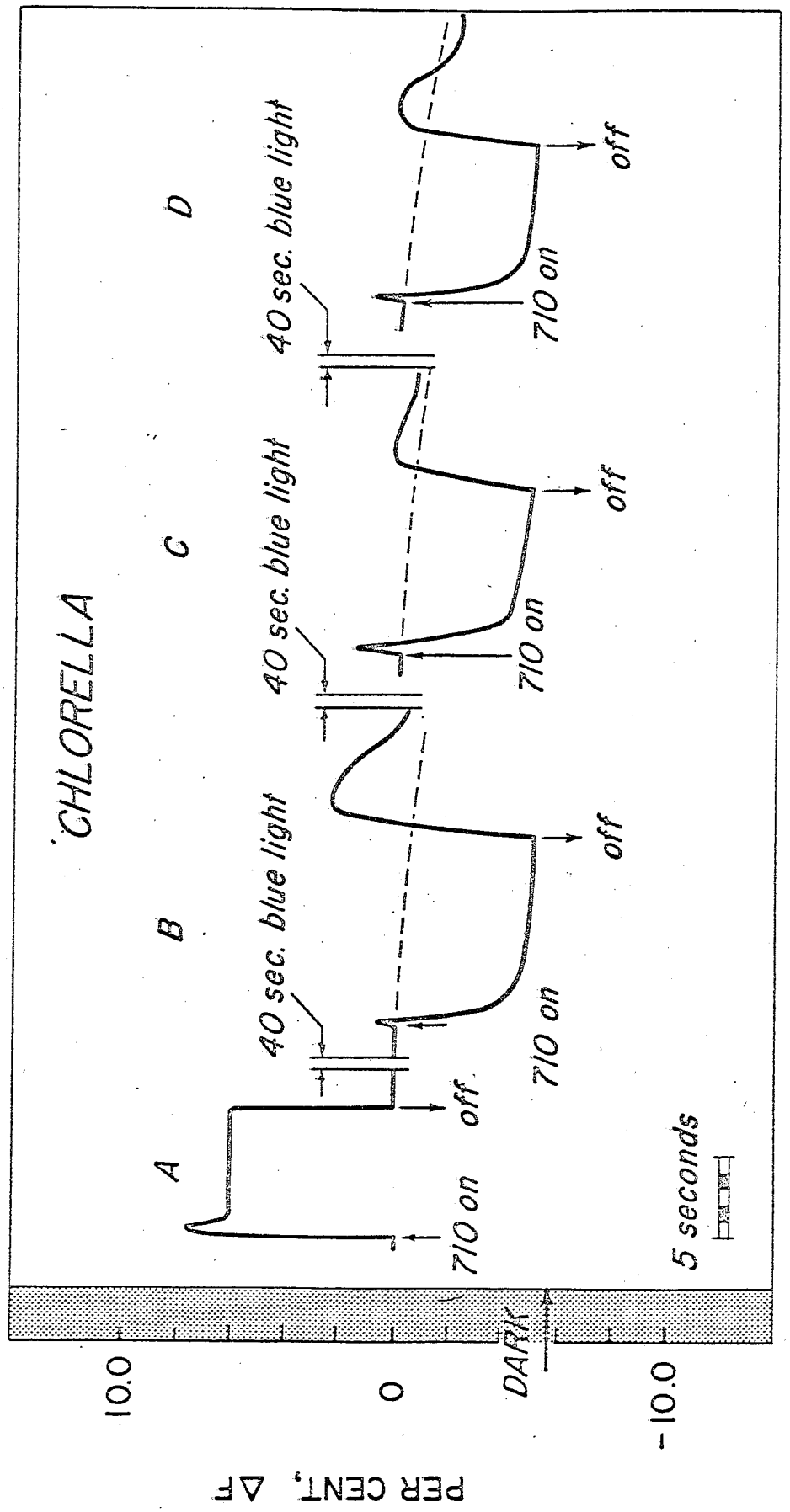

'CHLORELLA
A
B
C
D
40 sec. blue light
710 on
off
5 seconds
DARK
10.0
0
-10.0
PER CENT, ΔF

Figure 42 shows the traces of the transient imposed by system I light. System I light added after 5 seconds of blue illumination caused an enhancement (A) and the same light caused a quenching if added later (B, C, D). In all trials of 710 nm light the decrease in yield was stabilized only after about a second which suggests a slow interaction associated with the quenching process. Upon cessation of system I light (710 nm light) the fluorescence yield increased (1 to 4%) and then declined to the original steady state level. This transitory rise observed at the end of 710 nm illumination was completed within 1 to 2 seconds and recovered to original level with a half time of 5 seconds or more. (This confirms the observation of Murata (162) in Porphyridium and those of Bonnaventura and Myers (155) in Chlorella.)

We were surprised by the observation that such a weak system I light could bring about a large decrease in the fluorescence yield. But, a quenching was observed after 5-10 seconds of blue illumination in more than 20 separate cultures--only in two cases we did not observe any appreciable quenching. This quenching of Chl $\underline{a}_2$ fluorescence by system I light could, perhaps, be explained as a light induced acceleration of photoreaction I which in turn accelerates oxidation of Q, the quencher of fluorescence. On the other hand, the slow changes in the yield seen after the cessation of 710 nm illumination cannot be explained on the basis of changes in electron transport alone.

We observed that if the intensity of System II (blue) light was lowered to 24% of the maximum intensity (1.5×10^4 ergs $cm^{-2} sec^{-1}$) thereby putting the intensity near the lower portion of the F vs. I curve, there was no quenching. This is probably because larger amounts of Q remain in the oxidized state and the addition of PS I light causes no significant

change in the oxidation state of Q.

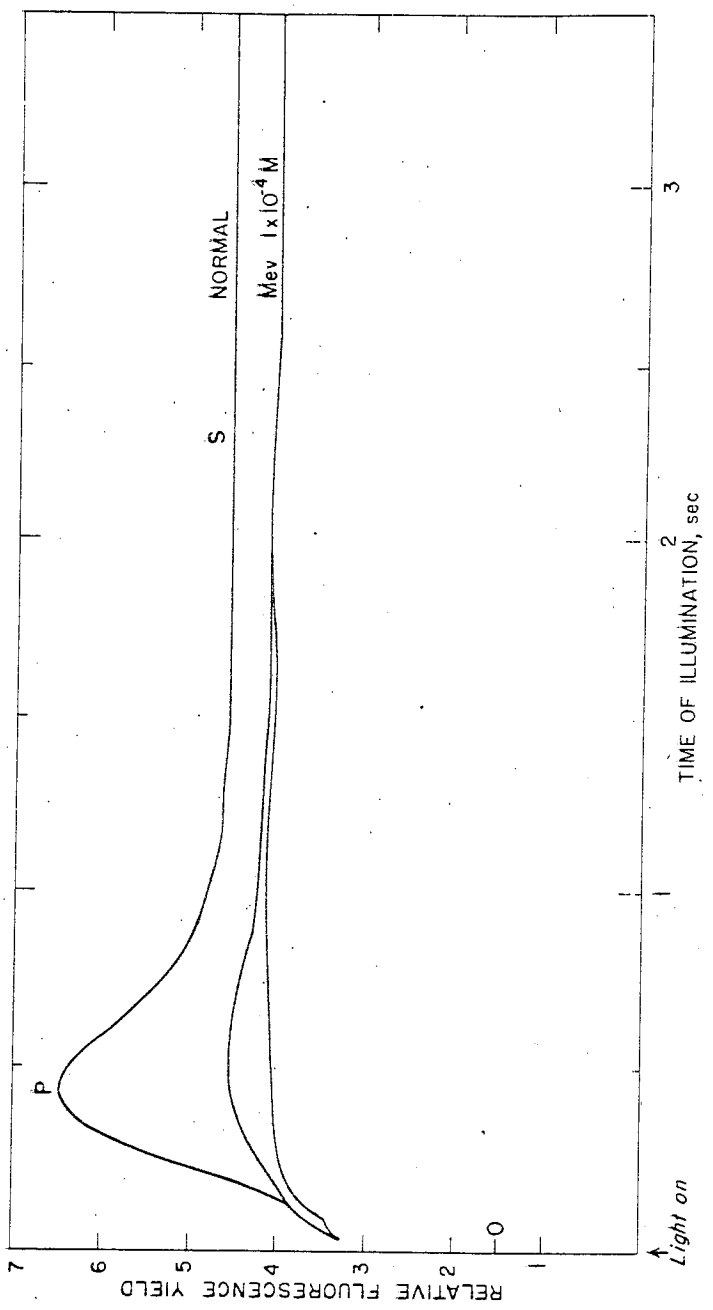
Addition of 10 μM DCMU abolished the quenching effect as well as the transitory increase after 710 nm light was turned off. A very small 1-2% increase was, however, observed. These results were confirmed in 4 different cultures. Addition of methyl viologen (0.1 mM) abolished the temporal fluctuations in the quenching but only small constant amount of quenching persisted. Methyl viologen caused a decrease in the yield at P and a suppression of the slow changes in the fluorescence yield (see later). Thus it appears that a complete photosynthesizing system and the presence of SMT changes are needed to show a variation in the quenching effect with time. It is clear that for an equal level of fluorescence yield at two different points (Figure 41 a and b) on the SMT phase, the amount of quenching caused by the same intensity of system I light is different. Thus, one cannot quantitatively correlate the extent of quenching by PS I light with the level of reduced Q. This together with the temporal increase in the yield after 710 nm system I light illumination (Figure 42) strongly supports the view that other factor(s) besides Q govern the level of fluorescence during the slow changes in the yield of Chl a fluorescence.

4. Effect of Electron Transport Cofactors and Uncouplers of Phosphorylation on the Fluorescence Yield Changes

4.1 Cofactors

Munday and Govindjee (85) showed that the low potential dye, methyl viologen, quenches the fast fluorescence transient of Chlorella. Figure 43 shows the effect of 0.1 mM methylviologen after 1-2 min (middle trace) and after 10 min of incubation (bottom trace). It is clear that this low potential dye (E_m = -440 mV) completely suppresses P and lowers S. In the presence of 0.1 mM methyl viologen, (Mev), the OI phase is not affected;

Figure 43. Recorder tracing of the fast Chl *a* fluorescence transient of Chlorella with and without methyl viologen. The cells were suspended in carbonate bicarbonate buffer (0.1 M; pH 9.2). Upper trace, untreated sample, middle trace 1 minute after addition of 0.1 mM methyl viologen (Mev); lower trace 10 min after addition of Mev. In each case the sample was kept in darkness for, at least, 7 min. Other details of measurement as in Figure 36.


RELATIVE FLUORESCENCE YIELD
7
6
5
4
3
2
1
0
P
S
NORMAL
Mev 1x10⁻⁴ M
Light on
1
2
3
TIME OF ILLUMINATION, sec

this was clearly shown by Munday and Govindjee (85). We observed a rapid restoration of the OI transient in the presence of MeV ($t_{\frac{1}{2}}$ = 0.5 sec). The O level was not affected at this concentration of MeV. As MeV is known to pick up electrons from the primary acceptor of photosystem I in isolated chloroplasts, Munday and Govindjee (85) interpreted their results of suppression of P by MeV to be due to an acceleration of the rate of electron flow. In their language, the P is due to a "traffic jam" of electrons beyond X and MeV, being highly autooxidizable, by-passes this limiting step.

Table IV.1 shows the effect of MeV in inhibiting photosynthesis (O_2 evolution). Preincubation with 0.1 mM MeV for 10-15 minutes is enough to cause inhibition of O_2 evolution; the latter is replaced by a small amount of O_2 uptake (as measured in saturating the white light). Higher concentrations of MeV increase the light stimulated oxygen uptake. The rate of O_2 uptake in light decreases with time. We could not restore the ability of cells to evolve oxygen by subsequent washing with buffer.

Table IV.1

Effect of Methyl Viologen (MeV) on the Net O_2 Exchange in Saturating White Light

Concentration of MeV	Net O_2 exchange relative units
0	+4.5
0.1 mM	-0.5
1.0 mM	-2.5
5.0 mM	-3.0

(+) O_2 evolution (-) O_2 uptake as measured on the rate electrode with PO_4^--KCl buffer (pH 7.0).

Similar observations have been made by Turner et al (183), using another viologen dye diquat in Chlorella vulgaris. Inspite of the difficulties of restoring the oxygen evolution ability, our results indicate that a light induced O_2 uptake occurs in the presence of methyl viologen in intact cells as in the case of isolated chloroplasts.

In a preliminary experiment we compared the extent of PS I light induced oxidation of Cyt f (measured at 419 nm; 3 mm slits, 10 nm half band width) in the presence and in the absence of 1 mM methyl viologen. In the presence of methyl viologen, the steady state extent of Cyt f oxidation by 710 nm light was higher (2 to 2.5 times) than control cells. Admittedly, this observation needs to be confirmed by constructing action spectra for Cyt f oxidation, with and without MeV. However, this preliminary observation, together with the observation that MeV causes oxygen uptake in whole cells of Chlorella, indicates that methyl viologen accelerates the electron flow and bypasses the $NADP^+$ reduction. Thus, the suggestion of Munday and Govindjee (84, 85) that the DP rise is brought about by an accumulation of QH, AH, and XH and that MeV prevents the accumulation of these reduced carriers, seems to be consistent with our observations. P to S decline, thus, may be regarded as a consequence of this transitory accumulation of the reduced electron transport carriers (QH, AH, XH, etc.) which cause the closure of PS II traps thereby facilitating a spill over of excitation energy from PS II to PS I (see later). Methyl viologen, on the other hand, accelerates electron transport and keeps the traps open. On the basis of this logic, one would expect a higher extent of spill over in the presence of DCMU; this does not happen, as the fluorescence yield remains high in the presence of DCMU. Moreover, even in the presence of methyl viologen addition of DCMU increases the

yield. We may speculate that an electron flow is, somehow, required to promote the spill over of quanta from PS II to PS I.

As discussed in the introduction, Duysens (86) recently reported a quenching of the Chl fluorescence by P by a bright microsecond flash of system I light. This result indicates that all the intersystem intermediates are not in the reduced state at P. The fluorescence yield at P in bright light is also lower than the yield in the presence of DCMU which may indicate that probably all Q are not reduced at P. It seems that even the fast fluorescence induction phase is also influenced by factors other than Q.

Figure 44 shows typical SMT transient (upper curve) and the effect of 1 mM MeV on these slow changes (lower curve). Most of the SMT variation is replaced by a slow decline to a low level within 10 seconds of illumination and then a slight rise. A similar result was observed in the case of the red alga Porphyridium (not shown). It seems that most of the slow transient is suppressed by this cofactor of electron transport.

We could not induce any alteration in the time dependent changes in the fluorescence yield of Chlorella by the addition of ferricyanide, even at a relatively high concentration (15 mM). Apparently, ferricyanide does not get into the cells.

Figure 45 illustrates the alterations in the time course of fluorescence by 0.1 mM parabenzoquinone (PBQ). PBQ completely suppresses the P and lowers the amplitude of S. PBQ also lowers (10-15%) the 0 level (not shown). But the maximum quenching occurs at P. This quenching of variable fluorescence seems to be due to the ability of quinones to act as typical Hill oxidants. The action spectrum of quinone photoreduction (as measured by O_2 evolution in Chlorella) shows the "red drop"

Figure 44. The slow SMT transient in the Chl *a* fluorescence yield with and without methyl viologen. Upper curve, untreated cells; bottom curve, 1 mM MeV. In both cases 7 min of dark time was interposed before illumination; illumination, blue light (C.S. 4-72 plus C.S. 3-73; intensity ~ 10 Kergs $cm^{-2}sec^{-1}$. Fluorescence was measured at 685 nm (half band width, 6 nm).

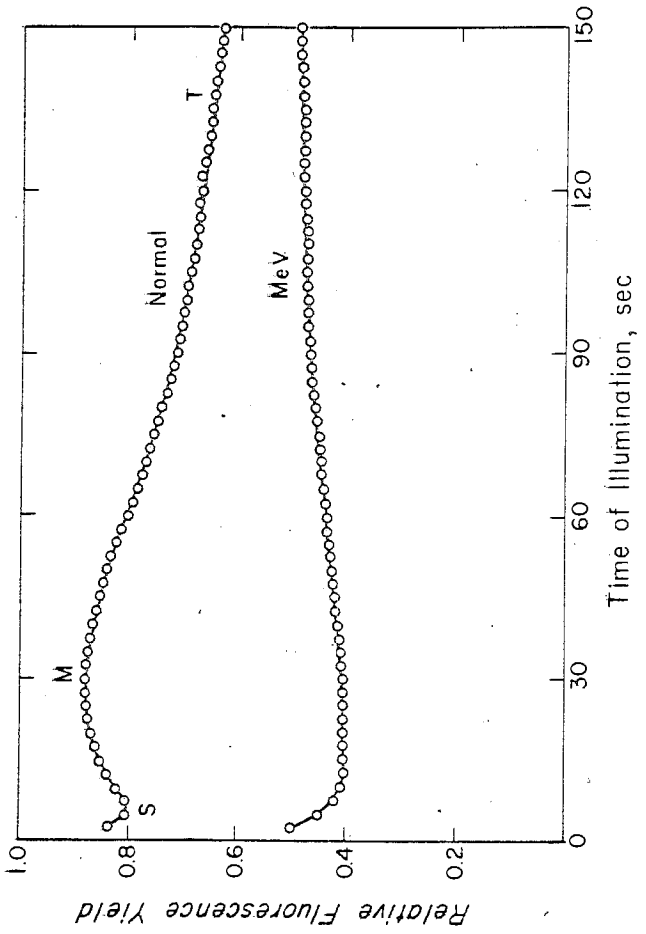
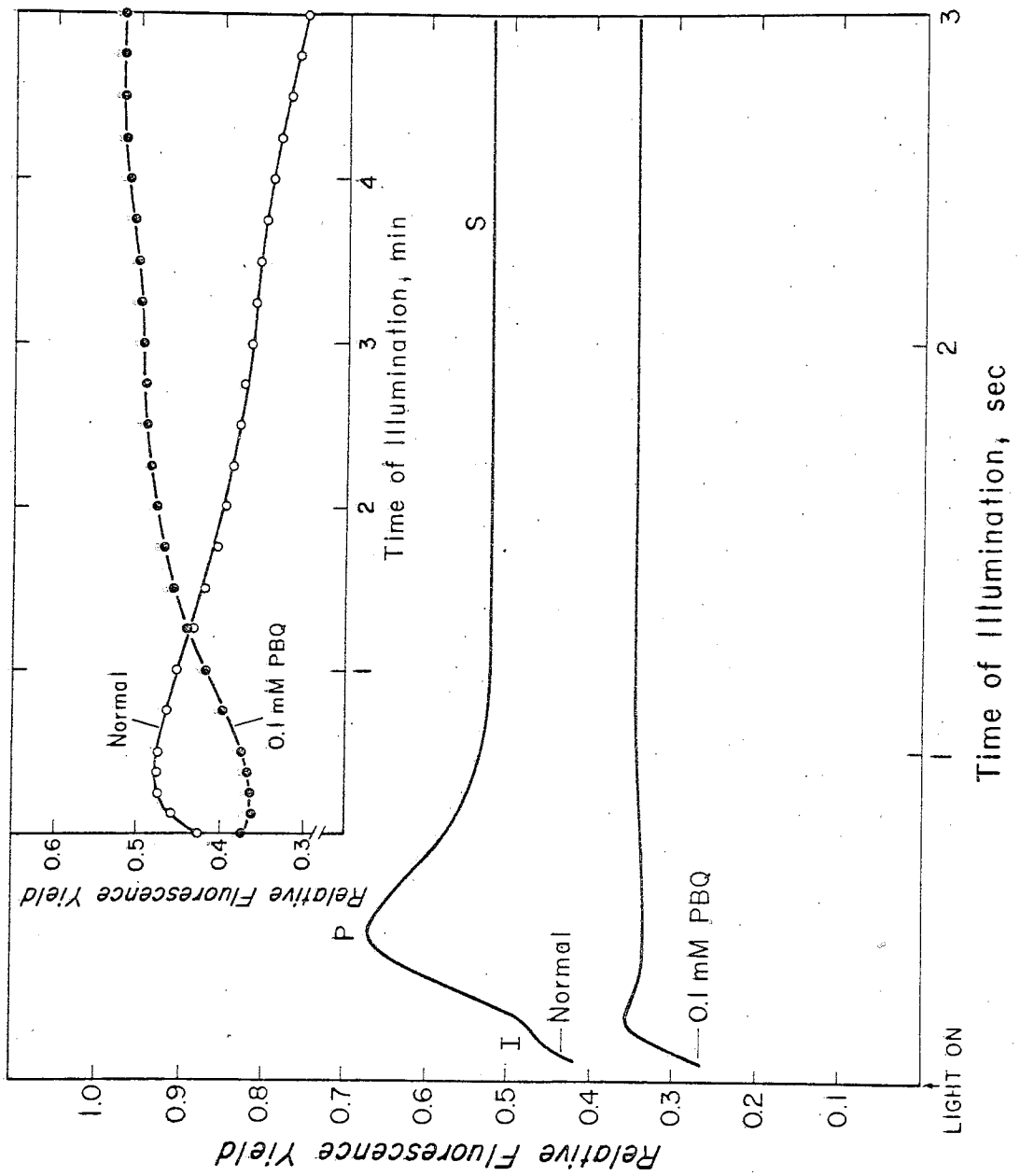

Relative Fluorescence Yield
1.0
0.8
0.6
0.4
0.2
0
30
60
90
120
150
Time of Illumination, sec
Normal
MeV
M
S
T

Figure 45. Time course of the relative fluorescence yield in Chlorella with and without parabenzoquinone (PBQ). Main figure shows the fast transient in untreated (upper curve) and in treated (with 0.1 mM PBQ) cells (lower curve). (The 0 level is not shown.) Insert: slow time course of fluorescence yield changes in normal (open circles) and parabenzoquinone treated (PBQ) cells. The samples were suspended in fresh growth medium of Chlorella (pH 6.8); excitation blue light (C.S. 4-72 plus C.S. 3-73) intensity, 14 Kergs $cm^{-2}sec^{-1}$. Observation λ = 685 nm; half-band width, 6.6 nm, Corning C.S. 2-58 filter before the photomultiplier.


Relative Fluorescence Yield
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
LIGHT ON
1
2
3
Time of Illumination, sec
Normal
0.1 mM PBQ
I
P
S
Relative Fluorescence Yield
0.6
0.5
0.4
0.3
Normal
0.1 mM PBQ
1
2
3
4
Time of Illumination, min

(R. Govindjee et al., 184). From these observations, we concluded that the suppression of the variable fluorescence at P is due to oxidation of Q by the quinones. The slow changes in yield that persist in the quinone treatment seem to be reminiscent of the SM rise in the normal, but this rise is slower than in the normal. With 0.1 mM PBQ, the S level is lower than in normal and it remains low (lag period) for 10-15 seconds and then it gradually increases to a higher level. The final yield of fluorescence was higher than the terminal (T) level of untreated cells. With a higher concentration of PBQ or with a longer period of incubation (20 to 30 min) the lag period was prolonged and the final intensity was decreased to a lower level (not shown). It is noteworthy that there is a slow rise in yield with PBQ. Such a significant extent of slow increase in fluorescence yield was not observed in the presence of MeV in Chlorella although MeV suppresses P and S like PBQ. We will discuss later the probable cause of the different effects of these two electron transport cofactors.

Table IV.2 shows the results of a typical experiment on the effect of various concentrations of PBQ on the amplitude of P and S. Progressive increase in concentration depresses P to a much greater extent than S. The ratio of P/S gradually dropped from 1.29 to 0.95. Fork and Amesz (185) have previously shown that quinones in general quench variable fluorescence of isolated chloroplasts to a larger extent than the constant f_o level. They have shown that this quenching occurs even in the presence of DCMU. Fork and Amesz (185) suggested that the quenching by quinones may be due to a dynamic type of quenching very similar to what has been observed for Chl a in solution. We observe that PBQ quenches fluorescence maximally at P level in Chlorella. Since the development of P is due

Table IV.2

Effect of Increasing Concentration of PBQ on the Amplitude of P and S and P/S Ratio

Concentration of PBQ added in μM	Amplitude at P	Amplitude at S	P/S	% Lowering of P	% Lowering of S
0	170	132	1.29	1.0	1.0
5	155	120	1.29	.91	.90
10	142	112	1.27	.83	.84
20	132	110	1.20	.78	.83
30	130	110	1.18	.76	.83

Fluorescence transient was measured at 685 nm after 7 minutes of incubation in the dark. A high concentration of ethanolic solution of PBQ was added by a lambda pipette. Maximum alcohol concentration was ~2%. Cells were suspended in phosphate buffer (0.05 M, pH 7.8) with 0.01 M NaCl.

mainly to the accumulation of QH and since PBQ is known to act as a Hill oxidant in Chlorella, we believe that the main cause of lowering of P by PBQ is due to its ability to act as a Hill oxidant (acceptor for PS II). Although PBQ suppresses P to S decline, it does not completely eliminate the slow SM rise in normal cells. Thus, it appears that slow rise is not dependent on the development of the first wave.

4.2 Uncouplers

The effect of uncouplers of photophosphorylation on the fluorescence yield changes has been reported by many investigators. One of the most studied uncouplers is carbonylcyanide phenylhydrazone (CCCP and FCCP). Bannister (177) and Bannister and Rice (89) showed that 10 μM FCCP suppressed the O_2 evolution and lowered the yield of fluorescence in

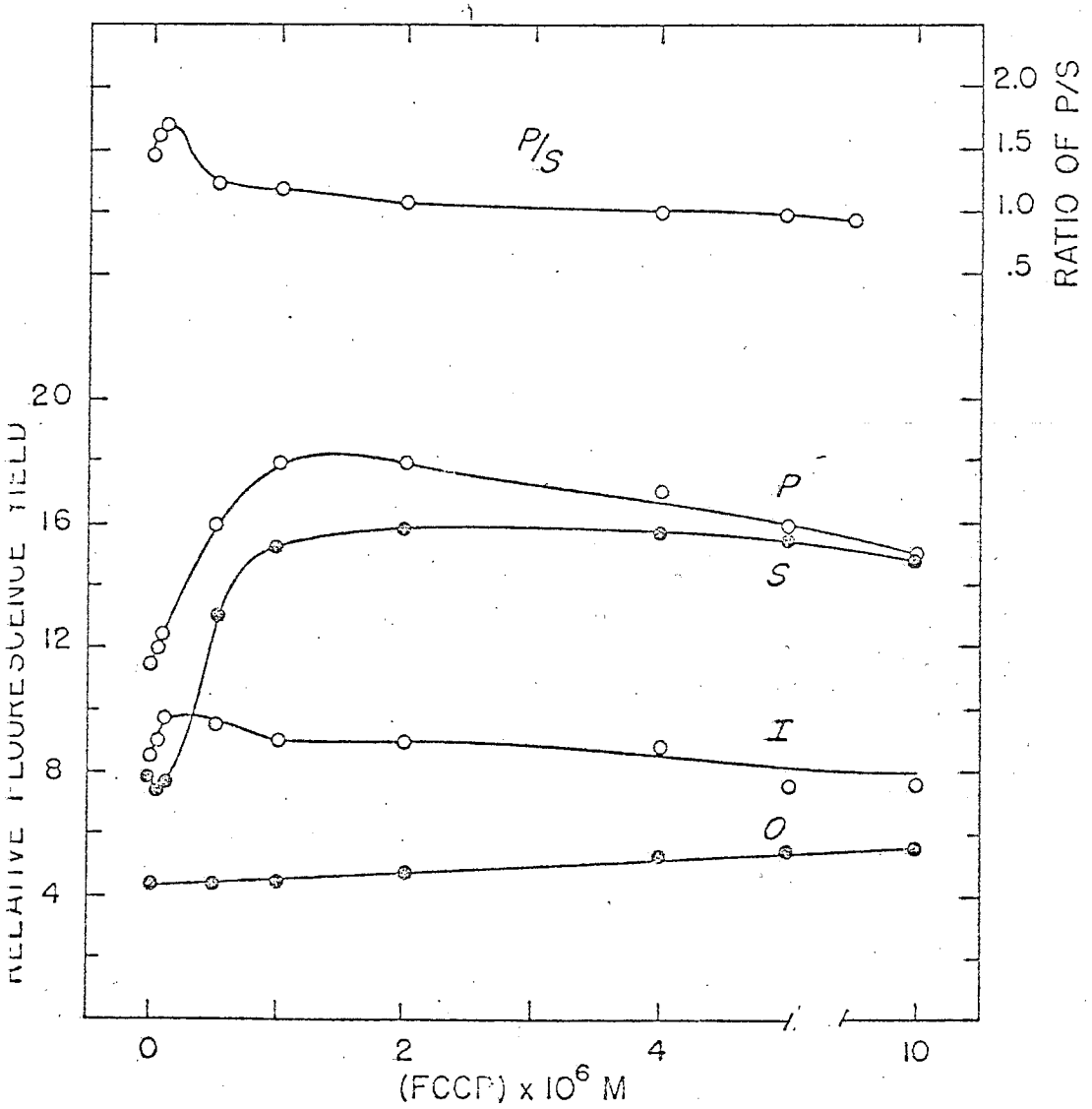
Chlamydomonas. These results are explained on the basis of the uncoupling activity (increased electron flow) of the chemical. However, Itoh et al. (186) suggested that CCCP induced lowering of fluorescence in isolated chloroplasts is due to inhibition of electron transport from water to PS II and not due to uncoupling of phosphorylation.

Recently two reports on the effect of CCCP on electron transport and on the chlorophyll fluorescence yield of isolated chloroplasts have appeared. Kimimura et al. (178) support the view of Itoh et al. (186) that low steady state yield of fluorescence is mainly due to an inhibition of electron transport while Homann (179) has shown that CCCP accelerates a Mehler type reaction even in previously uncoupled chloroplasts and causes a lowering of fluorescence yield. Thus, the quenching of fluorescence is due to a rapid rate of electron transport.

We have studied the effect of FCCP and CCCP mainly on the fluorescence yield changes in intact algal cells. Earlier, Papageorgiou and Govindjee (8) reported the suppressing effect of FCCP in Anacystis and Chlorella respectively on the slow SM rise and on MT decline of Chl a fluorescence. Recently, Govindjee and Delosme (personal communication, 187) studied the effect of FCCP on the fast transient of Chlorella. Results of Govindjee and Delosme are very similar to those that we obtained independently at Urbana.

Figure 46 shows the effect of various concentrations of FCCP on the amplitude of fluorescence at O, I, P, and S in the red alga Porphyridium. (We show this here for comparison with Chlorella.) At low (0.1 μM) concentration of FCCP there is an increase in I and P, but the fluorescence yield at O and S remains relatively unchanged. On increasing the concentration to 0.5 μM, the fluorescence yield at I declines while the

Figure 46. Changes in the relative fluorescence yield at various characteristic points of the fast transient in Porphyridium cruentum as a function of concentration of FCCP. Top curve, ratio of the yield P/S; ordinate scale on the right. Blue-green excitation light (C.S. 4-96 plus C.S. 3-73) intensity, 18 Kergs $cm^{-2}sec^{-1}$; observation λ = 685 nm (half band width, 10 nm); Corning C.S. 2-58 filter before the analyzing monochromator. Cells were suspended in carbonate-bicarbonate plus 0.25 M NaCl buffer, pH 8.5. FCCP was added in the dark and equilibrated for at least 5 minutes before the measurements.


P/S
P
S
I
O
RELATIVE FLUORESCENCE YIELD
RATIO OF P/S
20
16
12
8
4
2.0
1.5
1.0
.5
0
2
4
10
(FCCP) x 10^6 M

yield at P and S increases. At still higher concentrations of FCCP (4 μM) both P and S decline and we observe an increase in the O level fluorescence. Govindjee and Delosme (187) have obtained similar results for the effect of FCCP on Chlorella. (We have also obtained similar results on the effect of FCCP on Chlorella.)

Figure 46 (top curve) shows the variation in the ratio of P/S as a function of the concentration of FCCP. It is clear that the P/S ratio decreases at a relatively lower ($\sim$1 μM) concentration of FCCP when the yield at P is still high. Figure 47 is a similar plot of the variation in the amplitude of fluorescence at I, P and S as a function of the concentration of CCCP in Chlorella. At low concentrations, we observe a similar enhancement in the yield at I and P as well as at S and then the fluorescence yield declines on increasing the concentration of CCCP. The fluorescence yield at S level showed a larger enhancement in the yield at low concentration. But on increasing the concentration of CCCP it also was suppressed. Again, the ratio of P/S decreases at a concentration of CCCP when the yield of P is high. CCCP is known to be comparatively less potent than FCCP and thus a relatively higher concentration of CCCP was needed to cause similar effects. In these experiments we have used relatively higher concentrations of both CCCP and FCCP as compared to what is routinely used in the case of isolated chloroplasts or mitochondria. Such high doses of uncouplers are probably necessary as we do not know the exact fraction of the compound that permeates to the functional site of the chloroplasts in vivo.

Figure 48 shows the trace of the fast transient (up to 2 sec of illumination) with and without 10 μM CCCP in Chlorella. The extent of quenching at P war more than what was observed in Figure 47 as a separate culture of

Figure 47. Effect of various concentrations of CCCP on the amplitudes of various points of the fast fluorescence transient in Chlorella. The bottom P/S curve, scale to the right. Cells suspended in 0.1 M carbonate-bicarbonate buffer, pH 9.1 with 0.01 M NaCl; blue excitation light (C.S. 4-72 plus C.S. 3-73) intensity, 14 Kergs cm^{-2} sec^{-1}; C.S. 2-60 filter before the photomultiplier. CCCP was added in the dark approximately 5 min. before the measurements.

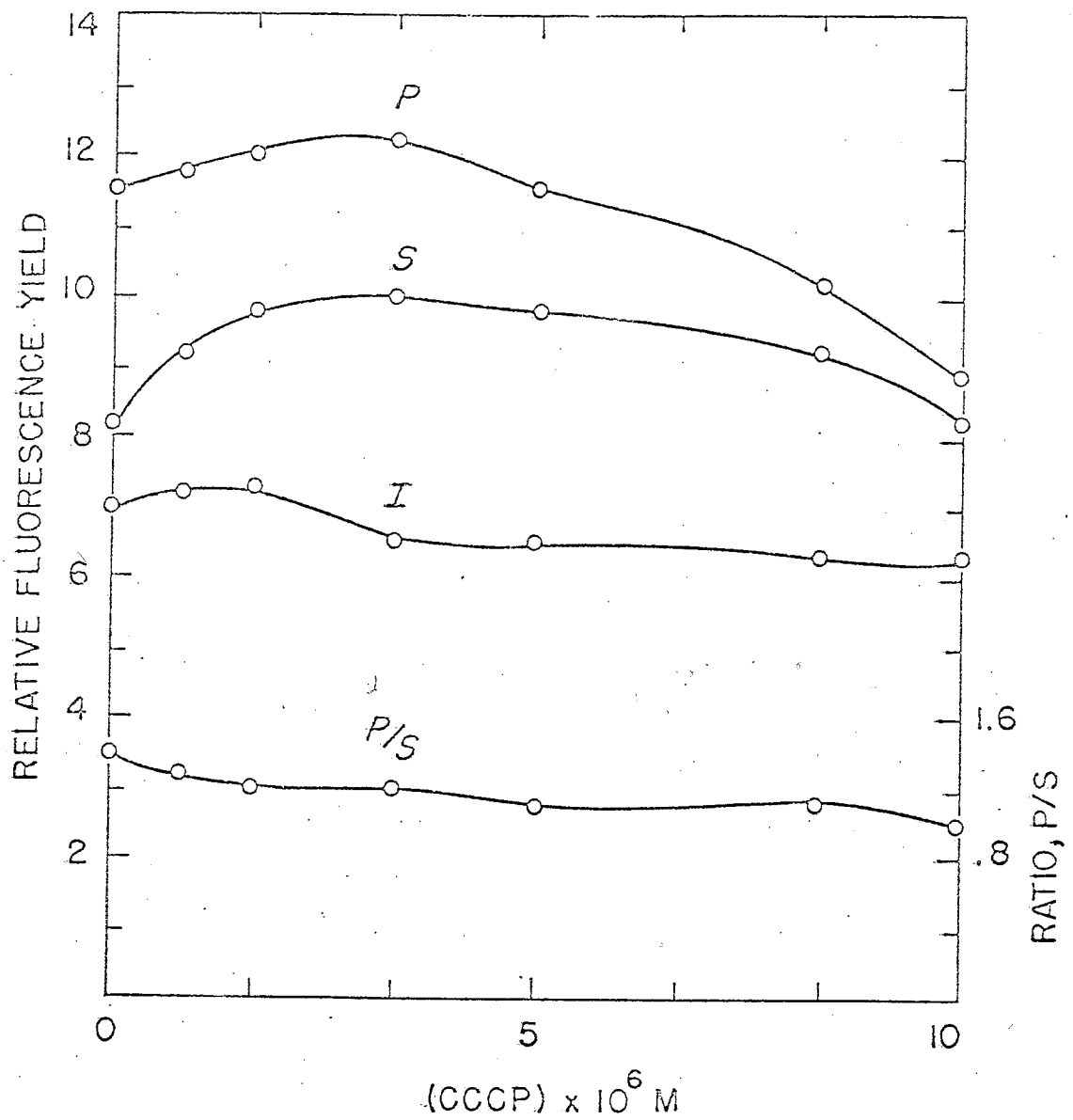
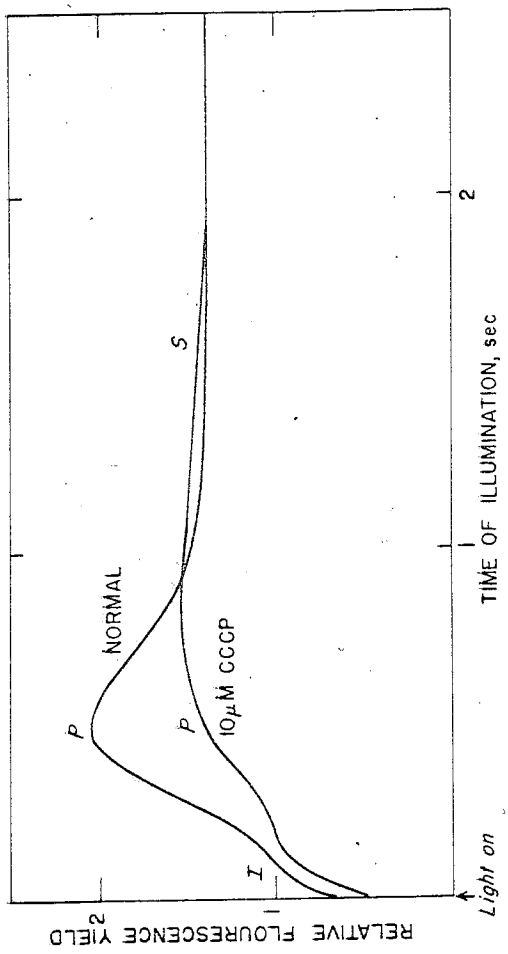

14
12
10
8
6
4
2
P
S
I
P/S
RELATIVE FLUORESCENCE YIELD
1.6
.8
RATIO, P/S
0
5
10
(CCCP) x 10^6 M

Figure 48. Time course of Chl *a* fluorescence yield in *Chlorella* with and without 10 μM CCCP. Cells were suspended in 50 mM Tris-Cl plus 0.01 M sodium chloride buffer, pH 7.8. λ observation, 685 nm; other conditions as in Figure 36.


RELATIVE FLOURESCENCE YIELD
2
1
Light on
TIME OF ILLUMINATION, sec
2
NORMAL
10μM CCCP
P
P
S
I

Chlorella was used in this experiment. Besides the suppression in P, there was a delay in time to reach P level (tp). Figure 49 is a plot of the time to reach P (tp) as a function of the concentration of FCCP in Porphyridium. Again, a different effect was observed at lower concentration of FCCP than at higher concentration of FCCP. At low concentration of FCCP the tp is hastened (not clearly shown in Figure 49), but on raising the concentration of FCCP the tp is progressively delayed. Very similar results were obtained on the effects of FCCP on tp in Chlorella (not shown). Figure 49 (right) is a similar plot of tp versus the concentration of FCCP obtained from an acidophilic alga Cyanidium. Although no clear hastening of tp at low concentrations of the uncoupler was noted here, a progressive delay of tp at higher concentration was observed (see bottom curve with open circles). Again, the amplitudes of fluorescence at various characteristic points of the fast transient, namely O, I, P, and S, showed similar variation with increasing doses of FCCP as it was observed in the case of Porphyridium or Chlorella. Thus the results obtained with three different algae suggest that observed results are a general manifestation of the action of this uncoupler. It has at least two modes of action; at lower concentration, it causes an enhancement of I, P, and the rate of DP rise and at higher concentration, it suppresses them.

Figure 50 shows a stimulation of the slow rise of fluorescence yield in Chlorella with FCCP. The P/S ratio (S measured at 3 sec) was lowered from 1.35 to 1.25 in this sample. The effect of stimulation of a slow S-M rise by FCCP is much greater at pH 6.8 than at pH 8.0 (not shown; but see Papageorgiou and Govindjee (87) for comparison). At pH 6.5 we observed a slow increase in yield (S-M type) in the presence of FCCP and

Figure 49. Effect of varying concentrations of FCCP on the time for development of peak fluorescence yield (P) in Porphyridium cruentum and on the fluorescence yield changes in Cyanidium caldarium. Left: time to reach p (tp) versus concentrations of FCCP in Porphyridium. Right: tp, open circles (and dashed line); scale to the right; relative yield at P (open circles and solid line), at I (solid triangles), and at 0 (closed circles); scale to the left. Porphyridium cells were suspended in carbonate bicarbonate buffer (pH, 8.5) plus sodium chloride; Cyanidium cells were suspended in growth medium (pH, 2.0); other details of measurement as in Figure 36.

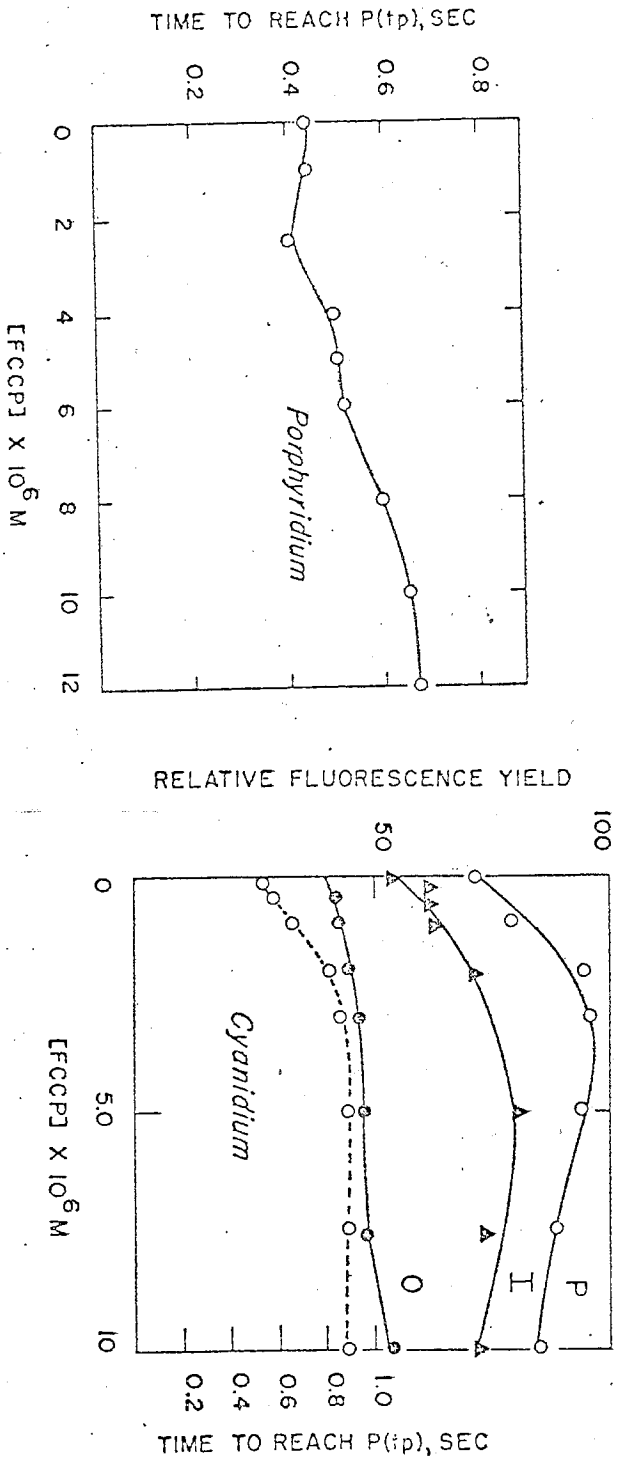
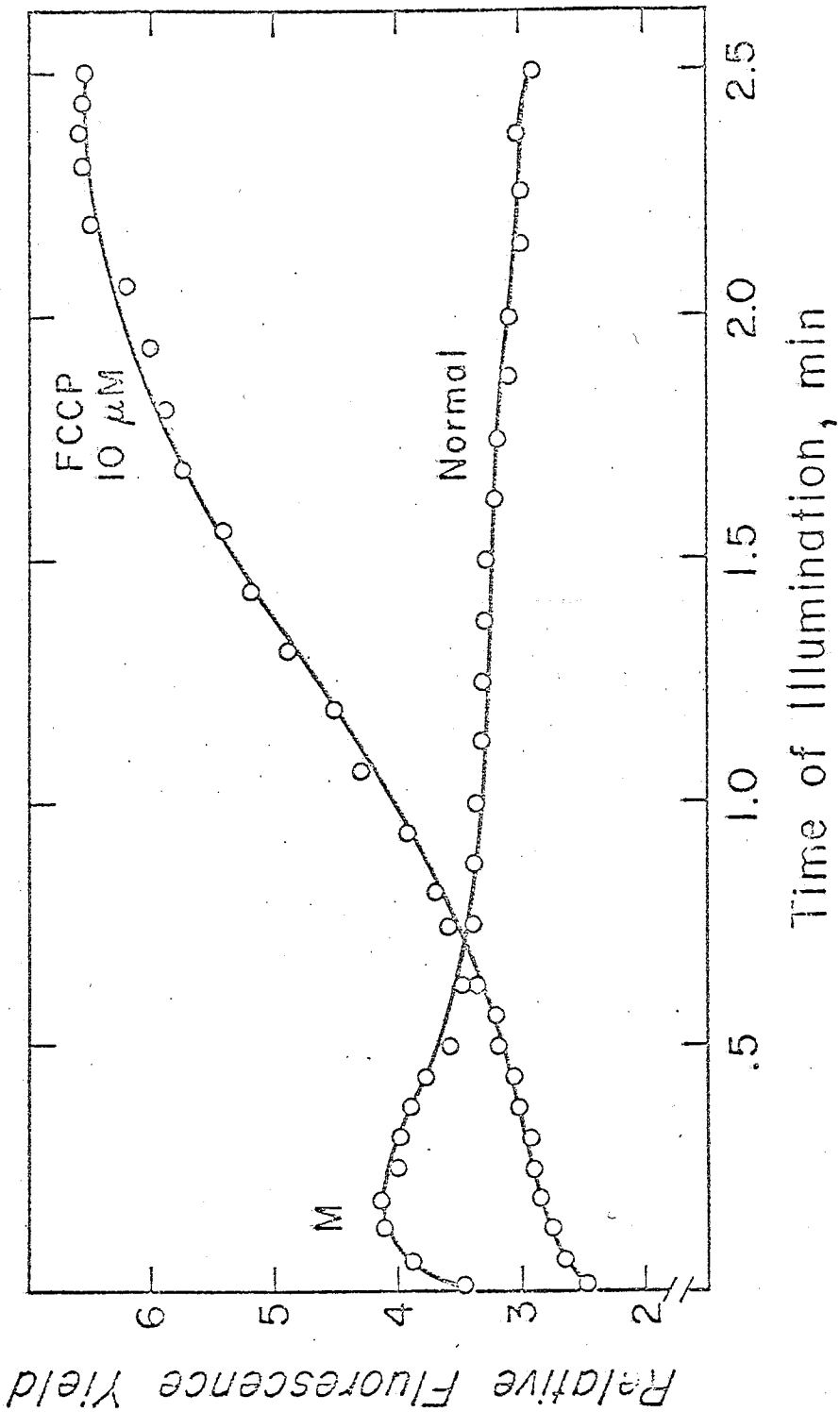

TIME TO REACH P(fp), SEC
0.2
0.4
0.6
0.8
0
2
4
6
8
10
12
[FCCP] X 10^6 M
Porphyridium
RELATIVE FLUORESCENCE YIELD
50
100
0
5.0
10
[FCCP] X 10^6 M
Cyanidium
O
H
P
0.2
0.4
0.6
0.8
1.0
TIME TO REACH P(fp), SEC

Figure 50. Time course of the slow changes in Chl *a* fluorescence yield in *Chlorella* with and without FCCP, 10 μM with phosphate buffer and NaCl, pH, 6.8; all other conditions for measurement as in Figure 46.


FCCP
10 μM
Normal
M
Relative Fluorescence Yield
Time of Illumination, min
2
3
4
5
6
.5
1.0
1.5
2.0
2.5

the final level of fluorescence was about 2-3 times higher than the normal (T) terminal level (Figure 50). Since CCCP and FCCP are weak acids, one would expect higher affinity of these uncouplers to the "energized state" at a pH around their pKa [the pKa of FCCP is not known with certainty but it has been estimated to be around 5.5 to 7.5 (see references in 188)]. In spite of the different kind of buffer used in this experiment, the results summarized in Table IV.3 indicate that FCCP is more effective in stimulating slow fluorescence rise at lower pH (6 to 7) than at a high pH (9). Although FCCP or CCCP suppress both PS and MT decline, they enhance the slow SM rise even in alkaline pH (8.0) as shown earlier by Papageorgiou and Govindjee (87).

Atebrin, another potent uncoupler of phosphorylation acts very similarly to FCCP (Govindjee and Delosme (187)), but unlike FCCP, atebrin was found to suppress the SM rise. We shall attempt to explain the probable cause of this differential effect observed with these two uncouplers later in our discussion.

The enhancement of I and P, and of the rate of D-P rise suggests that CCCP or FCCP at low concentrations brings about inhibition of the rate of back reoxidation of QH or Q^-. This would cause a hastening of tp. On increasing the concentration, CCCP or FCCP presumably accelerates the flow of electrons either due to the uncoupling effect as suggested by Itoh *et al.* (186) or increasing the O_2 uptake as suggested by Homann (179). Such an acceleration of electron flow will decrease P and delay the formation of P (Figure 49). FCCP has been known to stimulate light induced O_2 uptake in *Chlamydomonas* (89). (Also, see Chapter V.) Thus, it seems that FCCP or CCCP also stimulates O_2 uptake in whole cells as it does in isolated chloroplasts.

Table IV.3

Stimulation of Slow Rise in the Fluorescence Yield by 10 μM FCCP in Various Suspending Medium in Chlorella

Suspending Medium	Relative Fluorescence Yield at S	at T	Ratio of T/S
50 mM phosphate			
pH 6.0	25	60	2.4
pH 7.0	25	55	2.2
50 mM Tris-Cl			
pH 7.2	29	43	1.5
pH 8.0	29	40	1.4
100 mM carbonate-bicarbonate			
pH 9.2	26	47	1.9
pH 9.5	29	44	1.5

Broad band blue excitation; intensity ~10 Kergs $cm^{-2} sec^{-1}$; λ observation = 685 nm; the S/T ratio of normal cells suspended in growth medium for this particular sample was 0.89.

In summary, it appears that FCCP or CCCP has more than one mode of action. At low concentrations, it, perhaps, alters the environment of the primary donor and acceptor of PS II such that the back recombination between Z^{+} and Q^{-} is prevented and this causes a fast D-P rise. At higher concentrations, FCCP or CCCP accelerates the reoxidation of QH by intersystem intermediates. This may be due to uncoupling action of FCCP or acceleration of a pseudocyclic electron flow (Mehler reaction) as both

will cause a rapid electron flow.

The suppression of P to S decline by FCCP does not seem to be primarily due to the acceleration of electron flow, as we see a lowering of the P/S ratio at a concentration when the yield of P is high (Figures 46, 47). This suggests that suppression of PS decline is not solely due to an uncoupling activity but may be associated with the change in membrane permeability and structural changes linked to the uncoupling action.

4.2.1 Effect of Uncouplers in the Presence of DCMU

Poisons of electron transport, like CMU and DCMU or orthophenanthroline, eliminate most of the slow changes in the yield of Chl *a* fluorescence in *Chlorella* (Figure 51). Bannister and Rice (89) reported a DCMU resistant fluorescence change in the green alga *Chlamydomonas* at a relatively high intensity of excitation. They also observed slow changes in the fluorescence yield in a mutant strain of *Chlamydomonas* lacking an electron carrier on the reductant side of Photosystem I. However, in *Chlorella*, there is no significant slow change in the yield of Chl *a* fluorescence in the presence of DCMU. Figure 51 illustrates the maximal effect we have observed. From our experience with, at least 20 experiments, we conclude that there is no significant slow change in fluorescence yield in DCMU poisoned *Chlorella*.

Bannister (177) reported that FCCP depresses the increased fluorescence yield of DCMU treated *Chlorella*. Data in Table IV.4 confirms the findings of Bannister. Upon addition of FCCP, the fluorescence yield declines slowly to a lower value over a period of 2 to 3 min. The amount of quenching of fluorescence increases on increasing the concentration of CCCP (Table IV.4). Kimimura *et al*. (178) observed similar quenching of fluorescence at a high concentration of CCCP in isolated spinach

Figure 51. Time course for the slow fluorescence yield changes in Chlorella in the presence and absence of electron transport inhibitors. Upper curve, 10 μM DCMU; middle curve, 20 μM orthophenanthroline; lower curve, no addition; buffer (0.05 M phosphate plus 0.01 M NaCl; pH 6.8). Other conditions for measurements are as in Figure 46.

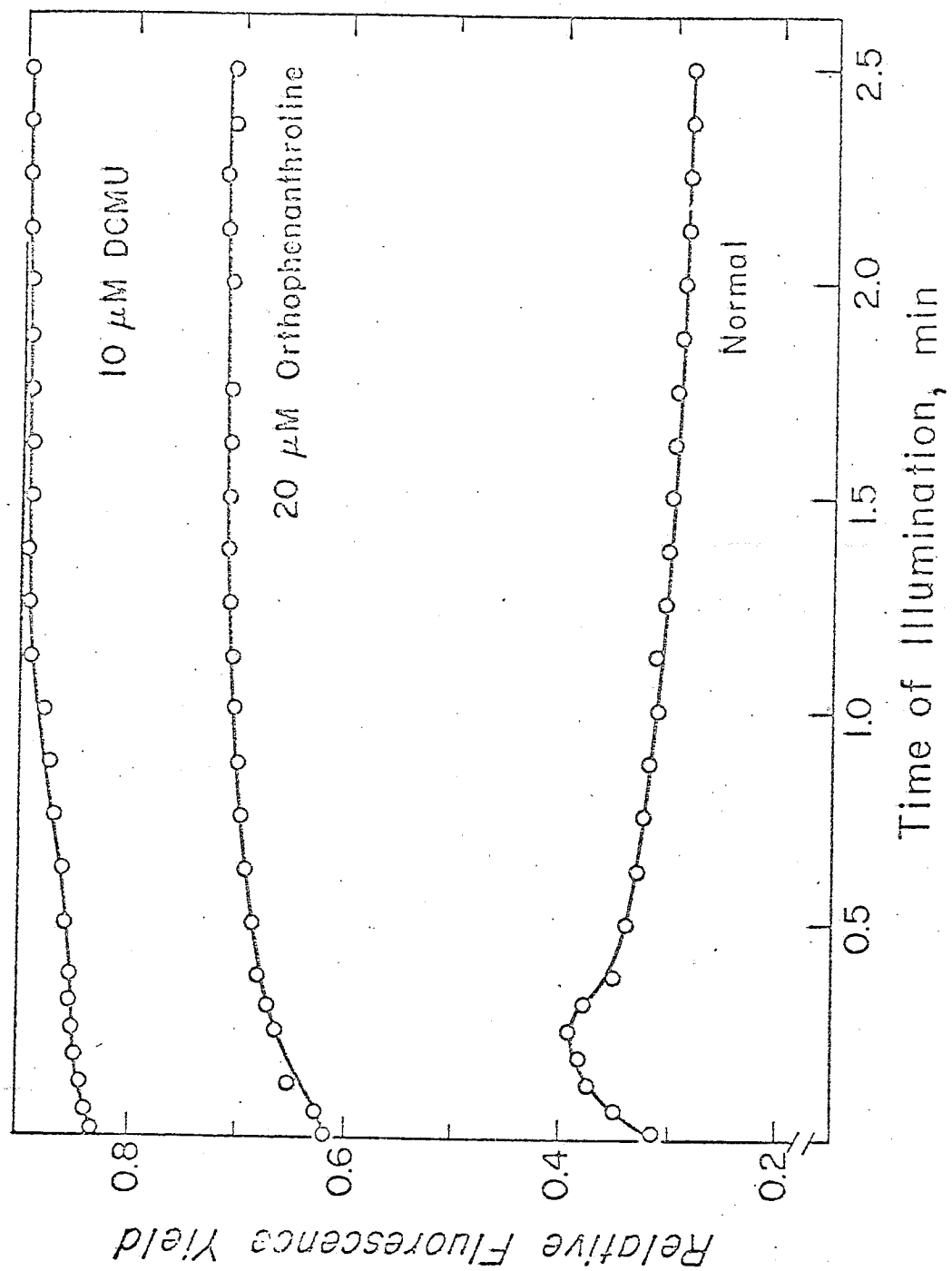

10 μM DCMU
20 μM Orthophenanthroline
Normal
0.8
0.6
0.4
0.2
0.5
1.0
1.5
2.0
2.5
Relative Fluorescence Yield
Time of Illumination, min

Table IV.4

Quenching of Chl a Fluorescence Yield of DCMU Poisoned Chlorella by CCCP

Concentration of CCCP	Steady State Fluorescence Yield in Relative Units	Fraction of Control
None	50.0	1.00
10 μM	45.5	0.91
30 μM	39.0	0.78
50 μM	29.0	0.58
100 μM	27.0	0.54

Fluorescence was measured at 685 nm; cells were suspended in fresh culture medium with 15 μM DCMU; blue excitation light, ~10 Kergs $cm^{-2}sec^{-1}$; C.S. 2-61 filter before the photomultiplier.

chloroplasts.

The amount of depression of Chl fluorescence yield by FCCP or CCCP varied depending on the culture conditions and the age of algal samples used (Govindjee and Bannister, unpublished data). The extent of quenching is greater in cells grown in high light (with four 15 watt lamps, 3.3" away) than the cells grown in low light (with a 40 watt lamp, 3.3" away). Depression of fluorescence was observed both in aerobic as well as in anaerobic samples (Figures 52 and 53). The room temperature emission spectra of control, DCMU treated and DCMU and FCCP treated samples (Figure 52), show that DCMU causes a preferential increase of the main band at 685 nm and FCCP quenches mainly this increased fluorescence band. Figure 53 shows similar results in anaerobic cells of Chlorella. The high light cells, in general, showed a greater extent of quenching by FCCP in the

Figure 52. Room temperature emission spectra of aerobic cells of D treated Chlorella with and without FCCP. Left: low lig cells; lower curve (open circles), control; middle curv (open squares), 80 μM FCCP; upper curve (solid circles) 30 μM DCMU. Cells were suspended in Warburg buffer #9 (pH 9.2); optical density of the sample was adjusted approximately to have 5% absorption at the red peak, sa were excited with bright blue light (C.S. 4-96 plus 3-7 (intensity ~ 10 Kergs $cm^{-2}sec^{-1}$). Observation monochro tor was guarded by a C.S. filter 2-64; slit width of th monochromator 5nm. (Right) emission in high light cell all additions as in previous measurements (after Govind and Bannister, unpublished observations).

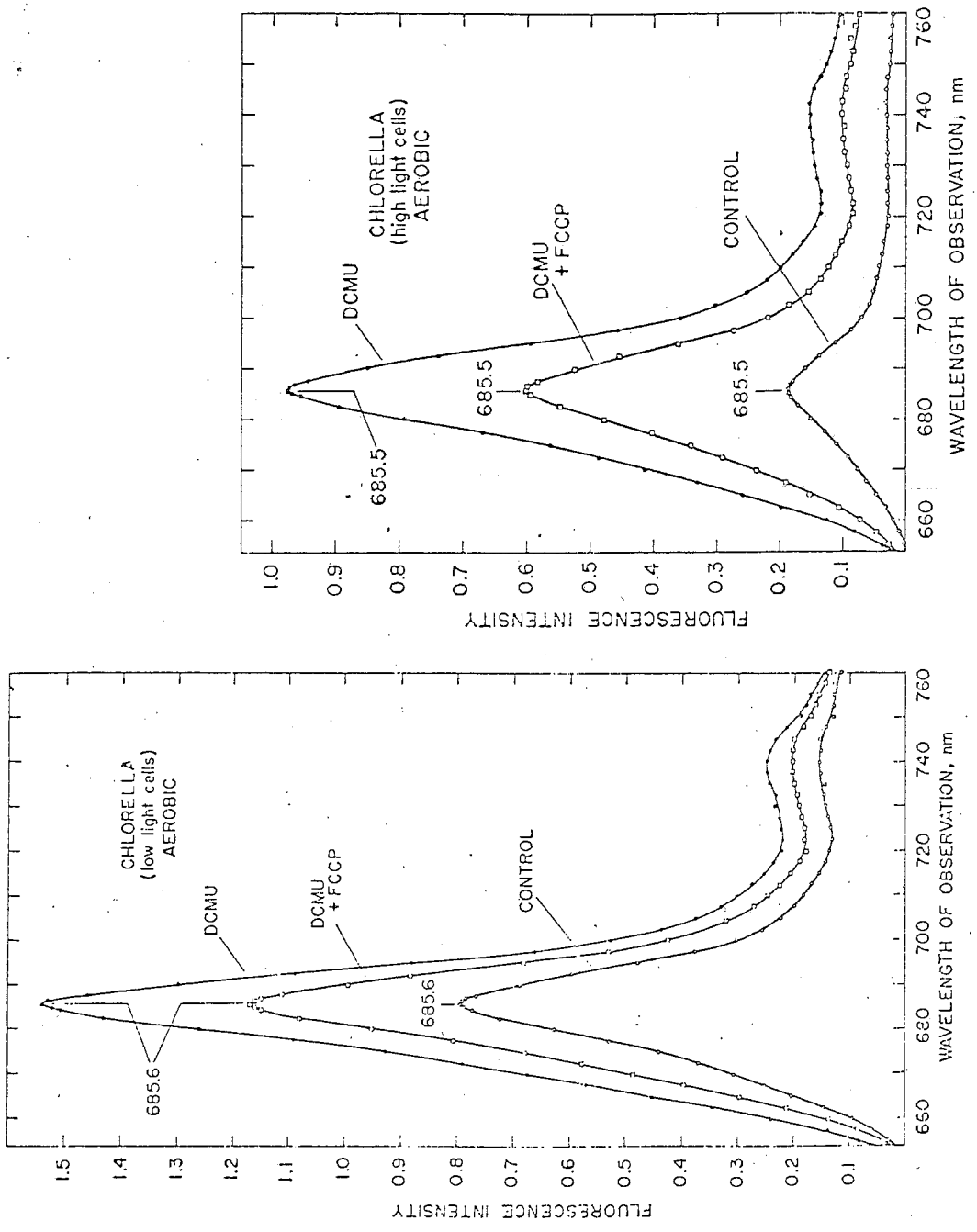
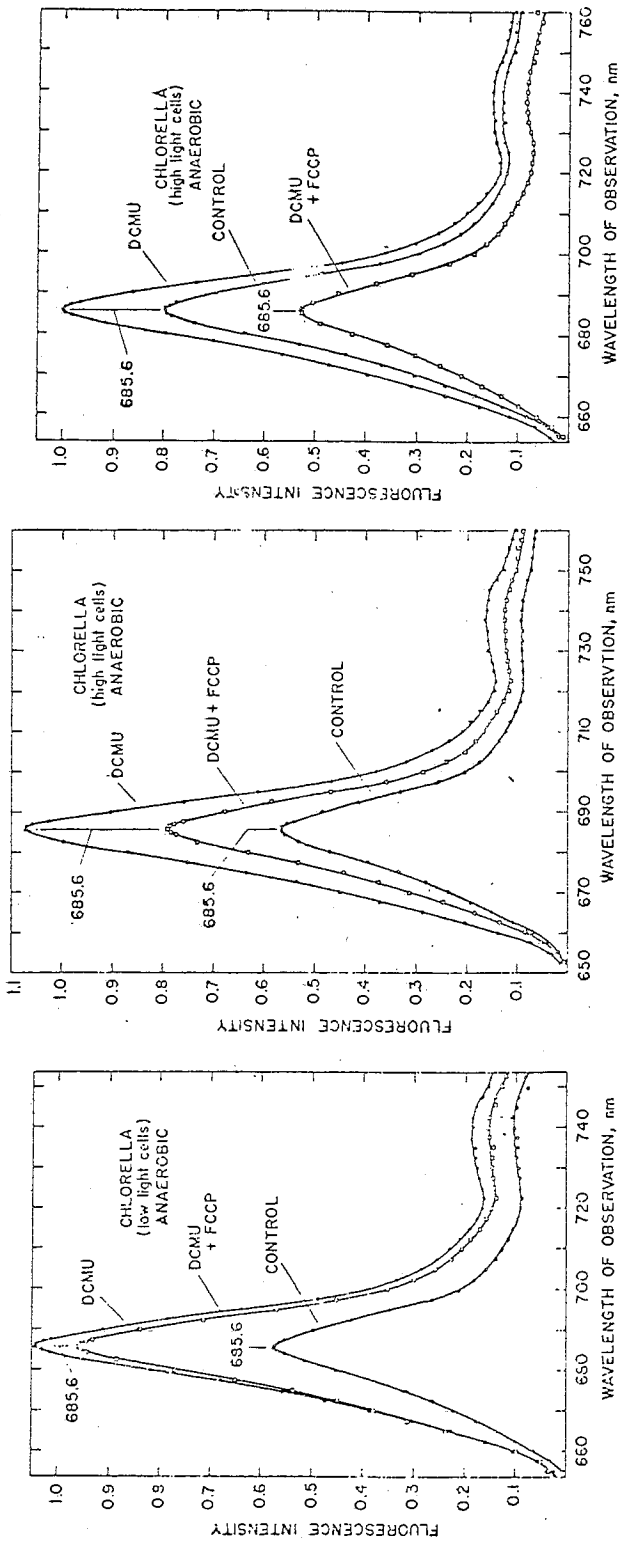

CHLORELLA
(high light cells)
AEROBIC
DCMU
DCMU
+ FCCP
CONTROL
685.5
685.5
685.5
FLUORESCENCE INTENSITY
WAVELENGTH OF OBSERVATION, nm
CHLORELLA
(low light cells)
AEROBIC
DCMU
DCMU
+ FCCP
CONTROL
685.6
685.6
685.6
FLUORESCENCE INTENSITY
WAVELENGTH OF OBSERVATION, nm

Figure 53. Room temperature emission spectra of anaerobic cells of Chlorella with and without FCCP in the presence of DCMU. Right: low light cells; Middle: high light cells from one batch of culture; Left: the same but from a separate batch of high light cells exhibiting pronounced lowering of peak emission with FCCP treatments. In each case, DCMU concentration was 30 μM, and FCCP 80 μM. Anaerobic condition was maintained by gasing 5% CO_2 in argon. All other conditions of measurements as in Figure 52 (after Govindjee and Bannister, unpublished observations).


CHLORELLA
(low light cells)
ANAEROBIC
DCMU
DCMU + FCCP
CONTROL
685.6
685.6
FLUORESCENCE INTENSITY
WAVELENGTH OF OBSERVATION, nm
CHLORELLA
(high light cells)
ANAEROBIC
DCMU
DCMU + FCCP
CONTROL
685.6
685.6
FLUORESCENCE INTENSITY
WAVELENGTH OF OBSERVTION, nm
CHLORELLA
(high light cells)
ANAEROBIC
DCMU
CONTROL
DCMU + FCCP
685.6
685.6
FLUORESCENCE INTENSITY
WAVELENGTH OF OBSERVATION, nm

presence of DCMU than the low-light cells. The high-light cells showed also a greater extent of quenching under anaerobic conditions. Perhaps these cells have a higher rate of cyclic photophosphorylation than the low light cells. However, growing cells under high light conditions may simply make them more permeable to the uncouplers. In any event, FCCP causes a quenching of Chl a fluorescence yield in the presence of DCMU both under aerobic and anaerobic conditions. Thus, the observed quenching cannot be solely associated with a Mehler type of reaction; it may be associated with the alteration of energy transfer between the two photosystems, or a physical alteration of the membrane structure causing a loss of quanta as heat.

Table IV.5 shows the results obtained with another uncoupler dinitrophenol (DNP). Like FCCP, DNP causes a quenching of the fluorescence yield.

Table IV.5

Quenching of Fluorescence Yield by DNP in DCMU Poisoned Chlorella

Concentration μM	Relative Fluorescence Yield at 685 nm	Fraction of Normal
None	145	1.0
40	145	1.0
80	120	0.83
120	110	0.75
160	85	0.59
200	80	0.55

DCMU, 8 μM; Buffer, 50 mM phosphate, pH 6.8; ethanolic stock solution of DNP was diluted with H_2O before use; all additions are in the dark; [Chl], 45 μg/ml; excitation, 633 nm; intensity, 25 Kergs $cm^{-2}sec^{-1}$.

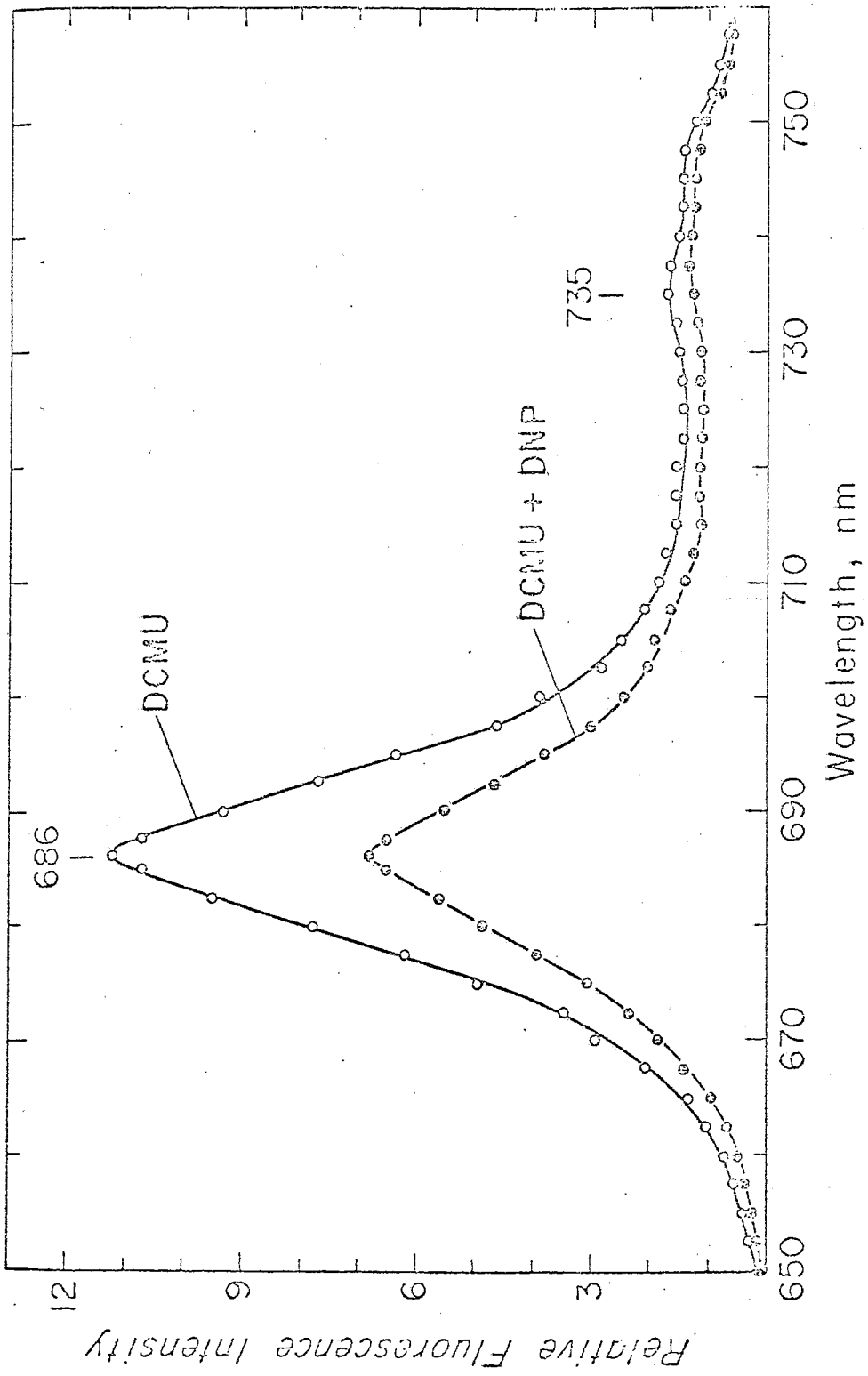
The percent-quenching remained the same at the low as well as the high light intensity. These results appear to be contradictory to those of Hoch and Randalls (163), who observed an enhancement of fluorescence yield upon addition of DNP at weak light intensity. However, the light intensity used by them was extremely weak, such that the fluorescence yield is low even in the presence of DCMU. The DNP uncoupler, probably, also inhibits the back recombination between Z^+ and Q^- and thus causes an increased yield in their experiments.

Figure 54 shows the room temperature emission spectrum of Chlorella in the presence of DCMU and DCMU plus DNP. As in the case of FCCP, the DNP causes a preferential lowering at the main 685 band without significant effect on the system I band. The ratio of F685 to F710 band was decreased from 6.2 to 4.9 by the addition of DNP. The quenching of fluorescence by DNP and FCCP seems to be very similar to the emission characteristics of Chlorella.

5. Induction of Slow Changes in Chl a Fluorescence Yield of DCMU Poisoned Cells by Cofactors of Electron Transport

It is well known that PMS (phenanzine methosulfate) is an efficient cofactor for cyclic electron transport in isolated chloroplasts (189). This cyclic flow of electrons, generating ATP, is mediated by photosystem I. Arnon et al. (190) observed that PMS quenches fluorescence of Chl a in chloroplasts, and Govindjee and Yang (191) observed that PMS could quench the fluorescence yield of DCMU poisoned chloroplasts; Cramer and Butler (192) also observed a similar quenching in isolated chloroplasts. Murata and Sugahara (193) reported a detailed analysis of this quenching of Chl a fluorescence in DCMU poisoned spinach chloroplasts by reduced PMS. Wraight and Crofts (194) made similar studies with another cofactor

Figure 54. Room temperature emission spectra of DCMU poisoned aerobic cells of Chlorella with and without DNP. Upper curve (open circles), 15 μM DCMU; low light cells suspended in fresh growth medium (pH 6.7); excitation λ, 633 nm; intensity, 20 Kergs $cm^{-2}sec^{-1}$. All other conditions as in Figure 53.


Relative Fluorescence Intensity
12
9
6
3
650
670
690
710
730
750
Wavelength, nm
686
735
DCMU
DCMU + DNP

DAD (diaminodurene) which was found to be more effective in bringing abou quenching of Chl a fluorescence in chloroplasts than PMS. The quenching was found to be sensitive to all uncouplers and ionophorous antibiotics like gramicidin and nigericine. Cohen and Sherman (195) have shown that the EDTA (ethylene diamine tetraacetate) treatment, which inhibits proton accumulation, also impairs the quenching of fluorescence yield by DAD.

Sybesma and Williams (196) and Williams et al. (197) reported a simi lar type of quenching of fluorescence yield with another cofactor (DCPIPH of electron transport in intact algal cells of Chlorella. We reinvestigated the effect of these cofactors on the fluorescence yield changes in intact algal cells with both PMS and DCPIP.

Figure 55 (A, C, D) shows a slow induction of a lowering of Chl a fluorescence yield in the presence of 10 μM DCMU by oxidized PMS. Figure 55 (B) shows that the recovery of the original fluorescence level occurre within 40-45 seconds of darkness. This restoration to the original level of fluorescence yield is slower than what has been seen in chloroplasts. Figure 55 (C) shows quenching of fluorescence with different concentrations of PMS. The quenching of PMS saturates at about 50 μM PMS. The quenching requires a relatively high intensity ($\sim 1.4 \times 10^4$ ergs $cm^{-2}sec^{-}$ of light. Figure 55 (D) illustrates the effect of 4 μM FCCP on the rate of quenching of the fluorescence yield by PMS. FCCP slows down the rate of lowering of yield and it also decreases the extent of quenching.

Figure 56 is a plot of a specific quenching activity represented as $-\Delta F/F_{DCMU}$ versus concentration of PMS. The extent of quenching was found to be greater at pH 8.0 than at pH 6.8. Although we have not studied the optimal pH for the maximal quenching, we assume from Figure 56 that the optimal is probably in the neighborhood of pH 8.0. (In some cultures we

Figure 55. Light induced quenching of Chl *a* fluorescence yield in *Chlorella* by PMS in the presence of DCMU. Upper left (A): upper curve (open circles) 10 μM DCMU alone; lower curve, 10 μM DCMU + 25 μM PMS. Upper right (B): dark recovery of the fluorescence yield (closed circles) as measured by weak intensity 1/10 sec flashes; lower left (C): various concentrations of PMS with 10 μM DCMU, lower right (D): 10 μM DCMU, 4 μM FCCP and 30 μM PMS, additions as shown. Buffer, 50 mM potassium phosphate, pH 8.0; [Chl], 60 μg/3 ml. Other details of measurement as in Figure 46.

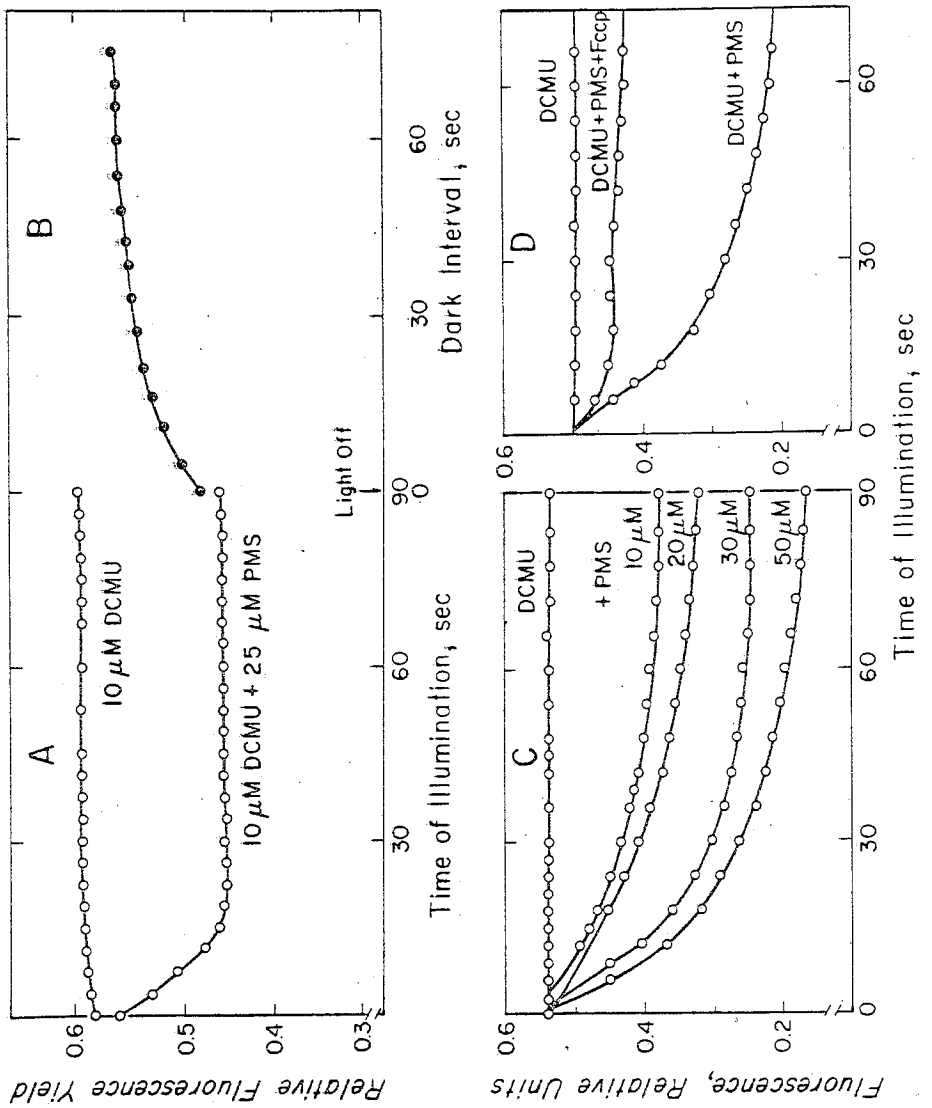
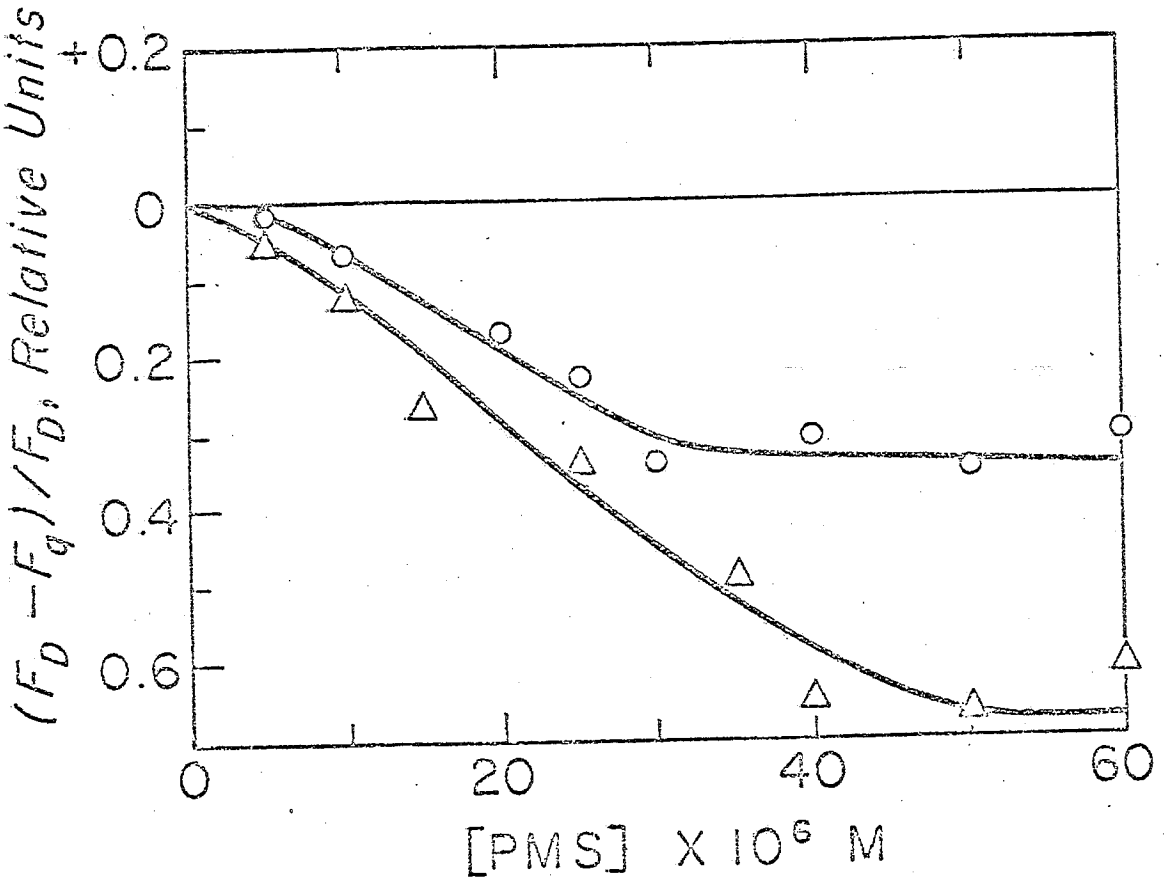

A
B
10 μM DCMU
10 μM DCMU + 25 μM PMS
Light off
Relative Fluorescence Yield
Time of Illumination, sec
Dark Interval, sec
C
DCMU
+ PMS
10 μM
20 μM
30 μM
50 μM
D
DCMU
DCMU+PMS+Fccp
DCMU+PMS
Fluorescence, Relative Units
Time of Illumination, sec

Figure 56. Effect of varying concentrations of PMS on the lowering of Chl *a* fluorescence yield in DCMU poisoned *Chlorella*. F_D, steady state fluorescence yield at 685 nm with 10 μM DCMU; F_q, steady state fluorescence yield at 685 nm with 10 μM DCMU plus 30 μM PMS; open circles, pH 6.8; open triangles, pH 8.0. Buffer, 50 mM phosphate buffer plus 0.01 M KCl; other details as in Figure 46.


$(F_D - F_q)/F_D$, Relative Units
+0.2
0
0.2
0.4
0.6
0
20
40
60
[PMS] X 10^6 M

found approximately the same extent of lowering with PMS or $PMSH_2$ at pH 6.8 or pH 8.0.) Although we are not aware of any study suggesting an optimal pH for photophosphorylation and electron transport in vivo, it may be that the optimal pH is probably around pH 8.0 as in the case of isolated chloroplasts (193). Figure 57 shows the emission spectra of Chlorella at liquid nitrogen temperature (77° K), in the presence of DCMU and DCMU plus PMS. Both the samples were pre-illuminated before cooling (rapidly) with liquid nitrogen. The emission characteristics are not significantly altered by PMS. Murata and Sugahara (193) reported a total depression of yield of fluorescence, without any alteration of emission characteristics by PMS at 77° K. Although it is difficult to ascertain the yield at 77° K as we do not know the exact path length of illumination after freezing with liquid nitrogen (45), it is clear that PMS does not seem to alter significantly fluorescence bands or their relative ratios. This suggests that lowering of yield by PMS is probably not associated with a change in spill over of quanta from PS II to PS I.

In collaboration with Professor C. Sybesma, I made some experiments on the effect of $DCPIPH_2$ and $PMSH_2$ on the chlorophyll yield changes in the red alga Porphyridium as well as in Chlorella. The data of a typical experiment with Porphyridium is presented in Figure 58 (with the permission of Dr. Sybesma). The details of these experiments and their possible relation to the recent findings of a light induced potential change across the chloroplast membrane by Vredenberg (198) will be elaborated on later by Dr. Sybesma (personal communication). Figure 58 shows that the quenching induced by $DCPIPH_2$ is reversed by an uncoupler, FCCP (3 μM) and an "energy transfer" inhibitor, phloridzin (1 mM). The quenching caused by the photosystem I cofactor $DCPIPH_2$ is faster than that caused by $PMSH_2$

Figure 57. Emission spectra of Chlorella at 77° K with DCMU and DCMU plus PMS. Curve with open circles, 10 μM DCMU; curve with closed circles, 10 μM DCMU plus 30 μM PMS; buffer, 50 mM phosphate, pH 7.8 plus 10 mM NaCl; excitation λ = 435 nm (half band width, 6.6 nm); observation slit, 3.3 nm. The samples were pre-illuminated for 3 min with broad-band blue light before cooling.

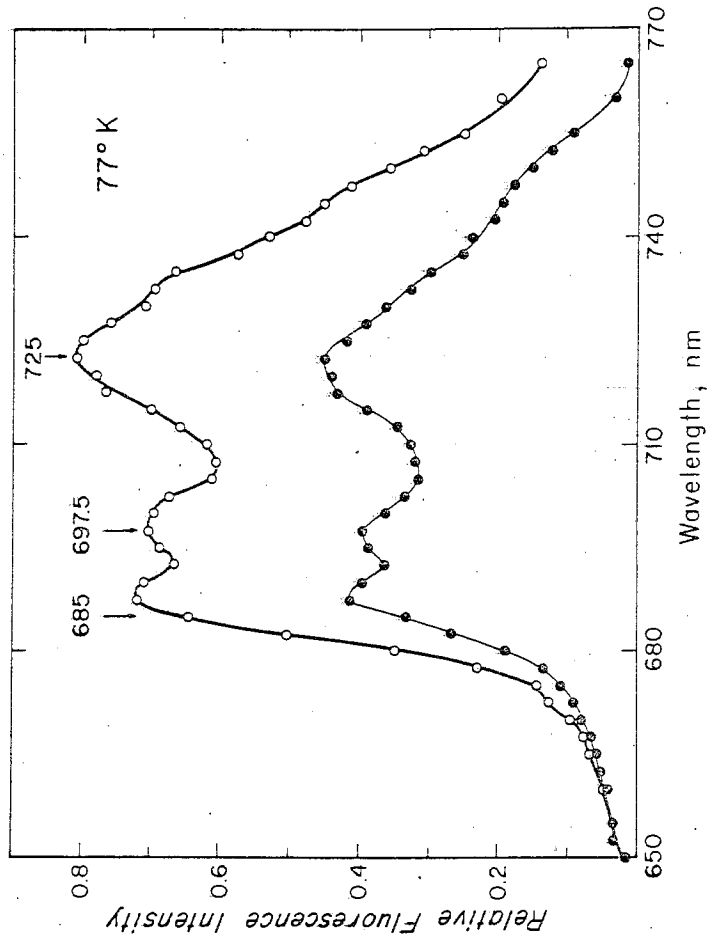
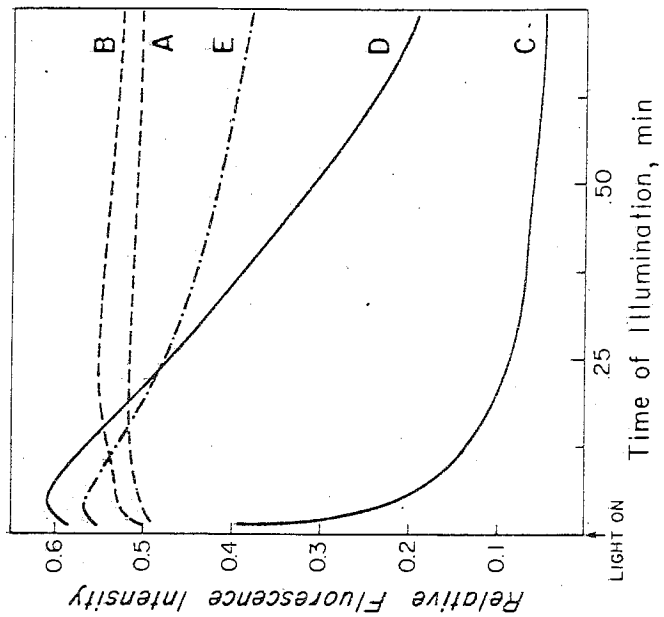

77° K
725
697.5
685
650
680
710
740
770
Wavelength, nm
0.2
0.4
0.6
0.8
Relative Fluorescence Intensity

Figure 58. Time course of slow fluorescence yield changes in the red alga Porphyridium cruentum. (A): 15 μM DCMU; (B): 15 μM DCMU + 1 mM Ascorbate (neutralized); (C): 10 μM DCMU + 0.1 mM DCPIP and 1 mM Ascorbate; (D): C plus 1 mM phloridzin; (E): C plus 3 μM FCCP; 4-day old cells in fresh culture medium, blue green excitation light (C.S. 4-96 + C.S. 3-73) intensity, 1.8 x 10^4 ergs cm^{-2} sec^{-1}. Observation λ, 685 nm (half band width, 24 nm); all additions were done in the dark 1-2 minutes before the measurements (C. Sybesma and P. Mohanty, unpublished observations).


B
A
E
D
C
0.6
0.5
0.4
0.3
0.2
0.1
LIGHT ON
.25
.50
Time of Illumination, min
Relative Fluorescence Intensity

(not shown). Ascorbate alone did not change the yield except for a slight increase. Low concentration of FCCP is more effective in reversing the lowering of yield by $DCPIPH_2$ than the glucoside phloridzin. But we did always observe a small inhibition with phloridzin.

We observed that the reoxidation of reduced DCPIP was accelerated in light by these intact cells which suggests that $DCPIPH_2$ feeds electrons, probably very similar to that in isolated chloroplasts. (We have not made a detailed study of this observation.) But because of this rapid reoxidation of the dye, the problem of inner filter effect of the indophenol dye gave rise to problems in repeating this transient repeatedly. In spite of these problems, we could observe a general quenching by $DCPIPH_2$ in Chlorella (not shown) as well as in Porphyridium (Figure 58) in the presence of DCMU. We shall discuss the probable nature of this type of system I mediated quenching or lowering of fluorescence yield later in our discussion.

6. Salt Induced Changes in the Fluorescence Yield in Chlorella

It has been recently shown that ionic environment influences yield in intact algal cells as well as in isolated chloroplasts. Papageorgiou and Govindjee (199) reported variations in Chl a fluorescence yield of intact Chlorella cells by change in pH of the bathing medium. Homann (56) first observed an increase in the fluorescence yield by the addition of salts. Murata (200) has recently catalogued the effect of various mono and divalent cations (notably Mg^{++}, on increasing the fluorescence yield in isolated spinach chloroplasts (also see Chapter VI). Murata (57) has also shown that these ions also enhance the photochemical activity of PS II reaction with a concomitant decrease in PS I activity.

In this section, we report the effect of various salts on the fast transient of Chlorella. These experiments wer carried out to investigate if the ions influence the change in fluorescence yield as they do in isolated chloroplasts (53, 201). Cells from a 4-6 day-old culture of Chlorella were washed in distilled water or in 0.005 M PO_4^- buffer (pH 6.8) several times (at least, 3 to 4 times). Very small quantities of salts were added in the dark and fluorescence transients were measured after a dark period of 7 to 10 minutes. (P. Mohanty and Govindjee presented the results of this section at the conference on the Primary Photochemistry of Photosynthesis, Argonne National Laboratory, November, 1970.)

Figure 59 shows the effect of KCl on the amplitude of 0, P and S. The data are plotted as the percent of control. The percentage of quenching at P varies between 10 to 20%, depending on the culture conditions. The quenching occurred only at a relatively high concentration of salts. We did not observe any increase in the relative yield of fluorescence at any concentration of salts used.

The quenching is greater at P than at S in the presence of KCl. The constant '0' level varied very slightly, and in some measurements, it remained constant. In the case of NH_4Cl the extent of quenching remains the same both at P and at S. There is a general depression of yield but P/S ratio did not change for NH_4Cl, while P to S decline is markedly affected at 0.3 to 0.4 M KCl in the bathing medium.

Since Mg^{++} ions are known to cause maximum enhancement of the yield of Chl a fluorescence in isolated chloroplasts, we have investigated the effect of $MgCl_2$ on the fast fluorescence transient. Again, the effect of $MgCl_2$ (Figure 60) is very similar to KCl or NaCl (not shown). $MgCl_2$ was found to be effective in lowering the yield at a relatively lower (0.3 M)

Figure 59. Salt induced fluorescence yield changes in Chlorella: Effect of various concentrations of salts of KCl and NH_4Cl on the P and S levels of the fast fluorescence transient. Upper 3 traces, fluorescence yield changes with KCl at S (solid squares), at 0 (solid circles), and at P (open circles); curve with large open circles (with small solid squares inside) represents the changes in yield at both P and S with NH_4Cl (0 level was not measured). Bottom traces, P/S ratio for KCl (solid circles); and for NH_4Cl (open circles); ordinate scale to the right. Fluorescence yield of cells, without salts was adjusted to read 1.0 in the upper 4 curves. Blue light excitation (C.S. 4-72, 3-73) intensity, 11 Kergs $cm^{-2}sec^{-1}$; λ observation, 685 nm (half band width, 6.6 nm). All additions were made in the dark.

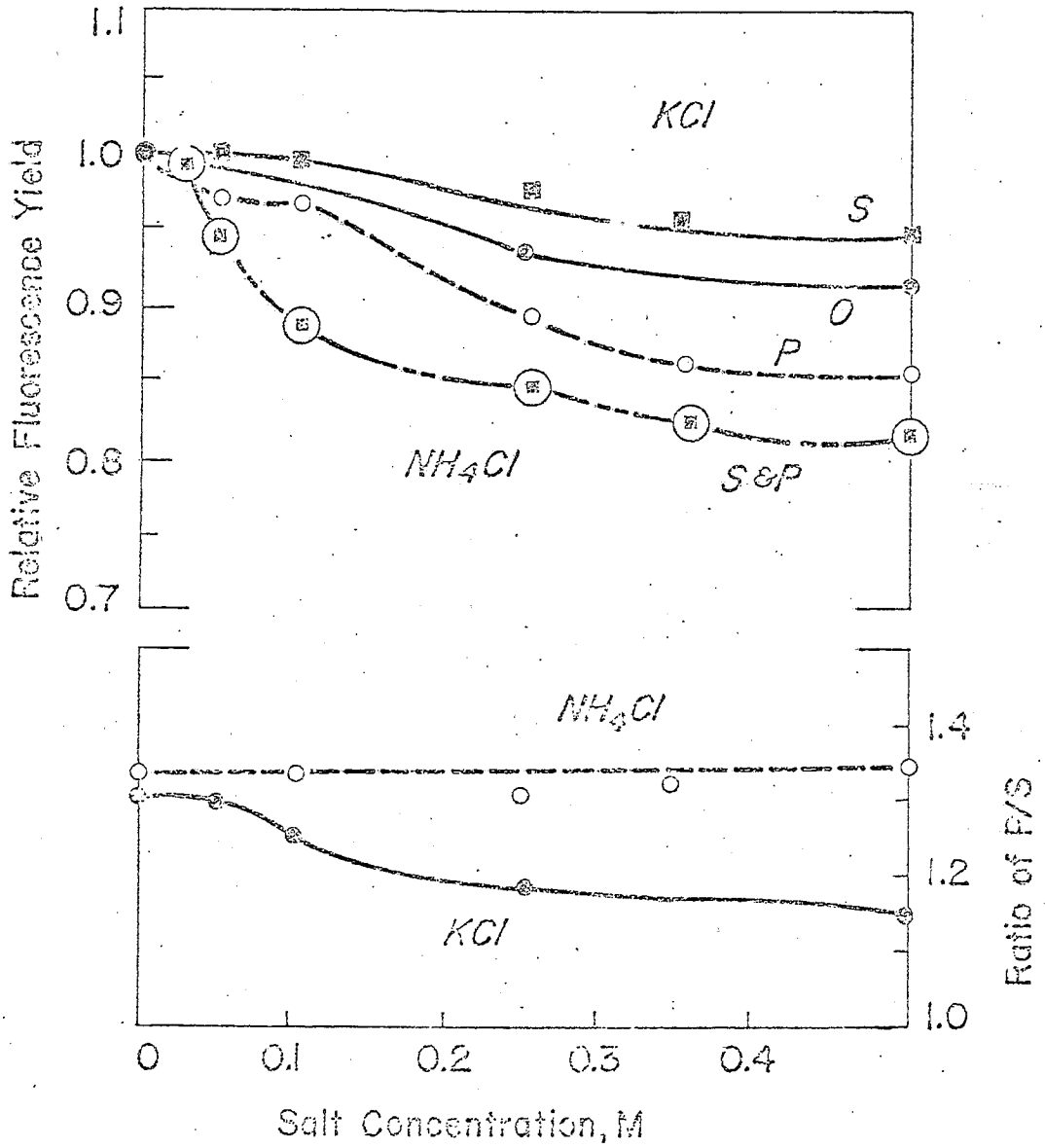
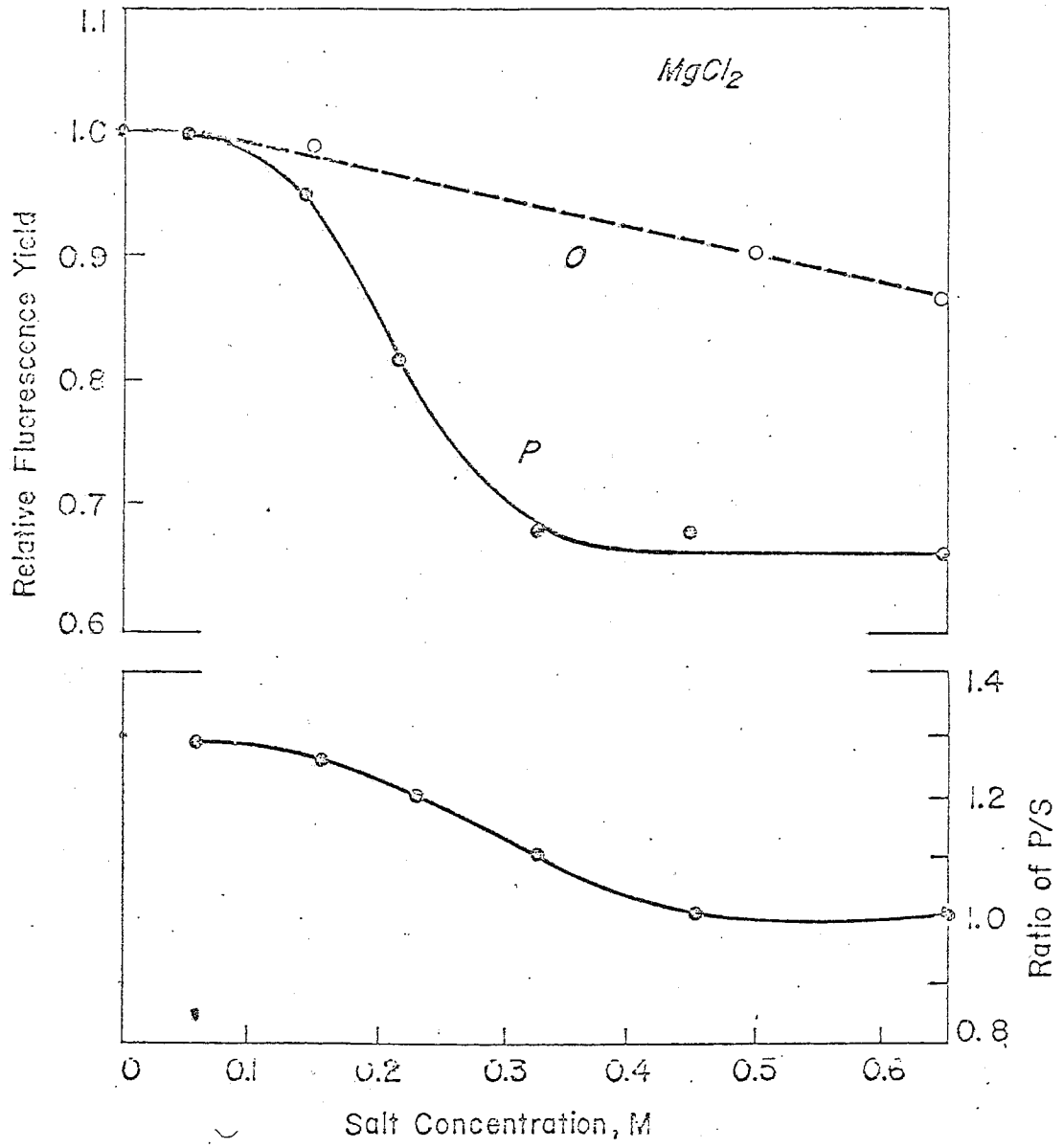

Relative Fluorescence Yield
1.1
1.0
0.9
0.8
0.7
KCl
S
O
P
NH4Cl
S&P
NH4Cl
KCl
1.4
1.2
1.0
Ratio of P/S
0
0.1
0.2
0.3
0.4
Salt Concentration, M

Figure 60. Salt induced fluorescence yield changes in Chlorella; effect of various concentrations of $MgCl_2$ on the fluorescence yield at the O and at the P levels of fluorescence transient. Bottom curve, P/S ratio; ordinate scale to the right. All other details of measurement as in Figure 59.


1.1
1.0
0.9
0.8
0.7
0.6
Relative Fluorescence Yield
$MgCl_2$
O
P
1.4
1.2
1.0
0.8
Ratio of P/S
0
0.1
0.2
0.3
0.4
0.5
0.6
Salt Concentration, M

concentration than KCl. We did not observe any increase in yield at any concentration of Mg^{++} ions used as observed by others in chloroplasts.

Figure 60 shows the relative changes in the yield at 0 and at P levels with the increasing concentration of $MgCl_2$. The relative yield at 0 declined only 9 to 10% while the P was suppressed approximately 30 to 35% compared to the untreated sample. The bottom curve shows the decrease in the P/S ratio with increasing concentrations of $MgCl_2$; a complete suppression of P to S decline occurred with 300 to 350 mM $MgCl_2$. The exact concentration of the salt necessary to suppress this decline varied with different cultures and, also at times, with the time of incubation with salt. (We usually used 10 minutes of incubation between the addition of salts and measurements.) Table IV.6 shows the relative fluorescence yield at P, when measured at 685 nm and at 720 nm with normal and $MgCl_2$ treated

Table IV.6

Relative Changes in Fluorescence Yield at P as Measured at 685 nm or at 720 nm in Chlorella With and Without $MgCl_2$

Addition	Yield at P λ Observation, 685 nm	Yield at P λ Observation, 720 nm
H_2O	1.00	1.00
0.15 M $MgCl_2$	0.98	1.00
0.3 M $MgCl_2$	0.79	0.89

Other conditions for measurements of relative yields as in Figure 59.

samples. (These data were collected from a separate experiment.)

The extent of lowering of the fluorescence yield was greater at 685 nm--the main band of the fluorescence spectrum--than at 720 nm band which

probably has a larger contribution from PS I than PS II. We observed a similar effect with KCl.

Figure 61 shows the effect of varying the intensity of illumination on the percent of quenching by 0.25 M $MgCl_2$. The percent quenching remained invariable at most of the intensities used (the decrease seen at the lowest intensity is due to an uncertainty in our measurements). We know that the yield at P does not remain constant at these intensities of excitation--the yield declines when the intensity is lowered to 40% of the maximum intensity used. Thus, the intensity independence of quenching by $MgCl_2$ suggests that this lowering of the yield is not associated with the primary acceptor of PS II.

This suggestion is further verified from the observation of a quenching in the presence of DCMU which keeps all Q in the reduced state at high intensity of illumination. With 350 mM $MgCl_2$ we observed 50-55% quenching by $MgCl_2$. Figure 62 is a plot of F_{DCMU}/F_{salt} versus concentration of added $MgCl_2$. This reciprocal plot indicates that the quenching by $MgCl_2$ is not a simple dynamic quenching as we do not obtain a straight line as predicted by the Stern-Volmer relationship. Thus, from our observations of quenching by KCl, NaCl or $MgCl_2$ in the presence of DCMU, we conclude that it is not influenced by the reduction level of the primary acceptor Q, and that the quenching is not of collisional type.

Since chloroplasts are known to shrink or swell depending on the ionic environment, it has been suggested that these macroscopic changes of structural state may govern the yield of fluorescence. However, the molecular mechanism of shrinking or swelling is not clearly defined for the salts of strong acids (125). On the other hand, the mechanism of structural changes brought about by salts of weak organic acids are very

Figure 61. Ratio of yield of fluorescence at the peak P or normal to that of $MgCl_2$ treated samples of Chlorella as a function of excitation intensity. Intensity 1.0 = 14 Kergs $cm^{-2}sec^{-1}$. All other details of measurement as in Figure 59 except that the cells were washed repeatedly in distilled water and suspended in distilled water (pH 6.8).

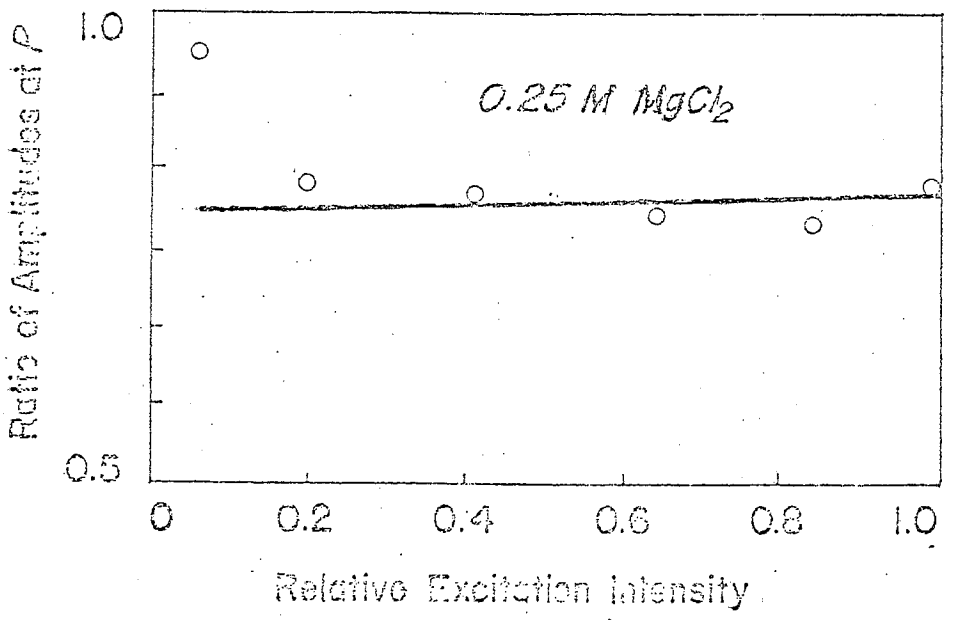
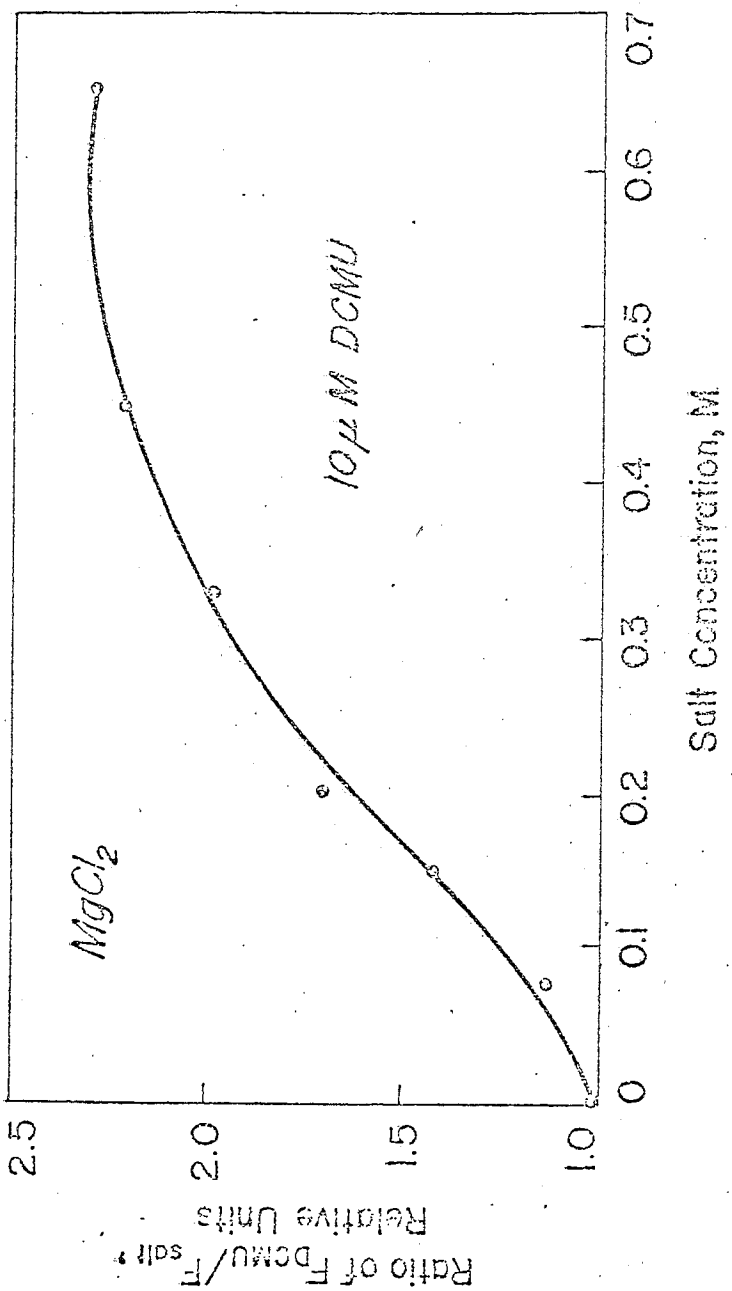

Ratio of Amplitudes at P
1.0
0.5
0.25 M MgCl2
0
0.2
0.4
0.6
0.8
1.0
Relative Excitation Intensity

Figure 62. Salt induced fluorescence yield changes in DCMU poisoned Chlorella. Ratio of steady state Chl a fluorescence yield of Chlorella cells treated with DCMU (F_{DCMU}) to that treated with DCMU plus $MgCl_2$ (F_{salt}) versus various concentrations of $MgCl_2$. Other details of measurement as in Figure 60.


$MgCl_2$
10 μM DCMU
Ratio of F_{DCMU}/F_{salt}
Relative Units
2.5
2.0
1.5
1.0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Salt Concentration, M

well defined by Crofts et al. (123) (see Introduction). We, thus, tested the effect of salts of weak acids (weak acid anions and weak base cations) on the fast fluorescence transient of Chlorella.

Figure 63 illustrates the effect of potassium acetate on the amplitudes of various characteristic points of the fast transient measured at 685 nm. Both fluorescence yield at P and S are lowered, but the yield at P is more suppressed than at S. The effective concentration is in about the same range as usually used to cause volume changes in the chloroplasts. At 50 to 100 mM K-acetate, P to S changes are abolished. Injection of 15 μM DCMU to the potassium acetate quenched samples causes an increase in the yield. But this increase in yield does not reach the level attained by DCMU poisoned sample; it is always lower than the normal sample treated with DCMU alone. Further addition of K-acetate quenches the fluorescence even in the presence of DCMU.

Figure 64 shows the effect of 100 mM NH_4-acetate and 100 mM K-acetate. K-acetate (also Na-acetate) completely abolishes most of the fast transient while the same concentration of ammonium acetate only hastens the D to P rise slightly; these salts either accelerate, or do not affect the P to S decline. The faster DP rise with NH_4-acetate could be explained to be due to a slowing down of the rate of electron transport by ammonium acetate, but PS decline is not affected. Similarly a suppression of the development of P by sodium and potassium acetate may be due to an acceleration of electron flow. We have not made oxygen exchange measurements to support or discard this suggestion. However, it seems very unlikely that ammonium and sodium salts of acetic acid would cause entirely opposing effects on the electron flow. (We also note that NH_4Cl did not alter the rate of DP

Figure 63. Effect of varying concentrations of potassium acetate on the O, P and S of the fast transient in Chlorella. Bottom curve (solid squares), P/S ratio; ordinate scale to the right. Other details as in Figure 59.

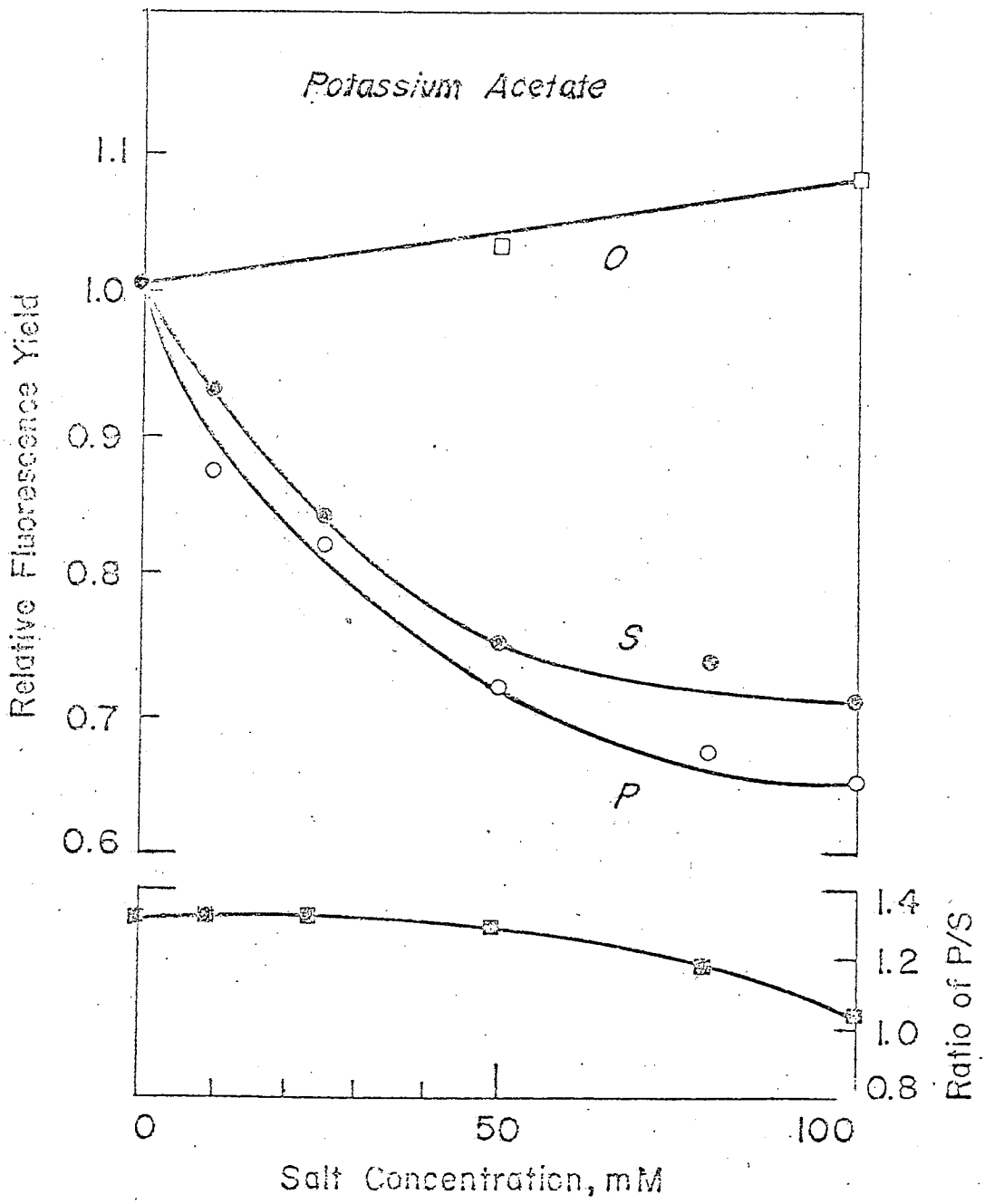
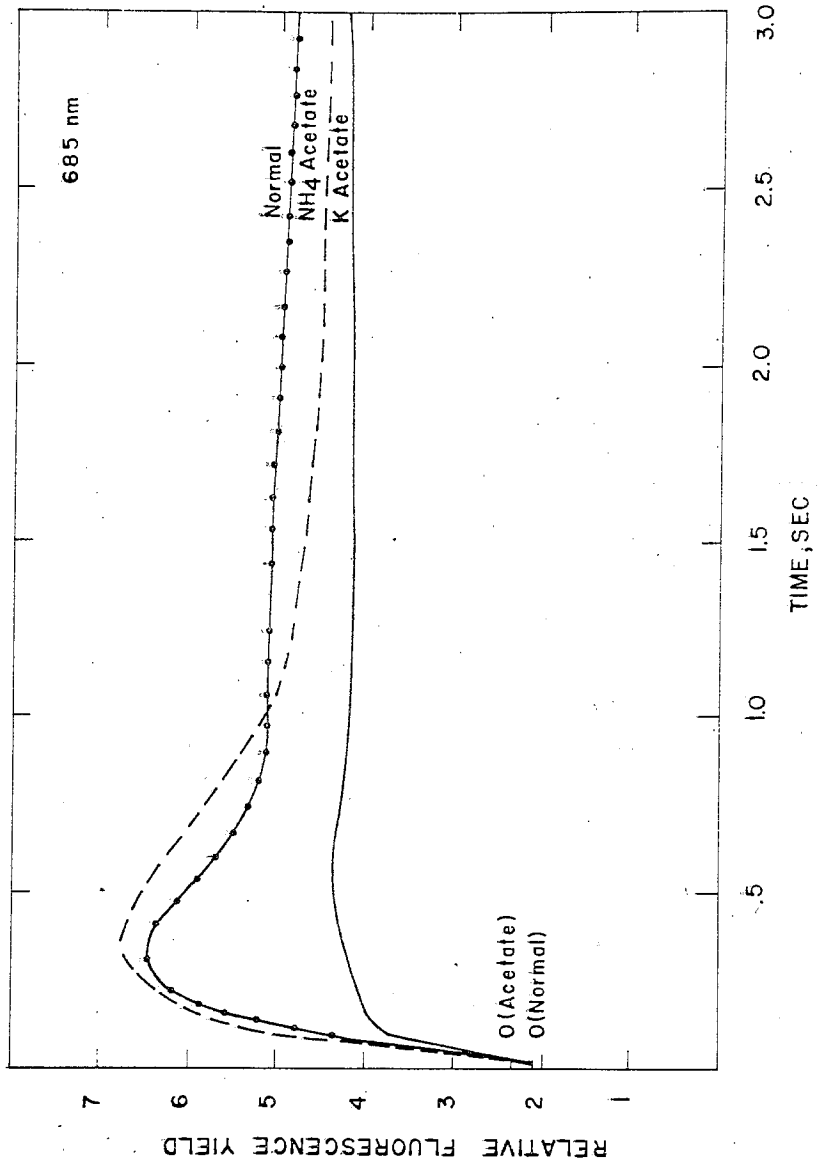

Potassium Acetate
1.1
1.0
0.9
0.8
0.7
0.6
O
S
P
Relative Fluorescence Yield
1.4
1.2
1.0
0.8
Ratio of P/S
0
50
100
Salt Concentration, mM

Figure 64. Time course of Chl *a* fluorescence yield of *Chlorella*. λ observation, 685 nm (half band width, 5 nm); excitation blue light (C.S. 4-72, plus C.S. 3-73) intensity, 14 Kergs $cm^{-2} sec^{-1}$; curve with solid circles, normal; dashed line, 100 mM ammonium acetate; solid line, 100 mM potassium acetate. Dark time, 7 min before illumination. Other details of measurement as in Figure 36.


685 nm
Normal
NH4 Acetate
K Acetate
O(Acetate)
O(Normal)
RELATIVE FLUORESCENCE YIELD
TIME, SEC
1
2
3
4
5
6
7
.5
1.0
1.5
2.0
2.5
3.0

rise to any measurable extent.) Furthermore, from the data in Table IV.7, it is clear that salts of sodium benzoate and sodium acetate; that they also lower the fluorescence yield even in the presence of DCMU although

Table IV.7

Effect of the Addition of 100 mM Salts on the Yield of Fluorescence in Chlorella

Salts	Relative Fluorescence Yield at P	Ratio of P/S
No addition	1.00	1.3 to 1.35
KCl	0.97	1.25
NaCl	0.98	1.27
NH_4Cl	0.90	1.30
K acetate	0.65	1.00
Na acetate	0.60	1.00
Na benzoate	0.70	1.00
NH_4 acetate	1.04	1.50

Fluorescence was measured at 685 nm; excitation, broad band blue light intensity 14 Kergs $cm^{-2}sec^{-1}$; salts added 5-7 min. before measurements.

to a lesser extent than $MgCl_2$. Thus, these data indicate that lowering of P by salts of sodium or potassium acetate is not due to a change in the electron transport. Instead of a quenching, we observed a slight increase in the constant '0' level by the addition of potassium or sodium acetate. This increase was found to be greater in sodium salts than potassium and the extent varied from culture to culture. (Particularly, in the case of Na-acetate we observed an increase of 0 on increasing the time of incubation.) We have not investigated the cause of this differential effect on

0 by Na-acetate and NaCl. Since the extent of enhancement at the 0 level is much smaller than the quenching observed at P level, we believe that this does not influence our conclusions to any great extent. The lowering of fluorescence yield by benzoates or acetate or other salts in the presence of DCMU may be interpreted to be due to an acceleration of back reactions (see discussion). Salts are known to stimulate delayed fluorescence or delayed light emission (105, 106). But, in these experiments, salts are added after the illumination to cause a shift in the ionic environment between the inside and outside of chloroplast membranes. We believe that the cause of the fluorescence yield decrease by the addition of salts is not due to a stimulation of back reactions as we observed no change in the percent of quenching at low and high intensity of illumination with $MgCl_2$. The back reaction should predominate at lower intensity of excitation.

Table IV.8 shows the effect of adding 100 mM salts of various kinds on the yield of fluorescence at P and on the ratio P/S. The weak acid

Table IV.8

Effect of Sodium Acetate and Sodium Benzoate on Lowering the Steady State Fluorescence Yield in DCMU Poisoned Chlorella

Concentration	Steady State Fluorescence Level, Arbitrary Units	Fraction of Control
10 μM DCMU (control)	7.0	1.0
+ 100 mM Na-acetate	6.6	0.94
+ 200 mM Na-acetate	5.2	0.74
200 mM Na-benzoate	5.8	0.83

Fluorescence was measured at 685 nm as described in the legends of Figure 61.

anions (acetate and benzoate) are very effective in causing a quenching in the Chl a fluorescence yield. These salts are known to cause very large magnitudes of shrinkage in isolated chloroplasts.

The lowering of fluorescence with salts reported above does not seem to result from an osmotic effect as the addition of 0.6 M sorbitol only slightly depressed the yield at P but the PS decline did not alter significantly.

6.1 Spectral Changes in the Presence of Salts

Figure 65 shows room temperature emission spectra of Chlorella in the presence and in the absence of $MgCl_2$; the ratio of F720/F685 is increased by $MgCl_2$. The same ratio at liquid nitrogen temperature increased from 0.9 to 1.4 by the addition of $MgCl_2$ (see Table IV.9). This indicates that

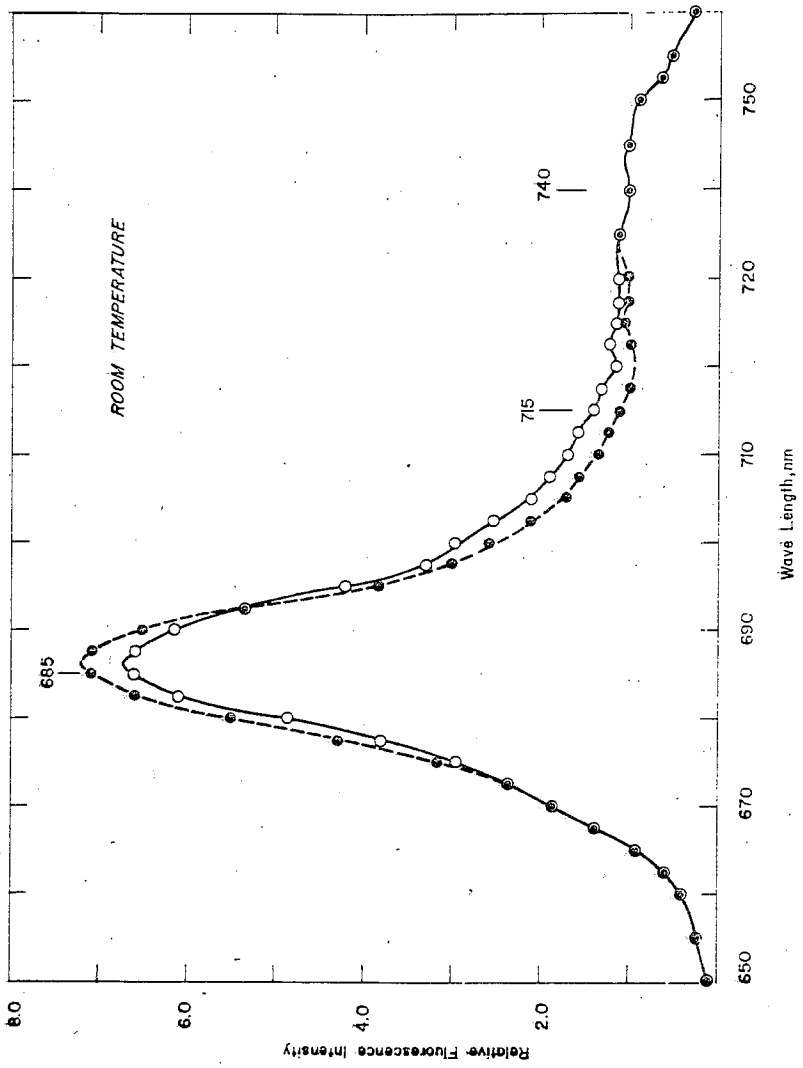
Table IV.9

Comparison of the Ratio of the Long Wavelength (F720) to the Short Wavelength Fluorescence Band (F685) at Room Temperature and at Liquid Nitrogen Temperatures in Chlorella

Addition	298°K	77°K
None	0.133	0.9
150 mM $MgCl_2$	0.140	1.1
300 mM $MgCl_2$	0.177	1.4

For liquid nitrogen spectra the samples were pre-illuminated for 5 minutes before cooling to 77°K. The ratios were calculated from uncorrected emission spectra.

Figure 65. Room temperature emission spectra of Chlorella with and without $MgCl_2$ (normalized at 660 nm). Solid circles, normal cells measured 5 min after illumination; open circles, 300 mM $MgCl_2$, also measured 5 min after illumination; excitation, broad band blue light (C.S. 4-72 and C.S. 3-73) intensity, 14 Kergs $cm^{-2}sec^{-1}$; λ observation, variable (half band width, 5 nm). A Corning C.S. 2-64 filter was placed before the analyzing monochromator.


ROOM TEMPERATURE
685
715
740
650
670
690
710
720
750
Wave Length, nm
2.0
4.0
6.0
8.0
Relative Fluorescence Intensity

$MgCl_2$ increases energy transfer from system II to system I. Figure 66 shows room temperature emission spectra of Chlorella in the presence and absence of 100 mM sodium acetate. The intensity of the two samples was matched at 685 nm to show the increase in the intensity of the long wavelength fluorescence (690-720) associated with PS I.

Figure 67 shows emission spectra measured at liquid nitrogen temperature (77°K) with and without potassium acetate. It is clear that F720 is enhanced with respect to F685 in both potassium and sodium acetate (not shown) treated samples. In these cases, 15 μM DCMU was added to the normal sample to avoid the complications of spectral changes that may be associated with SMT changes during illumination before freezing. However, we obtained almost similar results if DCMU was omitted. These results indicate that the fluorescence yield changes stimulated by addition of salts are mostly independent of the redox state of the primary acceptor of PS II. The decrease in the yield of fluorescence of chlorophyll a of system II may be caused by changes in the structural state of the chloroplasts. Our results suggest that the shrinkage of chloroplast volume in light causes a change in energy transfer from PS II to PS I. It is well known that chloroplasts shrink in light in the presence of Na-acetate or benzoate and swell in the presence of ammonium salts. Perhaps, chloroplasts in vivo also shrink in light in the presence of salts like NaCl, KCl, or $MgCl_2$ although both swelling and shrinkage have been shown to occur in light in the presence of salts of Cl ions (see references cited in 124). The mechanism of structural changes with chloride salts has not been clearly elucidated yet.

Although we believe that most of the quenching is due to an increase in energy transfer from the strongly fluorescent PS II to weakly

Figure 66. Room temperature emission spectra of Chlorella (normalized at 740 nm) with and without sodium acetate. Solid curve, 10 μM DCMU plus 100 mM sodium acetate; open circles with dashed line, 15 μM DCMU alone. Other details as in Figure 64.

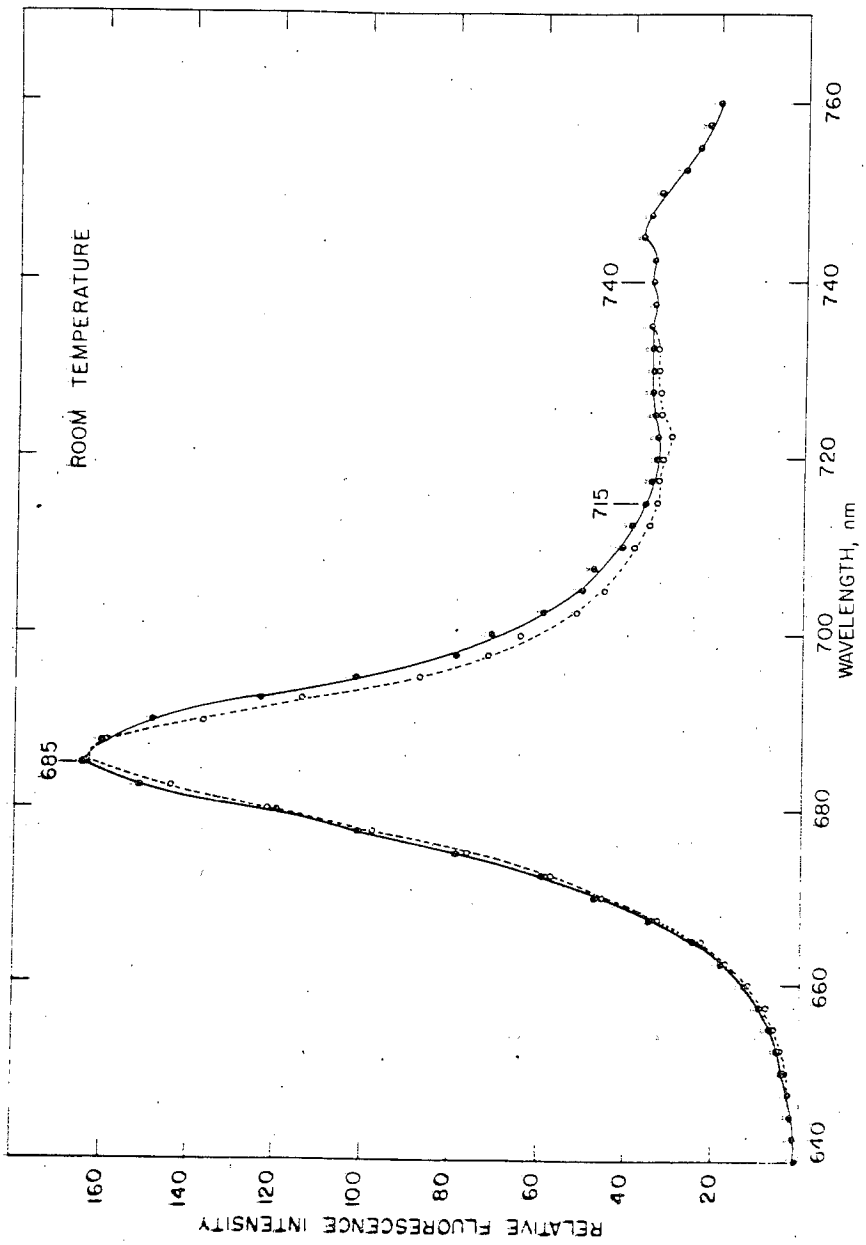
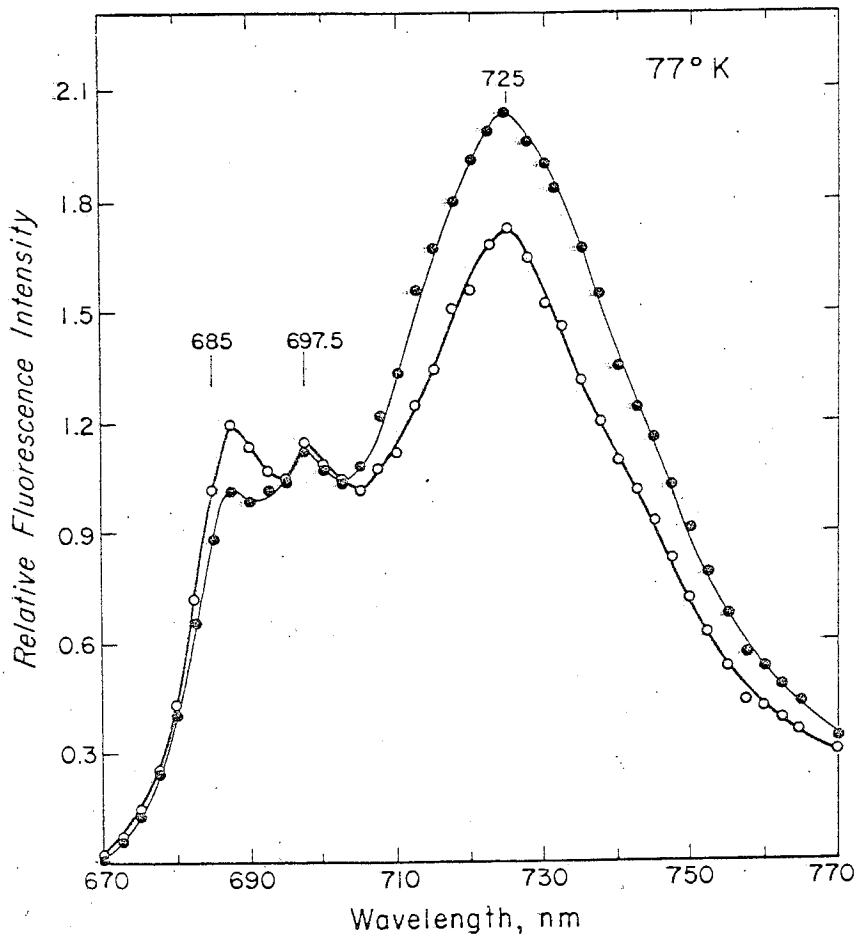

ROOM TEMPERATURE
685
715
740
RELATIVE FLUORESCENCE INTENSITY
160
140
120
100
80
60
40
20
640
660
680
700
720
740
760
WAVELENGTH, nm

Figure 67. Emission spectra of Chlorophyll a fluorescence of Chlorella at 77°K. Open circles, 15 μM DCMU; solid circles, 15 μM DCMU plus 100 mM potassium acetate. Both samples were pre-illuminated before cooling. λ excitation, 436 nm (half band width, 10 nm); C.S. 2-58 filter before the photomultiplier.


77° K
725
685
697.5
2.1
1.8
1.5
1.2
0.9
0.6
0.3
Relative Fluorescence Intensity
670
690
710
730
750
770
Wavelength, nm

fluorescent PS I, some of these salts may also affect, to some extent, the rate of electron transport. The concentration of salts used to observe the lowering of fluorescence yield is relatively high. But, in all likelihood, the actual concentration of salts in the vicinity of chloroplasts must be lower than the amount present in the bathing medium.

The effects of salts in intact algal cells seem contradictory to what has been observed in isolated chloroplasts. We believe this difference arises due to different limiting membranes in intact algal plastids and broken class II type chloroplasts usually used in such studies. In broken chloroplasts the thylakoid membrane may have been ruptured. This would allow salts to enter into the space between the outer and the inner portion of the membrane of the thylakoids and bring about a differential shrinkage and folding between these portions of the membranes thereby causing a spatial separation of two photosystems. In intact algal plastids the whole chloroplasts would shrink and cause stacking with salts and this would bring the two photosystems close to each other and thereby facilitate energy transfer. Obviously, these are speculative suggestions and we have no experimental support for it yet.

7. Summary and Conclusions

The results reported in this chapter confirm and amplify the suggestion (87, 88, 155) that the slow changes in Chl fluorescence yield are not affected by the redox state of Q, although a net electron transport may be necessary to support the slow changes in fluorescence yield.

The ability of system I light to quench the system II fluorescence yield was found to be complex. Our results (Figure 41 a, b) clearly indicate that the quenching by system I light is not the same for the same

amount of fluorescence yield (same level of QH) during the SMT transient. In other words, the fluorescence yield during SMT phase is not solely governed by the amount of QH. If it were so, we should have observed the same extent of quenching at times when the fluorescence yield during the SM rise and MT decline are about the same. System I light brings about an immediate decrease in the yield of fluorescence caused by system II illumination. This immediate decline in the yield seems to be associated with oxidation of QH. However, during the period when both lights are on, the fluorescence yield tends to increase slowly with time and appears as an overshoot when system I illumination is turned off. This slow increase in yield caused by PS I illumination is not linked with the oxidation-reduction state of Q. It may be associated with a shift in quantum distribution between the two photosystems as proposed by Murata (162), Bonaventura and Myers (155) and Duysens (157). Thus it seems that the SM rise portion of the fluorescence induction curve is due to suppression of energy transfer from strongly fluorescent PS II to weakly fluorescent PS I.

The S to M rise phase of the "second wave" of fluorescence induction was stimulated or remained unaffected by uncouplers (cf. Figure 50) and to the surrounding pH of the medium as shown by Papageorgiou and Govindjee (199), but MT decline was found to be very sensitive to these parameters (Figure 50). FCCP, CCCP, DNP and atebrin all caused greater suppression of the MT decline phase of the fluorescence transient than the SM rise phase. These results indicate that SM rise phase has different underlying causes than the MT decline portion of the slow fluorescence induction curve. Moreover, a comparison of the PS decline and the MT decline kinetics in Chlorella suggest that these two modes of the fluorescence

yield changes are not related. Furthermore, the O_2 transient and greater sensitivity of the PS decline to system I pre-illumination support this suggestion. The extent of MT decline was found to be greatly influenced by the cell culture and the nature of the suspending medium.

We have shown that Hill oxidants like quinones, although they abolish most of the fast fluorescence transient, still retain a slow SM rise in the fluorescence yield (Figure 45). With methyl viologen (MeV), which accepts electrons from the terminal acceptor of PS I, the extent of the slow fluorescence rise was smaller than with parabenzoquinone. It may be tempting to speculate that methyl viologen inhibits the cyclic photophosphorylation which may be related to the slow rise in the fluorescence yield. But, this does not seem to be the case as addition of MeV causes the net ATP content of the cell to increase over the control sample (Bedell, 145). Although it is difficult to ascertain the cause of the increase of ATP by methyl viologen, it is clear that ATP, and probably its precursors, are not limiting in the MeV treated sample. Thus, we believe that the depression of the extent of slow changes in the yield is probably due to some additional effect of MeV on the membrane structure. In any event, results obtained with parabenzoquinone indicate that a slow increase in fluorescence yield persists even when most of the primary acceptor would be expected to be in the oxidized state. Furthermore, our results with FCCP and parabenzoquinone indicate that a slow SM type rise persists when most of the DPS changes in yield are suppressed.

As in the case of Porphyridium most of the slow changes in the fluorescence yield in Chlorella were arrested by the addition of DCMU or orthophenanthroline (Figure 51). It has been assumed that cyclic photophosphorylation support slow changes in fluorescence (88, 89). Our

results indicate that there is probably a very limited rate and extent of cyclic photophosphorylation in Chlorella. Measurements of ATP with DCMU poisoned samples of Chlorella by Bedell (145) seem to agree with this suggestion. (We are aware that the ATP content of the cell may not reflect the true extent of the cyclic electron transport.) However, it was observed that a slow decline in yield and subsequent quenching can be induced by the addition of ionophorous chemicals like CCCP, FCCP and DNP in the presence of DCMU. (This confirms the observations of Bannister who first discovered that FCCP lowers the fluorescence yield in the presence of DCMU (177).) This kind of lowering in the fluorescence yield probably arises due to the change in the permeability of the thylakoid membranes to protons. However, our studies with these proton permeating uncouplers indicate, at least, three modes of action for these compounds depending on the concentration (Figures 46, 47, 49). This seems to be true, at least, for CCCP and FCCP and, maybe, also for DNP (Figures 52, 54). These chemicals cause an inhibition of the back reaction between Z^+ and Q^- in some unknown fashion, stimulate electron flow, and seem to alter structural states of the chloroplasts.

We have shown a lowering of the fluorescence yield in DCMU-treated intact algal cells by PMS (Figures 55, 56) which is very similar to what has been observed in isolated chloroplasts (191-194). Other photosystem I cofactors like $DCPIPH_2$ also lower the fluorescence yield (190; Figure 58). In the first approximation, the kinetics of these two types of lowering are similar to each other. The sensitivity of this lowering of fluorescence yield to uncouplers of phosphorylation both in chloroplasts as well as in intact algal cells strongly suggests that a high energy intermediate may also regulate the chlorophyll a fluorescence (see

Chapter VI).

Finally, we have shown that different ionic environments bring about modifications in the fast changes in Chl a fluorescence yield. de Kouchkovsky (201) has made independent observations on the effect of certain salts on Chl a fluorescence of Chlorella. However, we have provided experimental support for the conclusion that fluorescence yield changes induced by salts are independent of the redox state of Q (Figure 61 and Table IV.8); furthermore we found that the effect of some salts composed of weak acid anions (sodium or potassium acetate) and weak base cations (ammonium acetate) are different. In this respect our studies are quite different from that of de Kouchkovsky (201) who used salts of one type to modify the Chl a fluorescence in Chlorella. As discussed in the introduction, the molecular mechanism is better defined for the salt induced structural alterations in the presence of weak organic acids than for salts like KCl. Our results indicate that a shrinkage of chloroplast volume modifies the spatial relation between the two photosystems and causes a quenching of fluorescence. This provides circumstantial evidence that all fluorescence yield changes, even after 1 to 2 seconds of illumination, are not fully associated with the redox state of Q and A pool. In other words the yield at P seems to be governed not only by Q to QH transformation but also, to a lesser extent, by the so-called "state" transition associated with the quantum distribution between the two pigment systems, the P being a low spill over state. Changes in ionic environment seem to alter the state transition to a greater extent than the redox reactions.

V. FLUORESCENCE INDUCTION IN THE BLUE-GREEN ALGA ANACYSTIS NIDULANS

1. General

The prokaryotic cellular make-up of the blue-green algae constitute the most exciting aspect for the use of blue-green algae (Cyanophyceae) for studies in photosynthesis. From an evolutionary point of view the blue green algae are probably the progenitors of oxygen evolving capacity. Like bacteria, blue-green algae do not have organized plastids but unlike photosynthetic bacteria, they evolve molecular oxygen. The entire lamellar system constitutes both photosynthetic and respiratory thylakoid.

One of the best studied members, in photosynthesis research, is the unicellular alga Anacystis nidulans. Recently, Papageorgiou and Govindjee (88) documented some interesting characteristics of the slow changes in the fluorescence yield in this alga. The fast transient in blue-green alga is not well documented. Clayton (202) is the only one to report the fast fluorescence transient in a blue-green alga--a halophytic species Anacystis montana.

Duysens and Talens (203) reported the slow (long term) changes in the fluorescence yield in another genus of blue-green alga Schizlothrix calcicola. The results obtained by Duysens and Talens (203) are very similar to those obtained in Anacystis nidulans by Papageorgiou and Govindjee (88). We summarize below some of the interesting properties of the slow fluorescence induction observed by these investigators.

In these algae one observes a slow SM rise in the fluorescence yield attaining a maximum after 1 to 2 minutes of illumination (88). After attaining the maximum level the fluorescence level remains high and the

MT decline is very sluggish (204). Secondly, both Anacystis (88) and Schizlothrix (203) exhibit DCMU resistant slow changes in the fluorescence yield. During these time dependent variations of Chl fluorescence yield, the fluorescence yield of the accessory pigments (phycobilins) remains constant throughout the period of illumination (88). Papageorgiou and Govindjee (88) also documented a differential effect of the uncouplers on the slow changes in the fluorescence yield with and without DCMU. FCCP was found to suppress to a large extent the slow changes in fluorescence yield in normal Anacystis although the same concentration of FCCP did not alter the fluorescence yield changes that persist after DCMU poisoning. Because of a pronounced slow change in the fluorescence yield in the presence of DCMU it was believed that a product of cyclic phosphorylation "reactivates" the photosystem II reaction centers (cf. 88, 89, 203).

In this chapter we report experimental results characterizing in some detail the fast fluorescence transient in Anacystis nidulans. This was never done before. We have also employed a variety of metabolic inhibitors to characterize DCMU resistant fluorescence yield changes in blue-green algae. We will present evidence suggesting that the slow changes in the fluorescence yield are entirely independent of the redox state of photosystem II acceptor. We have also attempted to assess the contribution of cyclic phosphorylation as a source of energy for the slow changes in the fluorescence yield. We have also shown, in this study, that there is a slow change in the thylakoid structure upon illumination (with and without DCMU) and these slow structural changes, like slow changes in Chl a fluorescence, are sensitive to uncouplers.

2. Fast Changes in Chl Fluorescence Yield in Anacystis

Blue-green algae are believed not to show the fast wave of fluorescence transient. But actually all blue-green algae which I examined have a distinct but small fast (OIDPS) fluorescence transient. After attaining the S level the fluorescence yield rises slowly to another maximum level M. The MT decline in Anacystis, unlike in other algae, is extremely slow (204). M level, in blue-green algae, is much higher than the P level unlike Chlorella or Porphyridium where P level is the maximum level of fluorescence yield during the transient. We always observed a higher M than P level in Anacystis. Figure 68 is an oscilloscope tracing of the fluorescence transient in the dark adapted cells of Anacystis nidulans. After the onset of illumination, fluorescence yield jumps to an initial O level from where the yield increases to I, and then declines to D. From the minimum (D) level, the fluorescence yield increases to a high 'P' level. P to S decline is very small in Anacystis (Figure 69). From S the fluorescence gradually rises to another maximum M level (not shown in the trace). Figure 69 shows that the cells grown in high light intensity (dotted line) have a slower DP rise and PS decline compared to cells grown in low light. The O level is higher in the high light grown cells than cells grown in low light (not shown in the figure). (The OI rise is not recorded in this figure due to slow response time of the Keithley voltmeter used.)* Also, cells grown at 30°C gave more pronounced OPS transient than cells grown at room temperature (23°C). For most of our experiments we grew our cells in low light intensity (~80 foot candles; a 40 watt bulb placed 13 cm away from the flask) and at room temperature for 2-3 days and then transferred to high temperature (30°C) for 2-3 days before harvesting them. Usually, 5-7 day old cells were used. In spite

Figure 68. Oscillograph tracing of a typical Chl a fluorescence transient in Anacystis nidulans. λ observation = 685 nm (half band width, 6.6 nm), C.S. 2-58 filter before the photomultiplier; λ excitation, 633 nm (half band width, 8 nm; peak transmittance, 85%); intensity, 26 Kergs $cm^{-2} sec^{-1}$. A 7 day old culture of Anacystis was suspended in fresh culture medium after washing the cells in the same medium; 15 min. dark period preceded illumination. Ordinate, 1 volt/division; abscissa, 1 sec/division.

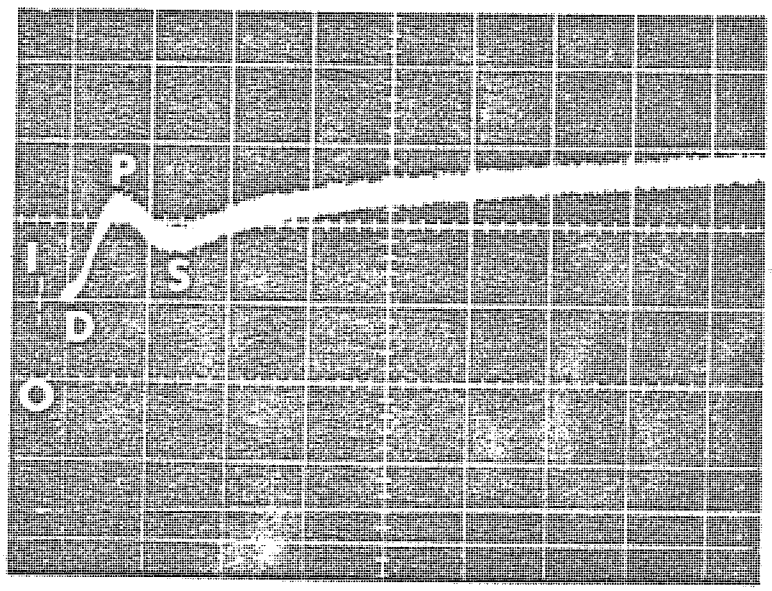
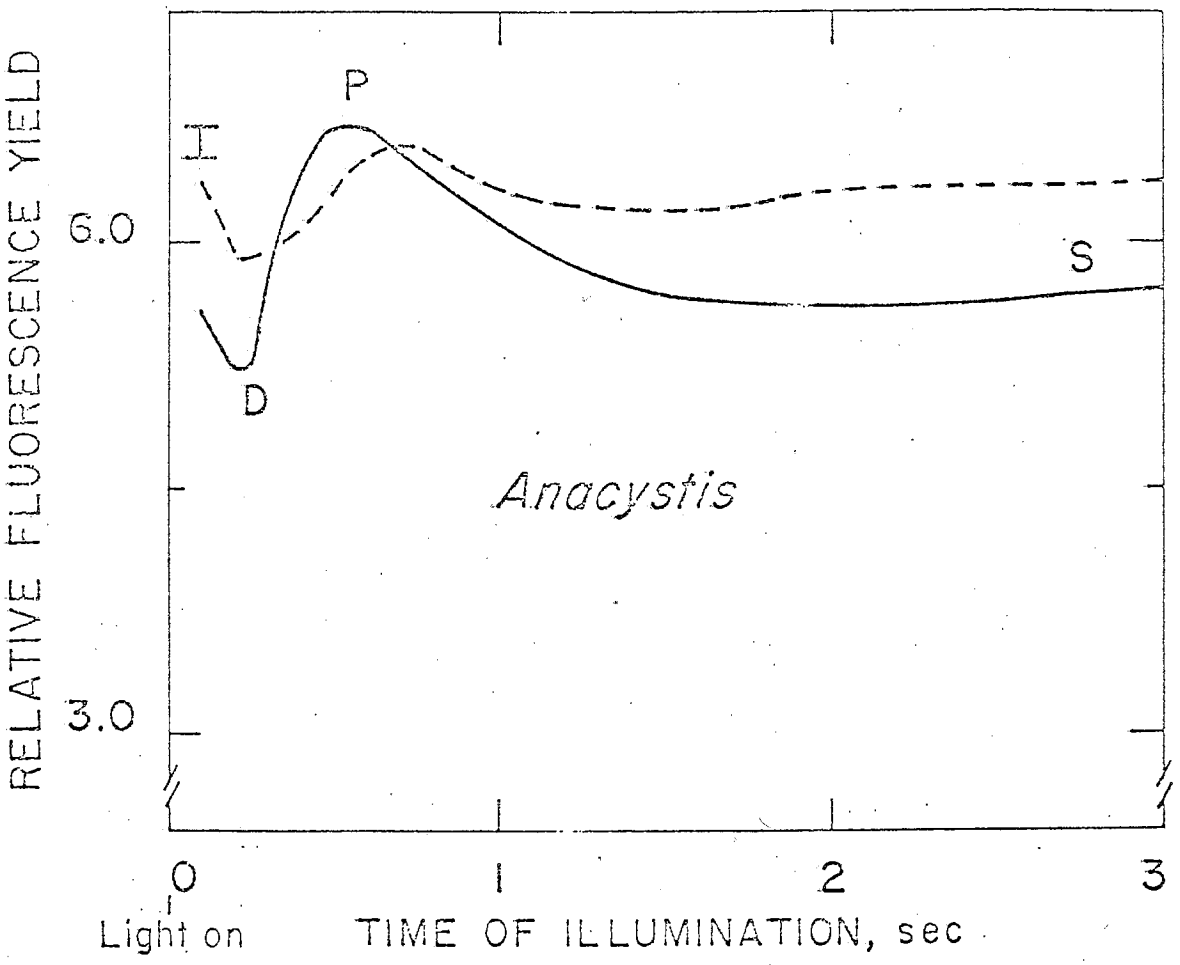

P
L
S
D
O

Figure 69. Time course of Chl *a* fluorescence in two different cultures of *Anacystis*. λ excitation, 633 nm; intensity, 26 Kergs $cm^{-2}sec^{-1}$; λ observation, 685 nm; C.S. 2-61 before the analyzing monochromator. Dashed line, cells grown in high light (100 watt bulb 13 cms away); solid line, cells grown in low light (40 watt, 13 cm away from the lamp, ~90 foot candles); 6 day old samples suspended in fresh growth medium, absorbance of both samples were adjusted at the red peak to 0.3 for 1 cm path length; OI rise not recorded.


RELATIVE FLUORESCENCE YIELD
6.0
3.0
H
P
D
S
Anacystis
0
1
2
3
Light on
TIME OF ILLUMINATION, sec

of these precautions we have observed occasional suppression of OIDPS transient in some cultures.

Figure 70 shows both the fast (sec) and the slow (min) changes in fluorescence yield in another uncillular blue-green alga, Microcystis. The fast IDPS transient is clear. With the intensity used, P occurred at about 0.7 to 0.8 seconds and the P/O ratio varied from 2.2 to 2.5. The top curve (open triangles) shows a slow SM rise of fluorescence yield, which is very similar to that of Anacystis. We have also observed similar OIDPS transient in two filamentous blue-green algae, namely, Plectonema and Phormidium (not shown). It seems that the fast fluorescence transient, though small, is always present in all blue-green algae. In order to observe a fast transient, a long dark adaptation (see below) and high intensity of illumination are necessary. In all blue-green algae the dip (D) is pronounced and the extent of P to S decline is small. The SM rise constitutes the largest variations in the fluorescence yield and this slow transient usually masks the OIDPS type fast oscillations in the fluorescence yield.

2.1 Intensity Dependence

The fluorescence transient in Anacystis is responsive to the wavelength and intensity of illumination. Blue light which preferentially excites Chl a does not induce any measurable transient while the orange light mainly absorbed by phycocyanin evokes a fluorescence transient. This is because in blue-green algae, most of the phycobilin pigments belong to system II and most of Chl a to system I.

The time course of Chl a fluorescence, recorded at various intensities of illumination, is shown in Figure 71. The orange light, obtained by the use of a narrow band interference filter peaking at 633 nm (half

Figure 70. Recorder trace of the time course of Chl *a* fluorescence yield in *Microcystis*. λ observation, 685 nm (half band width, 6.6 nm); C.S. 2-58 filter before the photomultiplier; λ excitation, 633 nm; intensity, ~25 Kergs cm^{-2} sec^{-1}; open circles, fast transient; open triangles, slow transient. Note the abscissa has two time scales; 7 day old culture was used. 15 min dark time between measurements; OI rise not recorded.

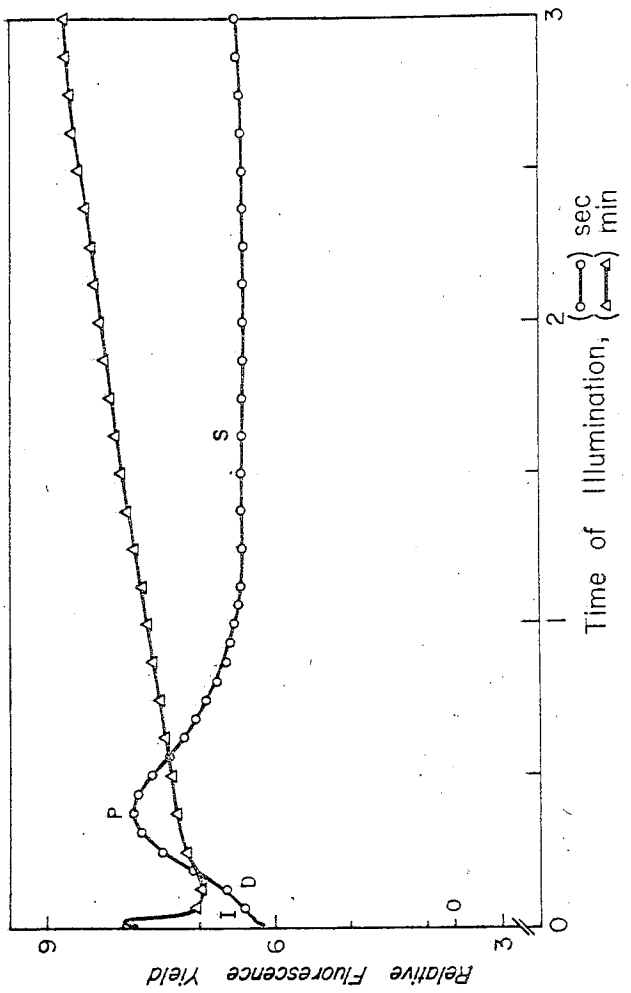
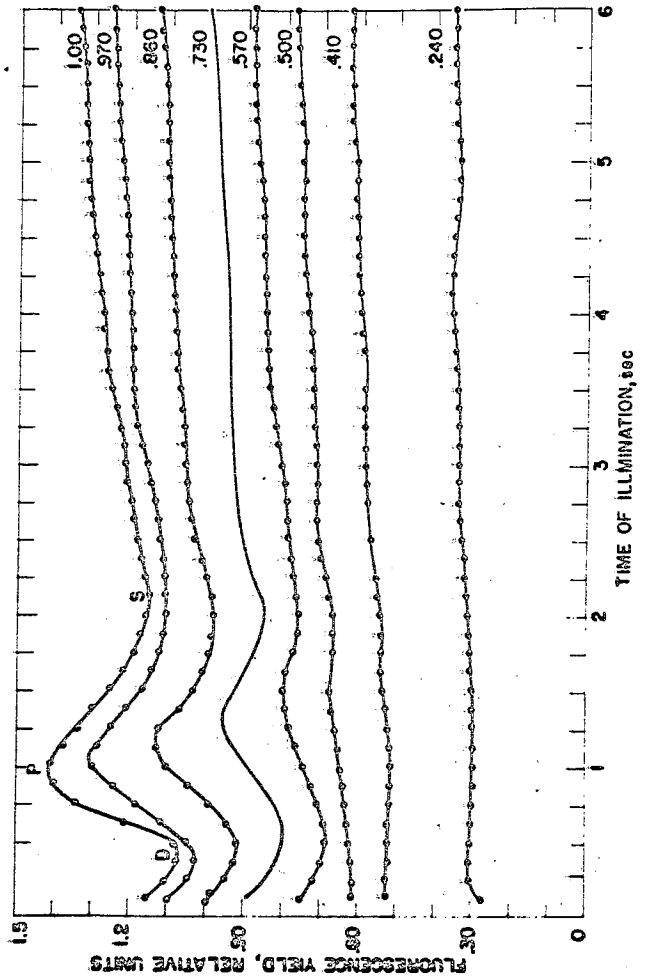

Relative Fluorescence Yield
6
3
0
P
I
D
S
0
1
2
3
Time of Illumination, sec min

Figure 71. Fast transient of Chl *a* fluorescence yield at different intensities of excitation. λ observation, 684 nm; C.S. 2-58 filter before the photomultiplier. The numbers on the curves are fractions of the maximum intensity used. Intensity 1.0 = 26 Kergs $cm^{-2}sec^{-1}$; 15 minutes dark period between measurements; cells were centrifuged and suspended in Warburg buffer #9 (pH 9.2) plus 0.01 M NaCl; absorbance at the "red" Chl peak, 0.35 for 1 cm path length; PC/Chl (peak absorbance) = 1.3. Signals were recorded on an Esterline Angus recorder.

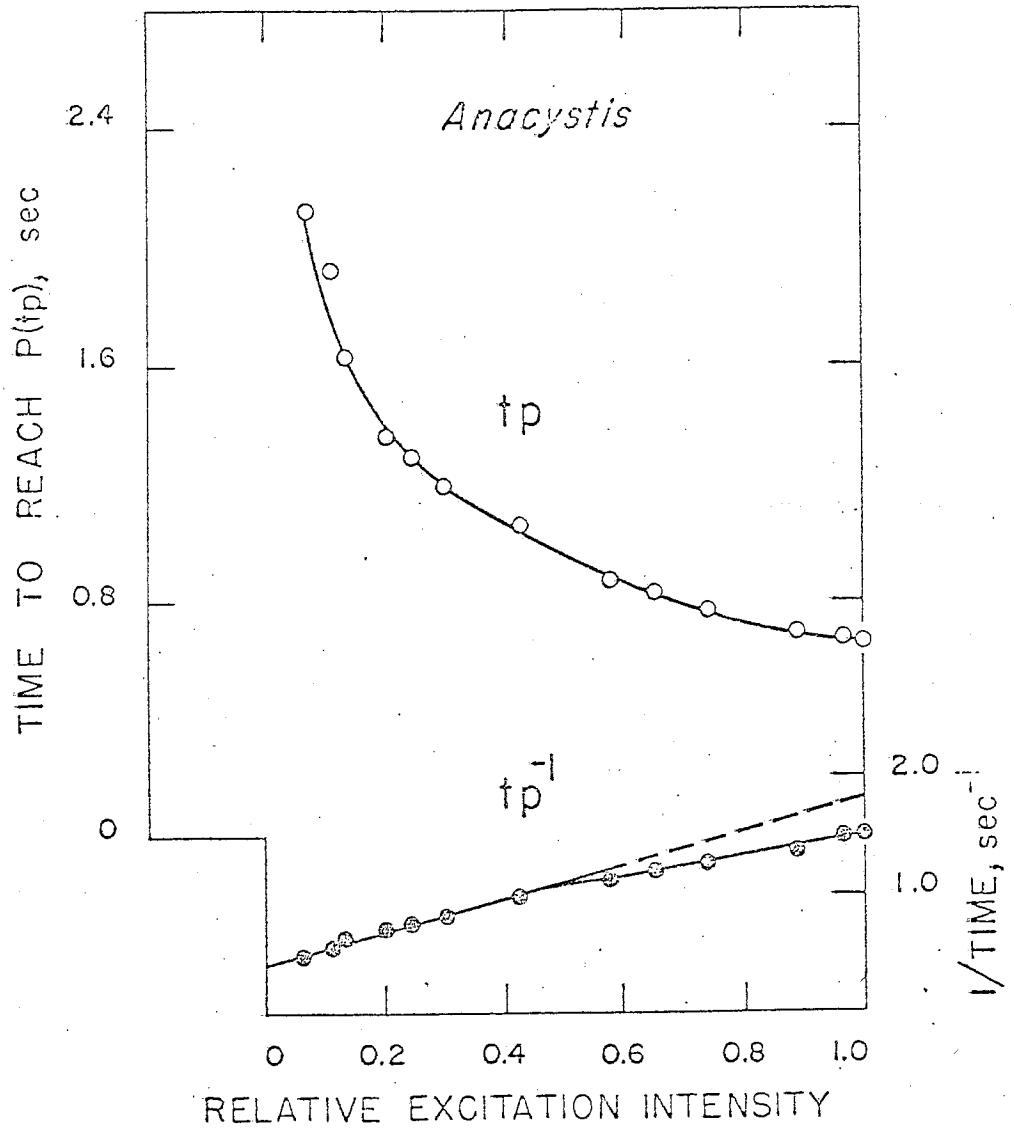

1.00
.970
.860
.730
.570
.500
.410
.240
P
S
D
1.5
1.2
.90
.60
.30
0
1
2
3
4
5
6
FLUORESCENCE YIELD, RELATIVE UNITS
TIME OF ILLUMINATION, sec

band width, 8 nm), excites mainly phycocyanin and causes fluorescence yield changes of Chl $\underline{a}_2$ (Chl $\underline{a}$ belonging to photosystem II). From Figure 71, it is evident that weak intensity of illumination fails to evoke the DP rise. On increasing the intensity of illumination both OI (not shown) and DP rise are accelerated and a distinct P is observed. It is also clear that the dip (D) remains pronounced even when excitation intensity is lowered by 40% to 60% of the maximum intensity used. Munday and Govindjee (83, 84) suggested that the ID decline is due to the antagonistic effect of two photosystems and thus, one would expect to observe a pronounced D at moderate intensity of system II light. PS decline is pronounced at high intensity of illumination and the extent of the decline would be lower as excitation intensity is lowered.

As shown in the case of Porphyridium (Chapter II; for Chlorella, see reference 83) the time interval between the onset of illumination and the peak P (tp) depends on the exciting light intensity. A graph of this time interval (tp) versus exciting intensity is shown in Figure 72. These data were plotted from an earlier experiment very similar to that shown in Figure 71. As intensity increases the tp becomes gradually smaller. The plot of 1/tp versus I shows that I x tp is constant at low intensities of excitation, but this relationship breaks down at higher intensities of illumination. Under light limiting conditions, the formation of P depends solely on the dose of light which is proportional to the absorbed quanta. At high light intensity some other factors besides light become limiting and delay the development of P.

The time to reach P (tp) in saturating light intensity seems to be characteristic of the organism. In Porphyridium, P occurs within 300-350 msec after illumination, in Chlorella it takes about 400-450 msec to

Figure 72. Time for the appearance of peak (P) versus intensity of exciting light. Open circles, time to reach P, scale to the left; solid circles, reciprocal plot (1/tp), scale to the right. Intensity 1.0 = 26 Kergs $cm^{-2}sec^{-1}$. Other conditions for measurement were as in Figure 71 except that an oscillographic recorder was used for the determination of the time interval between illumination and the development of peak (P).


Anacystis
2.4
1.6
0.8
0
TIME TO REACH P(tp), sec
tp
tp⁻¹
2.0
1.0
1/TIME, sec⁻¹
0
0.2
0.4
0.6
0.8
1.0
RELATIVE EXCITATION INTENSITY

reach P while in Anacystis, P develops after 500-600 msec of illumination. This, in all likelihood, reflects the size of the photosynthetic units and the limiting step in the electron transport chain in each organism. (It must, however, be mentioned that occasionally, in some Anacystis cultures, tp occurred at 500 msec, and a delay in tp was observed in some unused cultures of Porphyridium and Chlorella.)

Figure 73 shows the changes in the amplitudes at the points 0, I and P of the fast transient with increasing excitation intensity. The curve for "0" in Figure 73 shows no increase in the yield as light intensity is increased. But, both I and P assume a steeper slope at high intensity of illumination. The ratios of high intensity to low intensity yields were about 1.3 for I, and 2.0 for P. The values are lower than that for Chlorella, where we observed a 3-4 fold increase in yield at P.

As mentioned earlier, a dark adaptation of several minutes is necessary for the development of a complete OIDPS transient. Figure 74 shows the amplitude of I and P as a function of dark time between illuminations. At shorter dark times, there is a rapid OI rise and a small DP rise. As dark period is increased, the level of fluorescence at I gradually decreases and the DP transient becomes prominent. When the dark interval between illumination is short, some of the electron transport carriers reduced by the previous illumination remain in the reduced state; this probably causes a high level of fluorescence at I upon second illumination. But, as discussed earlier, the maximum fluorescence level remains low and does not attain the P level of the long dark adapted sample. If the dark period is, however, increased, the amplitude of fluorescence at P increases and attains a maximum value after 10-12 minutes of darkness. These results as originally indicated by the observations of Duysens and

Figure 73. Amplitude of fluorescence at points O, I and P of the fast transient as a function of excitation intensity. Intensity 1.0 = 26 Kergs $cm^{-2}sec^{-1}$; other details as in Figures 71 and 72.

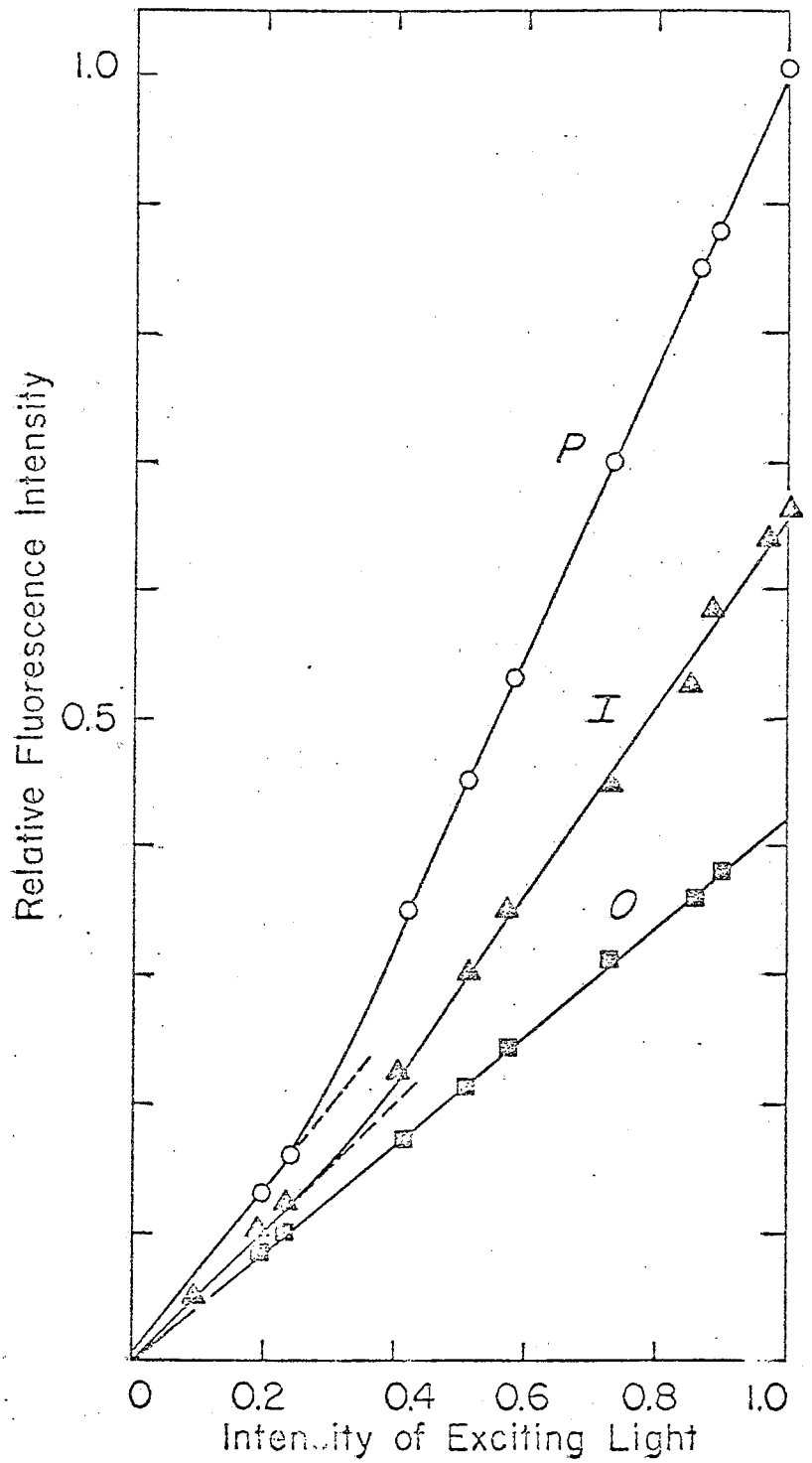
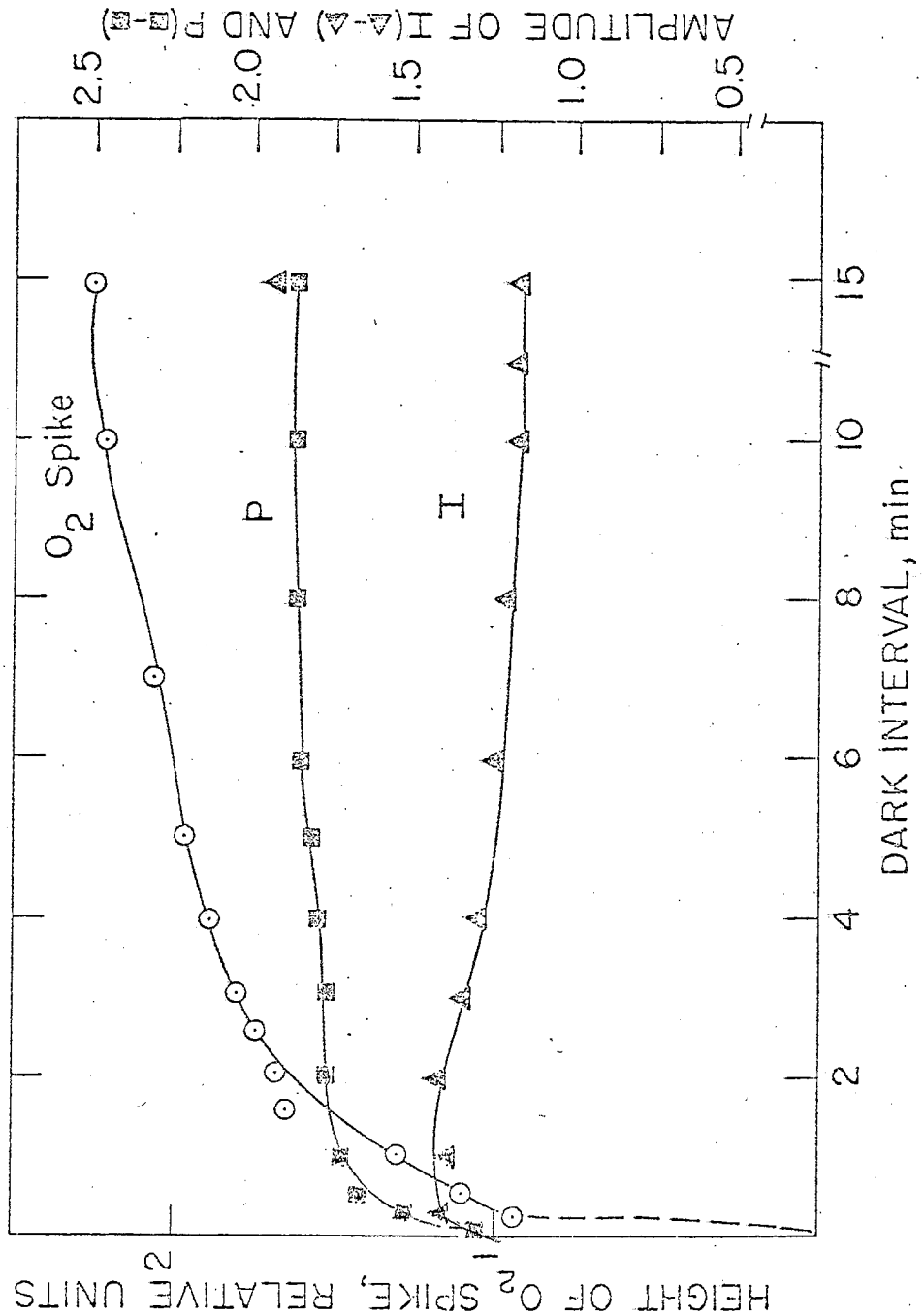

1.0
0.5
Relative Fluorescence Intensity
P
I
O
0
0.2
0.4
0.6
0.8
1.0
Intensity of Exciting Light

Figure 74. Dark recovery of the fast transient of Chl a fluorescence and oxygen spike in Anacystis. Fluorescence was measured as described in the legend of Figure 68, but the cells were suspended in 50 mM phosphate plus 0.01 M KCl, pH 7.8; ordinate scale to the right. Open circles, height of the O_2 spike measured on the same sample with a Haxo-Blinks type rate electrode; illumination, orange light (K-5 Balzer filter; intensity ~20 Kergs $cm^{-2}sec^{-1}$. O_2 measurements with 5 sec flashes; ordinate scale to the left.


AMPLITUDE OF I(▲-▲) AND P(■-■)
2.5
2.0
1.5
1.0
0.5
O_2 Spike
P
I
2
4
6
8
10
15
DARK INTERVAL, min.
HEIGHT OF O_2 SPIKE, RELATIVE UNITS
1
2

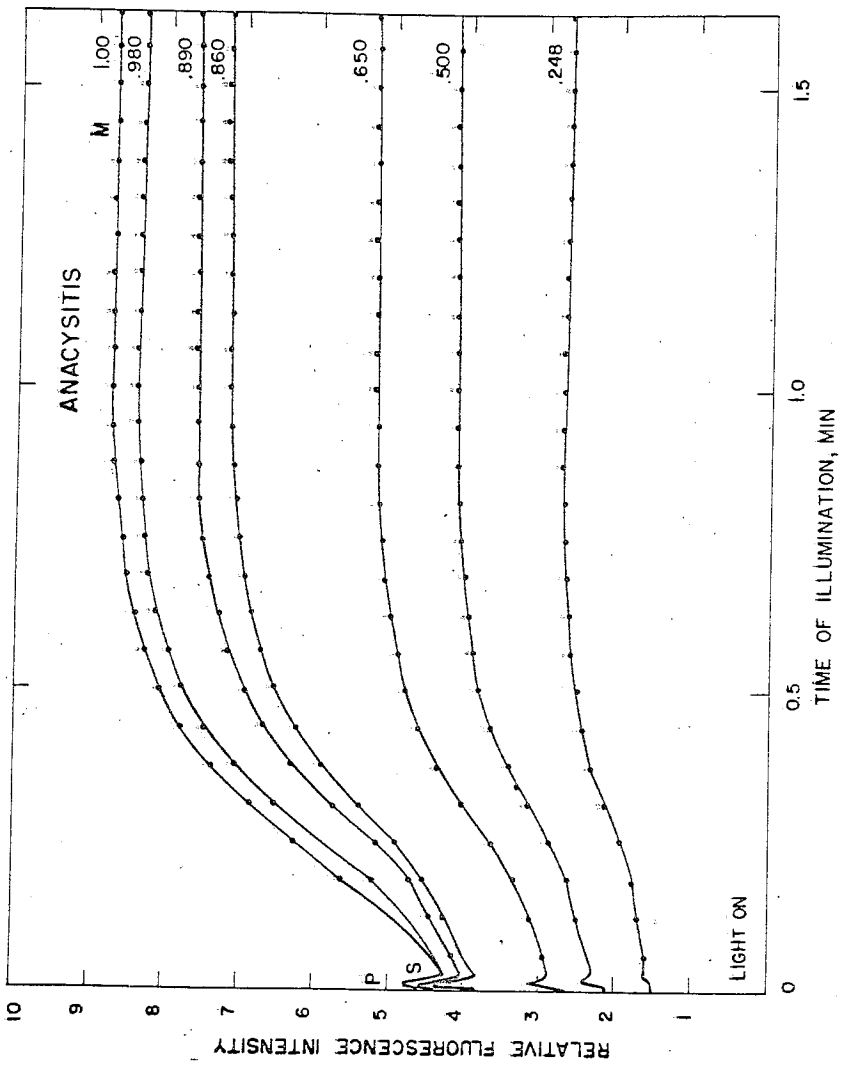
Sweers (10) in Porphyra suggest that the requirement of a dark period for the development of OIDPS transient is not associated with the redox state of Q. In the darkness, besides oxidation of Q some other transition related to high fluorescence yield occurs. From Figure 74 it is clear that rise of P in darkness is biphasic, there is a rapid phase occurring within 1 to 2 minutes of darkness and then a slow asymptotic rise in the yield at P with an increase in dark time. On the other hand, the O_2 gush (O_2 spike) which has been shown to be complementary with the DP fluorescence rise (and which, also like fast transient, requires darkness for its development) does not show such a biphasic rise. This indicates that besides the redox state of the pool A, which affects the O_2 gush, there is some other factor that influences the development of the fast (DPS) fluorescence transient.

3. The Slow Changes in Fluorescence Yield in Anacystis

With continuous system II illumination, a gradual increase in fluorescence yield to a higher level (M) is observed. In subsequent darkness the fluorescence yield declines to a lower level. The half time of this dark decline of fluorescence, monitored by a weak blue 436 nm light (~ 0.8 Kergs $cm^{-2}sec^{-1}$) varied from 20 to 40 seconds. These results (not shown) are very similar to those obtained by Papageorgiou (59) who interposed short (2 sec) flashes. These results suggest that the dark decay of the M level is comparatively slower than that of P (Figure 74).

Figure 75 shows the effect of various intensities of illumination on the slow SM rise of fluorescence yield. Very weak excitation causes only a slight increase in the yield of fluorescence with time--starting only after a lag phase. With increasing intensity the rate of rise of fluorescence increases. At moderate to low--but not high--exciting intensity,

Figure 75. Slow transient of Chl *a* fluorescence at various intensities of excitation. λ excitation, 633 nm; λ observation, 684 nm; C.S. 2-61 filter before the photomultiplier to guard the light leak. The numbers on the curves represent the fraction of the maximal intensity (1.0 = 29 Kergs $cm^{-2}sec^{-1}$) used. Cells were centrifuged, washed, and suspended in Warburg buffer #9 (pH 9.2) with 0.1 M NaCl. [Chl] = 72 μg/3 ml; Phycocyanin/Chl *a* peak ratio = 1.4; 15 min dark period between measurements. Transients were recorded by a Keithley microvoltammeter and an Esterline Angus recorder.


ANACYSITIS
M
1.00
.980
.890
.860
.650
.500
.248
P
S
LIGHT ON
RELATIVE FLUORESCENCE INTENSITY
TIME OF ILLUMINATION, MIN
0
0.5
1.0
1.5
1
2
3
4
5
6
7
8
9
10

the SM rise curve is biphasic. This biphasic SM rise suggests that the light induced increase in fluorescence yield is opposed by a dark decay. Figure 76(A) shows the amplitude of P and S as a function of exciting intensity. At low light intensity, the P and S levels are indistinguishable; separation of P and S occurs at an intensity which is 30% of the maximum intensity used. Figure 76(B) is a similar plot of fluorescence level at M and at S obtained from a separate experiment. It is clear that the separation of S and M occurs at a relatively low intensity ($\sim$ 5% of the maximum intensity used). The M level rises much faster than the S level. These results are similar to what has been observed in the case of Porphyridium (see Chapter III). We have also observed that the variable increase in yield at M (represented as (M-S)/S) saturates at lower intensity than variable fluorescence yield at P (not shown). These data indicate a lack of direct correlation between the electron flow during photosynthesis and the slow rise of fluorescence yield.

4. Changes in Emission Spectra During Fluorescence Induction

Papageorgiou and Govindjee (204) showed that a large spectral distribution accompanies long term fluorescence induction in Anacystis. However, spectral changes during the first few seconds of illumination were not reported. Figure 77 shows the emission spectra at three characteristic points (D, P and S) in the fast transient in Anacystis. The fluorescence band peaking at 655 nm represents the emission from phycocyanin, and that at 683 nm Chl *a*. The ratio of fluorescence intensity at 683 nm to that at 710 nm is 4.32 for "P" spectrum, 3.89 for "S" spectrum and 3.76 for "D" spectrum. It is clear that P is comparatively enriched in Chl a_2 fluorescence relative to S and conversely S is comparatively enriched in

Figure 76. Amplitudes of fluorescence at P, S, and M as a function of exciting intensity. Left (A): λ excitation, 633 nm; intensity, 1.0 = 29 Kergs $cm^{-2}sec^{-1}$; λ observation, 685 nm (half band width, 4.8); carbonate-bicarbonate (0.1 M) Warburg buffer #9 plus 0.01 M NaCl; [Chl], 60 μg/3 ml. Left (B): [Chl], 20 μg/2 ml; other conditions as in (A). Fluorescence transient was recorded with a Keithley microvoltammeter and a Brown recorder.

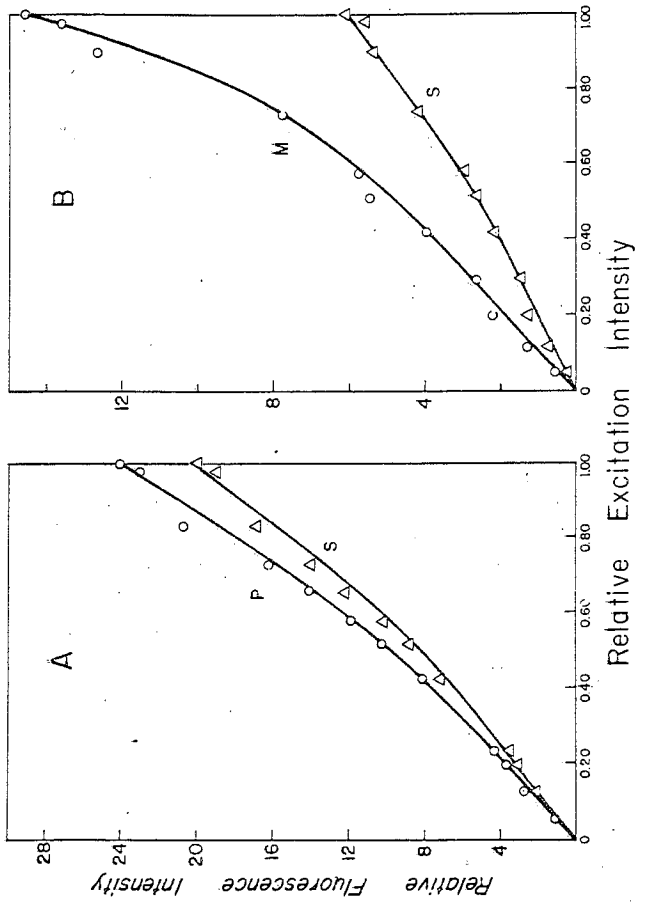
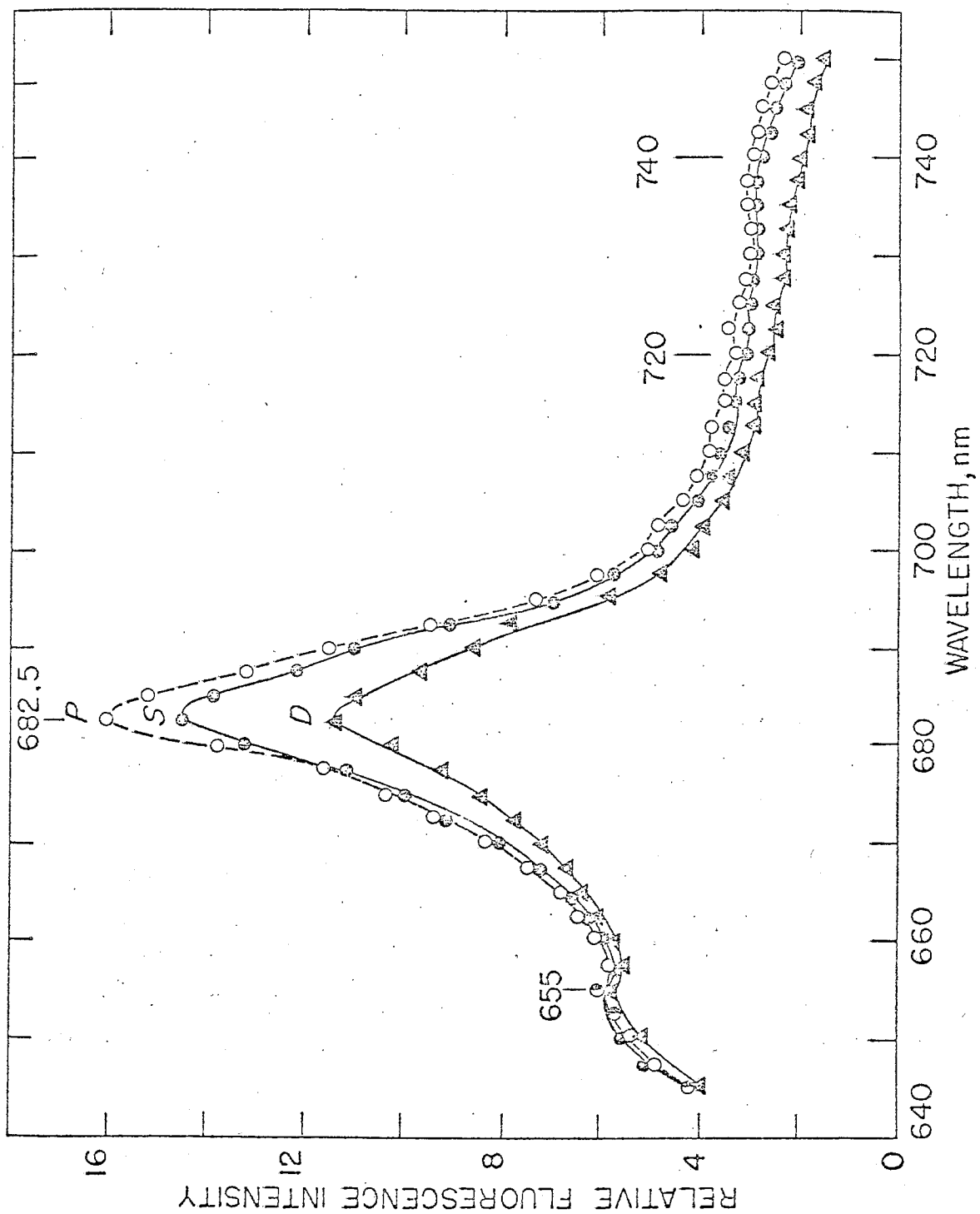

A
B
P
S
M
S
C
Relative Fluorescence Intensity
Relative Excitation Intensity
0
4
8
12
16
20
24
28
0.20
0.40
0.60
0.80
1.00

Figure 77. Room temperature emission spectra at the stage D (solid triangles), S (solid circles), and P (open circles). 15 min darkness between measurements; λ excitation, 633 nm; intensity, 26 Kergs cm^{-2} sec^{-1}; Schott red filter (645) was placed before the analyzing monochromator; 6 day old culture in carbonate-bicarbonate buffer (0.1 M) plus 0.01 M NaCl, pH 9.2; [Chl], 6.5 μg/ml; Phycocyanin/Chl *a* Peak ratio = 1.2.

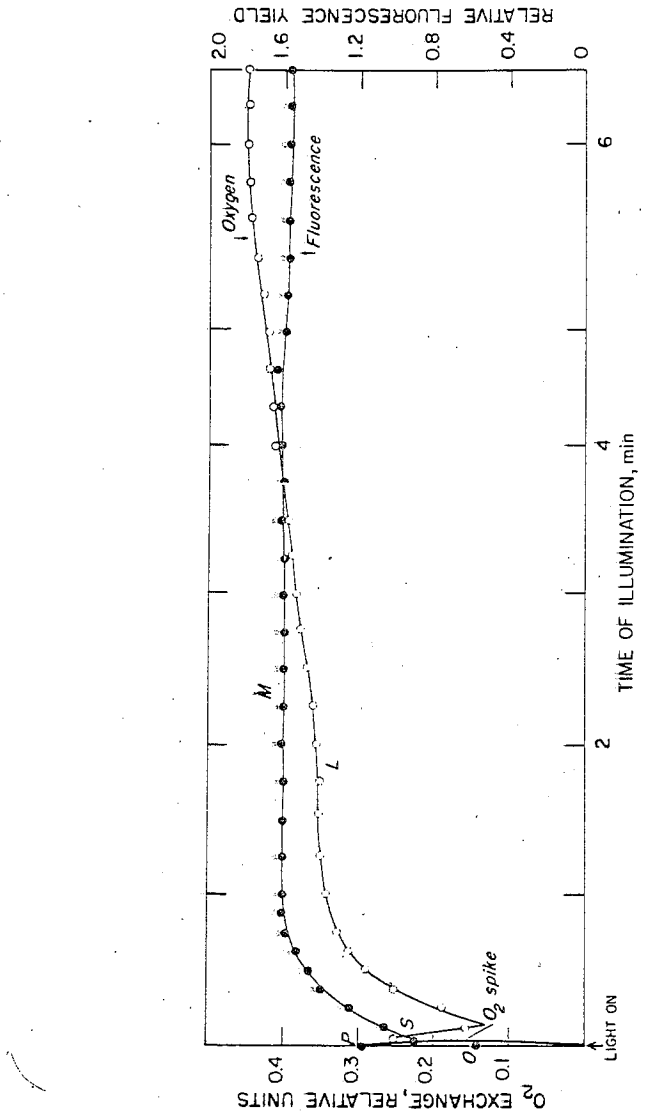

RELATIVE FLUORESCENCE INTENSITY
0
4
8
12
16
640
660
680
700
720
740
WAVELENGTH, nm
682.5
655
P
S
D

Chl $\underline{a}_1$ fluorescence. These results are similar to that of Lavorel (161) on other algae; he was the first to show the heterogeneity in emission spectra at "O" and "P". We also observe here a striking enrichment of system I fluorescence in the "D" spectrum. Since Papageorgiou and Govindjee (88) have made an extensive study of the spectral changes during the slow fluorescence induction, we did not measure point by point spectra for the SM rise phase of the induction curve. But on several occasions we have compared the ratio of intensity at F684 to that at F710 at S and at M. This ratio for S varied from 3.0 to 3.5 and for P it ranged from 3.5 to 4.5 depending on the culture conditions. These variations in the ratio are mainly due to changes in the energy transfer from phycocyanin to Chl $\underline{a}_2$ as shown by Ghosh and Govindjee (205). In spite of these variations, we always observed an enrichment of Chl $\underline{a}_2$ fluorescence in M compared to that in S. Thus it can be safely concluded that the S to M rise accompanies an enhancement of Chl $\underline{a}_2$ fluorescence just as we have shown in the case of Porphyridium (Chapter III). Since the fluorescence yield of phycocyanin does not change during the induction period (88) the SM rise must be a suppression of energy transfer to weakly fluorescent Chl $\underline{a}$ of system I.

5. Time Course of Fluorescence Yield Changes and of Oxygen Evolution

In the case of Porphyridium, we observed that the SM rise portion of the fluorescence parallels the rise in oxygen evolution. Figure 78 shows the time course of oxygen evolution and of fluorescence yield after a 15 minute period of darkness. For both of these measurements a bright saturating red light was used to excite the sample. Both transients were recorded on identical samples. The lag in the appearance of the O_2 spike

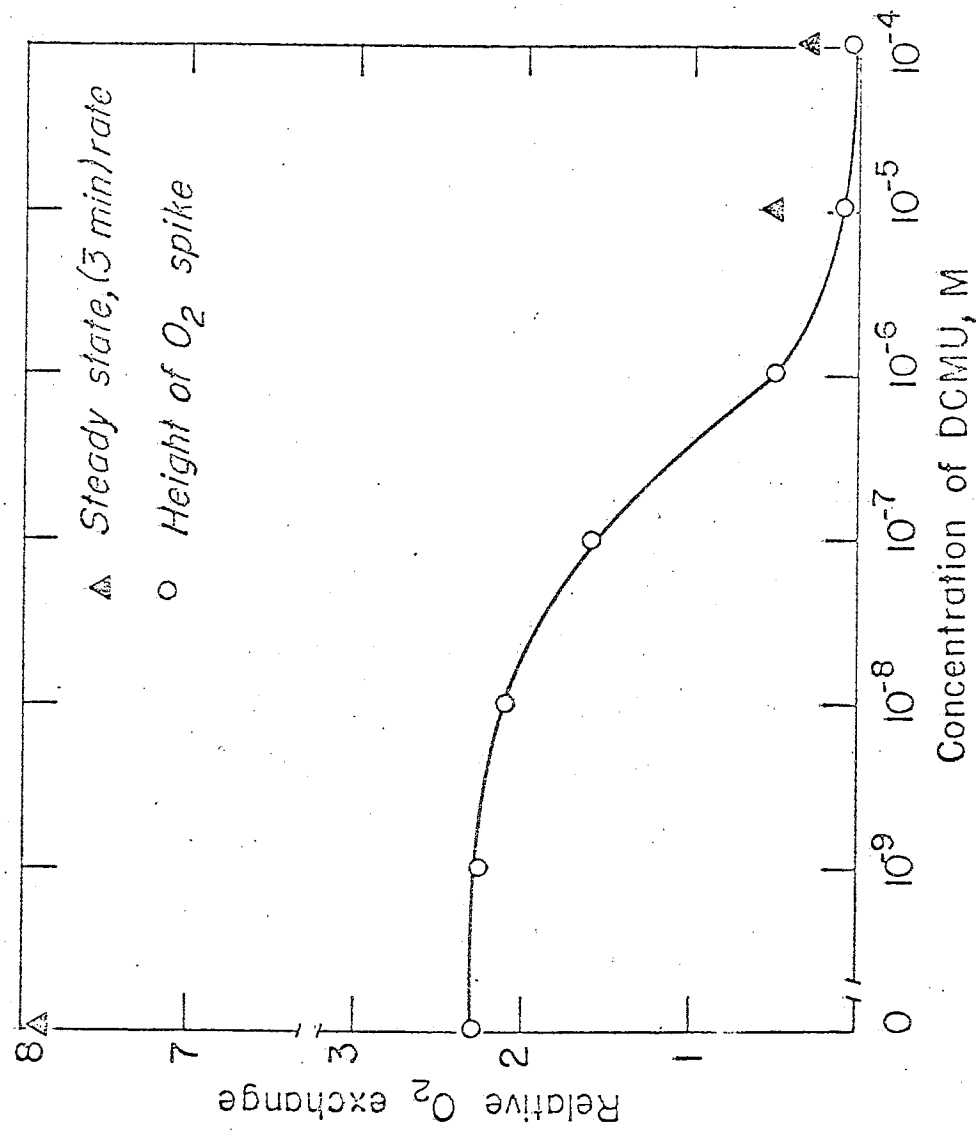
Figure 78. Time course of the fluorescence yield of Chl *a* and the rate of oxygen exchange (evolution) in *Anacystis*. Recorder trace of the fluorescence transient (solid circles); conditions for the fluorescence measurements are as described in the legend of Figure 75 except that the cells were suspended in 50 mM phosphate plus 10 mM KCl, pH 7.8; intensity of excitation, 26 Kergs $cm^{-2}sec^{-1}$; the fast OI phase is not shown. Oxygen exchange (open circles) was measured with a Haxo-Blinks type rate electrode; samples were illuminated from the top with red light (C.S. 2-58); intensity, 15 Kergs $am^{-2}sec^{-1}$; electrolyte, 50 mM phosphate plus 10 mM KCl, pH 7.8, 2% CO_2 in air in gas phase; a 15 min dark time preceeded the measurements.


RELATIVE FLUORESCENCE YIELD
2.0
1.6
1.2
0.8
0.4
0
Oxygen
Fluorescence
M
T
S
P
O
O2 spike
LIGHT ON
0.4
0.3
0.2
0.1
O2 EXCHANGE, RELATIVE UNITS
2
4
6
TIME OF ILLUMINATION, min

with reference to the P level of fluorescence is due to a diffusional lag of the polarograph to oxygen. But, it is very clear that the S to M rise in the fluorescence induction is parallel to the increase in the rate of oxygen evolution and the latter continues to increase even after fluorescence reaches the maximum intensity (M). This parallel increase in the rate of 0_2 evolution and the yield of fluorescence was earlier observed by other investigators (88) in Anacystis. Furthermore, this parallel rise during this interval of induction seems to be an intrinsic characteristic of all 0_2 evolving organisms as it has been observed in Chlamydomonas (89), Chlorella (87) and Porphyridium (Chapter III). In Anacystis, the 0_2 spike is very prominent (Figure 78); in anaerobic samples, the spike is more pronounced and the rate of 0_2 evolution declines after the spike to the base level from where it again rises gradually to a steady state level (not shown).

Figure 79 shows the effect of varying concentrations of DCMU on the height of the oxygen spike measured by a 5 sec flash of saturating white light. At 10^{-9} M and 10^{-8} M DCMU the 0_2 gush (the height of the spike) remains unchanged, then declines rapidly to almost zero level at 10^{-5} M DCMU. But, some 5 to 10% steady state 0_2 evolution persisted at this concentration of DCMU even after 30-40 minutes of incubation with DCMU (see Figure 79, solid triangles). Since 0_2 gush is a true indication of photosynthetic oxygen evolution, we believe that this concentration of DCMU completely suppresses all photosynthetic electron transport in Anacystis. In all our experiments we have used 10 to 15 μM DCMU to inhibit electron transport. The residual apparent evolution that resisted DCMU poisoning is probably due to suppression of 0_2 uptake in light.

Figure 79. Height of the oxygen spike versus concentration of DCMU in the bathing medium. Experimental conditions as described in the legend of Figure 74. For each measurement the electrolyte containing the required concentration of DCMU was incubated for at least 15 minutes before assaying the spike with a 5 sec flash. Solid triangles represent the steady state O_2 evolution level after 3-5 min of illumination.


Steady state, (3 min) rate
Height of O_2 spike
Relative O_2 exchange
Concentration of DCMU, M
0
1
2
3
7
8
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}

6. Action of Cofactors and Inhibitors of Electron Transport on the Slow Changes in Fluorescence Yield

10 μM DCMU inhibits oxygen evolution and electron transport. 10-15 μM DCMU also completely abolishes the fast DPS transient in Anacystis (Figure 80). On the other hand, the slow fluorescence rise resists DCMU poisoning (see below). We have also used a variety of other known electron transport inhibitors and in all cases the slow changes in yield resisted the poisoning. We also tested the effect of HOQNO which is assumed to inhibit cyclic electron flow in chloroplasts. At low concentrations of this inhibitor (1-2 μM), the fast fluorescence was completely inhibited, but unlike DCMU it did not cause a rapid OI rise of fluorescence. Both HOQNO and Antimycin A did not alter significantly the slow fluorescence rise. Table V.1 and Table V.2 catalogue the effect of some of the electron transport inhibitors and cofactors on the ratio of final (fm) and the initial (fi) fluorescence levels in Anacystis. It seems from the results in Tables V.1 and V.2 that all the inhibitors of electron transport give similar results in that they do not suppress to any great extent the slow changes in the yield while they depress the fast DPS transient. In the case of DCMU and orthophenanthroline the maximal yield was slightly enhanced compared to the untreated sample. Salicilaldoxime and phenylmercuric acetate (10^{-4}M) showed a gradual inhibition of the yield with the time of incubation.

Although HOQNO and antimycin A are claimed to inhibit cyclic electron transport (see review by Good et al., 206) it appears from our studies that they probably inhibit the electron transport chain between the two photosystems. Although a slight inhibition of the fluorescence yield change was observed with HOQNO, we do not know if this is due to some

Figure 80. Time course of the yield of Chl *a* fluorescence in *Anacystis* with and without DCMU. λ excitation, 633 nm; λ observation, 685 nm; intensity, ~25 Kergs $cm^{-2}sec^{-1}$; Corning C.S. 2-61 filter before the photomultiplier; cells were suspended in Warburg buffer #9; 15 minutes dark period before illumination.

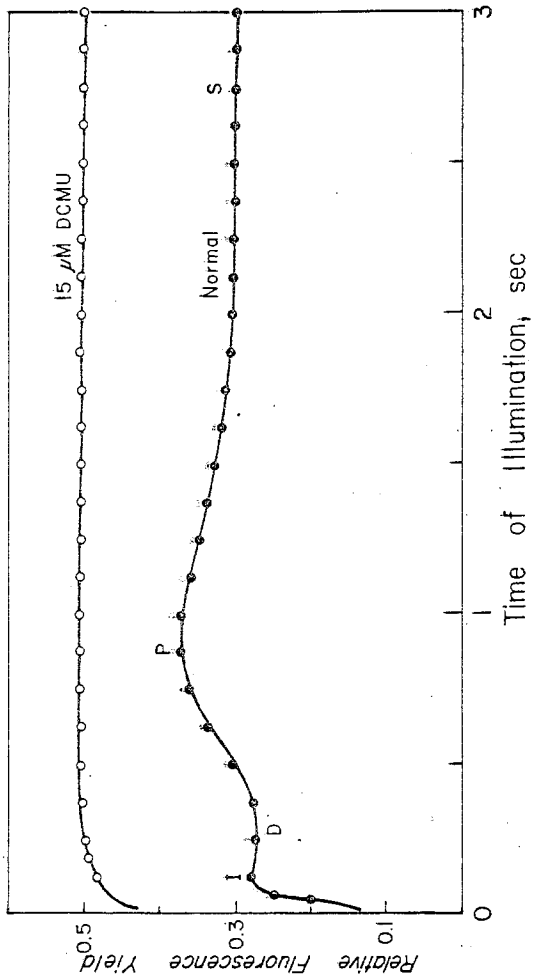

Relative Fluorescence Yield
0.5
0.3
0.1
0
1
2
3
Time of Illumination, sec
15 μM DCMU
Normal
I
D
P
S

Table V.1

Effect of Various Concentrations of Antimycin A on the Initial (fi) and the Final (fm) Level of Fluorescence Yield in Anacystis

Treatment	Relative Fluorescence Yield fi	fm	Ratio fm/fi
No addition	75	125	1.6
15 μM DCMU	90	165	1.8
1 μM antimycin A	92	151	1.64
5 μM antimycin A	91	150	1.64
10 μM antimycin A	90	151	1.66
30 μM antimycin A	90	155	1.7

Samples were incubated in 50 mM phosphate plus 10 mM NaCl buffer (pH 8.0) with the inhibitors for 10 minutes before each measurement. (Antimycin A was obtained from Sigma Chemicals, St. Louis, Missouri.)

uncoupling effect of HOQNO or due to inhibition of electron flow.

We were, however, surprised to note the differential effect of the addition of 1.0 mM MeV with and without DCMU. In normal cells, MeV abolished the development of P as in the case of Chlorella (Figure 81). Methyl viologen depressed the maximal fluorescence (M) level in the absence but not in the presence of DCMU. Papageorgiou and Govindjee (88) have shown that slow changes in fluorescence yield are resistant to treatments of uncouplers in the presence of DCMU, while the same concentration of uncouplers suppresses the slow changes in normal cells. It seems that methyl viologen (MeV) exhibits somewhat similar differential behavior. It is logical to assume that MeV is more effective in suppressing cyclic electron transport as a non-cyclic flow is favored. Although methyl

Table V.2

Effect of Various Electron Transport Inhibitors on the Maximal Steady State Fluorescence Yield (fm) of Anacystis

Additions	Maximal Change fm/fi
None	1.60
10 μM DCMU	1.8
5 mM Salicilaldoxime	1.67
10 μM HOQNO	1.5
0.1 mM orthophenanthroline	1.7
1 mM MeV	1.47
15 μM DCMU + 1 mM MeV	1.75

Conditions of measurement as in Table V.1. HOQNO and orthophenanthroline were obtained from Sigma Chemicals, St. Louis, Missouri; Salicilaldoxime from Kodak Chemicals, Rochester, N.Y.; and methyl viologen (MeV) from Mann Research Chemicals, Orangeburg, New York. HOQNO and orthophenanthroline were dissolved in ethanol, salicilaldoxime in methanol and MeV in distilled H_2O. Salicilaldoxime was added to the empty Dewar flask and was allowed to evaporate a few seconds before the addition of cells. All additions were made in dim room light.

viologen, due to its low redox potential (E_o' = 0.44 volt) may be expected to disrupt the cyclic flow, this may not occur in all cases.

Figure 82 shows the effect of adding 20 μM $PMSH_2$ on the slow changes in fluorescence yield in normal Anacystis. $PMSH_2$ abolished the OPS transient (or simply delayed the PS decline). Upon illumination, the fluorescence yield rises to P level and then remains constant for 10-15 seconds and then gradually rises to a maximum value higher than the M level of normal cells. Both reduced and oxidized PMS gave approximately similar results. But with DCMU treated samples, $PMSH_2$ brought about a small depression in the final f_m level in Anacystis. This was somewhat

Figure 81. Time course of Chl *a* fluorescence in normal and methyl viologen treated cells of *Anacystis*. λ observation, 684 nm (half band width, 6.6 nm); intensity, 26 Kergs $cm^{-2}sec^{-1}$. A 6 day old culture was suspended in 50 mM phosphate NaCl buffer, pH 8.0. MeV was allowed to incubate for 15-20 minutes before each measurement.

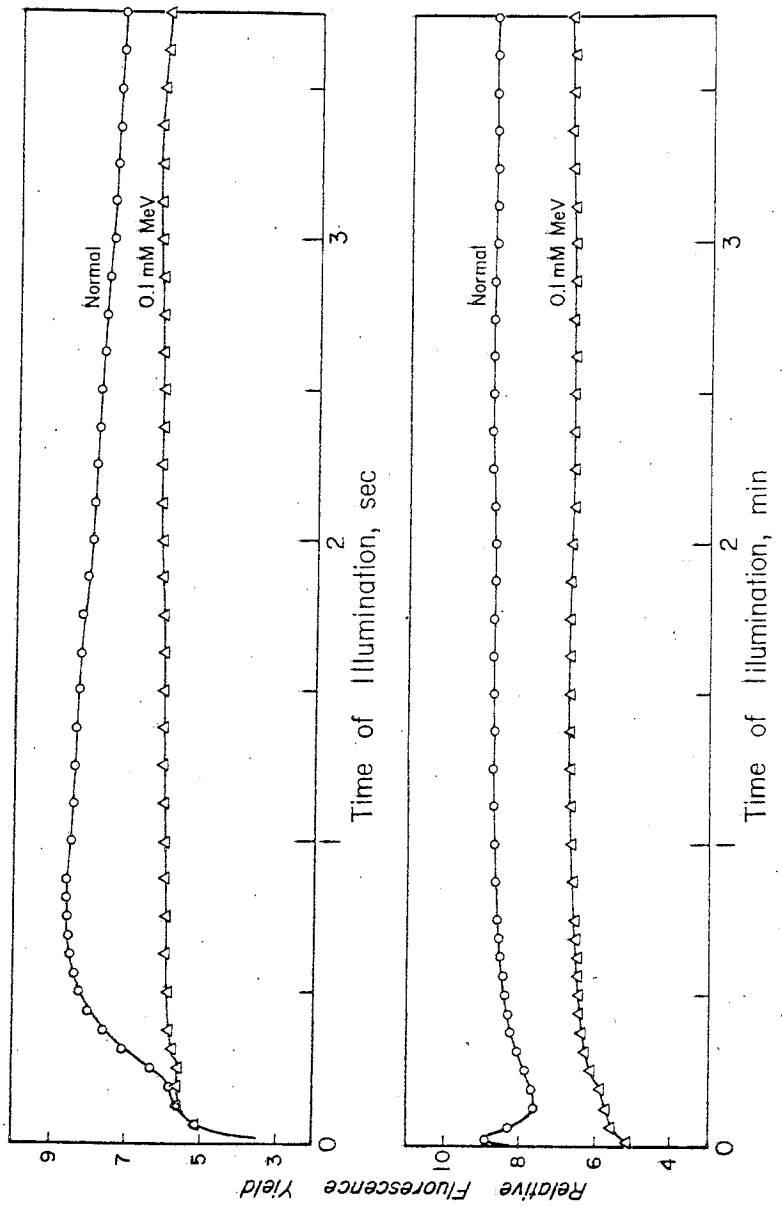
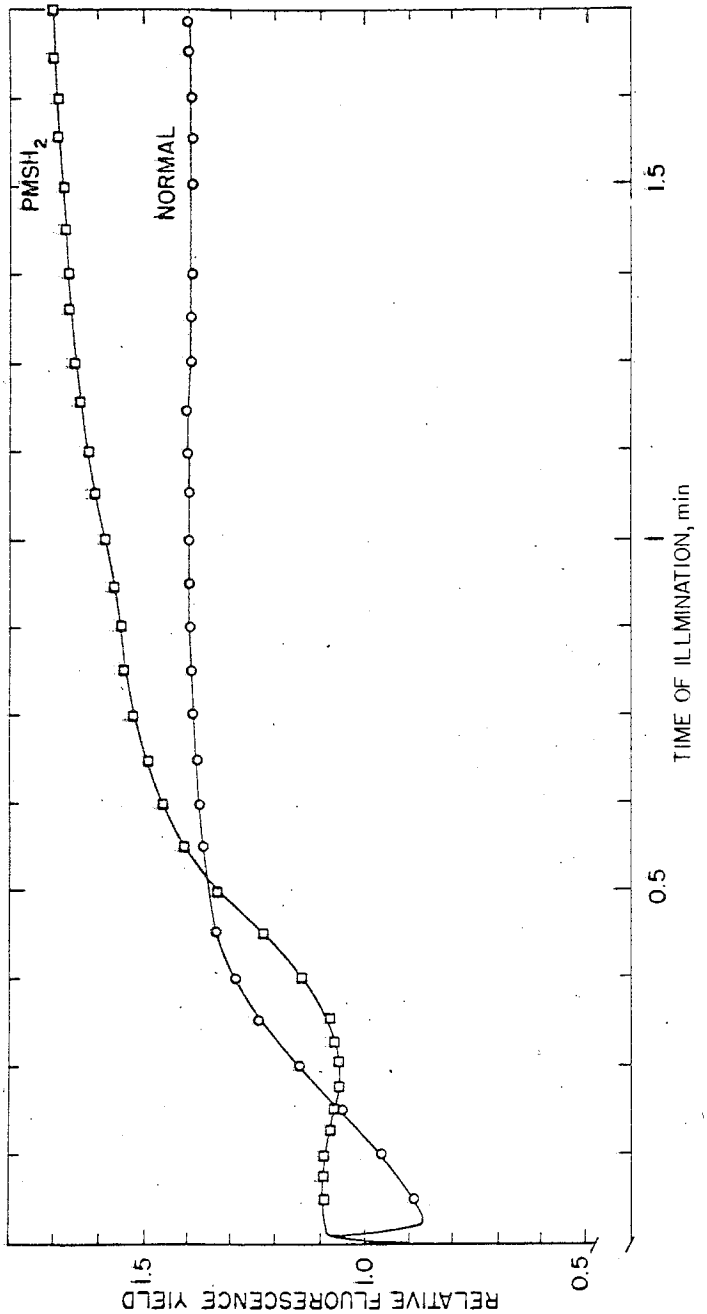

Normal
0.1 mM MeV
Yield
Time of Illumination, sec
Normal
0.1 mM MeV
Relative Fluorescence
Time of Illumination, min

Figure 82. Slow transient in the yield of Chl *a* fluorescence in *Anacystis* in the presence and in the absence of $PMSH_2$. λ excitation, 633 nm; intensity, 26 Kergs $cm^{-2}sec^{-1}$. 20 μM PMS with 0.1 mM Na ascorbate (neutralized); buffer, 50 mM Tris-Cl, pH 8.0, with 10 mM NaCl. [Chl], 21 μg/ml. All additions were made in the dark.


PMSH2
NORMAL
RELATIVE FLUORESCENCE YIELD
1.5
1.0
0.5
0.5
1
1.5
TIME OF ILLMINATION, min

unexpected.

In normal cells, PMS could act as a Hill oxidant. If it were so, one would expect results similar to methyl viologen which depressed the slow rise in Chl a fluorescence in normal Anacystis and did not stimulate the extent of SM rise. It may be tempting to assume that PMS accelerated cyclic electron flow. But one would expect, from an analogy with results in chloroplasts, that PMS would accelerate cyclic electron flow even in the presence of DCMU. If slow fluorescence yield is triggered by PS I mediated electron transport, we would also expect to see a stimulation in slow fluorescence rise with DCMU poisoned cells of Anacystis. Our results show that this is not the case. It may be possible that the enhanced SM rise, seen in the case of normal Anacystis with PMS, could be due to an increase in light induced swelling. However, DCMU poisoned samples show a greater extent of swelling as measured by the 540 nm absorbance change (see later). Thus, we suggest that the observed depression in the yield in the presence of DCMU, noted above, may be due to an additional effect of PMS in causing an enhanced dissipation of heat.

7. Slow Changes in Fluorescence Yield in DCMU Poisoned Cells

As in the case of Chlorella, addition of DCMU abolished the fast (OIDPS) fluorescence yield changes in Anacystis (Figure 80). But, unlike Chlorella, fluorescence yield rises slowly from a low initial level to a maximal level upon illumination. The fluorescence yield attains a maximum value only after 1-2 min. of illumination.

In normal Anacystis the ratio of Fm/Fi (also referred to as fm and fi at certain portions of this thesis) varied from 1.4 to 1.6 depending on the condition of the culture of the algae. DCMU treatment, in most cases,

but not always, gave only 10 to 15% increase in maximal yield over normal cells. Mar et al. (45) obtained similar results in their fluorescence life time measurements in that the life time of Chl a fluorescence (τ) in both normal and DCMU poisoned Anacystis cells are within 10-15% (~0.8 nsec).

7.1 Intensity of Illumination

Figure 83 shows the time course of slow fluorescence yield changes as a function of light intensity in DCMU poisoned cells. Very weak light intensity does not cause any appreciable increase in yield with time of illumination. Increase in intensity evokes a large enhancement in fluorescence yield with time. In Figure 84 the initial (Fi) and the final (Fm) levels of fluorescence yield are plotted as a function of exciting intensities. Both initial (Fi) and maximal (Fm) intensity increase linearly with intensity, although with different slopes, but at a high intensity of excitation, the deviations from linearity is much steeper for Fm than Fi levels. (Our results are slightly different from that of Papageorgiou and Govindjee (88) who observed a completely linear relationship between fluorescence intensity and excitation intensity in DCMU poisoned samples. However, the maximum excitation intensity used by them was lower than that used in this experiment.) The relative fluorescence yield increment represented as (Fm-Fi)/Fi shows a saturation at a relatively low light intensity (top curve).

The increase in the rate of rise of fluorescence yield with illumination intensity indicates that it originates from photochemical events and an early saturation suggests its relationship with the PS I mediated cycle which is also known to saturate in vivo at a relatively low light intensity (207).

Figure 83. Slow transient of Chl *a* fluorescence in DCMU poisoned *Anacystis* at different intensities of excitation; numbers on the curves are fractions of maximum intensity used. 1.00 = 29 Kergs $cm^{-2} sec^{-1}$; cells were suspended in Warburg #9 buffer pH 9.2, with 0.01 M NaCl; PC (Phycocyanin)/Chl *a* peak ratio = 1.35; other conditions as in Figure 80.

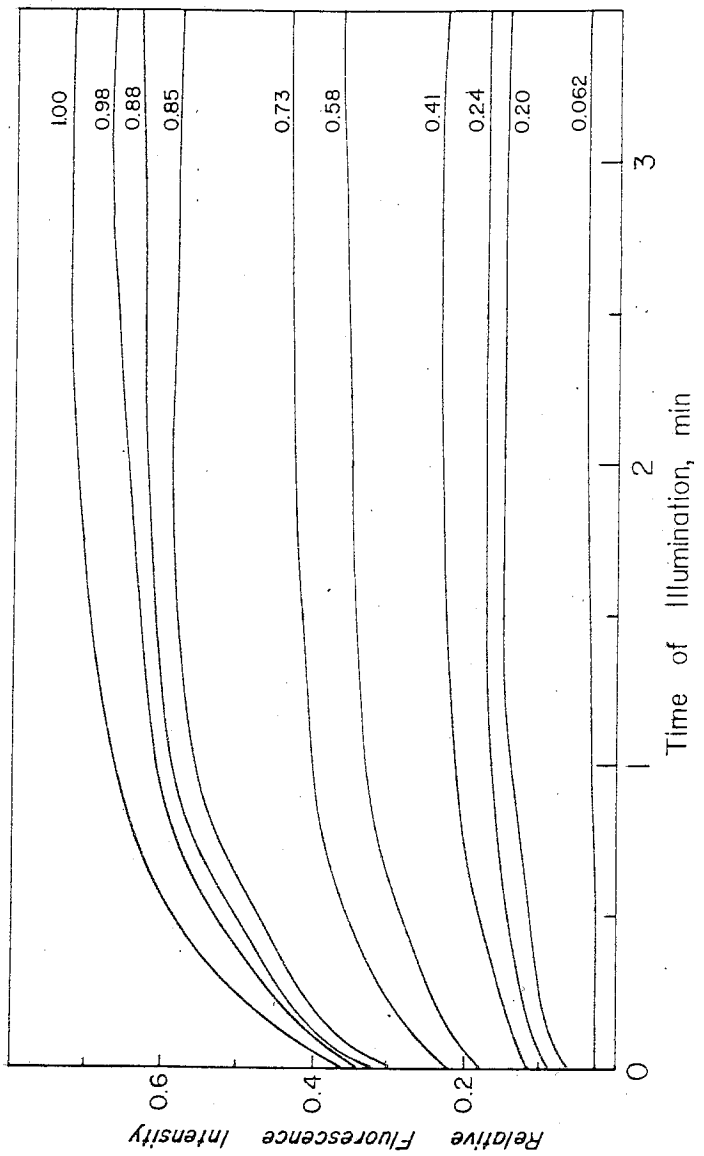

100
0.98
0.88
0.85
0.73
0.58
0.41
0.24
0.20
0.062
0.6
0.4
0.2
0
1
2
3
Relative Fluorescence Intensity
Time of Illumination, min

Figure 84. Amplitude of the initial (Fi) and final level (Fm) of Chl *a* fluorescence in DCMU poisoned *Anacystis* at various exciting intensities. Intensity of 1.00 = 29 Kergs $cm^{-2}sec^{-1}$. Top curve (stars), specific increase in variable fluorescence yield (Fm-Fi)/Fi; ordinate scale to the right. 4-day old culture was centrifuged and resuspended in 50 mM Tris-Cl with 10 mM KCl and 15 μM DCMU (pH, 7.9); [Chl], 18 μg/ml; Phycocyanin/Chl *a* peak ratio = 1.25.

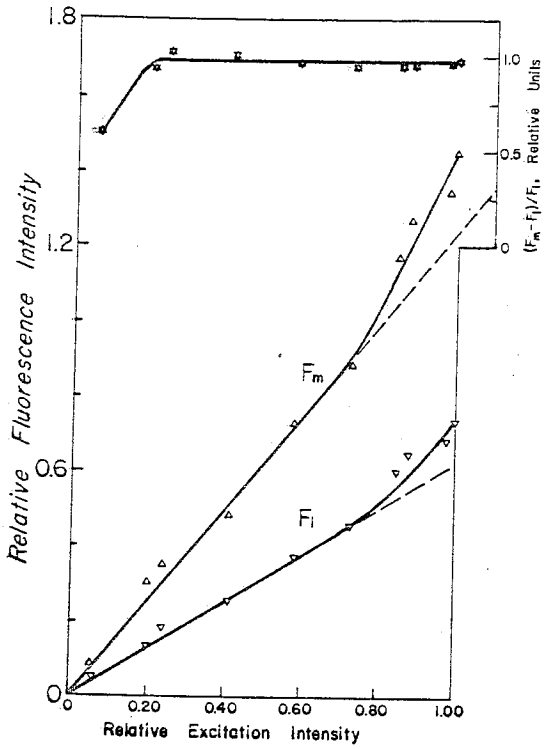
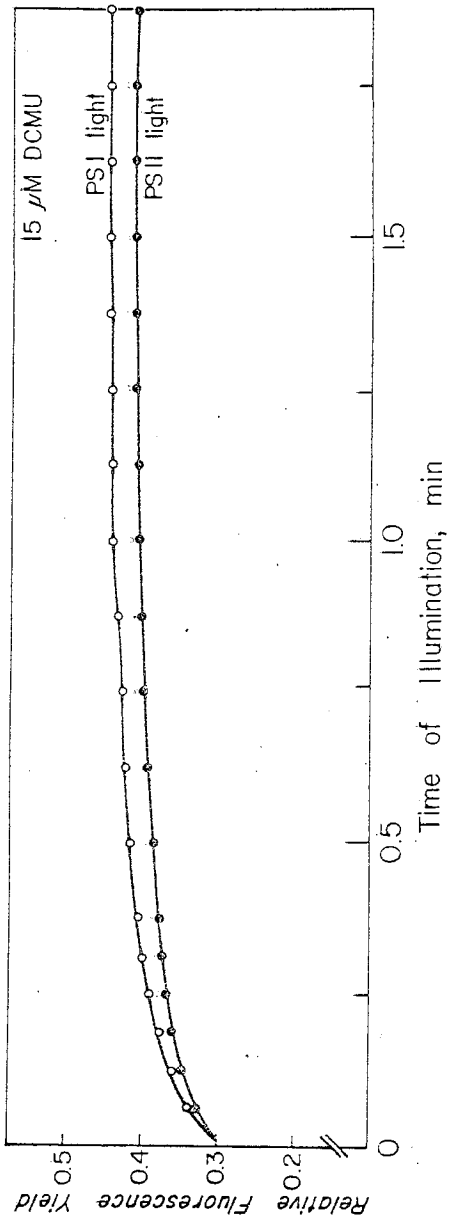

1.8
1.2
0.6
0
Relative Fluorescence Intensity
1.0
0.5
0
$(F_m-F_i)/F_i$, Relative Units
F_m
F_i
0
0.20
0.40
0.60
0.80
1.00
Relative Excitation Intensity

Figure 85 shows comparison of the effect of system I illumination (λ, 436 nm) and system II (mainly) illumination (λ, 633 nm) in causing slow induction of fluorescence in DCMU poisoned cells. In this figure, we adjusted both the curves at initial level (Fi) to compare the relative extent of the rise in fluorescence yield. It is clear that system I is quite effective in causing slow changes in fluorescence yield in DCMU poisoned cells. Duysens and Talens (203), using a dual beam spectrofluorometer, have shown that system I illumination causes a larger magnitude of slow change than system II light, in Schizothrix calicola if the initial rate of rise is matched. Our results with Anacystis also show a small increase in relative yield at stationary level in system I light over system II illumination. We cannot meaningfully compare our results with those of Duysens and Talens since different techniques of measurement and different organisms were used by these authors. Nevertheless, system I illumination is quite effective in causing the slow changes in fluorescence yield. We did not use system I illumination for routine measurements since the intrinsic yield of fluorescence is very low. Thus the large variations in the fluorescence yield in the presence of DCMU (when all oxygen evolution is completely inhibited) indicates that PS I mediated electron flow somehow supports these variations in the fluorescence yield.

7.2 The Influence of Back Reaction Between Z^+ and Q^-

The slow (long-term) rise of fluorescence yield in the presence of DCMU is taken as evidence that these changes are not related to the redox level of the acceptor Q. However, one may argue that in these organisms there is a faster turn over of Q due to dark back-recombination of Z^+ and Q^-, causing a slow rate of rise in the fluorescence yield. To test if a

Figure 85. Time course of the slow rise in the yield of Chl *a* fluorescence in DCMU poisoned *Anacystis*. Open circles system I blue light; solid circles, system II orange light; the initial fluorescence levels were normalized to the same level. λ observation, 685 nm (C.S. 2-62 filter before the photomultiplier). 5 day old culture suspended in 50 mM Tris-Cl buffer with 10 mM NaCl, pH 7.8 was used. Phycocyanin/Chl *a* peak ratio = 1.39; 15 min. dark adaptation before illumination.


15 μM DCMU
PS I light
PS II light
Relative Fluorescence Yield
0.2
0.3
0.4
0.5
0
0.5
1.0
1.5
Time of Illumination, min

back reaction at all influences the slow changes in fluorescence yield, the following experiments were done.

Figure 86 shows the decay of delayed light emission observed after 0.1 second of switching off of the illumination in normal, DCMU and DCMU plus hydroxylamine treated cells of Anacystis. As reported earlier (110), the peak intensity of the slow (sec) component of DLE is smaller in DCMU poisoned cells than in normal cells. Hydroxylamine in the presence of DCMU completely abolished all DLE suggesting a complete elimination of a back recombination reaction. But from Figure 87 it is clear that NH_2OH suppresses the development of the DPS transient. As in the case of Porphyridium, we observe that NH_2OH causes an ID decline and from D, the fluorescence increases slowly, suggesting a feeding of electrons by NH_2OH to PS II centers.

Figure 88 shows that NH_2OH alone or in the presence of DCMU did not inhibit the slow long-term rise in the fluorescence yield in Anacystis. Measurements of DLE of identical cells and under identical conditions showed no measurable amount of DLE in the presence of these two inhibitors. (The slight rise in fluorescence level seen in normal cells treated with 10 mM NH_2OH (open squares) is probably due to a small rise in the constant (0 level) fluorescence.) In any event, most of the slow changes persisted in the presence of both DCMU and NH_2OH (Figure 88) when all back reactions seem to have been inhibited completely (Figure 86). This experiment strongly supports the contention that the slow changes in yield are independent of the level of Q and thus there must be other factor(s) that also govern fluorescence yield besides Q.

Figure 86. Slow delayed light (DLE) intensity in Anacystis in the presence of DCMU and DCMU plus hydroxylamine. 1 ml algal sample was illuminated with a broad band blue light (C S. 4-96 plus C.S. 3-73; intensity, 21 Kergs $cm^{-2} sec^{-1}$) for 2 minutes. DLE was measured 0.1 sec after illumination. Cells were suspended in carbonate-bicarbonate buffer (0.1 M) pH 9.2 plus 0.01 M NaCl; absorbance at the red peak; 0.3; Phycocyanin/Chl a peak ratio = 1.4; 15 μM DCMU; 10 mM NH_2OH.

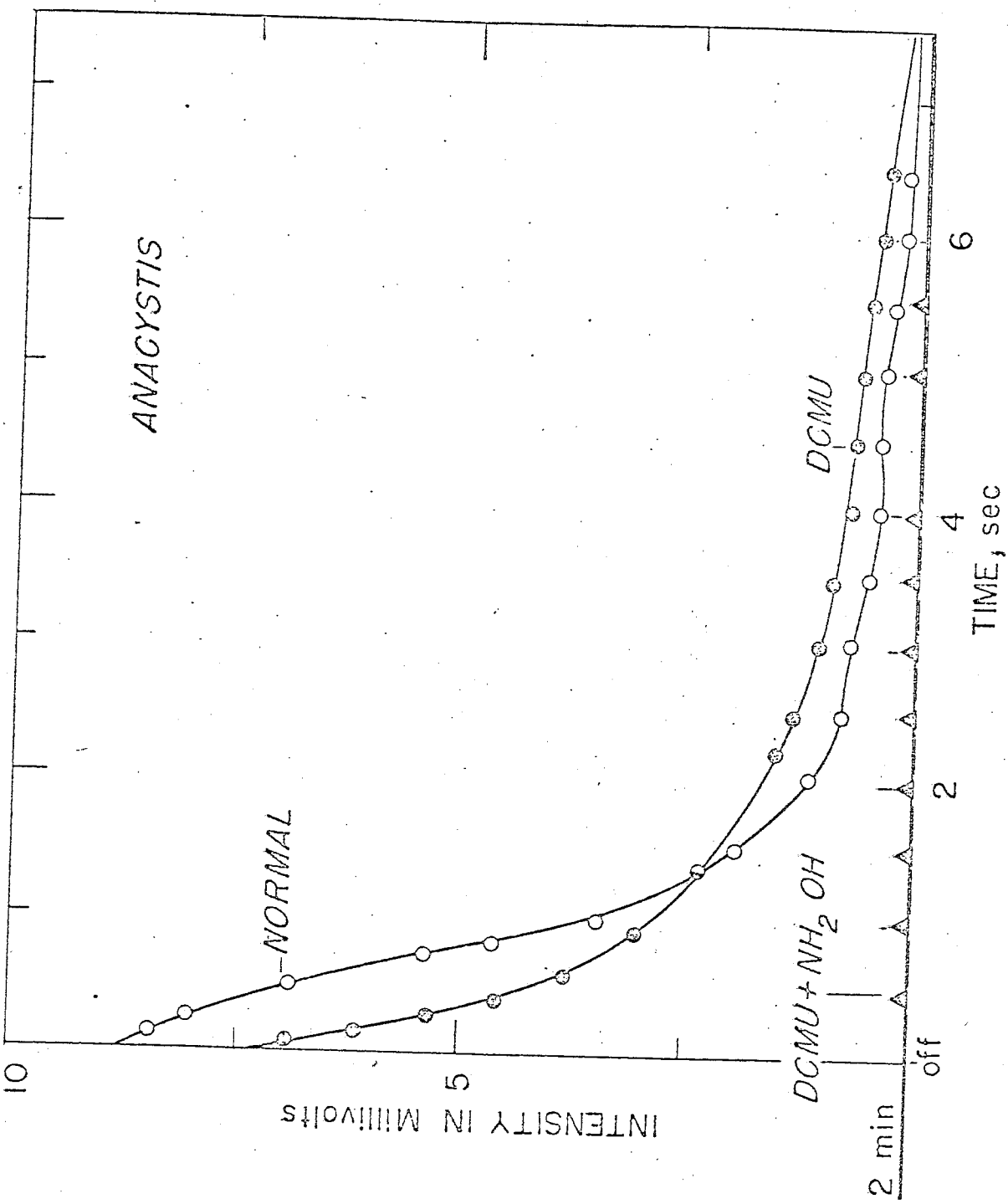

ANACYSTIS
NORMAL
DCMU
DCMU+NH2 OH
INTENSITY IN Millivolts
10
5
TIME, sec
2
4
6
2 min
off

Figure 87. Recorder traces of the fast transient of the Chl *a* fluorescence yield in the presence and the absence of NH_2OH. Open circles, no addition; closed circles, 5 mM NH_2OH. λ observation, 684 nm (half band width, 2.7 nm); C.S. 2-62 filter before the photomultiplier. λ excitation, 633 nm; intensity, 25 Kergs $cm^{-2}sec^{-1}$ cells were suspended in phosphate, sodium chloride buffer, pH 8.0.

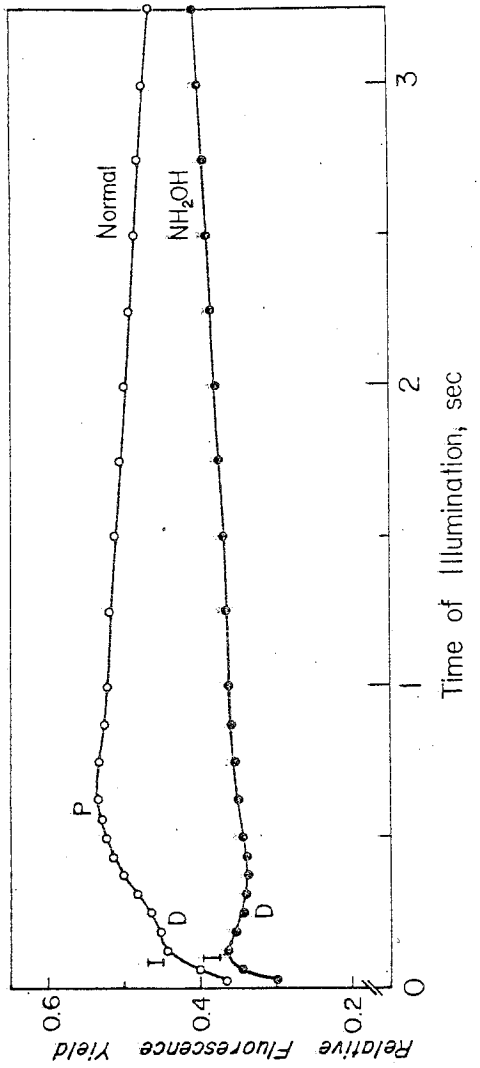
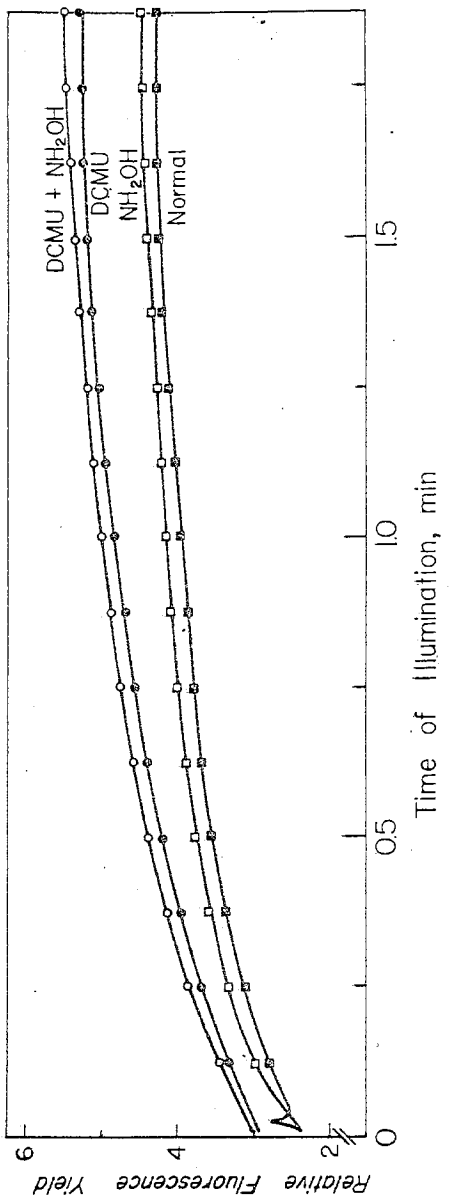

Normal
NH_2OH
P
I
D
I
D
0.6
0.4
0.2
Relative Fluorescence Yield
0
1
2
3
Time of Illumination, sec

Figure 88. Time course of the Chl *a* fluorescence yield in the presence and the absence of DCMU and DCMU plus hydroxylamine. Cells were suspended in Warburg buffer #9, pH 9.2, with 0.01 M NaCl; absorbance at the red peak of Chl *a* = 0.3; PC/Chl *a* peak ratio = 1.4. Additions, 15 μM DCMU; 10 mM NH_2OH. 15 min dark period before illumination; λ excitation, 633 nm; intensity, 26 Kergs $cm^{-2} sec^{-1}$.


DCMU + NH2OH
DCMU
NH2OH
Normal
Relative Fluorescence Yield
6
4
2
0
0.5
1.0
1.5
Time of Illumination, min

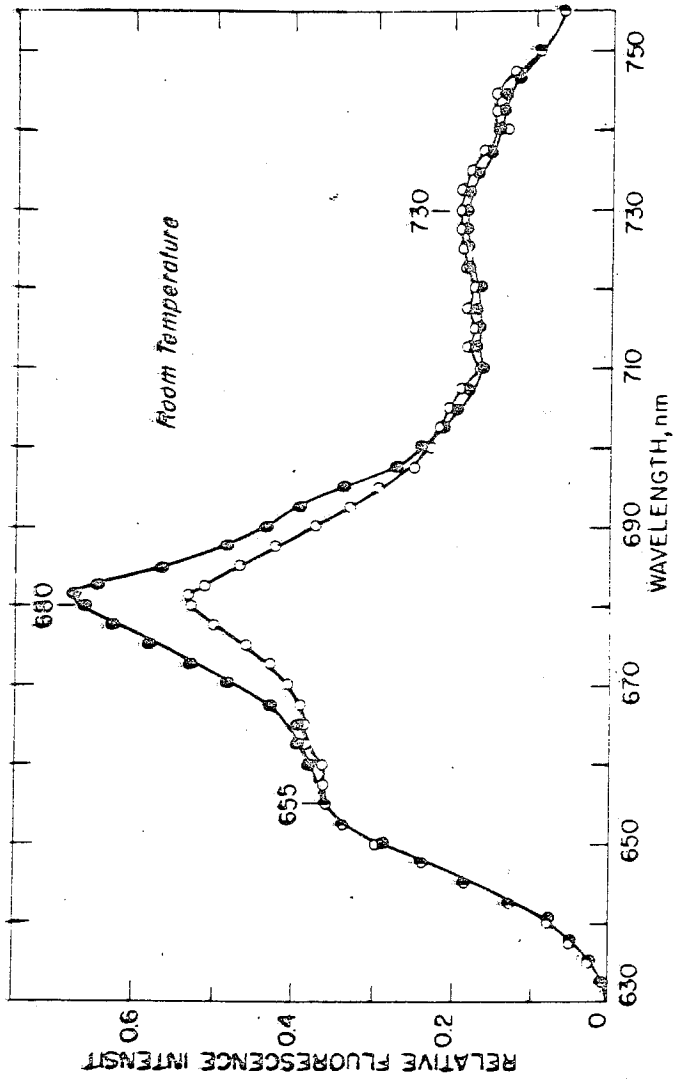
7.3 Emission Spectra

In Figure 89, the room temperature emission spectra of DCMU poisoned Anacystis, excited by low and high intensity of 570 nm light are compared. The two curves are adjusted at the invariable phycocyanin fluorescence band for comparison. At low intensity, used in the experiment, there is no change in fluorescence yield, and thus, the emission spectrum would be comparable to that of the yield at initial low level (Fi). At high intensity of illumination, the recorded spectrum is for the maximum steady state level (Fm). It is clear from the figure that an increase in fluorescence at high light intensity is due to enhancement in system II fluorescence band. As in the case of normal Anacystis (88), the DCMU poisoned samples show an extensive change in the emission spectra during the slow fluorescence induction in DCMU poisoned cells. This type of enhancement in fluorescence intensity in the main Chl a fluorescence band at the high emissive (Fm) state compared to low emissive (Fi) state indicates a suppression of energy transfer from PS II to PS I during the SM rise. The higher fluorescence yield and parallel rise in the rate of oxygen evolution in unpoisoned cells are indicative of change in distribution of quanta in favor of PS II. This, however, occurs even when the PS II reactions are blocked by DCMU.

8. Effect of Inhibitors of Phosphorylation on the Slow Changes in Fluorescence Yield in Anacystis

Papageorgiou and Govindjee (88) found that uncouplers like FCCP suppress the slow changes in Chl a fluorescence in normal cells of Anacystis but not as much as in DCMU poisoned samples. In this section, we report results obtained with uncouplers like CCCP and FCCP, and those with tetrachlorosalicylanide derivatives (S_6 and S_{13}, ref. 208). Our results

Figure 89. Room temperature emission spectra of Anacystis in the presence of DCMU. Solid circles, excitation with high intensity of 570 nm light (half band width, 15 nm), 9.3 Kergs $cm^{-2}sec^{-1}$; open circles, with low intensity of 570 nm light, 0.11 Kergs $cm^{-2}sec^{-1}$. 15 μM DCMU in carbonate-bicarbonate buffer, pH 9.1, plus 0.01 M NaCl. Both spectra are adjusted at 650 nm to read equal intensity.


Room Temperature
680
655
730
630
650
670
690
710
730
750
WAVELENGTH, nm
0
0.2
0.4
0.6
RELATIVE FLUORESCENCE INTENSITY

indicate that these latter potent uncouplers inhibit the slow rise in fluorescence in both normal as well as DCMU treated samples. We have also employed some ATPase inhibitors to study their effect on the slow changes in the Chl a fluorescence yield.

Figure 90 (top) depicts the effect of two concentrations of CCCP on the fast fluorescence in Anacystis. 5 μM CCCP lowered the development of P and thus the PS decline, but the slow fluorescence rise was not altered at this concentration (open triangles). On doubling the concentration to 10 μM (open squares), fluorescence yield was further depressed at the level of P and the SM rise was also lowered. The bottom curve of Figure 90 shows the effect of 5 and 10 μM CCCP in the presence of DCMU. 10 μM CCCP did not cause the same extent of suppression in the slow rise of fluorescence in the presence of DCMU as it did in its absence. Papageorgiou and Govindjee (88) observed a similar, but more dramatic differential behavior with normal and DCMU treated Anacystis with 10 μM FCCP which is a more powerful uncoupler compared to CCCP.

Figure 91 illustrates the effect of FCCP and S_{13} on the SM rise of Chl a fluorescence in unpoisoned Anacystis. 10 μM FCCP inhibited DPS change and also suppressed the extent of SM rise (solid squares). Upon increasing the concentration to 10 μM the S level was slightly lowered, compared to normal (open circles), the SM rise was slowed down, the rate of the fluorescence rise was depressed and the maximal level of the fluorescence yield was lower than that of normal. S_{13} was found to be comparatively more effective in inhibiting the slow fluorescence changes in Anacystis than FCCP. At a concentration of 1 to 2 μM, most of the slow changes were abolished. On increasing the concentration, it stimulated a decline in the yield of fluorescence upon illumination. Both S_6 and

Figure 90. Effect of the uncoupler CCCP on the fluorescence yield of Chl *a* in normal and DCMU poisoned *Anacystis*. (Top figure): fast changes in the fluorescence in normal cells. (Bottom figure): poisoned samples with 15 μM DCMU; λ observation, 685 nm; C.S. 2-62 filter before the analyzing monochromator; buffer, 50 mM Tris-Cl, pH 7.8, with 10 mM NaCl. (Ethanolic solution of CCCP was diluted with water, ethanol concentration 1%.)

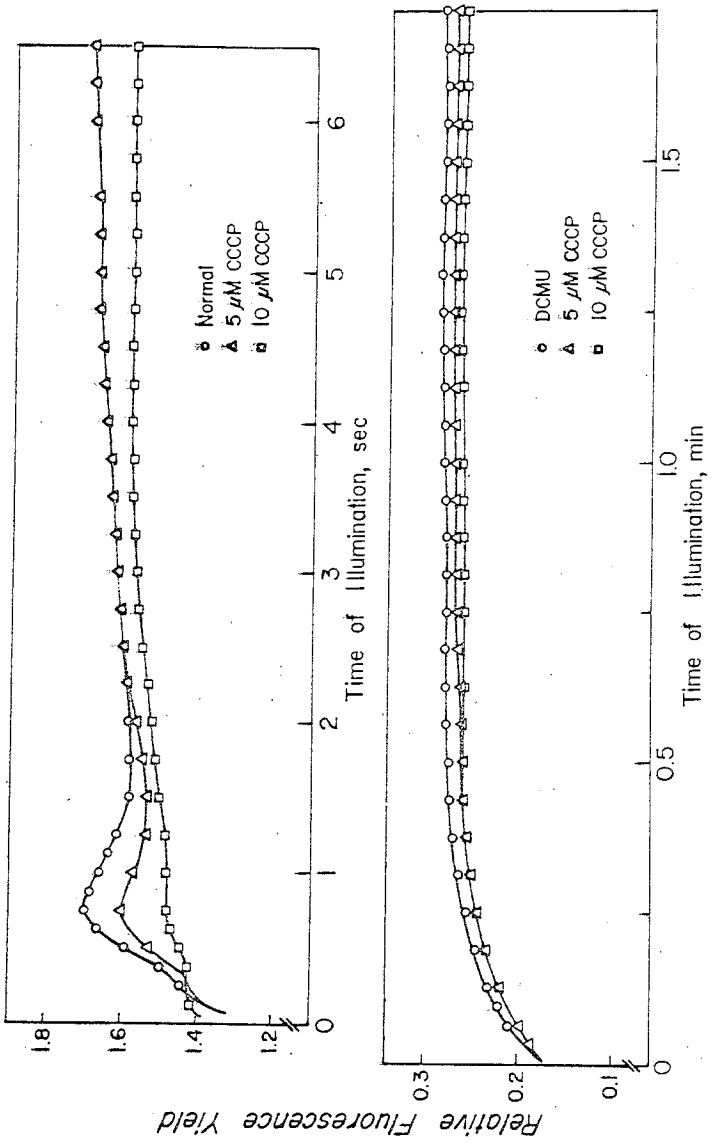
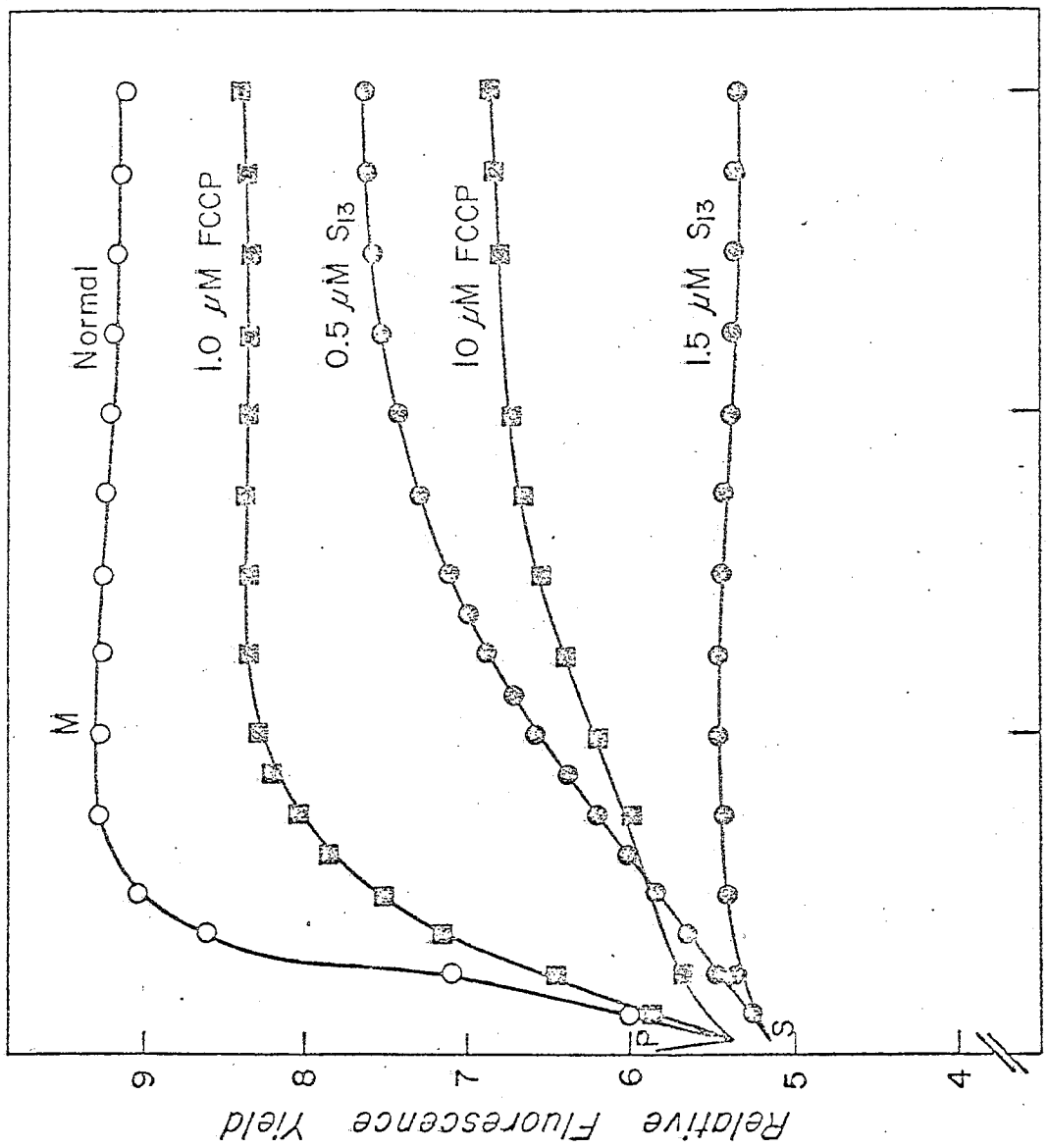

Relative Fluorescence Yield
1.8
1.6
1.4
1.2
0
1
2
3
4
5
6
Time of Illumination, sec
Normal
5 μM CCCP
10 μM CCCP
0.3
0.2
0.1
0
0.5
1.0
1.5
Time of Illumination, min
DCMU
5 μM CCCP
10 μM CCCP

Figure 91. Effect of uncouplers on the slow fluorescence transient of normal Anacystis. λ observation, 685 nm (half band width, 6.6 nm); λ excitation, 633 nm; intensity, 23 Kergs $cm^{-2}sec^{-1}$. A 6 day old culture, centrifuged and suspended in 50 mM Tris-Cl plus 10 mM NaCl buffer, pH 7.8, was used. Phycocyanin/Chl a peak ratios = 1.3; ethanolic solution of S_{13} and FCCP were added; ethanol concentration 2%.


Normal
1.0 μM FCCP
0.5 μM S13
10 μM FCCP
1.5 μM S13
M
P
S
Relative Fluorescence Yield
Time of Illumination, min
0
1
2
3
4
5
6
7
8
9

S_{13} were found to exhibit similar effects on the long term fluorescence yield changes as S_{13}. However, the former seems to be more effective in suppressing the fluorescence yield in Anacystis.

Figure 92 shows the effect of FCCP on the rate of oxygen evolution in Anacystis. Addition of FCCP (5 μM) depressed the oxygen spike and caused uptake (decline in the rate of evolution). With time, the rate of O_2 evolution increases, but at a slower rate than in the normal. The steady state O_2 evolution was approximately 15 to 20% less than in normal Anacystis. It seems that FCCP partly accelerates O_2 uptake in light although steady state photosynthesis was not inhibited to any great extent. With 10 μM FCCP, there was a further suppression of steady state photosynthesis, but photosynthesis was not totally inhibited. This suppression of measured photosynthesis may either be due to loss of ATP synthesis (and thus of complete photosynthesis) or photo-induced O_2 uptake. No measurements of O_2 evolution with S_{13} were made.

Figure 93 shows the effect of S_6 (208), another derivative of salicylanilide, on the fluorescence transient of DCMU poisoned Anacystis. With 2 μM S_6 most of the fluorescence transient was suppressed. Upon doubling the concentration, a further lowering of both initial and maximal levels of fluorescence was observed. S_{13} gave about the same results as S_6; FCCP was found to bring about less inhibition of the slow changes in the fluorescence yield.

Figure 94 shows room temperature emission spectra of DCMU poisoned Anacystis in the presence and in the absence of the uncoupler S_{13}. The sample was excited with a broad band 570 nm light (half band width, 20 nm; intensity, ~10 Kergs $cm^{-2}sec^{-1}$). After 5 minutes of illumination, the

Figure 92. Time course of oxygen exchange in Anacystis in the presence of FCCP. Values above the baseline are taken to be due to oxygen evolution. Other conditions for the measurement of the rate of O_2 exchange as in Figure 75. Open circles, normal sample; solid circles, with 5 μM FCCP; after the measurement of the normal sample the electrolyte was quickly drained out and was replaced with buffer containing FCCP, incubated for 20 minutes and bubbled with 2% CO_2 in air before the FCCP curve was recorded.

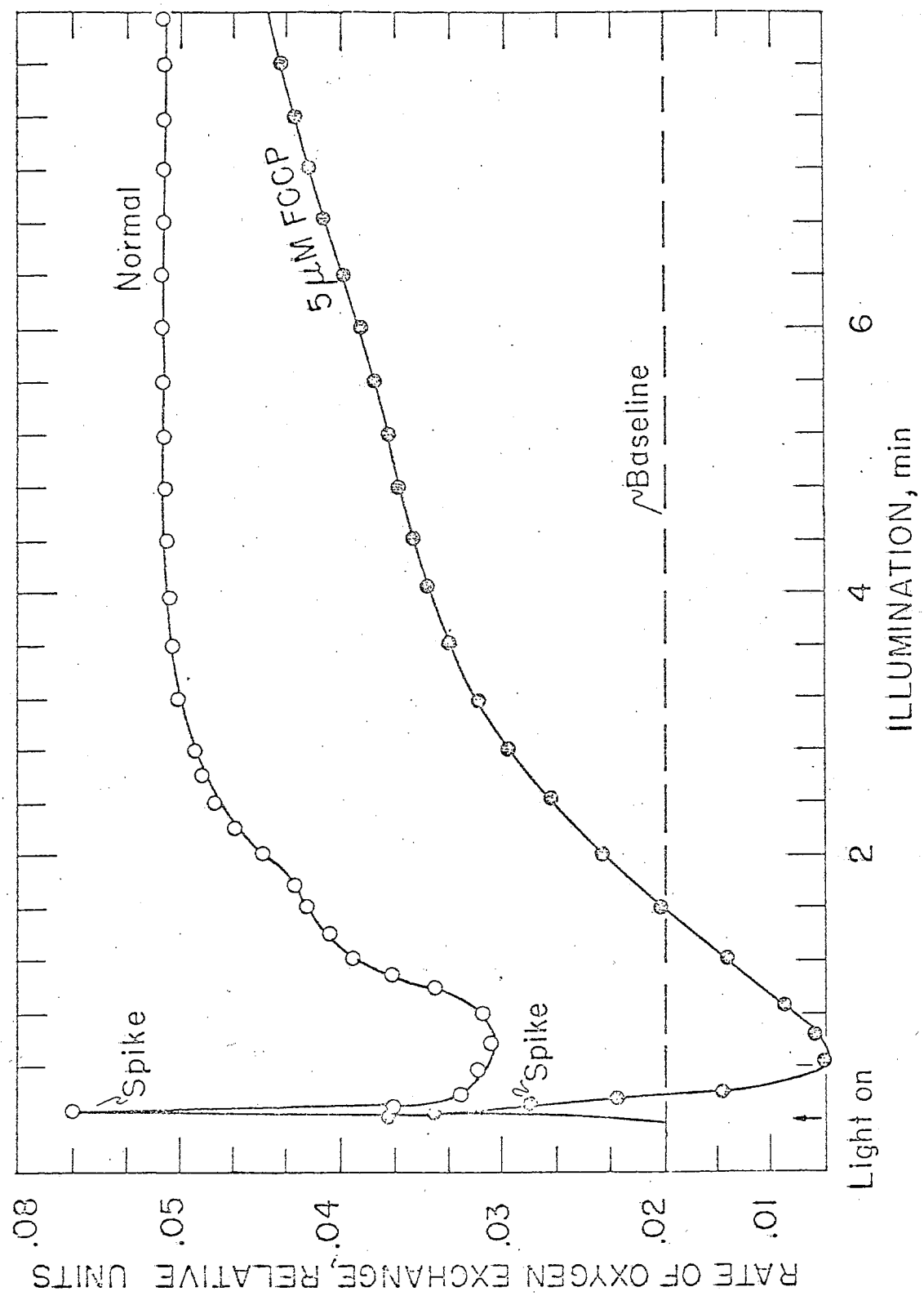

RATE OF OXYGEN EXCHANGE, RELATIVE UNITS
.08
.05
.04
.03
.02
.01
Normal
5 μM FCCP
Spike
Spike
Baseline
Light on
2
4
6
ILLUMINATION, min

Figure 93. Time course of the slow rise in Chl a fluorescence yield in DCMU poisoned Anacystis with and without uncoupler S_6. Other conditions for measurement as described in the legend for Figure 91.

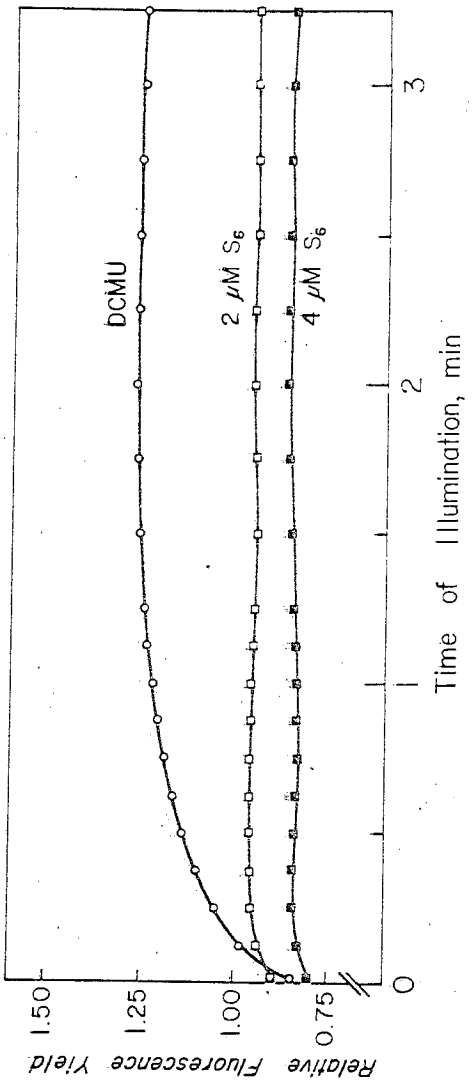
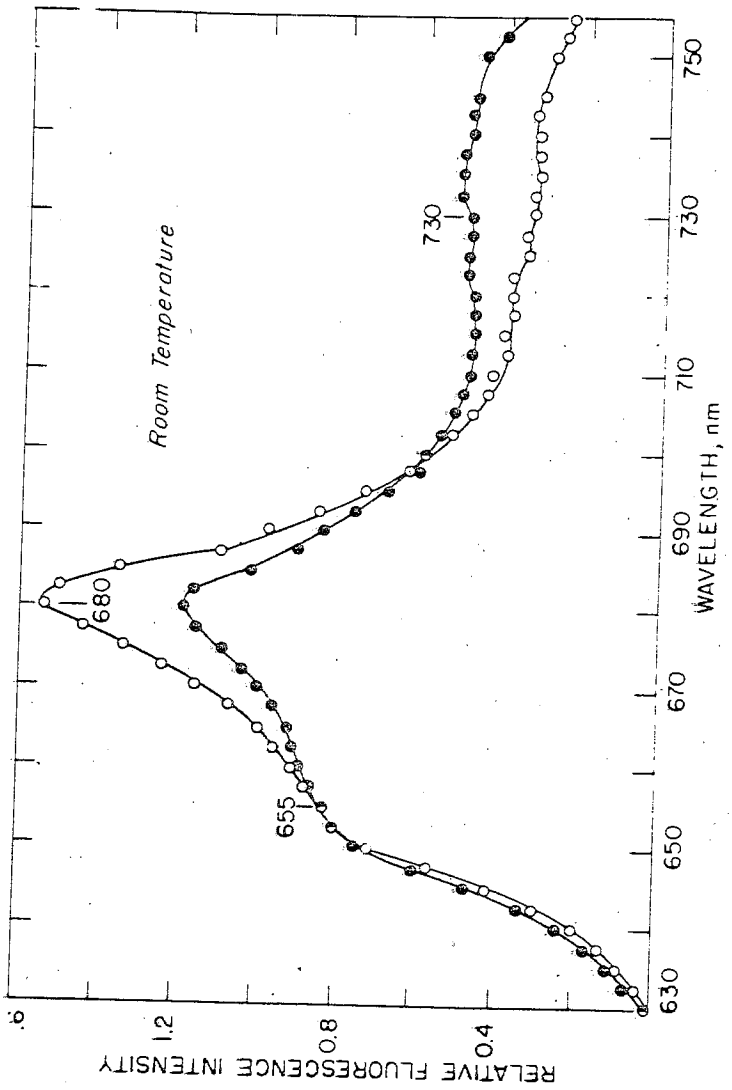

DCMU
2 μM S6
4 μM S6
1.50
1.25
1.00
0.75
0
1
2
3
Relative Fluorescence Yield
Time of Illumination, min

Figure 94. Room temperature emission spectra in the presence and the absence of S_{13} in DCMU poisoned cells of Anacystis. Cells were suspended in growth medium containing 15 μM DCMU; open circles, DCMU poisoned sample; closed circles, DCMU + 5 μM S_{13}. Other details as in Figure 89.


Room Temperature
680
655
730
630
650
670
690
710
730
750
WAVELENGTH, nm
0.4
0.8
1.2
RELATIVE FLUORESCENCE INTENSITY

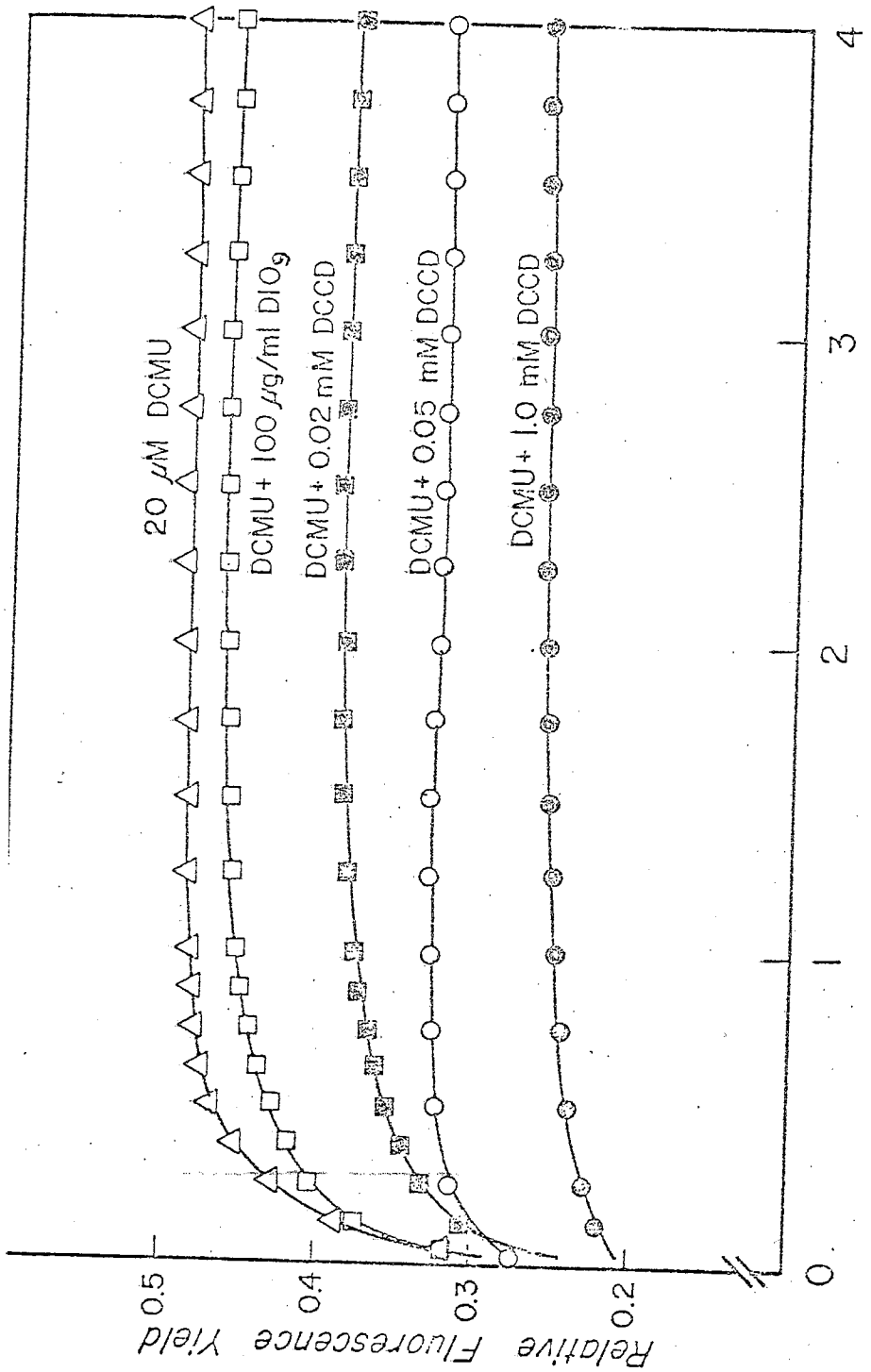
emission spectra were recorded and the two spectra were arbitrarily adjusted at 655 nm with the assumption that phycocyanin fluorescence remains unchanged. A depression of system II fluorescence (680 nm peak) accompanied by an increase in the long wavelength fluorescence was caused by S_{13}. In other words, during the slow SM rise system II fluorescence increases and system I fluorescence decreases, a parameter which is indicative of a shift in the energy distribution between the two photosystems.

Figure 95 illustrates some of the effects of so-called energy transfer inhibitors of phosphorylation on the slow changes in fluorescence yield. Dio-9, an experimental antibiotic, is known to inhibit ATP synthesis in chloroplasts (209, 210). Dio-9, at a concentration of 120 μg/ml, did not inhibit significantly the slow changes in fluorescence yield in the presence of DCMU. Phloridzin, which is also a terminal inhibitor of phosphorylation, did not cause any inhibition of slow change in fluorescence yield even at 1 mM concentration (not shown). DCCD (N, N' dicyclohexylcarbodiimide), also considered as an energy transfer inhibitor of chloroplasts and mitochondria (211, 212) suppressed fluorescence yield changes in the presence of DCMU. At a concentration of 1.0 mM, most of the slow changes in yield were inhibited.

We have also tested the effect of some respiratory inhibitors like azide and arsenate and found them to be ineffective in inhibiting the slow changes fluorescence in yield in DCMU poisoned samples.

Our results reported in this section appear contradictory to those of Papageorgiou and Govindjee (88) who found that DCMU resistant fluorescence yield changes are also resistant to uncouplers and energy transfer inhibitors. Admittedly, the slow changes in fluorescence yield in the

Figure 95. Effect of Dio-9 and DCCD on the slow rise in Chl *a* fluorescence in the presence of DCMU in *Anacystis*. Other conditions as described in the legend of Figure 91.


20 μM DCMU
DCMU + 100 μg/ml DIO9
DCMU + 0.02 mM DCCD
DCMU + 0.05 mM DCCD
DCMU + 1.0 mM DCCD
Relative Fluorescence Yield
0.5
0.4
0.3
0.2
0
1
2
3
4
Time of Illumination, min

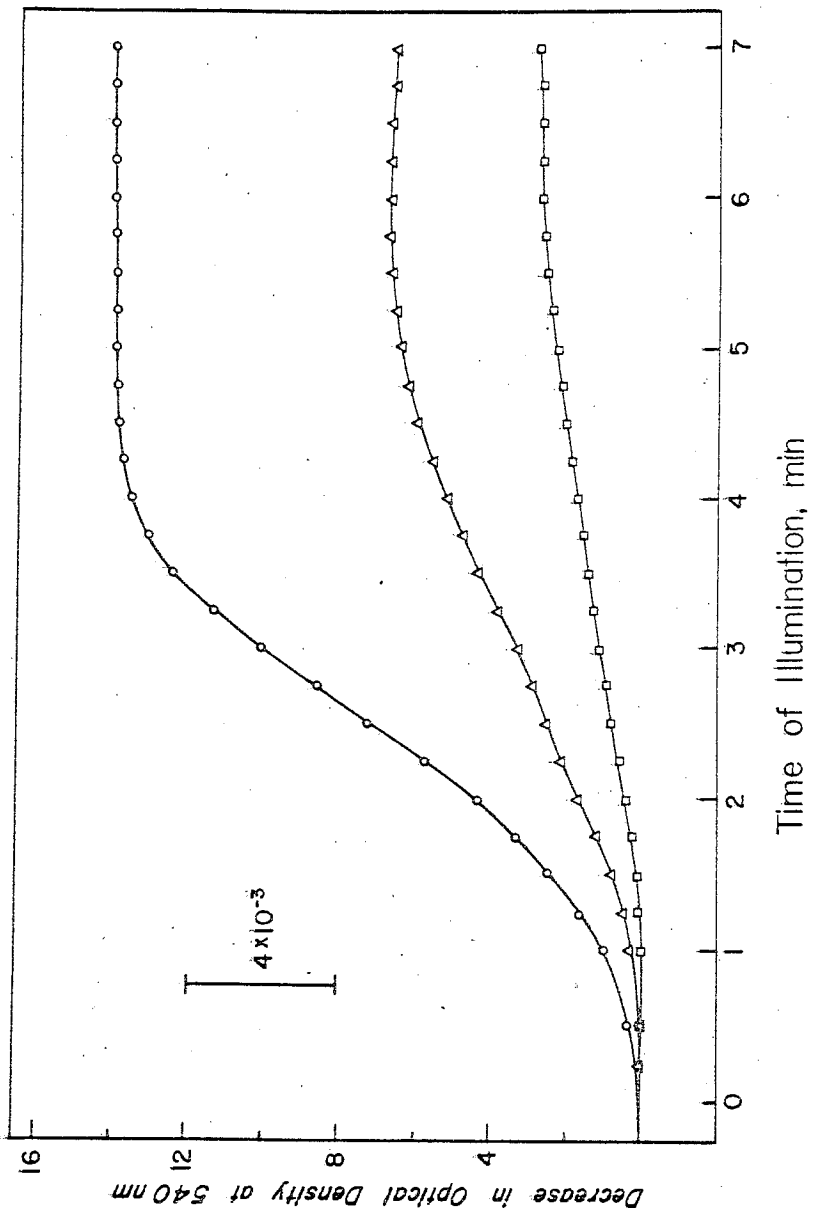
presence of DCMU is rather resistant to inhibitors and was only lowered at relatively high concentrations of compounds. We shall discuss later in this chapter the differential effect of uncouplers on the fluorescence yield changes.

9. Structural Changes in Anacystis

It has been proposed that light induced structural changes which accompany phosphorylation modify fluorescence yield (87, 88). If Anacystis cells suspended in phosphate buffer (0.05M, pH 8.0) with 0.01M NaCl or in the growth medium (pH 6.8) were illuminated by red light (Schott R.G. 665 or C.S. 2-64), a decrease in optical density at 540 nm was observed suggesting a swelling of the thylakoid membranes; this decrease in absorbance at 540 nm was observed both in the absence and in the presence of 15 μM DCMU. However, DCMU treated samples exhibited slightly greater change than normal cells (not shown).

Figure 96 illustrates the decrease in optical density at 540 nm measured in Professor C. Sybesma's difference absorption spectrophotometer in Anacystis induced by three intensities of red illumination. For the bottom trace the actinic illumination was 1.8×10^4 ergs $cm^{-2}sec^{-1}$, for the middle trace, 2.4×10^4 ergs $cm^{-2}sec^{-1}$, and for the upper trace 3.0×10^4 ergs $cm^{-2}sec^{-1}$. Upon illumination there was a lag for about 15 to 20 seconds and then absorbance at 540 nm began to decline slowly and finally a maximal decrease in optical density was obtained after 4 to 5 minutes of illumination. On turning off the actinic illumination (not shown) there was again a lag of 15-20 seconds after which the optical density increased and was restored to the dark level after about 5 to 7 minutes of darkness. This sequence could be repeated. Decrease in the

Figure 96. Decrease in the 540 nm absorbance induced by three different intensities of red actinic illumination in normal Anacystis. The numbers on the ordinate should be multiplied by 10^{-3}; λ measuring, 540 nm; slit width, 6.6 nm; C.S. 4-72 filter before photomultiplier. Actinic filter, Schott R G 665. Open circles, intensity = 1.00; open triangles, intensity = 0.80; open squares, intensity = 0.60 (1.00 = 3.0 x 10^{4} ergs $cm^{-2}sec^{-1}$). Cells were suspended in phosphate sodium chloride buffer; absorbance at the red peak of Chl a for 1 cm path = 0.35; PC/Chl peak ratio = 1.32.

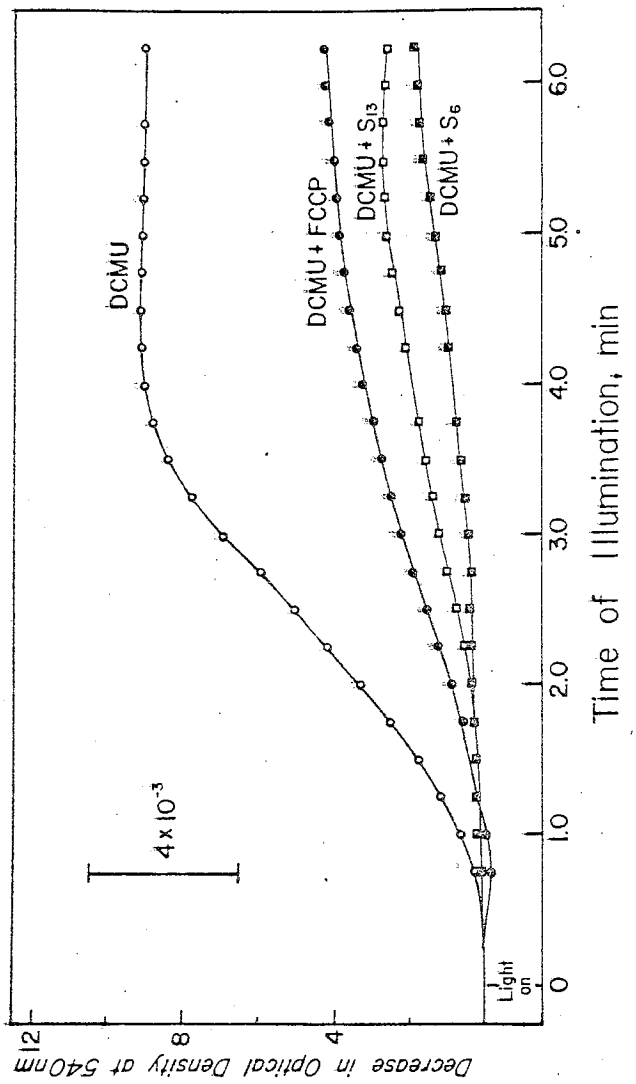

Decrease in Optical Density at 540 nm
16
12
8
4
0
1
2
3
4
5
6
7
Time of Illumination, min
4 x 10⁻³

intensity of actinic illumination pronounced the lag period and lowered the rate and extent of decrease in absorbance at 540 nm. However, time to attain saturation did not alter very much.

The intensity of actinic light used for these measurements was higher than that used for the fluorescence measurements. However, the time course of decrease in 540 nm optical density is much slower than that of slow changes in fluorescence yield (Figure 96). We do not expect a correspondance in time of these two events. The decrease in optical density reflects the large macroscopic volume changes of the membraneous vesicles and changes in conformation of the individual thylakoid membrane should preceed gross macroscopic changes in structure; and we expect only the former to alter spatial relations or orientations of the two pigment systems. Also, the extent of swelling is not proportional to the intensity of illumination although the relative fluorescence yield remains constant at these intensities. This observation does not pose any restriction on the hypothesis that structural changes are related to Chl a fluorescence yield changes. Macroscopic changes in volume may not be proportionately related to a photochemical events while fluorescence yield is dependent on the excitation intensity. (Volume changes are light triggered phenomena but photochemical reactions are not.)

Figure 97 shows the effect of uncouplers (10 μM FCCP, 5 μM S_{13} and 5 μM S_6) on the changes in transmission at 540 nm in DCMU-poisoned cells. The concentration of chlorophyll used here is slightly higher than the concentration used for fluorescence measurements. Interestingly, 10 μM FCCP did cause some 50-60% inhibition while S_{13} and S_6 suppressed 80-90% decrease in optical density at 540 nm. In some measurements, a complete suppression in change in absorbance at 540 nm was observed.

Figure 97. Time course for the decrease in 540 nm absorbance. The numbers on the ordinate should be multiplied by 10^{-3}; λ measuring, 540 nm (slit width 6.6 nm); C.S. 4-72 filter before the photomultiplier; sample was illuminated with red actinic light (Schott R.G. 665; intensity 3.0×10^{4} ergs cm^{-2} sec^{-1}); cells were suspended in phosphate buffer with 15 μM DCMU. Uncouplers (FCCP, S_6 or S_{13}) were added to the sample and pre-incubated for 10 min. in the dark before each measurement. Absorbance of the sample at the red Chl *a* peak = 0.35 for 1 cm path length; phycocyanin/Chl *a* peak ratio = 1.2.


DCMU
DCMU + FCCP
DCMU + S13
DCMU + S6
4 x 10-3
Light on
0
1.0
2.0
3.0
4.0
5.0
6.0
Time of Illumination, min
12
8
4
Decrease in Optical Density at 540nm

FCCP did not cause as large an inhibition in fluorescence yield changes as it did for structural changes in DCMU-poisoned Anacystis, although 10 to 20 μM FCCP did suppress (10-20%) fluorescence yield. (We do not know the reason for this difference.) But, qualitatively our results indicate that both salicilanilide and FCCP suppress the light induced volume changes as well as slow changes in fluorescence; S_6 and S_{13} are more effective than FCCP.

9.1 Fixation with Formaldehyde

We have earlier shown (Chapter III) that fixation of Porphyridium cells causes a loss of the slow fluorescence transient and loss of CO_2 fixation although a net electron transport capacity is retained in these preparations.

Anacystis cells were fixed with 2% (w/V) paraformaldehyde in 0.05M phosphate buffer, pH 7.4, according to the method of Ludlow and Park (213). These authors have shown that formaldehyde fixed cells can support both PS I and PS II activity.

Figures 98 and 99 illustrate that formaldehyde fixation caused a complete loss of slow changes in fluorescence yield both in the presence and in the absence of DCMU. This result was similar to that observed in Porphyridium. From Figure 99 it is clear that the addition of DCMU caused an increase in the fluorescence yield in fixed cells due to inhibition of electron transport in spite of bright illumination used in these experiments. However, the long term slow changes in yield were completely arrested by aldehyde fixation.

The results reported in this section support a correlation between light triggered structural and slow changes in fluorescence yield. However, the probable cause of alteration in fluorescence yield must be

Figure 98. Time course of Chl *a* fluorescence yield in formaldehyde fixed cells of *Anacystis*. Fixation was done according to Ludlow and Park (213). [Fixed cells could reduce 28 μmoles of dichlorophenol indophenol / mg Chl hr; there was no reduction of dye by unfixed cells.] Fluorescence transient was measured as in Figure 87. OI rise phase was not recorded; [Chl]; 15 μg/ml.

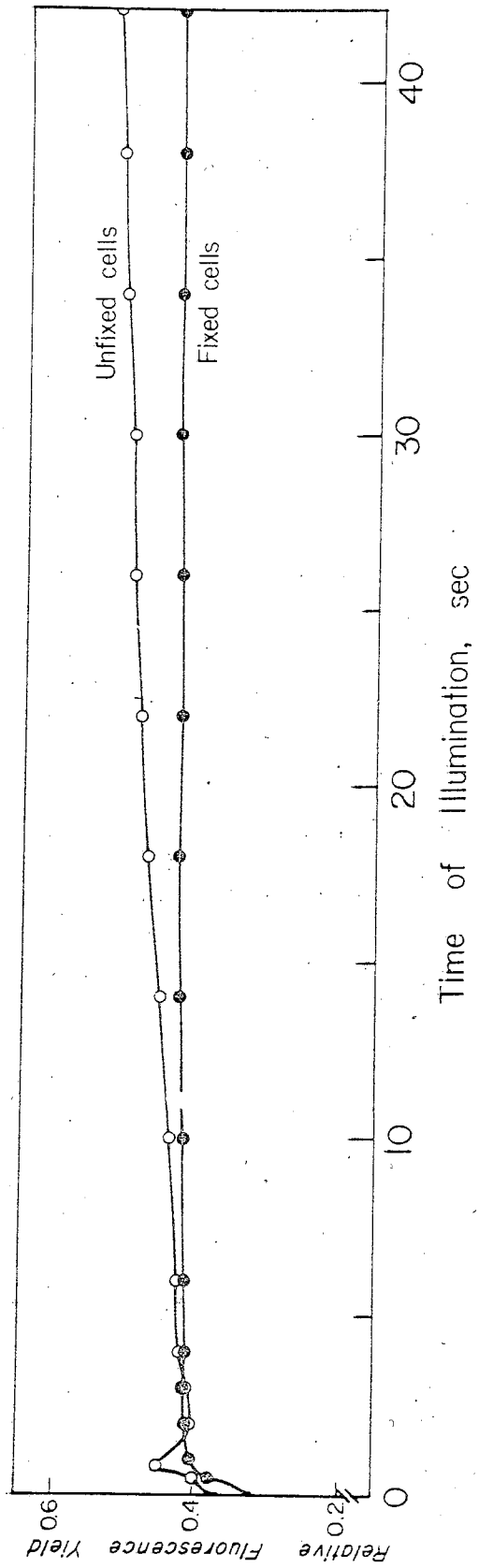
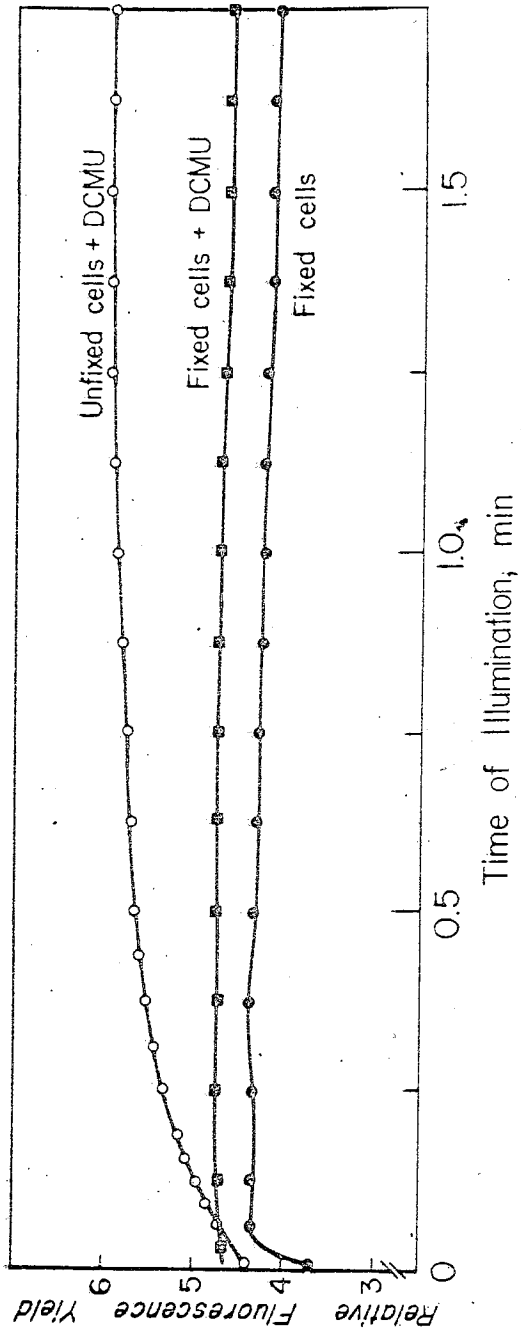

Unfixed cells
Fixed cells
0
10
20
30
40
Time of Illumination, sec
Relative Fluorescence Yield
0.2
0.4
0.6

Figure 99. Time course of Chl *a* fluorescence in formaldehyde fixed cells of *Anacystis* in the presence of 15 μM DCMU. Other conditions as in Figure 87.


Unfixed cells + DCMU
Fixed cells + DCMU
Fixed cells
Relative Fluorescence Yield
3
4
5
6
0
0.5
1.0
1.5
Time of Illumination, min

associated with microscopic alteration in membrane structure and not with the gross swelling or shrinkage of the entire organelle. This later phenomenon is the net terminal result of a change in membrane conformation and is associated with energy conservation processes (120).

10. Estimation of ATP Levels in Anacystis

Studies with uncouplers and other inhibitors of phosphorylation suggest that the energy dependent structural alteration is associated with fluorescence yield changes. A time course of ATP level or more importantly some precursor of it would yield a meaningful comparison. (However, any hypothetical precursor of ATP (like XE) is difficult to measure in whole cells.)

The cells were suspended in carbonate-bicarbonate buffer (0.1M, pH 8.2) and incubated with additives in the dark for 45 minutes. The samples had an absorbance of 0.3 at the red Chl a peak corresponding to chlorophyll concentration of approximately 30 μg/ml. Other details of measurements are as in reference 145; 50 units of luminescence signal corresponds to 5×10^{-8} M ATP.

We have not measured the time course of light induced ATP formation in Anacystis. In the absence of such experiments, we have only estimated the total ATP content before and after illumination. Such determination of total ATP content will enable us to assess the source of energy dependent structural changes that presumably alter the fluorescence yield.

Table V.3 shows the relative changes in the ATP triggered luminescence signal* in normal and treated Anacystis before and after 30 seconds of

*(These experiments were done with the collaboration of Mr. G. W. Bedell using the assay procedure developed by him (145).)

Table V.3

Changes in ATP Level in Treated and Normal Anacystis

(Experiments made in collaboration with G. W. Bedell, 145.)

Additions	1 Relative ATP Level before illumination (D)	2 Relative ATP Level after 30 sec illumination (L)	3 (L-D)
None	38.1	36.7	-1.4
10 μM FCCP	23.4	36.0	+12.6
15 μM DCMU (normalized to control)	38.0	46.7	+8.7
15 μM DCMU + 10 μM FCCP	23.9	30.5	+6.6
15 μM DCMU + 5 μM S_{13}	28.2	24.6	-3.6

Cells were suspended in Warburgs buffer pH 8.2; kept in dark for 45 minutes before illuminating for 30 sec with bright (~100 Kergs $cm^{-2}sec^{-1}$) orange light; 50 units = 50 nm ATP.

illumination with a bright saturating orange light (~100 Kergs $cm^{-2}sec^{-1}$). In these determinations, variations in the dark level introduced a large problem. Evidently, all these poisons, including DCMU, affected dark ATP levels. Since dark incubation period exceeded that of light illumination, we can conceive of an accumulation of ATP in dark. Both 5 μM S_{13} and 10 μM FCCP lower the dark level, perhaps, due to uncoupling of respiration. Upon illumination, there is an increase in ATP level, presumably due to cyclic electron transport in DCMU treated cells. It seems that S_{13} uncouples the cyclic photophosphorylation to a greater extent than FCCP.

In normal unpoisoned cells, there is no net accumulation of ATP, probably, due to rapid utilization of ATP in light. Bedell (145) has observed similar results in Chlorella. The accumulation of ATP in FCCP treated samples of Anacystis, in all likelihood, represents the inhibition of these utilization steps; we have shown earlier that FCCP, at this concentration, also inhibits photosynthetic oxygen evolution.

11. Summary and Conclusions

In this chapter we have characterized, for the first time, the fast fluorescence transient in blue green algae. Blue green algae have a diminished OIDPS transient in comparison to the red alga Porphyridium and green alga Chlorella. Anacystis shows a pronounced dip (D) and this dip arises, perhaps, due to a rapid oxidation of QH by PS I light.

Unlike red and green algae, blue green algae exhibit a very pronounced SM rise, the M level being much higher than the P level, and the MT decline is extremely slow. The M level was 60 to 80% higher than the P level. This type of fluorescence seems to be characteristic for all blue green algae examined.

We have confirmed the earlier observation of Papageorgiou and Govindjee (88) that during the SM rise there is also a parallel increase in the rate of net O_2 evolution (Figure 78). DCMU inhibits both O_2 evolution and the fast induction wave; however, the rapid OI rise and the slow changes persist. Other cofactors of electron transport like PMS and MeV and some other inhibitors of electron transport abolish the fast transient without causing any significant effect on the slow fluorescence yield changes (Figures 81, 82). It seems that the slow transient is not entirely dependent on the fast transient. We have provided evidence that

the DCMU resistant slow changes in the fluorescence yield are not influenced by the acceleration of a back reaction between the oxidized primary donor (Z^+) and the reduced acceptor (Q^-). Hydroxylamine abolished the DLE which presumably originates from such back reactions but did not inhibit the slow changes in fluorescence yield in Anacystis (cf. Figures 86 and 87 and 88).

In the presence of DCMU, the steady state O_2 exchange was negligible. (DCMU inhibited more than 80-90% of O_2 evolution.) But, the slow fluorescence change is still present. It appears that photoinhibition of respiration does not influence the slow changes in fluorescence yield. From Table V.3 and from a comparison of the data of Bedell (145) on Chlorella, it appears that the extent of cyclic phosphorylation is greater in Anacystis than in Chlorella. This observation prompts us to believe that DCMU-resistant cyclic flow and DCMU resistant slow changes in fluorescence are correlated. It is, however, difficult to assess whether or not DCMU treatment induces a cyclic electron flow. If the slow changes in fluorescence yield are assumed to reflect the cyclic electron flow it seems there is not too large an enhancement in the slow fluorescence yield in the DCMU treated over normal cells. This poses a dilemma as the observation of Papageorgiou and Govindjee (88), and some of our own, show differential suppression of the slow rise in the presence and in the absence of DCMU. We do not have a satisfactory answer to this problem at this time. But the slow changes in fluorescence yield, both with and without DCMU, are not dependent on the path of electron transport but on the source and supply of high energy intermediate that alters the lamellar conformation and thereby electronic excitation energy transfer process in the pigment bed.

It is conceivable that different uncouplers may have differential affinity for the high energy intermediates. We have shown that slow changes in fluorescence yield in the presence of DCMU are not immune to uncouplers. S_{13} and S_6 abolished the slow changes in the fluorescence yield. These uncouplers also abolished the cyclic ATP formation (Table V.3) and light induced scattering changes at 540 nm (Figure 97). FCCP, in comparison to S_6 or S_{13}, was found to be less potent for blue green algae. (See Kraayenhof, 214, 215.)

Our results with the so called energy transfer inhibitors posed another problem. Phloridzin exerted no effect on DCMU resistant slow changes in fluorescence yield. We do not think that this result is simply due to non-penetrability of this glucoside as it has been shown both in our work as well as in ref. 216 (also see ref. 210) to get into intact cells. Dio-9 has been known to inhibit active K^+ influx (217, 218) and thus may cause alteration in structure. Dio-9, unlike phloridzin, slightly lowers the extent of slow rise in fluorescence yield. DCCD (N, N' Dicyclohexylcarbodiimide) which is also known to inhibit energy transfer in mitochondria and chloroplasts (210) (see reference 212) inhibited the slow changes in fluorescence yield (Figure 95). However, Harold et al. (219) and Harold and Baarda (220) have observed that DCCD, in intact cells of Streptococcus faecilis affects a number of energy dependent processes like accumulation of K^+ by exchange with Na^+ or H^+. Their results also indicate that DCCD is a potent inhibitor of a membrane bound ATPase both in isolated membrane fragments and in intact cells. These authors suggest that the ATPase plays a key role in the maintenance of a pH gradient. Conceivably, DCCD may bind to specific sites on the

membranes to inhibit indirectly ion transport, ATP hydrolysis and osmotic changes of lamellar conformation.

We, therefore, do not consider our results with DCCD or Dio-9 to be inconsistent with the suggestion that a high energy intermediate rather than ATP is the source of energy for light induced changes in lamellar conformation. Chemicals like DCCD or Dio-9, besides being energy transfer inhibitors, also interfere with energy dependent ion uptake processes (217, 218). Similarly, Smith (221) has shown that uncouplers affect solute uptake which requires pH gradient. Thus, it seems that although the structural alterations that modify the fluorescence yield is mostly driven by a high energy intermediate, it is also partly modified by other ATP requiring processes like ion or solute transport.

VI. FLUORESCENCE TRANSIENT IN ISOLATED CHLOROPLASTS

1. Fluorescence Transient in the Presence of Electron Acceptor (Methyl Viologen) and Donor (Hydroxylamine)

As discussed in the introduction, the isolated broken chloroplasts from higher plants exhibit only the fast (Fo, Fi, F_∞) type of transient (Figure 100). Figure 101 shows the typical transient of isolated spinach chloroplasts without any added acceptor. Upon addition of DCMU, the fluorescence rapidly rises to a maximum (OI phase). If, however, an excess of an acceptor like MeV (0.1 mM) is added before illumination, the fluorescence level remains low. Addition of MeV would keep Q in the oxidized state and thus the fluorescence yield remains low.

Figure 102 shows a fluorescence transient obtained from oat chloroplasts. Here, we see typical transients of chloroplasts with and without DCMU, and hydroxylamine. The addition of 10 mM NH_2OH does not affect the rapid (Fo-Fi) increase of fluorescence yield. However, the final steady state fluorescence is higher than in the normal. This confirms Izawa *et al.* (168) who first observed this phenomenon in spinach chloroplasts.

Table VI.1 shows the effect of the addition of 50 mM and 100 mM NH_2OH on the steady state fluorescence yield of oat chloroplasts treated with 15 μM DCMU. We observe a small but significant increase in the steady state level of fluorescence by the addition of NH_2OH to the DCMU treated chloroplasts. Similar results were obtained with both oat and spinach chloroplasts. (We have also observed a small increase in fluorescence level by the addition of NH_2OH to the DCMU poisoned *Anacystis* (Chapter V).) An increase in the steady state level of fluorescence would be expected if NH_2OH were to inhibit the back reaction between Z^+

Figure 100. Time course of chlorophyll a fluorescence in isolated broken chloroplasts with and without DCMU. λ observation, 685 nm (half band width, 6.6 nm); excitation, broad band-blue light (C.S. 4-96 plus C.S. 3-73), intensity, 1.6×10^4 ergs $cm^{-2} sec^{-1}$; oat chloroplasts; [Chl], 10 μg/ml; buffer, 40 mM phosphate, pH 6.8, with 10 mM KCl; 10 minute dark time between measurements.

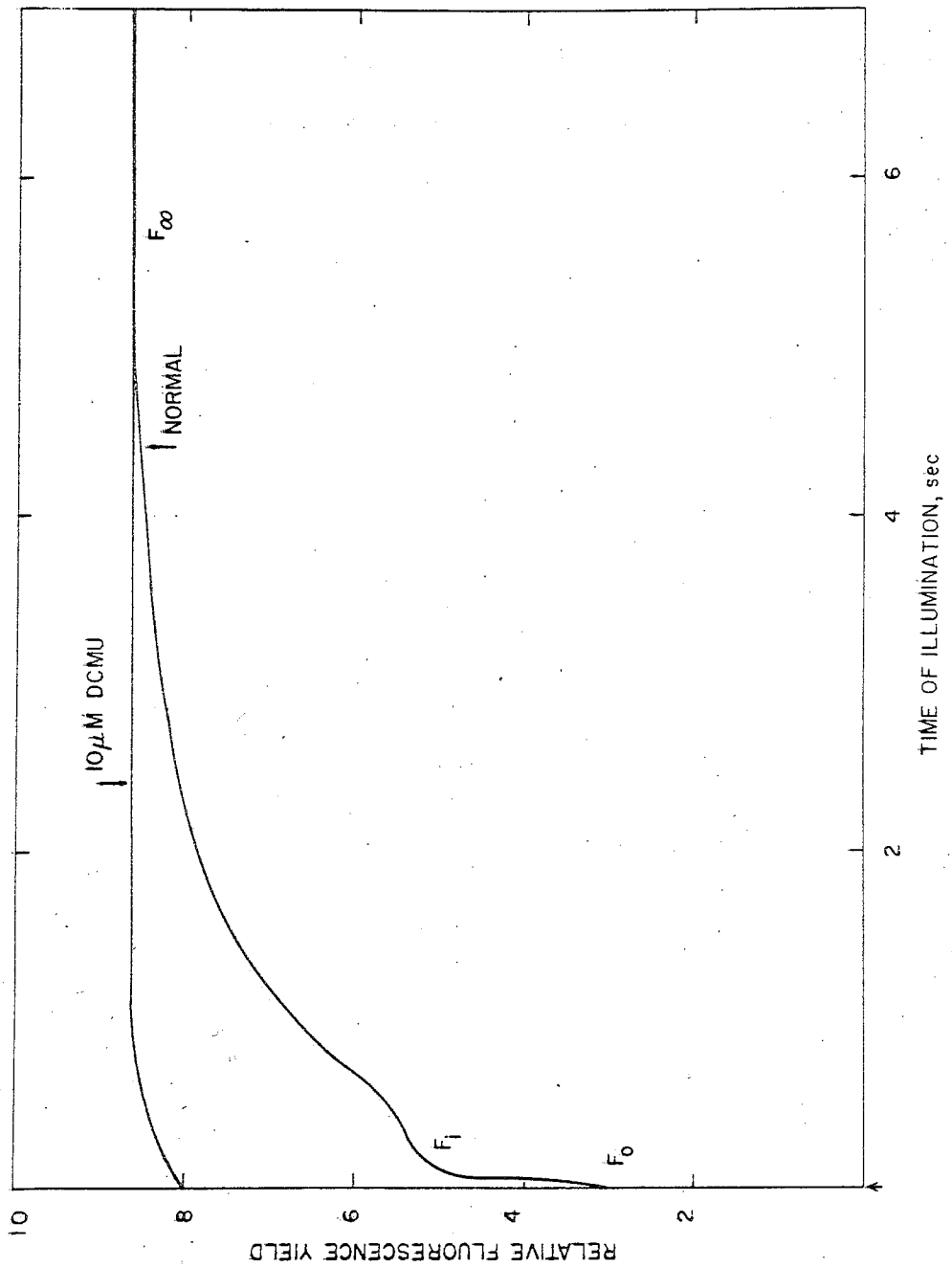

RELATIVE FLUORESCENCE YIELD
10
8
6
4
2
TIME OF ILLUMINATION, sec
2
4
6
10μM DCMU
NORMAL
F∞
Fi
Fo

Figure 101. Time course of Chl *a* fluorescence in broken spinach chloroplasts with and without terminal electron acceptor. Fluorescence was measured at 685 nm (half band width, 6.6 nm); solid circles, normal chloroplasts without any addition; open circles, 15 μM DCMU; open triangles, 0.1 mM methyl viologen (MeV); 0 level not recorded in these plots. Excitation, broad band blue light (C.S. 4-96 and C.S. 3-73), intensity, 1.4×10^4 ergs $cm^{-2} sec^{-1}$; [Chl], 40 μg in 3 ml; buffer, 40 mM phosphate plus 10 mM KCl, pH 6.8; dark time 10 minutes before each measurement.

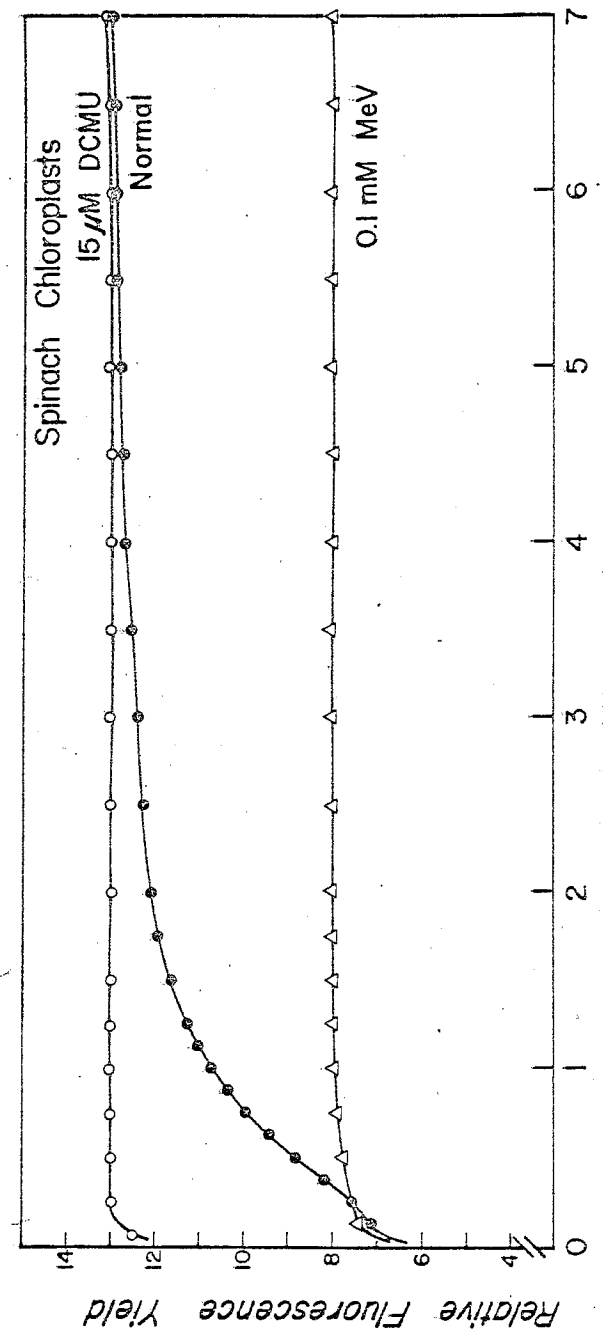

Spinach Chloroplasts
15 μM DCMU
Normal
0.1 mM MeV
Relative Fluorescence Yield
Time of Illumination, sec
14
12
10
8
6
4
0
1
2
3
4
5
6
7

Figure 102. Time course of Chl fluorescence in broken oat chloroplasts in the presence of an excess of hydroxylamine. All conditions as in Figure 100 except that chloroplasts were previously stored frozen in dry ice and acetone. [Chl], 40 μg/3 ml; buffer, 40 mM phosphate, with 10 mM KCl, pH 7.4.

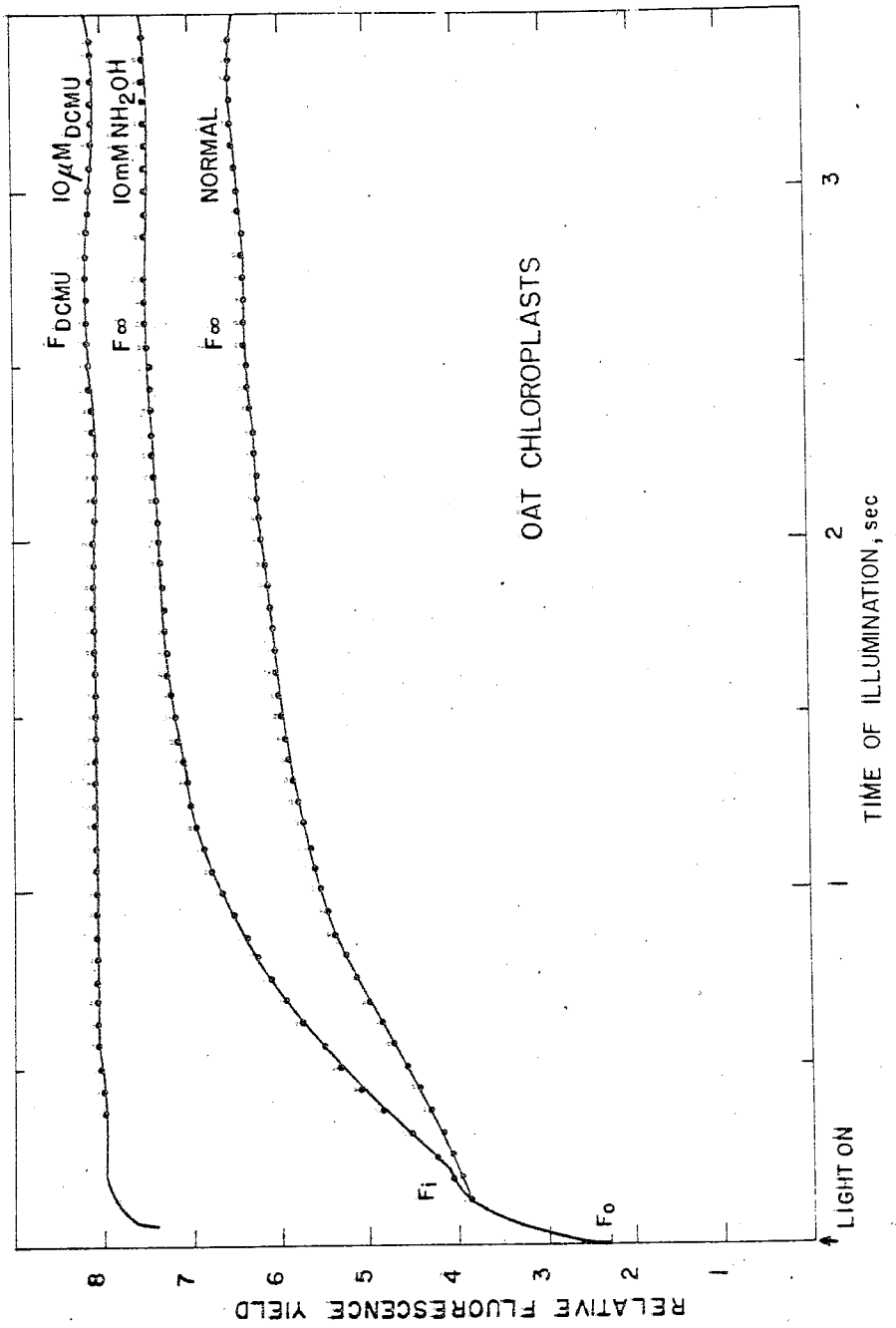

F DCMU
10μM DCMU
F∞
10mM NH2OH
F∞
NORMAL
OAT CHLOROPLASTS
Fi
Fo
LIGHT ON
TIME OF ILLUMINATION, sec
RELATIVE FLUORESCENCE YIELD
1
2
3
4
5
6
7
8

Table VI.1

Effect of the Addition of NH_2OH on the Steady State Fluorescence Yield of Oat Chloroplasts in the Presence of 15 μM DCMU

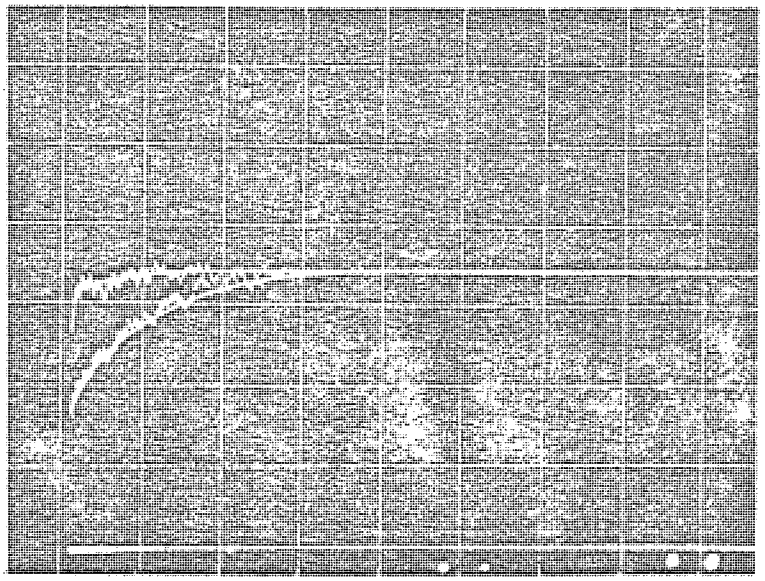
Treatment	Relative Fluorescence Yield	Ratio of Yield of NH_2OH + DCMU/DCMU
None	45.2	----
15 μM DCMU	48.0	----
50 mM NH_2OH + 15 μM DCMU	49.5	1.03
100 mM NH_2OH + 15 μM DCMU	50.0	1.04

Three ml of chloroplast samples containing 30 μg of chlorophyll in 0.05 M phosphate buffer, pH 7.4, were used. Fluorescence was measured at 685 nm with a 6.6 nm slit width. A Corning filter C.S. 2-61 was used to guard the light leak. The sample was excited by blue light (C.S. 4-96 + C.S. 3-73) with an approximate intensity of 1.4×10^4 ergs $cm^{-2}sec^{-1}$. NH_2OH (neutralized) was added 5 minutes before measurements. Chloroplast samples were previously frozen with dry ice and acetone and then stored for later use.

and Q^-. The extent of increase in the fluorescence yield would be expected to be the same in the normal as well as the DCMU poisoned sample. But this is not the case. We do not have any simple explanation for this difference. The higher level of F_∞ in the presence of NH_2OH compared to normal chloroplasts may be due to an elimination of quenching by oxygen.

Figure 103 shows an oscilloscope picture of fluorescence rise curve in the presence of NH_2OH and DCMU (added in the dark) in oat chloroplasts. The initial transient (lower trace) is very similar to chloroplasts treated with DCMU alone, but after a dark period of 2 minutes, the transient does not recover and remains high (upper curve). This is because, as proposed earlier (Chapter III), NH_2OH reduces the oxidized Z^+, and QH

Figure 103. Oscilloscope tracing of the fluorescence recovery transient in the presence of DCMU and hydroxylamine. Lower trace, initial transient; upper trace, after 2 min. of dark interval; [Chl] = 11 μg/ml; buffer, 40 mM phosphate with 10 mM KCl, pH 7.4; hydroxylamine, 10 mM; DCMU, 15 μM. Abscissa, 0.2 volt/division; ordinate, 50 msec/division; intensity of blue excitation light, 1.2×10^{4} ergs $cm^{-2} sec^{-1}$; oat chloroplasts.



remains reduced due to the blocking of electron flow by DCMU. As both QH and Z remain reduced, the back reaction between them is prevented and fluorescence remains high--as seen in the second illumination. As mentioned before, Bennoun (167) and Mohanty et al. (154) have independently obtained similar data with other systems. (Furthermore, Bennoun (167) has shown a gradual loss of the fluorescence transient with various concentrations of NH_2OH in the presence of DCMU.)

Figure 104 shows a set of fluorescence transients reproduced from another set of experiments with spinach chloroplasts. Here we observe a decrease in fluorescence yield by washing the chloroplasts with (0.8 M, pH 8.0) tris-Cl. Addition of DCMU to the tris washed chloroplasts increases the fluorescence yield. These observations are similar to that of Yamashita and Butler (222) who suggested that tris-washing inhibits the flow of electrons from H_2O to $NADP^+$. Figure 105 shows that additions of 10 mM NH_2OH increases the fluorescence yield both of normal as well as of tris-washed chloroplasts to the almost same level. According to Yamashita and Butler (222), NH_2OH feeds electrons to PS II at a site closer to "Z" and farther from the site of inhibition by tris.

One would expect, from a simple competitive relationship, that any inhibition of electron transport would cause a concomitant rise in the fluorescence yield. But inhibition of electron transport by tris-washing brings about a decrease in fluorescence yield. Furthermore, addition of DCMU to tris-washed chloroplasts should not cause any further change in the fluorescence yield. But the fluorescence rises (Figure 104). No satisfactory explanation has been given for this phenomenon. We have explored some of the possibilities for the cause of the low yield in

Figure 104. Time course of Chl *a* fluorescence in normal and tris washed chloroplasts without any electron acceptors or donors. Excitation, broad-band blue light; intensity, 1.4×10^4 ergs $cm^{-2} sec^{-1}$; λ observation, 685 nm (slit width, 6.6 nm); C.S. 2-58 filter before the photomultiplier. Tris-extraction as in reference 222. Spinach chloroplasts were incubated in 0.8 M Tris, pH 8.0, for 20 min. in dim white light; buffer, 0.05 M, Tris-Cl, pH 7.8; 7 min. dark time before illumination.

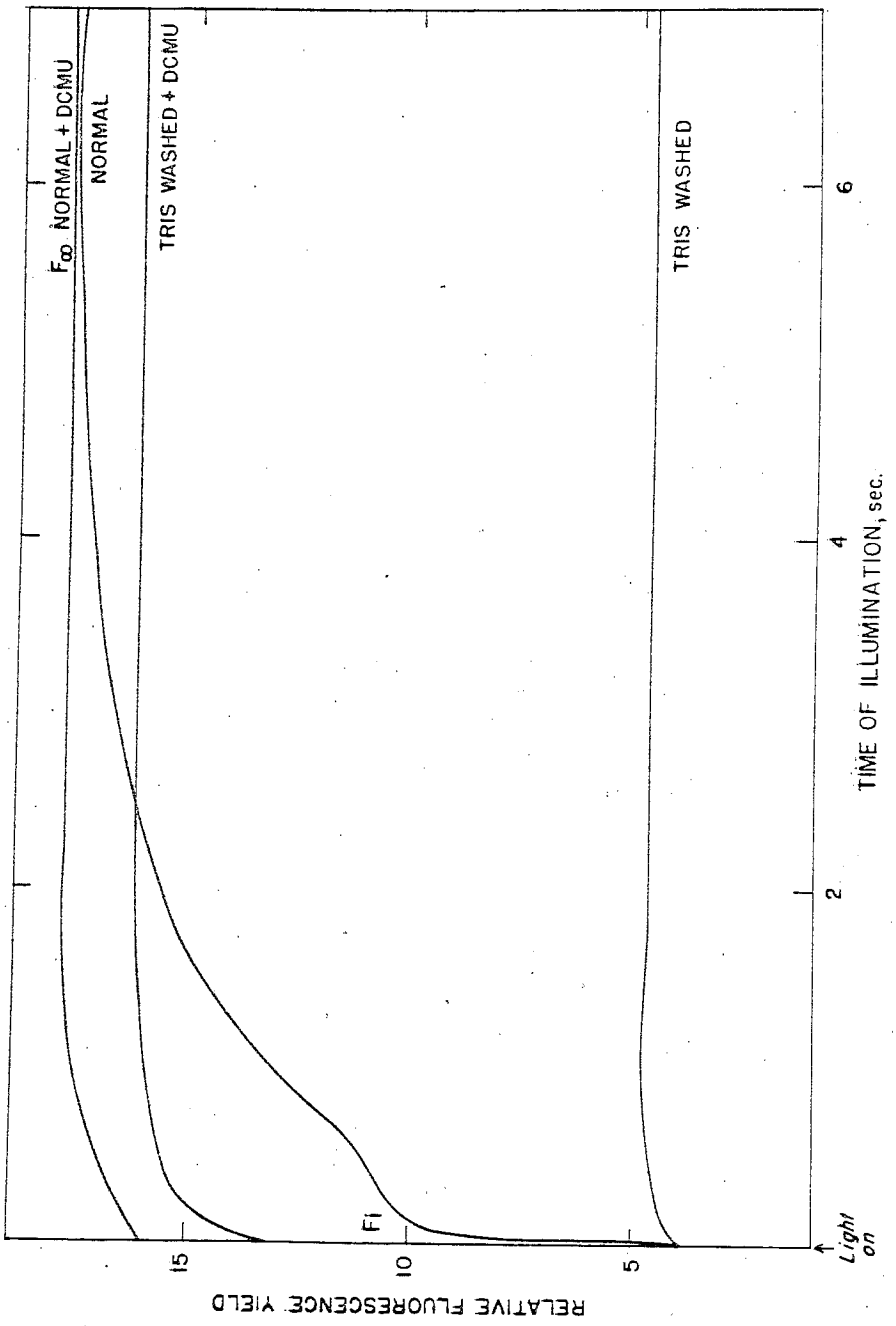
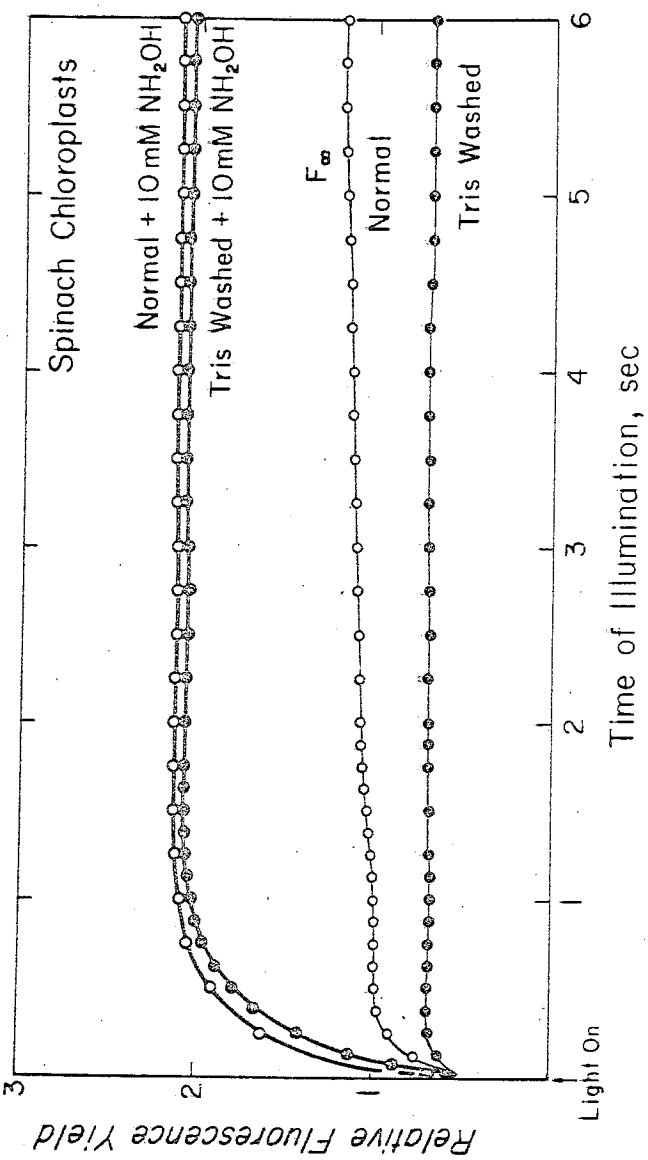

RELATIVE FLUORESCENCE YIELD
15
10
5
Fi
Light on
2
4
6
TIME OF ILLUMINATION, sec.
F_∞ NORMAL + DCMU
NORMAL
TRIS WASHED + DCMU
TRIS WASHED

Figure 105. Time course of Chl *a* fluorescence in normal and tris-washed chloroplasts in the presence and the absence of 10 mM hydroxylamine hydrochloride. Stored (frozen) chloroplasts were thawed and diluted to give 14 μg Chl/ml; buffer, 50 mM Tris-Cl, pH 7.4; intensity, 1.0×10^4 ergs $cm^{-2}sec^{-1}$; other conditions as in Figure 100.


Spinach Chloroplasts
Normal + 10 mM NH_2OH
Tris Washed + 10mM NH_2OH
F_∞
Normal
Tris Washed
Light On
Relative Fluorescence Yield
Time of Illumination, sec
1
2
3
4
5
6

tris-washed chloroplasts and the increase of it with DCMU; these results will be discussed later. All the results described above are well-known phenomena. We showed them here to illustrate that fluorescence yield changes in isolated chloroplasts are, in most cases, dependent on redox level of Q.

2. Cation Induced Changes in Fluorescence Yield and Emission

Homann (56) discovered that cations like Mg^{2+} can enhance the yield of Chl a fluorescence in isolated chloroplasts. Figure 106 illustrates the effect of 5 mM $MgCl_2$ on the fluorescence yield in chloroplasts both in the presence and in the absence of DCMU. In both cases the yield was enhanced by Mg^{2+}. In this particular experimental sample the variable fluorescence yield was low; also, a low intensity (8×10^3 ergs $cm^{-2} sec^{-1}$) of illumination was used. Additions of 5 mM of $MgCl_2$ causes a greater enhancement of yield than the addition of DCMU. Also, the extent of enhancement of the yield with Mg^{2+} was the same both in the presence and the absence of DCMU. The fluorescence level slowly increases after the addition of Mg^{2+} and it takes about 2 to 3 minutes to reach the maximal level. In our experiments, we allowed 5 minutes of incubation before each measurement. Figure 107 shows a fluorescence transient in lettuce chloroplasts, with and without Mg^{2+}, measured at a faster time scale; this was done to record the influence of Mg^{2+} on the constant level (Fo) fluorescence. This experiment shows that Mg^{2+} does not affect significantly the constant (Fo level); the steady state yield, however, increases (30-80%) by the addition of Mg^{2+}.

This enhancement of fluorescence yield by Mg^{2+} cannot be due to an inhibition of a back recombination reaction as in the case of NH_2OH

Figure 106. Time course of Chl *a* fluorescence in the presence of $MgCl_2$. Excitation, blue light (C.S. 4-72 plus C.S. 3-73); intensity, 0.8×10^4 ergs $cm^{-2} sec^{-1}$. Spinach chloroplasts suspended in 0.005 M Tris-Cl, pH 7.9; [Chl], 15 μg/ml; 5 mM $MgCl_2$; 10 μM DCMU.

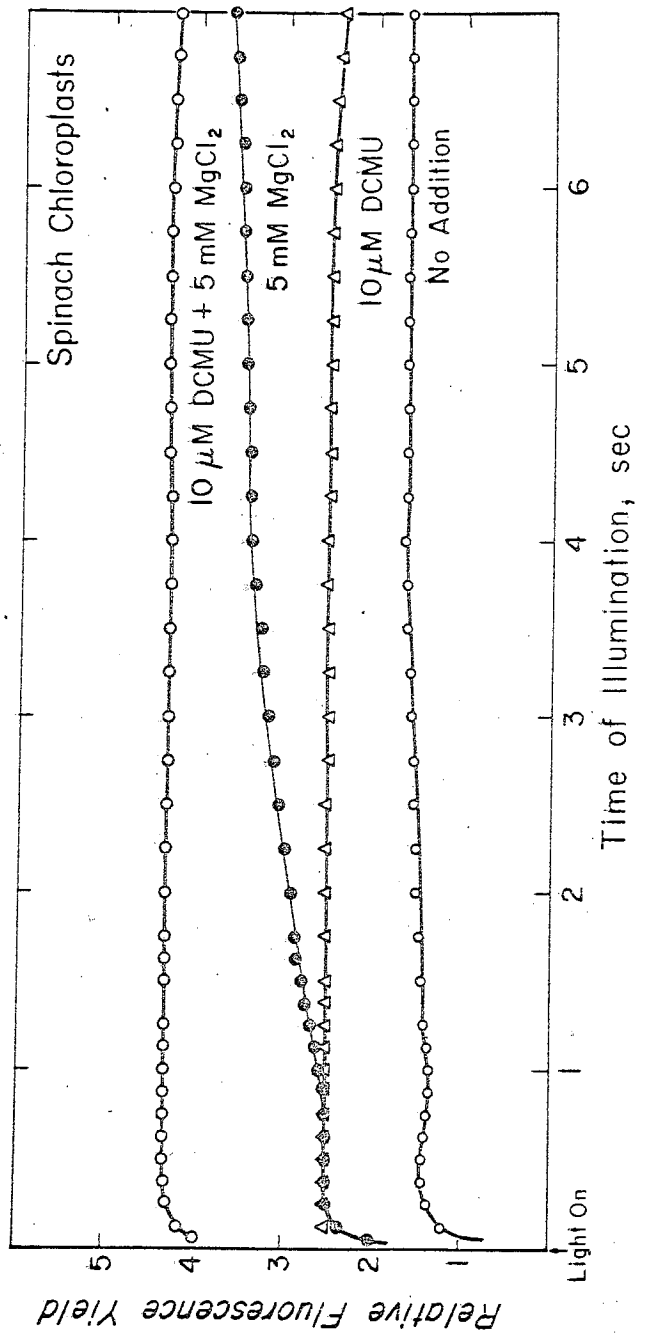
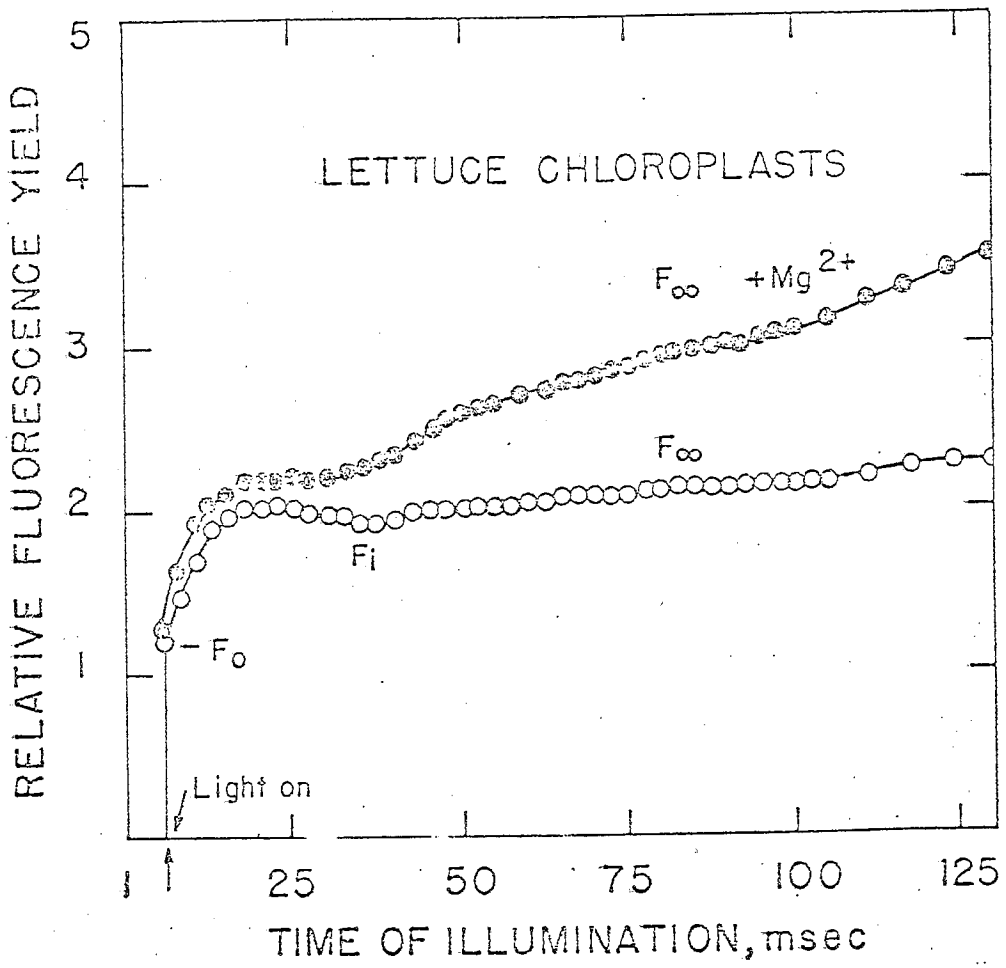

Spinach Chloroplasts
10 μM DCMU + 5 mM MgCl2
5 mM MgCl2
10μM DCMU
No Addition
Light On
Relative Fluorescence Yield
Time of Illumination, sec
1
2
3
4
5
6

Figure 107. Oscillograph recording of fluorescence yield changes, with and without $MgCl_2$, in lettuce chloroplasts, λ observation, 685 nm. Note the absence of change in Fo upon the addition of Mg^{2+}; buffer 0.001 M tris-Cl pH 7.8, [Chl], 12 μg/ml. All other conditions as in Figure 100.


LETTUCE CHLOROPLASTS
RELATIVE FLUORESCENCE YIELD
5
4
3
2
1
F_∞ +Mg^{2+}
F_∞
Fi
F_o
Light on
25
50
75
100
125
TIME OF ILLUMINATION, msec

because in the case of the latter we did not observe any change in the ratio of F685/F730 emission bands at 77° K, while Mg^{2+} caused a large change in the relative ratio of these two emission bands, as shown by Murata (57) (also see below).

Although changes in 77° K emission spectra were recorded before (57), changes in the room temperature emission characteristics caused by cations were not measured earlier. The latter become important because of possible errors that may occur with 77° K spectra. Figure 108 shows the room temperature emission spectra of lettuce chloroplasts with and without 10 mM $MgCl_2$. There is a decrease in the ratio of F720/F685 band, by the addition of Mg^{2+}, from 0.15 to 0.13, suggesting an enhancement of F685 band with Mg^{2+} treatment. Figure 109 shows similar room temperature spectra of chloroplast fragments (broken chloroplasts) with and without 10 mM $CaCl_2$. Here, the fluorescence level at 685 nm has been adjusted to give the same value; a decrease in the fluorescence level at 720-740 nm bands by the addition of cations is observed.

Figure 110 shows the emission spectra, at 77° K, of lettuce chloroplasts with and without $MgCl_2$ added prior to cooling. Again we observe a relative decrease in the height of the long wavelength band and an increase in the 685 nm band by the addition of Mg^{2+}; the ratio of F730/F685 band decreases from 2.75 to 1.70 suggesting an enrichment of system II fluorescence with Mg^{2+}. Hence, the type of enhancement of fluorescence yield does not reflect the reduction level of Q and like A pool but a decrease in the energy transfer from PS II to PS I. From the enhancement of fluorescence yield by cations, we estimated that the rate constants (probability per unit time) for the electronic excitation transfer from

Figure 108. Room temperature emission spectra of Chl *a* fluorescence with (solid circles) and without (open circles) $MgCl_2$. λ excitation, broad-band blue light (C.S. 4-72 and C.S. 3-73); C.S. 2-58 filter before the analyzing monochromator; 1.6 nm slits, small amount of concentrated $MgCl_2$ added to give final concentration of 15 mM; [Chl], 5 μg/ml. All other conditions as in Figure 106.

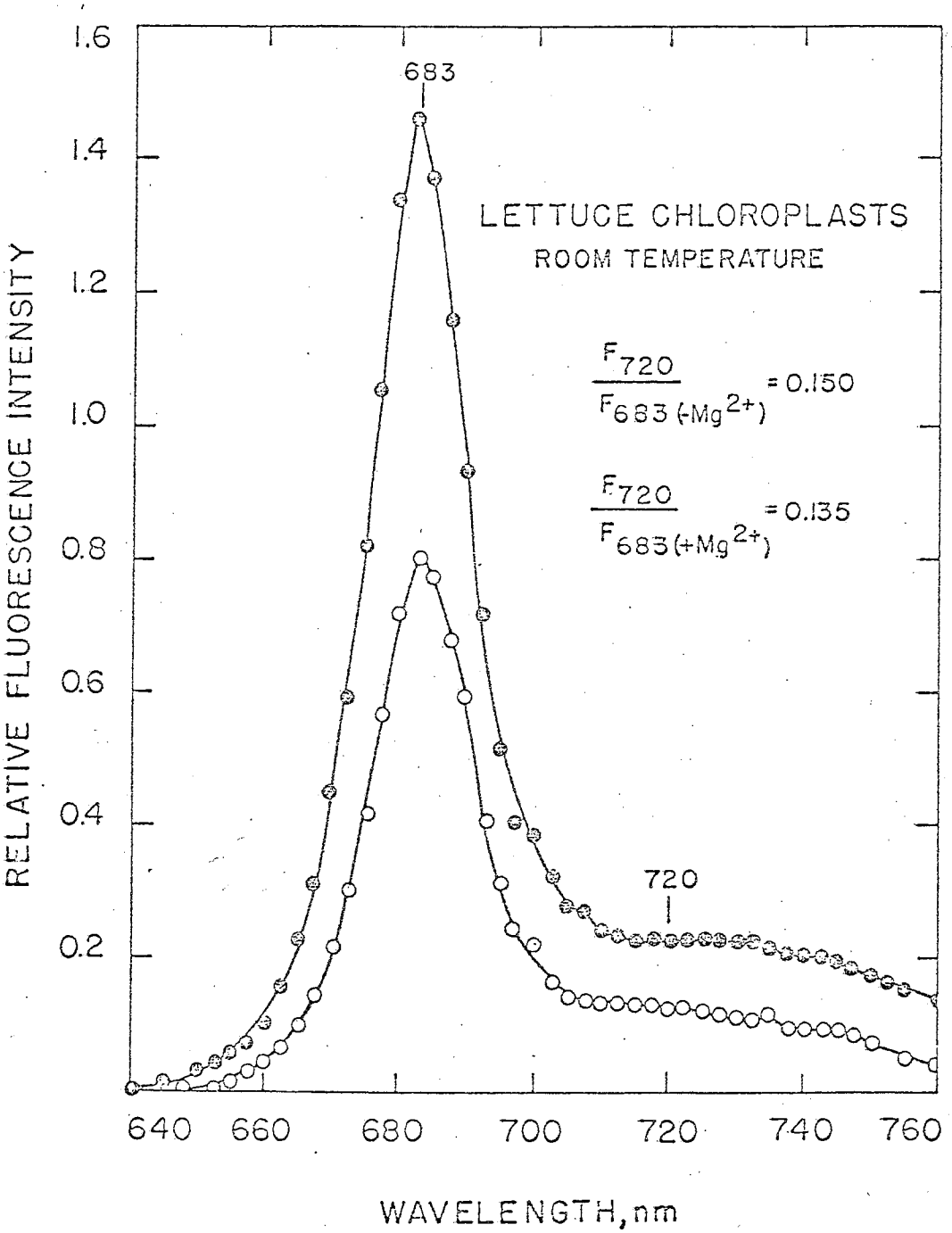

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
RELATIVE FLUORESCENCE INTENSITY
683
720
LETTUCE CHLOROPLASTS
ROOM TEMPERATURE
F720 / F683 (-Mg2+) = 0.150
F720 / F683 (+Mg2+) = 0.135
640
660
680
700
720
740
760
WAVELENGTH, nm

Figure 109. Room temperature emission spectra of Chl *a* fluorescence in the presence and the absence of $CaCl_2$; the fluorescence yield at 683 nm has been adjusted to give the same value; open circles, no addition, closed circles, with 10 mM $CaCl_2$; λ excitation, 435 nm with 5 nm slits; λ observation, variable; 1.5 nm slits. Corning C.S. 2-61 before the photomultiplier; [Chl] = 5 μg/ml; buffer, 0.05 M Tris-Cl, pH 7.4.

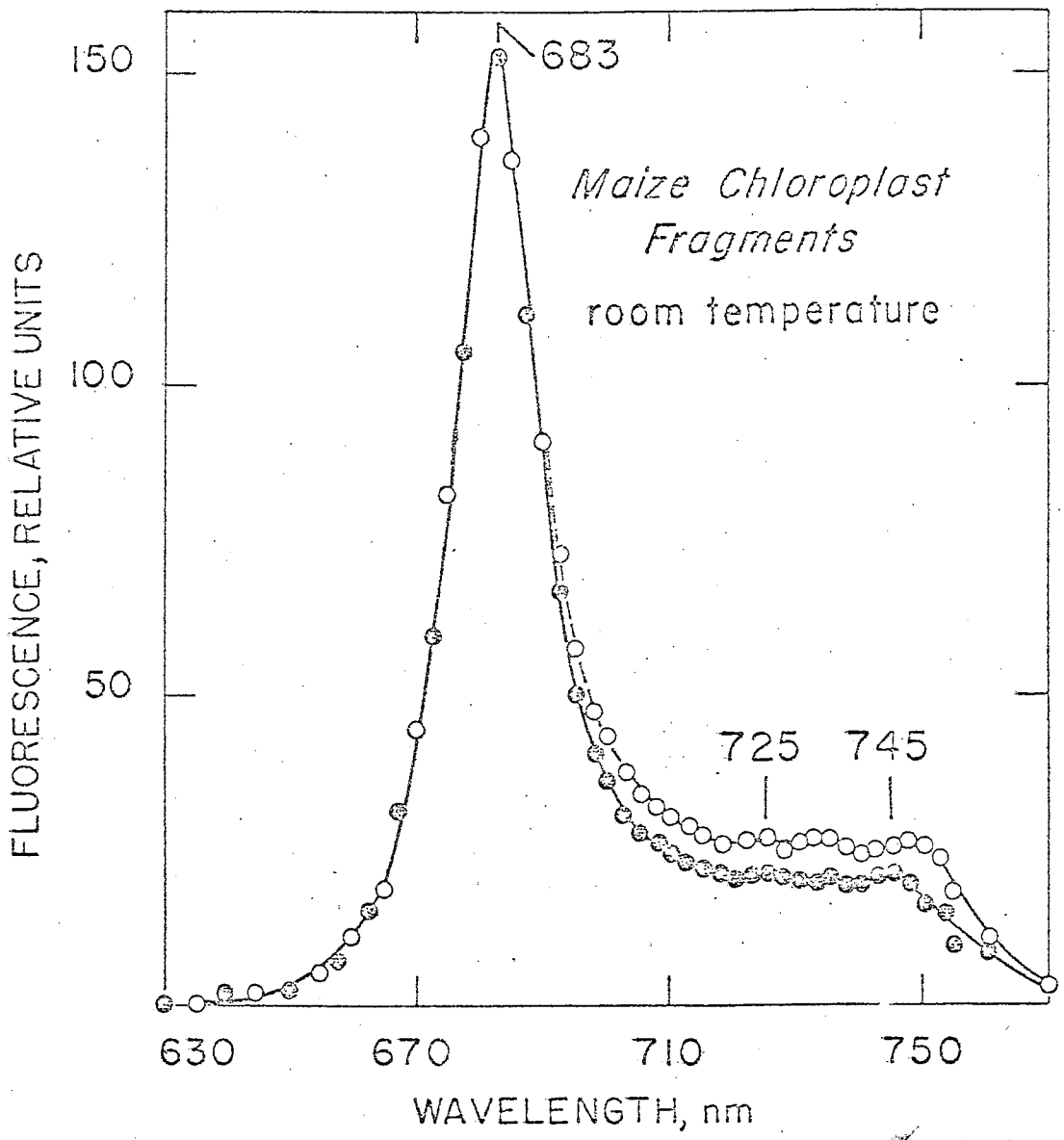
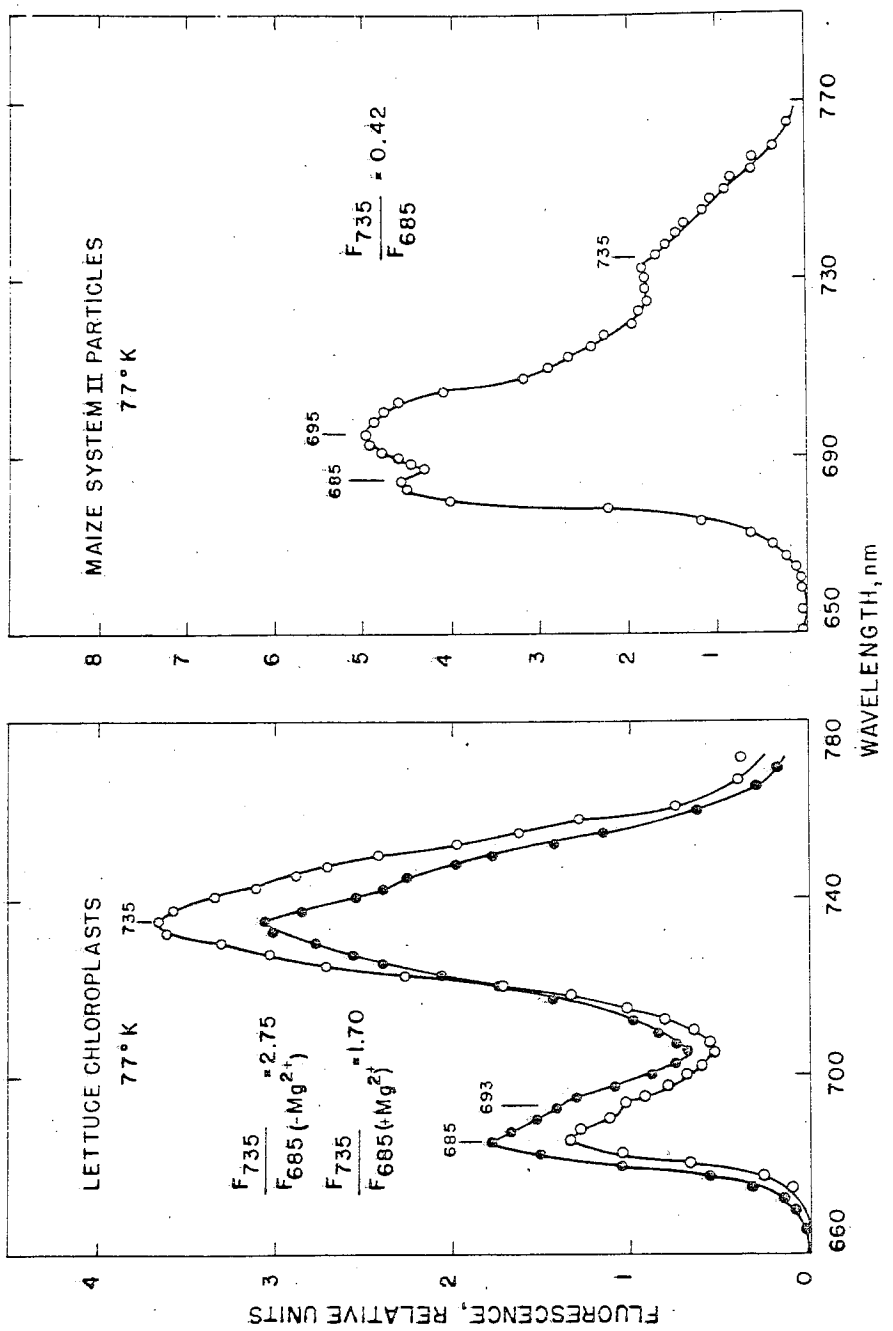

683
Maize Chloroplast Fragments
room temperature
725
745
150
100
50
FLUORESCENCE, RELATIVE UNITS
630
670
710
750
WAVELENGTH, nm

Figure 110. Emission spectra of Chl a fluorescence at liquid nitrogen temperature. (Left): Solid circles, normal ($-Mg^{2+}$); open circles, with Mg^{2+}. λ excitation, 435 nm; samples were pre-illuminated with a 60 watt bulb, 10 cm away for 2 minutes before freezing. (Right): emission spectra of system II particles (Huzisige type) from maize chloroplasts given here as an illustration of the emission characteristics of system II particles (74). These system II particles do not show any Mg^{2+} induced alteration in the yield; the solid line, with Mg^{2+}; open circles, without Mg^{2+}.


LETTUCE CHLOROPLASTS
77° K
735
693
685
$\frac{F_{735}}{F_{685}(-Mg^{2+})} = 2.75$
$\frac{F_{735}}{F_{685}(+Mg^{2+})} = 1.70$
MAIZE SYSTEM II PARTICLES
77° K
695
685
735
$\frac{F_{735}}{F_{685}} = 0.42$
FLUORESCENCE, RELATIVE UNITS
WAVELENGTH, nm

PS II to PS I are approximately 4 to 5 times greater than that of de-excitation by fluorescence (see Appendix).

3. Fluorescence Yield in Tris-Washed Chloroplasts*

As shown in Figure 104, tris extraction causes a lowering of fluorescence yield. It has been clearly established that tris extraction inhibits electron flow from H_2O to PS II traps (222). On the basis of a simple complementary relation between photosynthesis and fluorescence, one would expect a high yield of fluorescence but one observes a low yield of fluorescence. Addition of DCMU, however, increases the fluorescence yield to almost the level in the normal sample.

Yamashita and Butler (222) suggested that chloroplasts contain limited quantities of endogenous donors sufficient enough to reduce all Q but insufficient to reduce all the secondary pools of acceptors in the chloroplasts. This suggestion, although apparently explaining the observations, has two difficulties: (a) repeated washing in tris, as it was used in this method, should deplete the pool of this hypothetical endogenous donor that may be present in the "parent" chloroplasts; (b) the photo-oxidation of a hypothetical donor in the presence of DCMU would be expected to inhibit the recovery of fluorescence rise curve in the presence of DCMU. Our results indicate that this is not the case (see below).

Another suggestion (224, 225) to explain the cause of the low yield is the acceleration of cyclic flow by tris-washing; DCMU, according to one hypothesis (224) would have to intercept this cyclic flow of

*The following studies were made with the collaboration of Mrs. Barbara Z. Braun; a preliminary report has already been published (223).

electrons, causing an increase in fluorescence yield. A third possibility is that tris-washing may have caused a conformation change so as to increase the spill over of the quanta from strongly fluorescent PS II to weakly fluorescent PS I. Thus, the fluorescence yield will be low.

Another alternative is that both the oxidized donor and the acceptors accumulate in light in tris-washed preparations. This possibility arises from the fact that 1) more than one equivalent of positive charges can accumulate on Z (81), and 2) there is a greater abundance of secondary pool acceptors than Q, and 3) the interaction between Q and A is rather fast (31).

Our results with the fluorescence transient and the delayed light emission (DLE) measurements suggest the last possibility is the most likely cause of the low fluorescence yield in tris-washed chloroplasts.

It has recently been suggested that the slow component of delayed light emission is due to the formation of excited chlorophylls by the back recombination reactions between Z^+ and Q^- (see 108). If tris-washing were to induce a strong back recombination between Z^+ and Q^-, and DCMU were to inhibit this flow, we would expect (a) a higher intensity of DLE in tris-washed chloroplasts than in normal and (b) complete elimination of DLE in the presence of DCMU. Our experiments (Table VI.2) show that the intensity of DLE (sec) in tris-washed chloroplasts is approximately the same as in normal chloroplasts. However, see ref. 225 for different results in the msec. DLE. Addition of 10 μM DCMU considerably lowers the intensity of DLE in both cases; it is approximately the same in both cases. This lowering of DLE by DCMU has been reported in Chlorella cells (110). Increasing the DCMU concentration to 20 μM did not lower the DLE

Table VI.2

Relative Intensities of Delayed Light Emission (1 Sec) in the Presence and Absence of DCMU in Normal and Tris-Washed Chloroplasts (after Mohanty et al., 223)

Treatment	Normal Chloroplasts	Tris-Washed Chloroplasts
None	70 ± 5	76 ± 5
10 μM DCMU	41 ± 5	41 ± 5
20 μM DCMU	45 ± 5	48 ± 5
10 μM DCMU + 1 mM NH_2OH	0	0

Two ml of the sample, containing 40 μg Chl in 0.05 M tris-Cl buffer (pH 7.8), was illuminated for 15 sec with blue light (C.S. 4-96 and 3-73 filters) with an intensity ~10 K ergs $cm^{-2}sec^{-1}$. DLE was measured after 1 sec. of the cessation of illumination. Add additions were made in the dark. DLE values, given above, represent an average of 10 measurements except in the case of 20 μM DCMU where two measurements were made.

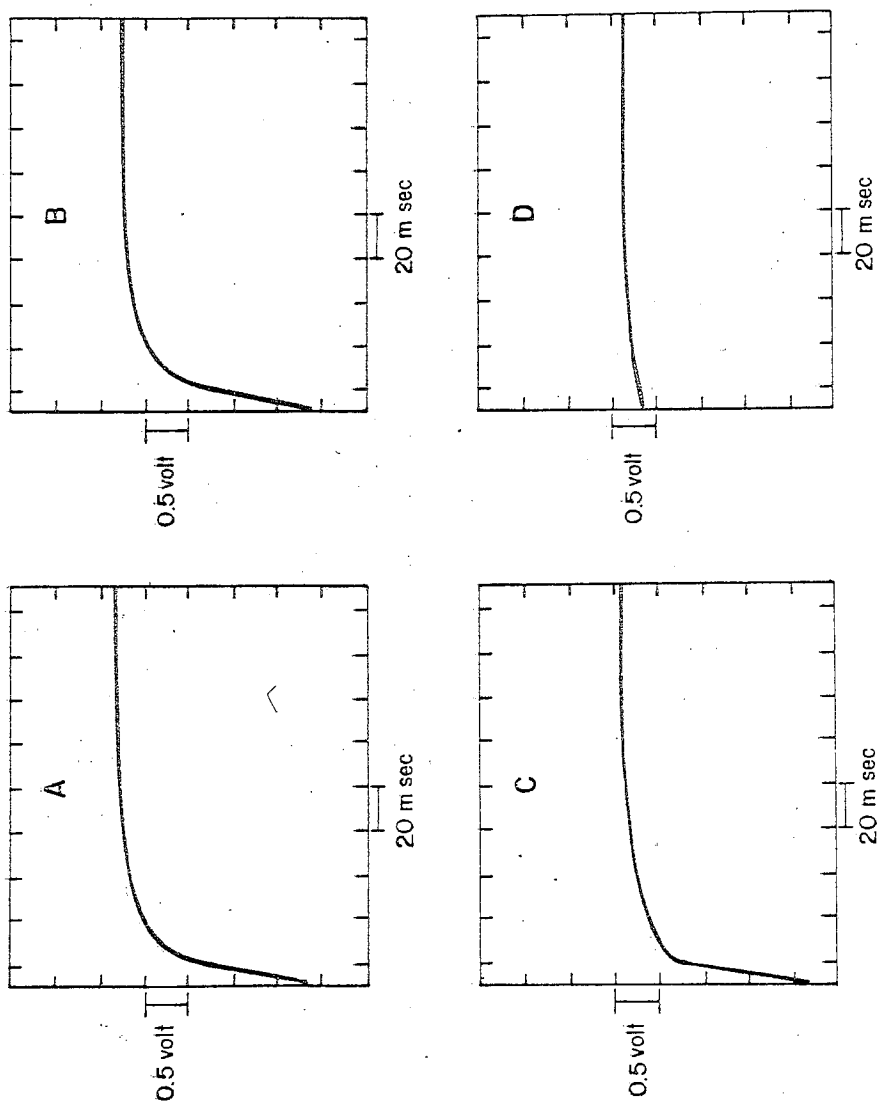
any further. However, addition of 1 mM NH_2OH, which is known to feed electrons very close to PS II, completely abolished DLE. The elimination of the slow component of DLE by the addition of both NH_2OH and DCMU has been shown to occur in intact cells of Porphyridium, Anacystis and Chlorella and in isolated chloroplasts, and has been interpreted to be due to the inhibition of back reaction between donor Z^+ and reduced acceptor Q^- of PS II. If the back recombination reaction between Z^+ and Q^- is indeed the source of slow component of DLE, our results show that DCMU does not inhibit most of it. Thus, our results in Table VI.2 indicate that an acceleration of cyclic electron flow cannot be the cause of low fluorescence yield in tris-washed chloroplasts.

It is well known that in the presence of DCMU the fluorescence

increases from a low level (f_o) to a high level (f_∞) upon illumination, and that this transient could be repeated if a dark period is interposed between measurements (226). This means that both Q^- and Z^+ recover to Q and Z in the dark period. The addition of both DCMU and hydroxylamine eliminates the restoration of the fluorescence transient as shown and discussed earlier (also see refs. 109, 154, 167). This observation supports the hypothesis that hydroxylamine inhibits the reoxidation of Q^- in the dark and the possible mechanism of the block in the restoration of the transient is due to the reduction of Z^+ by NH_2OH. If DCMU were to block the recombination between Z^+ and Q^- in tris-washed chloroplasts, no fluorescence transient would be observed after the first illumination and the fluorescence level would remain high during subsequent exposures. Figure 111 shows the oscilloscope tracings of the recovery of the fluorescence rise curve after a 2 minute dark period in tris-washed chloroplasts; more than 90% of the fluorescence transient was recovered. Thus, DCMU does not seem to block the recombination between Z^+ and Q^- in tris-washed chloroplasts. If donors such as reduced TMPD (that feed electrons to PS II) are added, fluorescence remains high and the transient does not recover after the first illumination (Figure 111, C and D).

Incidentally, our measurements of DLE (Table VI.2) and the recovery of Chl fluorescence transient (Figure 111) rule out the possibility that the functional site of DCMU is on the water side of PS II; and that it must act on the Q side of PS II--in order to stop the flow of electrons from H_2O to $NADP^+$. This, of course, is the accepted site of DCMU action (see ref. 10 and 86). If DCMU inhibits the flow of electrons from Q to the intersystem carriers (A), the back reaction from Q^- to Z^+ will not

Figure 111. Restoration of Chl *a* fluorescence transient in tris-washed spinach chloroplasts in the presence of DCMU. (A), initial transient after 7 min of darkness; (B), recovery of transient after 2 min. of dark time after A was measured; (C), initial transient from experiment with reduced TMPD after 7 min. of darkness; (D), absence of recovery of the fluorescence transient measured 2 min. of darkness after C was measured. For A and B, [Chl] = 45 μg/3ml; buffer, Tris-Cl, pH 7.8; for C. and D., 0.02M TMPD and 0.2 M ascorbate (neutralized). λ observation, 685 nm; excitation, broad band blue light; intensity, 0.6×10^4 ergs $cm^{-2}sec^{-1}$. All additions are in dark.


A
B
C
D
0.5 volt
20 m sec

be hampered and we will expect both the recovery of the fluorescence transient as well as DLE. Our data show that both the DLE and the recovery of the transient persist in tris-washed chloroplasts. Thus, there does not seem to be any endogenous PS II donor providing electrons as proposed by Yamashita and Butler (222); if there were endogenous donors these donors like other PS II donors, would cause a loss of DLE and restoration of the transient.

Table VI.3 shows the results of an experiment designed to test if there is a difference in the energy spill over from PS II to PS I upon

Table VI.3

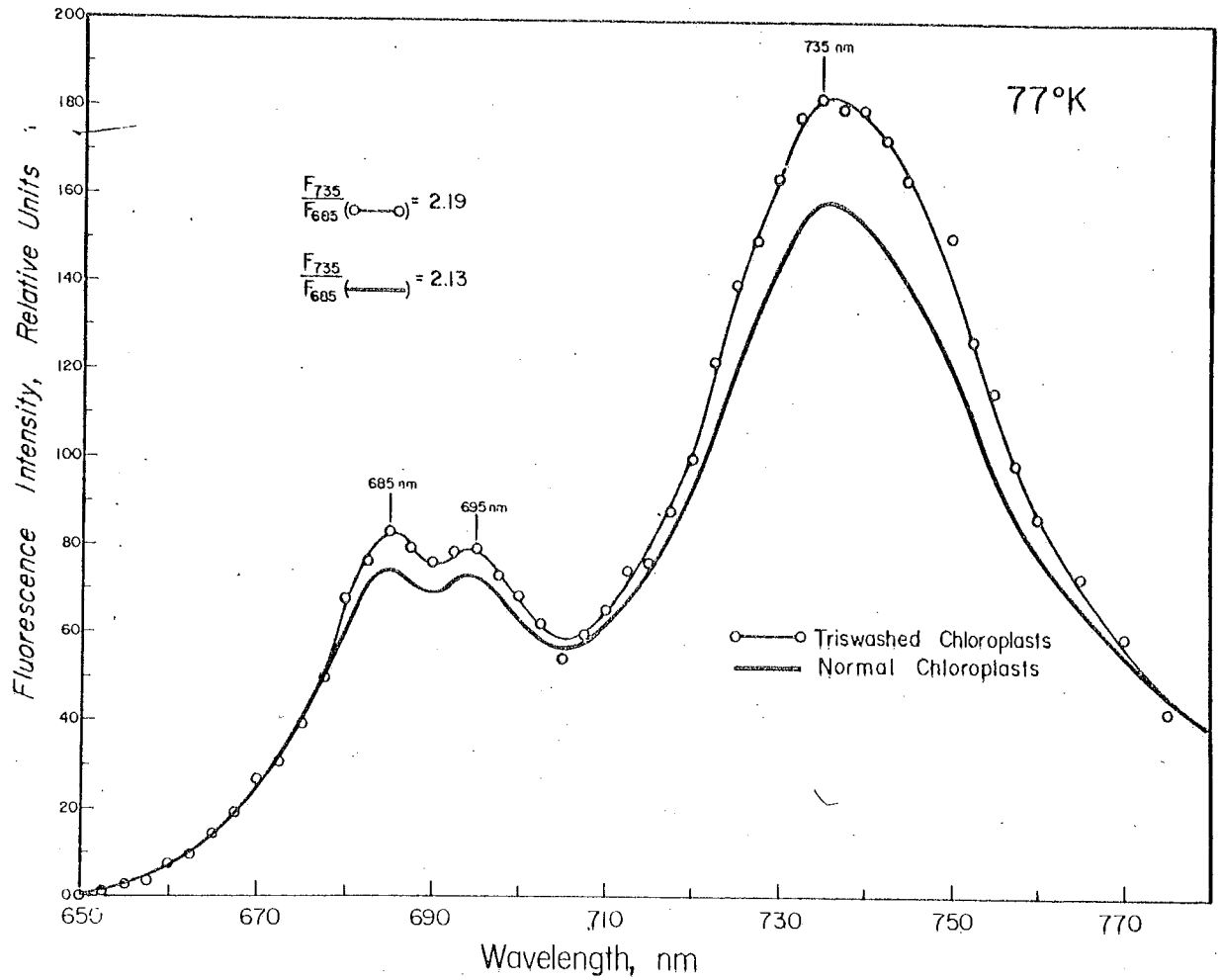
Relative Fluorescence Yield (Final Steady State) Level Upon the Addition of 5 mM $MgCl_2$ to Normal and Tris-Washed Chloroplasts

Treatment	Normal Chloroplasts	Tris-Washed Chloroplasts
None	0.96 ± 0.04	0.70 ± 0.04
5 mM $MgCl_2$	1.82 ± 0.06	1.20 ± 0.05

Spinach chloroplasts were washed and suspended in low molarity (0.005M) Tris-Cl buffer. Other details as in reference 223.

the addition of Mg; this spill over decreases in both tris-washed and normal chloroplasts. Furthermore, the liquid nitrogen emission spectrum of tris-washed chloroplasts had about the same ratio of F730/F685 bands as the control (Figure 112). One would expect the F720 band to increase and the F685 band to decrease if there were any appreciable enhancement, in the extent of energy flow from PS II to PS I by washing with tris. These results rule out the possibility that the cause of low fluorescence

Figure 112. Emission spectra of Chl *a* fluorescence in tris-washed chloroplasts, at 77° K (with and without DCMU). 3-5 µg Chl/ml; 0.2 ml total volume, preilluminated for 3 min, and then cooled. λ excitation, 435 nm (slit width 10 nm); λ observation, variable (5 nm slit width); C.S. 2-58 filter before the photomultiplier.


77°K
735 nm
685 nm
695 nm
$\frac{F_{735}}{F_{685}}$ (o—o) = 2.19
$\frac{F_{735}}{F_{685}}$ (——) = 2.13
o—o Triswashed Chloroplasts
—— Normal Chloroplasts
Fluorescence Intensity, Relative Units
Wavelength, nm
0
20
40
60
80
100
120
140
160
180
200
650
670
690
710
730
750
770

yield in tris-washed chloroplasts is a massive spill over of energy from PS II to PS I.

The results in Table VI.4 suggest that the addition of DCMU, after a long illumination of tris-washed chloroplasts, does not cause an

Table VI.4

Relative Changes in the Steady State Fluorescence Yield Upon the Addition of DCMU in the Dark and in the Light to the Normal and Tris-Washed Chloroplasts (after Mohanty et al., 223)

Treatment	Normal Chloroplasts	Tris-Washed Chloroplasts
None	1.04 ± 0.04	0.65 ± 0.05
10 μM DCMU added in the dark before illumin.	1.30 ± 0.05	1.25 ± 0.05
10 μM DCMU added in light after 2-3 min. of illumination.	1.26 ± 0.05	0.80 ± 0.05

Three ml samples containing 15 μg Chl/ml in tris-Cl buffer (0.05 M, pH 7.8). The blue excitation light (C-S 4-72 and 3-73); intensity, 10 Kergs $cm^{-2}sec^{-1}$. DCMU was added either in the dark or in the light to give a final concentration of 10 μM.

enhancement of the yield of fluorescence to the same extent as DCMU added in the dark prior to illumination (also see Figure 105). In light, Q is reduced and the donor Z is oxidized to Z^+. Although the oxidized Z^+ cannot be re-reduced due to inhibition of electron flow from the H_2O, QH is quickly re-oxidized by secondary pool of acceptora (A Pool). Thus, in light, without DCMU, Z^+ and Q, accumulate and the addition of DCMU after a long period of bright illumination would not cause any further change in the steady state oxidation or reduction level of either Z or

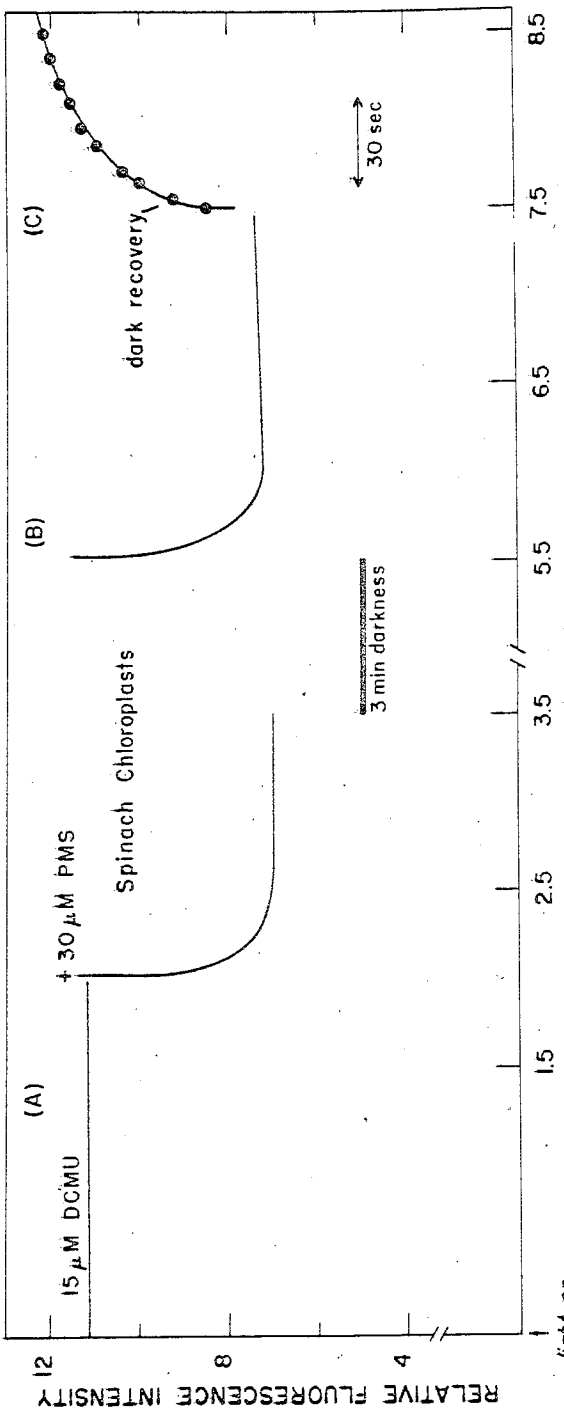
Q. If, however, DCMU is added in the dark periods before illumination, Z will be oxidized and Q will be reduced upon excitation with bright light. But in this case, QH would not be available to A pool and thus Z^+ and Q^- would be the predominating species at the PS II traps. Thus, it seems that interception of electron flow on the oxidant side of PS II (acceptor or Q^- side) causes an accumulation of reduced acceptors while a block of electron flow on the reductant or Z (water side) causes an accumulation of oxidized donors. But, it is the oxidation-reduction level of the acceptor Q that mostly determines the fluorescence yield.

4. Fluorescence Yield Changes in the Presence of DCMU

DCMU poisoned chloroplasts do not exhibit slow changes in the fluorescence yield upon illumination. With a moderately bright light, the yield increases rapidly within 30-50 msec to a maximal level. This transient rise of fluorescence yield from a low level (Fo) to the maximum level (F_∞) recovers if a dark period of about 1 to 2 minutes is given between illuminations. After reaching the maximum level, fluorescence yield remains fairly constant with prolonged periods of illumination.

Murata and Sugahara (193) showed that fluorescence yield declined to a lower level if PMS was added to DCMU poisoned chloroplasts. Figure 113 shows the effect of PMS in effecting such a decline in the yield. This decline of fluorescence is slow, with a $t_{\frac{1}{2}}$ of approximately 10-15 seconds. The lowering of the fluorescence yield by PMS occurs only in the light; the fluorescence yield recovers in the dark to its original level. The recovery of fluorescence to the original level required a 30-40 sec dark period (Figure 113, C). The extent of quenching decreased on successive illuminations.

Figure 113. PMS induced quenching of Chl *a* fluorescence in the presence of DCMU. (A) First, 15 μM DCMU was added and then chloroplasts were illuminated for 2 min., then 30 μM PMS was added and again chloroplasts were illuminated until a new steady state level of fluorescence was reached. (B) recovery of light induced PMS quenched fluorescence after 3 min. dark time. (C) Dark rise of fluorescence yield monitored by short (0.05 sec) flashes. Spinach chloroplasts; 14 μg/ml; 3 ml total volume; buffer, 50 mM Tris-Cl, pH 7.8. Excitation, blue light; 1.8×10^4 ergs $cm^{-2} sec^{-1}$.


(A)
15 μM DCMU
+ 30 μM PMS
Spinach Chloroplasts
(B)
3 min darkness
(C)
dark recovery
30 sec
light on
RELATIVE FLUORESCENCE INTENSITY
12
8
4
1
1.5
2.5
3.5
5.5
6.5
7.5
8.5
TIME, min

We measured the effect of PMS, $PMSH_2$, and reduced $DCPIPH_2$ on fluorescence yield of DCMU-treated oat chloroplasts. Reduced PMS and oxidized PMS caused about the same amount of quenching (Figure 114). $DCPIPH_2$, unlike in whole algal cells of Chlorella, did not cause any appreciable quenching. Ascorbate alone (not shown) also did not alter the yield. PMS at a concentration of 100 μM suppresses the initial fluorescence level, but the extent of slow lowering in the yield remains fairly constant, both in the presence or in the absence of DCMU if the exciting light was bright enough.

Table VI.5 summarizes the extent of quenching by 30 μM PMS in the presence and absence of $MgCl_2$, both in normal and in tris-washed

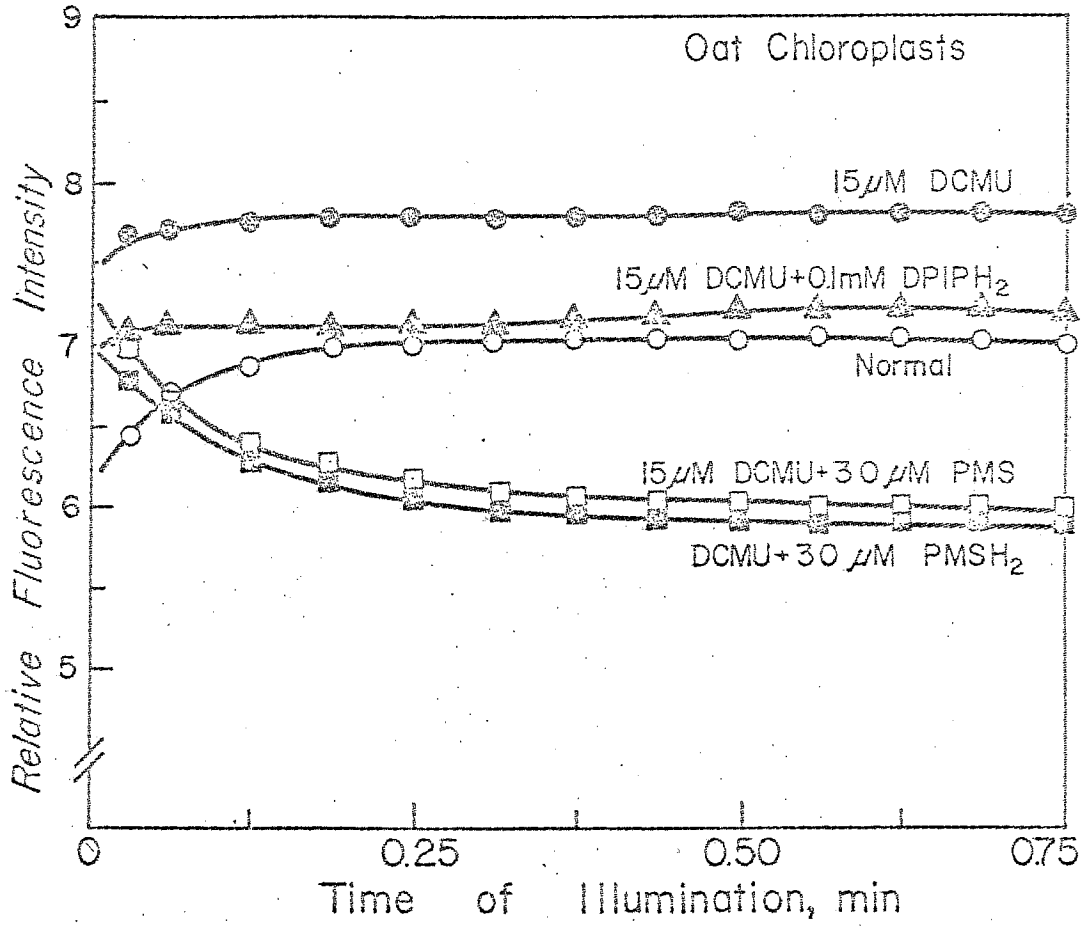
Table VI.5

Effect of Mg^{2+} on the PMS-Induced Lowering of Steady State Fluorescence Yield in Normal and Tris-Extracted Chloroplasts in the Presence of 10 μM DCMU

Treatment	Initial Intensity of Fluorescence	Final Intensity of Fluorescence After Addition of 30 μM PMS	Amount Quenched	Percent Quenched
1. Normal Chloroplasts	1.00	0.59	0.41	41.0
+ 5 mM $MgCl_2$	1.20	0.76	0.44	36.6
2. Tris-Washed Chloroplasts	0.70	0.45	0.25	34.1
+ 5 mM $MgCl_2$	1.02	0.75	0.27	26.4

Three ml samples, containing 18.5 μg Chl/ml, were suspended in Tris-Cl buffer (0.005 M, pH 7.8) containing 10 μM DCMU. PMS was added, after the initial fluorescence measurement, to give a final concentration of 30 μM. Fluorescence was again measured after 3 minutes of equilibration with PMS. Fluorescence was excited by blue light (C.S. 4-72 + C.S. 3-73); intensity, 10 Kergs $cm^{-2}sec^{-1}$. λ observation, 685 nm (half band width, 6.6 nm). Corning C.S. 2-61 filter was used to guard the entry of stray excitation light into the photomultiplier.

Figure 114. Time course of Chl *a* fluorescence in DCMU poisoned oat chloroplasts in the presence of cofactors of the system I reaction. 12.5 μg Chl/ml; (preparation buffer 0.05 M phosphate, 0.4 M sucrose, and 0.01 M NaCl, pH 6.8) in 0.05 M tricine-OH, pH 7.8. Additions as shown; PMS and DCPIP were reduced with 0.02 M neutralized ascorbate. Excitation, blue light; intensity, 2.0×10^{4} ergs $cm^{-2} sec^{-1}$.


Oat Chloroplasts
15μM DCMU
15μM DCMU+0.1mM DPIPH2
Normal
15μM DCMU+30μM PMS
DCMU+30μM PMSH2
Relative Fluorescence Intensity
9
8
7
6
5
0
0.25
0.50
0.75
Time of Illumination, min

chloroplasts. The extent of quenching remained approximately the same, although we observed a small decrease in the extent of quenching in the presence of Mg^{2+}. Mg^{2+} does not exert any significant effect on PMS induced quenching of Chl a fluorescence.

Results of Table VI.5 and Table VI.6 indicate that the quenching in fluorescence intensity by PMS is not associated with a shift in the

Table VI.6

Comparison of the Ratio of the Long Wavelength (Mainly System I) Emission Band to the Short Wavelength Emission Band (Mainly System II) at Liquid Nitrogen Temperature

Treatment	Ratio of F735/F685
None	2.4
5 mM $MgCl_2$	1.8
PMS 15 μM	2.2

0.2 ml of chloroplasts, containing 3 μg Chl/ml, were illuminated before cooling quickly to 77° K. Emission spectra were measured with an excitation at 435 nm (half band width, 6.6 nm).

distribution of quanta between the two photosystems.

Table VI.7 shows the effects of uncouplers (CCCP, S_{13}) and an ionophorous antibiotic (gramicidin) on the PMS-induced quenching. These compounds reverse the effect of PMS. The susceptibility of the PMS induced quenching of fluorescence to these compounds suggests that the quenching may be related to a cyclic electron flow mediated energy conservation process.

Murakami and Packer (121) have shown that PMS induced cyclic electron flow causes a decrease in thickness and spacing of the thylakoid membrane.

Table VI.7

Reversal of Quenching of Fluorescence Induced by 20 μM PMS by Uncouplers of Phosphorylation

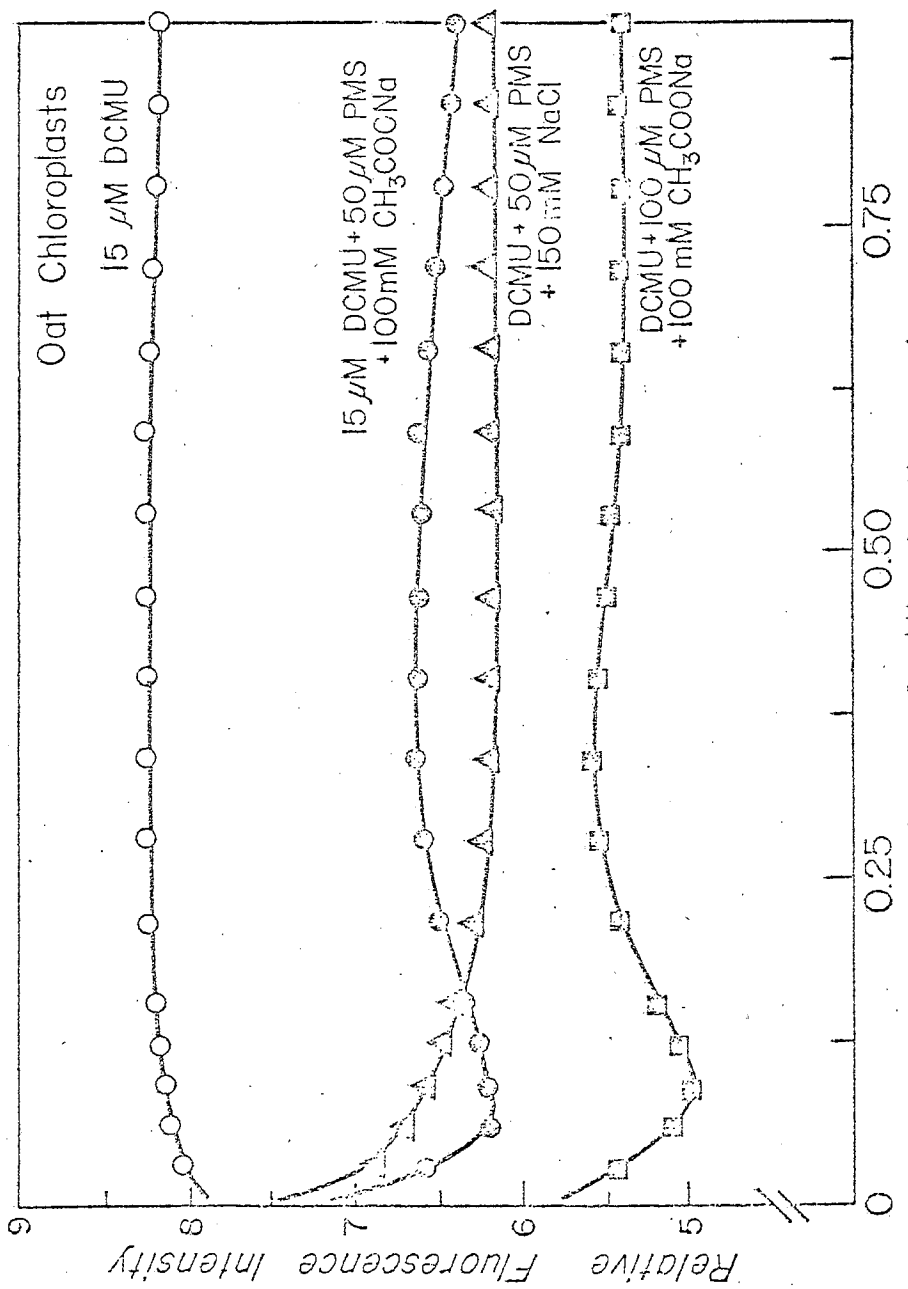
Addition	Relative Steady State Fluorescence Intensity	Fraction of Control
None	115.5	1.000
PMS	83.5	0.722
+PMS + 5 μM CCCP	105.2	0.919
+PMS + 1 μM S_{13}	111.8	0.968
+PMS + 4 μM gramicidin D	98.7	0.855

Three ml of oat chloroplasts, containing 20 μg Chl/ml, were suspended in phosphate buffer (0.05 M, pH 8.0) with 10 μM DCMU. Chloroplasts used in this experiment were previously frozen in dry ice and acetone and then stored. Fluorescence was measured at 685 nm as excited by blue light (intensity, 10 K ergs $cm^{-2}sec^{-1}$). The uncouplers alone, at the concentrations used, had no appreciable effects on the steady state fluorescence yield. All other conditions were as in Table VI.5.

This kind of shrinkage becomes pronounced if the chloroplasts are incubated with weak acid anion solutions. Figure 115 shows the effect of 100 mM sodium acetate on the PMS induced quenching in chloroplasts. In the presence of sodium acetate, the fluorescence yield declines to a low level, but unlike sodium chloride, the yield increases slowly to some intermediate level. We consider this small but significant rise in the fluorescence level to be due to structural changes associated with a sodium acetate action. In other words, PMS causes a quenching of fluorescence, and a sodium acetate-induced shrinkage probably causes a slow increase in the fluorescence yield.

The extent of quenching by PMS could be reduced by adding excess of

Figure 115. Time course of Chl *a* fluorescence in oat chloroplasts. Open circles, 15 μM DCMU; solid triangles, 15 μM DCMU + 50 μM PMS + 150 mM NaCl; solid squares, 15 μM DCMU + 100 μM PMS + 100 mM Sodium acetate. Other conditions as in Figure 114.


Oat Chloroplasts
15 μM DCMU
15 μM DCMU + 50 μM PMS + 100mM CH3COCNa
DCMU + 50 μM PMS + 150 mM NaCl
DCMU + 100 μM PMS + 100 mM CH3COONa
Relative Fluorescence Intensity
9
8
7
6
5
0
0.25
0.50
0.75
Time of Illumination, min

(0.1 mM) MeV. But if the PMS concentration was now increased, the extent of quenching could again be restored. (At high concentrations of PMS, the initial level of fluorescence is also very low. This effect is not solely due to be an inner filter effect, as we obtain similar results with blue (480 nm) and red light (633 nm) excitation. From the absorptions spectrum of PMS we expect negligible absorption with red light. Thus, a very high concentration of PMS may have some nonspecific inhibitory effect.)

Preliminary results on the life time of fluorescence (τ), obtained in collaboration with Mr. J. Hammond, show that PMS induced quenching is a true decrease in the fluorescence yield (ϕ). Thus, the 30% decrease in yield induced by PMS, in the presence of DCMU, results from an increase in the rate constant (probability per unit time) of heat losses. We suggest that this acceleration in loss arises due to a structural modification of the membrane structure. Glutaraldelyde fixed chloroplasts are unable to show PMS-induced quenching of fluorescence. Such chloroplasts are shown to retain the ability to show light-induced pH changes (173). Thus, the latter cannot be directly responsible for slow fluorescence changes.

5. Fluorescence Transient in Intact Leaf Segments and Class I Chloroplasts*

Although the fluorescence transients are very well characterized in broken Class II chloroplasts, no fluorescence studies have been made with Class I chloroplasts. We expected that these whole chloroplasts would exhibit fluorescence transients similar to that of intact algal cells.

*The experiments reported in this section were done in collaboration with Drs. W. S. Vredenberg and Govindjee.

But, unfortunately, we observed slow irreversible decline of the yield in dark as well as in light (see below, and reference 134 for a discussion of this topic).

Figure 116 shows a transient of Escarole leaf. The fast fluorescence transient is similar to that of isolated chloroplasts. Class I chloroplasts prepared, essentially, according to Nobel (129), showed a fast transient similar to that of the leaf segment (not shown).

Figure 117 shows that there are no large fluctuations in the fluorescence yield in broken Class II (maize) chloroplasts even if illumination is prolonged up to 3-4 minutes. Figure 117 (right) shows that the uninjured leaf segment exhibits reproducible PSMT transients in the fluorescence yield. However, if the leaf surface is gently scratched (see ref. 134) these variations in the yield were suppressed but a slow decline persisted. Depending on the extent of injury the extent of the slow decline in the yield varied.

From this type of observations Vredenberg (227) suggested that the slow changes in fluorescence yield are associated with ion transport between chloroplasts and cell mellieau. Figure 117 also illustrates a slow irreversible decline of the fluorescence yield in isolated Class I chloroplasts. This decline was irreversible and it occurred both in dark as well as in light. Addition of $NaHCO_3$ did not inhibit the irreversible decline in the yield. We prepared and used Class I chloroplasts from a variety of leaf materials by a variety of preparative procedures (227, 228, 229, 230). In each case we were faced with the problem of an irreversible slow decline in the yield, except in the case of cotledonary leaves of pumpkin where this slow transient could be repeated for a few

Figure 116. Fluorescence transient of a leaf segment of Escarole. 2 mm x 2 mm segment of the leaf kept wet with drops of buffer (50 mM phosphate, 0.4 M sucrose 0.01 M NaCl) and covered with a cover slip; 5 min dark time before illumination. λ observation, 685 nm (half band width, 6.6 nm).

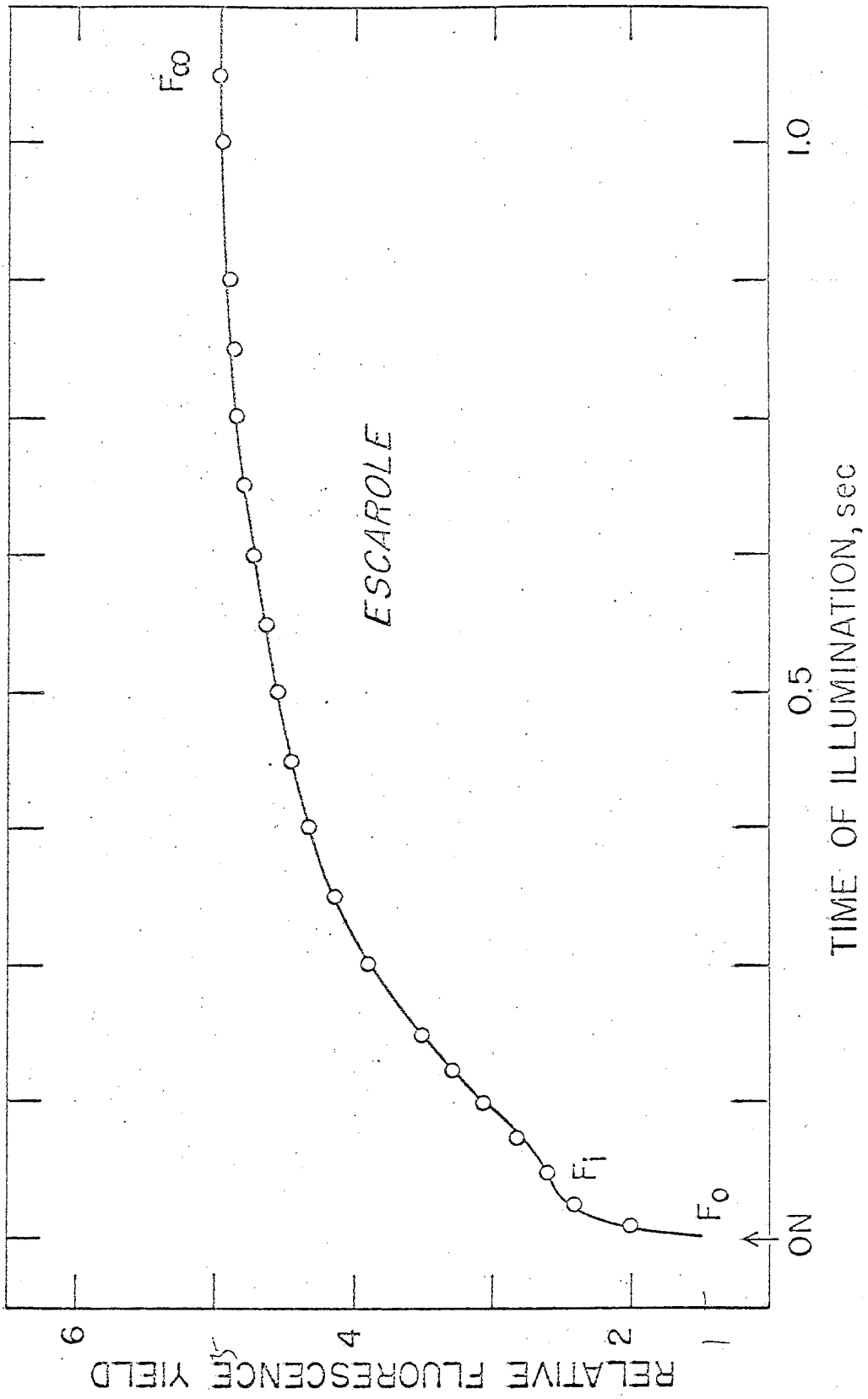
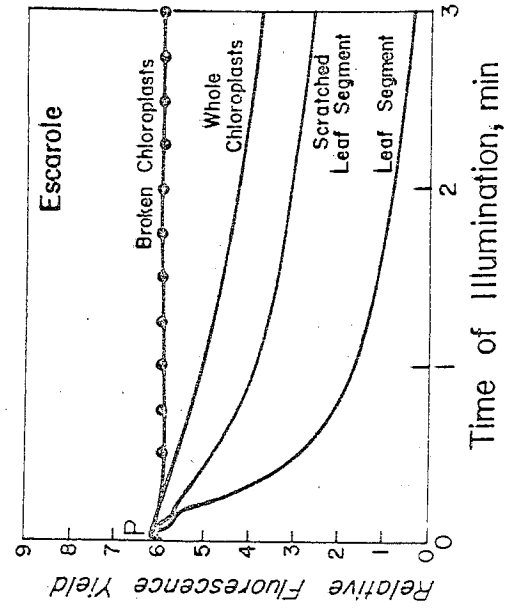
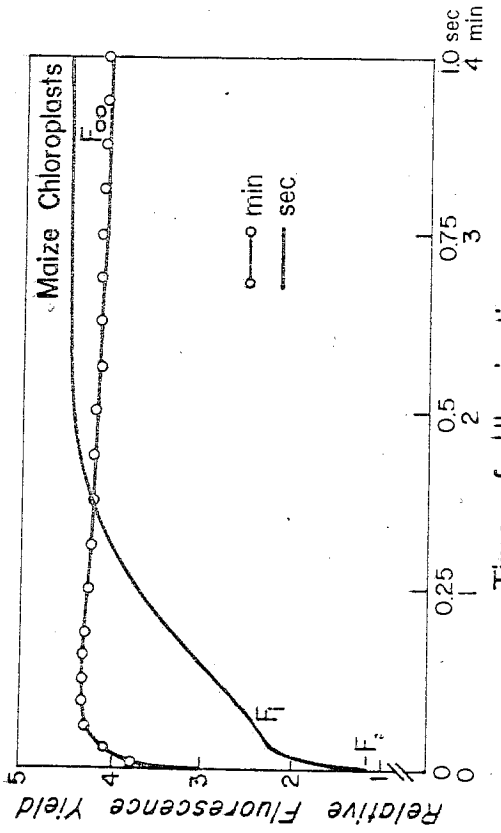

RELATIVE FLUORESCENCE YIELD
1
2
4
6
ON
0.5
1.0
TIME OF ILLUMINATION, sec
F_0
F_i
F_∞
ESCAROLE

Figure 117. Time course of Chl *a* fluorescence in chloroplasts and leaf segments. (Left): maize chloroplasts, previously stored, frozen in dry ice and acetone, thawed and suspended in 50 mM phosphate buffer (pH 7.2); 30 μg Chl/3ml; illumination, broad band blue light; intensity, 1.4×10^4 ergs $cm^{-2}sec^{-1}$; 7 min dark time; abscissa has two scales. (Right): Escarole (2 mm x 2 mm) leaf segment immersed in 0.3 M sorbitol, 0.01 M NaCl, 5 mM tricine-NaOH (pH 7.9), and 1 mM $MgCl_2$. Scraping of leaf segments was done with a spatula. Class II broken chloroplasts (solid circles) were prepared by suspending chloroplasts in 0.05 M phosphate buffer without any sugar (pH 7.8); class I (whole) chloroplasts were made according to reference (129).


Escarole
Broken Chloroplasts
Whole Chloroplasts
Scratched Leaf Segment
Leaf Segment
P
Relative Fluorescence Yield
Time of Illumination, min


Maize Chloroplasts
min
sec
F_∞
F_1
F_2
1.0 sec
4 min
Relative Fluorescence Yield
Time of Illumination

successive illuminations. Since biochemical aspects of these pumpkin chloroplasts are not known, we do not want to draw any conclusions from these results. Our results from the above studies, at this stage of the investigation, are difficult to interpret. We need more data to reach a general conclusion.

It is possible that the slow decline of fluorescence yield may just be due to a leakage of some enzymes, thus causing a loss of CO_2 fixation ability by these chloroplasts. It is well known that the CO_2 fixation ability of chloroplasts indeed is ephemeral (229, 230). Attempts should be made to repeat these measurements with chloroplasts having good CO_2 fixing ability.

6. Summary and Conclusions

The 'OIDP' type fast fluorescence changes in broken chloroplasts reflect the reduction of the A pool. In some preparations a very slow long term decline has been observed (Figure 117). This decline may be associated with the Mehler reaction. Class I type intact chloroplasts showed an irreversible decline in yield, both in light and in darkness. The cause of this decline could not be ascertained.

Addition of cations caused a gradual enhancement of fluorescence yield and also changes in emission characteristics of the chloroplasts. Preliminary studies, made in collaboration with Mr. J. Hammond, indicate that Mg^{2+} causes an increase in the lifetime of fluorescence (τ). Thus, the increase in fluorescence intensity by cations (231) is a true increase in yield and is caused by a shift in the extent of spill over from PS II to PS I. (The latter is now placed on a sound basis as we also observe an increase in the F685/F720 ratio even at room temperature.)

The nature of PMS induced quenching of Chl a fluorescence in chloroplasts is not well understood. It has been suggested that the PMS-induced lowering of fluorescence is due to a high energy intermediate (XE) induced quenching. A positive correlation with PMS induced quenching and pH formation was suggested (195). It is known that at a high pH and in the presence of a relatively high concentration of PMS, a post-illumination high energy potential (XE) persists (231), although no proton uptake can be measured.

On the other hand studies of Rottenberg et al. (232) suggest that light induced proton uptake measured by the disappearance of protons from the outside medium is not the same as the pH change inside the thylakoid membrane due to the buffering capacity of the chloroplasts. Also, at alkaline pH of the medium, the extent of Δ pH (proton uptake) is higher than at a low or neutral pH (232). It is possible that during cyclic electron flow some PMS gets reduced inside the thylakoid membrane and the reduced $PMSH_2$ and oxidized PMS develop a gradient. Such a gradient would apparently replace the formation of proton gradient due to proton uptake from the medium (231). It is, however, very unlikely that there would be any appreciable rate of electron flow in poisoned samples. It may be that a proton gradient or a high energy intermediate (XE and X-I) or both are associated with the quenching of fluorescence of Chl a by PMS. We do not know as to how the proton gradient or XE cause a lowering of this fluorescence yield. We may assume that quenching of Chl a fluorescence by PMS may be somewhat similar to the quenching of atebrin fluorescence (see refs. 233, 234 and 235 for discussion). It is assumed that uptake of atebrin through the membrane brings about a decrease in absorption

but not a decrease in fluorescence yield (234). Although it is not yet certain if this type of screening effect is the real cause of decline of atebrin fluorescence (233, 234) a similar kind of mechanism might be possible for the PMS induced lowering of fluorescence yield. Secondly, PMS may induce a thermal degradation of excited Chl a molecules (an increase in k_h). Measurements of the lifetime of Chl a fluorescence (τ) indicate that PMS induces a real decrease in the yield. Since k_p is zero and k_t remains unaltered, it seems that PMS enhances the k_h alone. Whatever the mechanism of the lowering of fluorescence yield by PMS is, we believe that it is associated with changes in the structural state of the pigment bed. Studies made in collaboration with Mrs. Barbara Z. Braun show that fixation of chloroplasts with glutaraldehyde abolishes the PMS-linked quenching as well as enhancement of yield by Mg^{2+}. It is well known that these fixed chloroplasts can carry out PS II and PS I partial reactions (213) and possibly have the ability to transfer H^+ from outside to inside (173). Thus, structural changes, not pH changes, seem to control the fluorescence yield.

In our studies with isolated chloroplasts we have also shown that low fluorescence yield caused by tris-inactivation of electron donation ability of water is due to the incomplete reduction of A pool and not due to an acceleration of a back reaction between Z^+ and Q^-. Furthermore, it was observed that 0.8 M tris extraction, although known to extract Mn^{2+} from chloroplasts, did not depress the F695 band. (It has been shown before (166) that Mn^{2+} deficiency causes a quenching of F696 band.) It is possible that the sites of inhibition by these two factors are different.

In summary, at least two main categories of fluorescence yield changes could be induced in chloroplasts (i) by the oxidation level of Q (Figures 101 and 104) (ii) by the structural state of the membrane (cation induced, Figures 106 and 107, and PMS-induced, Figures 114 and 115). The cation induced changes in the fluorescence yield result in an alteration of energy transfer between the two photosystems, but PMS-induced changes in fluorescence are perhaps, due to an increase in the rate constant of internal conversion (heat). Interaction of both photosystems is necessary to exhibit cation induced and PMS induced changes in the fluorescence yield as System II particles did not show any alteration of the yield by the addition of Mg^{2+} or PMS. A structural modification is definitely associated with this type of modification in the fluorescence yield. Our observations suggest that under some conditions the probability of losses by internal conversion is altered and does not remain constant as is often assumed. We speculate that a structural adaptation may be a protective device against high intensity illumination.

VII. GENERAL DISCUSSION, SPECULATIONS AND CONCLUSIONS

1. General

The chlorophyll a fluorescence transients of dark-adapted aerobic cells of a green alga Chlorella pyrenoidosa, a red alga Porphyridium cruentum, a blue green alga Anacystis nidulans and of isolated chloroplasts from a variety of plants (oats, maize, spinach, etc.) were studied in this investigation. It was the purpose of this investigation to find more information about the relationship between fluorescence yield changes and electron transport, energy conservation, and structural alterations during photosynthesis. It was our aim to assess the role of different factors that modify the chlorophyll a fluorescence yield during illumination.

A few years ago, it was believed that all fluorescence yield changes were strictly related to the redox level of the primary acceptor Q, and that system I had no role in regulating the fluorescence emission from photosynthetic organelles. In recent years observations from a number of laboratories, including ours, indicate that redox changes during photosynthetic electron transport are inadequate to explain all the observed fluorescence yield fluctuations during illumination.

2. Comparative Statements

We have, in this investigation, made comparable studies with isolated chloroplasts from higher plants and intact algal cells (or intact leaf segments). This should allow us to make more meaningful comparisons between in vitro and in vivo systems. Although fluorescence yield changes were measured earlier by Munday and Govindjee (83, 84, fast) and

Papageorgiou and Govindjee (87, 88, slow) in our laboratory and also by other investigators elsewhere (see ref. 1) we have attempted to characterize and compare both the fast and slow fluorescence yield changes in three representative algae and in isolated chloroplasts.

The fluorescence transient of dark adapted aerobic samples of all three algae, when excited by an intense system II (+ I) light has a base level (0) followed by three rapidly occuring sequential phases within one second of illumination: a hump (I), a dip (D) and a peak (P). From P, the fluorescence yield shows a slow decline to a quasi-steady state level S. After reaching the S level, the fluorescence yield slowly increases to a second maximum M level and then declines after a few minutes of illumination to a low fluorescence (T) level. The T level indicates the terminal level as no significant change in the yields seem to occur after that. From a comparative point of view, the blue green algae have a slightly higher base (0) fluorescence level and a lower P and a higher M level compared to Chlorella, the yield at M is much greater than that at P. In blue-green algae, the PS decline is smaller and the MT decline is either small or negligible. A comparison between the green algae Chlorella and the red alga Porphyridium shows that the fluorescence transients are very similar except perhaps for a smaller SM rise in the latter. In both cases the P level is the maximum level of fluorescence yield attained during the course of the transient. Intact leaf segments of higher plants have almost the same transient as that in Porphyridium; however, the MT to decline is comparatively faster in leaves than in algae.

3. OIDPS Rise

Isolated broken chloroplasts (without added electron acceptor) exhibit a biphasic, monotonic rise of fluorescence yield to a steady state level upon illumination. After attaining a maximum F_∞ level (comparable to the P level) the yield either slightly declines or remains constant throughout the period of illumination. In the case of intact whole chloroplasts (Chapter VI), the fast transient is identical to broken chloroplasts but an ephemeral slow decline in fluorescence yield is observed both in light and in darkness. Although this decline may be a simple time dependent loss of activity, the reason for this slow change in fluorescence yield remains to be investigated.

It has long been known that artificial physiological electron acceptors of PS I added exogenously can cause a quenching of the fluorescence yield in isolated broken chloroplasts. After a time proportional to the amount of added electron acceptor the fluorescence rises back to its maximum level (F_∞ or P level). The autooxidizable low potential viologen dyes under aerobic conditions are a perennial source of electron acceptor, and, thus in their presence the fluorescence yield remain very low (Figure 101) in isolated chloroplasts.

Addition of DCMU, which blocks the electron flow after the primary acceptor Q, causes a rapid OI rise of fluorescence yield to a maximum level. In bright light, the maximum fluorescence yield in the presence and in the absence of DCMU is same (Figure 100). Therefore, the fluorescence yield changes in isolated broken chloroplasts are explained to be due to the reduction of electron carriers (A) between the two photosystems. Darkness or a system I pre-illumination oxidizes these carriers and subsequent system II light reduces them. Without any added exogenous

acceptor only a O_2 gush is observed, suggesting a reduction of the pool of intermediates (A). Thus all fluorescence yield changes are considered to be governed solely by the redox level of Q.

In intact algal cells or in leaf fragments, one observes a fast OIDP rise of fluorescence yield comparable to the fast transient of chloroplasts. But after attaining the peak P, the yield gradually declines to a lower level S (Figures 12, 37, 68 and 117). This OIDPS transient may be considered to consist of two overlapping components of the time dependent yield changes: (a) a fast rising component and (b) slowly decaying component. The fast rising component may be regarded, as in case of isolated chloroplasts, to arise due to the reduction of pool of electron carriers between two photosystems. Again, the more reduced the state of the hypothetical primary acceptor of PS II (Q), the higher the yield of fluorescence. The slow declining component comprises both reoxidation of carriers and structural changes of the chloroplasts (Chapter III, IV and V).

In the past, many mathematical formulations (see literature cited in 1) have been made to explain the OIDP rise of the fluorescence transient. For our purpose, these mathematical treatments are of limited value as these formulations did not attempt to characterize the complete fluorescence transient. It is, however, important to note that most of the formulations recognize the quencher property of the primary acceptor Q.

Munday and Govindjee (83) suggested that the ID decline results due to a push and pull effect of two interacting photosystems on the redox state of Q and DP results from an accumulation of reduced carriers arising from a slow limiting step beyond the primary acceptor of system I. That

the DP rise occurs owing to a limiting step beyond Q is supported by the evidence that an exogenous supply of system I electron acceptors, as in case of isolated chloroplasts (Figure 101), eliminates the DP rise. Methyl viologen (or diquat) which is a terminal electron acceptor for Photosystem I, eliminates P in all three algae tested (Figures 43 and 81). Thus, it seems that DP rise is mostly affected by the redox state of electron transport carriers connecting the two photosystems and by the primary acceptor of Photosystem II, the Q being the major determinant of fluorescence yield during this phase of induction.

It is also interesting to note that the time for the development of P, at a high intensity of illumination, is a characteristic of the system II units and the relative rates of electron transport. It is possible to estimate from the time to reach P (tp) the number of oxidants molecules reduced per system II unit. This is a rather poor estimate, as it will vary depending upon the assumed values for the various parameters. Nevertheless, this gives a rough idea of the number of equivalents reduced.

The average time ($\bar{t}$) between absorption of quanta by a single chlorophyll molecule is calculated by the relation:

$$(\bar{t}) = \frac{\text{Number of chlorophyll molecules/ cm}^2}{\text{Number of absorbed quanta/cm}^2 \text{ sec}}$$

to be about 5 sec (at I = 10 nanoeinsteins/cm^2 sec, and $E_{av} = 2 \times 10^4$ cm^2/mole).

Assuming that a system II unit contains roughly 200 chlorophyll molecules, each unit encounters a quantum about every 15 milliseconds. Thus every 300-400 milliseconds, the time to reach P, approximately 20

oxidizing equivalents (A) per unit are destroyed (reduced). This value is slightly greater than that has been measured for chloroplasts (92, 93). We realize that the above number has been derived from a number of assumed factors and that this value is probably an overestimate.

Munday and Govindjee (84) have shown that a system I pre-illumination causes a decrease of and delay in P in Chlorella. We have also observed a similar delay in the development of P by PS I illumination in Porphyridium (Figures 20 and 39). It is assumed that weak system I pre-illumination causes a transfer of electrons from reduced A to X. In other words, 30 seconds of system I pre-illumination keeps the pool of intermediates in a more oxidized state than does 10 minutes of dark adaptation. Thus after a system I pre-illumination more oxidizing equivalents are available for reduction by the subsequent system II illumination and this causes a delay in P. This suggestion is also in conformity with the observation of a higher O_2 spike in system I illuminated sample than in the samples that are kept in darkness. But, if one assumes that at P all Q are reduced, then the fluorescence yield at P would be maximum and would remain unaffected by the system I pre-illumination. This is not the case. System I pre-illumination decreases the yield at P. Bannister and Rice (89), using a high intensity of system I pre-illumination, have observed an increase in the yield at P over the dark adapted samples. Unfortunately, we have not made any detailed study to decipher the cause of this discrepancy. In spite of this difficulty, it appears that at P, if all Q is reduced as our data with methyl viologen indicates (Figures 43, 81, 101) the fluorescence yield should be the maximum attainable yield (Figures 17 and 38) and should not vary. Furthermore, in intact algal cells, unlike

isolated chloroplasts, even with bright system II illumination the fluorescence yield at P saturates at a lower value than in the presence of DCMU, although in both cases all Q is presumably reduced. These results indicate that even during the first few seconds of illumination the fluorescence yield does not seem to be solely determined by the level of reduced Q. (The reduction level of the primary acceptor, however, may have been the major factor in determining the yield of Chl a fluorescence during this interval of illumination.)

4. The PS Decline

The slow PS decline component of the fast fluorescence transient is very complex. During the PS decline part of the transient, the rate of O_2 evolution increases, but the PS decline does not seem to reflect the reoxidation of Q, as shown by Duysens and Sweers (10). Our results with Porphyridium (Figure 22) indicate that the decay of the yield at P occurs more rapidly in the dark than in System II light. But in both cases a dark period is necessary to restore the original transient. A brief period of system I illumination restores the OIDP transient but slows down the PS decline and enhances the yield at the S level over the dark adapted control (Figures 20 and 39). Again, this effect of system I pre-illumination in delaying P and enhancing the S level decays in darkness (Figure 20, insert). These complex results suggest that during this part of transient the redox state of Q is not the only governor of the fluorescence yield. System I pre-illumination restores the electron carriers to the oxidized state but it also alters some other factor(s) that influences the fluorescence yield such that the yield at S is higher than in the normal dark adapted samples. Similarly if the light is turned on again,

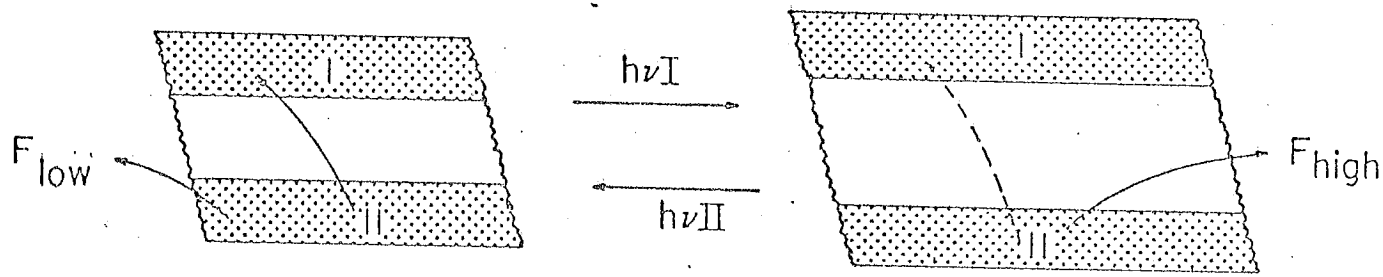
the maximum fluorescence yield during second illumination is lower than that of the first illumination (Figure 40). Sybesma and Duysens (236) suggested that System I light probably acts the same way as prolonged darkness in re-activating the fluorescence yield.

It appears that system I and system II illuminations, besides oxidizing or reducing the pool of electron carriers, alter some factor(s) in an antagonistic way that also governs the fluorescence yield.

In recent years, it has been proposed by Murata (151) and others that the PS decline is caused by the light-induced increase in excitation transfer from system II to system I, thus the availability of excitation quanta to photosystem II decreases. The terms, state 1 and state 2 were coined to describe the physical states for the regulation of excitation transfer between the two pigment systems (Figure 118). In 'state 1' a greater proportion of quanta is available to photosystem II than in 'state 2'. Consequently, 'state 1' is more fluorescent than 'state 2' and the PS decline reflects the 'state 1' to state 2 transition. System I light favors the transition to 'state 1' and system II to state 2. (In terms of the equation $\phi_f = k_f/(k_f + k_h + k_p)$ during the P to S decline system II light increased k_p as the O_2 yield increased but k_h may also increase.)

The above speculative model has received support mainly from two kinds of experimental observations: (1) a higher rate of O_2 evolution or electron transport in state I than in state II during the SM transition (see later); this is, however, not true during the PS decline; and (2) state II has a higher ratio of system I to system II emission than state I.

Figure 118. A diagrammatic picture of the two conformational states of the chloroplast membrane. State 2, a low fluorescent but a high (favored) spill over state; State 1, a highly fluorescent but low (unfavored) spill over state (after Govindjee and Papageorgiou (1) based on the ideas of Bonaventura and Myers, Murata and Duysens).



STATE 2
(high spillover state)

STATE 1
(low spillover state)

HYPOTHETICAL STATE TRANSITIONS IN CHLOROPLASTS

From the above discussion, it follows that in the presence of DCMU state I to state II transition does not occur. The state I is always preserved both in light and in darkness with DCMU. This seems, at least, to be true in the green alga, Chlorella and in the red alga Porphyridium, but this does not hold true in the case of the blue-green alga Anacystis (Figures 83, 89) and isolated broken chloroplasts (Figure 106). In the latter cases the Q independent fluorescence yield changes occur even in the presence of this powerful electron transport inhibitor.

5. The SM Rise

The slow SM rise of fluorescence yield in Chlorella, Porphyridium and Anacystis accompanies the parallel rise in 0_2 evolution. This parallel rise of oxygen evolution and fluorescence yield was shown earlier by Papageorgiou and Govindjee (87, 88) in Chlorella and Anacystis, and by Bannister and Rice (89) in Chlamydomonas. We have observed a similar parallel rise in 0_2 evolution and fluorescence emission in Porphyridium and have confirmed it in Anacystis. Bonaventura and Myers (155), using a fast amperometric technique (which senses only the photosynthetic oxygen evolution), showed that Chlorella cells change their fluorescence yield without any change in the rate of 0_2 evolution. They observed that cells pre-illuminated with system II light showed a parallel slow rise in the rate of 0_2 evolution and in the yield of Chl a fluorescence. Although these experiments were done in a different context, these results also show a slow parallel rise of photosynthetic 0_2 evolution and fluorescence yield. In our studies with Chlorella we observed that a superimposition of weak system I illumination at S caused a small increase in the yield of fluorescence rather than the expected Q-related decrease (Figure 41).

This would suggest that system I light, besides oxidizing the intersystem intermediates, caused a shift from the state II to state I. During the SM rise part of the transient, the quenching effect of system I illumination is low (Figure 42) probably due to the transition of the so-called 'states.' We have further shown that during the SM rise of fluorescence yield there is a shift in emission characteristics (Figure 25 and Table III.1). At M, there is relatively more system II to system I emission, and at S, less. This suggests that at M a larger fraction of absorbed quanta are delivered to PS II than at S. Thus, the SM rise, like the PS decline (Figure 77), indicates a light-induced control of distribution of absorbed energy between the two photosystems. This may be more so because of the parallel rise in O_2 evolution observed during SM rise. It is interesting to note that the fast IDP fluorescence change represents a balanced distribution between the two photosystems at the electron transport level, while the slow PSM change reflects another control mechanism in sharing the electronic excitation energy.

Thus, from the above discussion, we would like to interpret that the P to M change constitutes a full oscillation of state transitions from state I to state II and then back to I. There is, however, a differential sensitivity to added chemicals between the PS decline and the SM rise. The PS decline, in spite of its lack of correspondance with the redox state of the quencher Q, seems to be closely linked to the non-cyclic electron transport associated with accumulation of redox carriers. This is shown by the complementarity of O_2 evolution and Chl fluorescence yield. Additions of methyl viologen or benzoquinones (Figure 45), which prevents accumulations of QH, eliminate the shift from state I to II. In

fact, state I is not allowed to form. Under these conditions, however, a slow SM rise--although suppressed--persists (Figures 44, 45). It was also observed that the PS decline was very sensitive to uncouplers of phosphorylation (Figures 32, 48) while the SM rise was relatively insensitive to these treatments (87) (also see Figure 50). This differential sensitivity to uncouplers indicates that these state transitions are related to specific configurations of the pigment bed.

6. The MT Decline

The last phase of the fluorescence transient, the MT decline, was found to be most sensitive to external perturbations. Change in external pH and change in the growth conditions of algae exert the maximum influence on this slow decline of fluorescence. During this slow MT decline part of the transient, oxygen evolution remains constant (Figure 13). In blue-green algae (prokaryotes), this slow MT decline is significantly very low (Figure 75). Although this decline may also result from a shift in the distribution of absorbed photons similar to that in PS decline, it is kinetically different from it.

7. General Remarks

From the preceeding discussions, it is clear that the ratio of P/S or M/S is a rough parameter for measuring the ratio of the hypothetical states I to II. From our results the P/S ratio ranges from 3:1 to 5:1 in Chlorella and Porphyridium, but this ratio is lower (1:1 and 2:1) in Anacystis. On the other hand, the ratio of M/S is maximum in Anacystis, (5:1 to 6:1) while this value in Chlorella varies from 2:1 to 4:1; it is slightly lower in Porphyridium. Although these values give some estimate

of the extent of the interconversion of states, it must be realized that they are not simple to interpret. If we assume that during the PS decline more and more quanta become available to photosystem I and fewer photons reach system II reactions centers, this will also shift the balance between the oxidation-reduction levels of Q to a more oxidized state, which will also result in decreasing the fluorescence yield. Similarly, during the SM rise, as more and more quanta become available to system II, the balance of the oxido-reduction level of the pool of carriers is shifted toward the more reduced Q causing an additional enhancement in fluorescence yield. Therefore, it is important to realize that, both redox state changes accompany the "state" changes. We recall our results (Figures 41, 42) on the quenching effect of system I light during the SMT transient of Chlorella which clearly indicates that more than one factor influences the fluorescence yield.

The preceeding discussions lead to the dilemma of deciding between the cause and the effects: whether change in the state imposes a change in the redox reactions or vice versa. It seems logical to assume that electron transport processes preceed the interconversion of states. As we have discussed earlier, an accumulation of QH leads to the development of the DP rise and this, in turn, seems to lead to the state transition resulting in the P to S decline. But in the presence of DCMU, which also causes accumulation of QH, a state I to II transition is arrested, at least, in Chlorella and Porphyridium.

Greater influx of quanta to system I from system II would presumably promote a greater system I activity. One may envisage that this may ensue a higher rate of cyclic electron flow. Since cyclic electron flow is

mainly an energy conserving reaction, it leads to the accumulation of precursors for ATP. This, in turn, may promote a structural alteration resulting in the SM type of fluorescence rise and an associated transition to the original 'state 1'.

8. Possible Relation of Chlorophyll Fluorescence to Phosphorylation

Our results with uncouplers (Figures 32, 96, 91) and those of Papageorgiou and Govindjee (87, 88) suggest that energy conservation processes are interlinked with the slow SMT fluorescence change. The suggestion of a cyclic phosphorylation fostering the slow SM rise, however, meets with two difficulties: (a) it is claimed that in vivo cyclic photophosphorylation saturates at low light intensity and thus, direct absorption by PS I unit should saturate it; and (b) system I illumination alone does not cause appreciable change in the fluorescence yield. Although we have shown that the relative increase in the yield a M (represented as (M-S)/S) saturates at low light, the above hypothesis suggests that a greater delivery of quanta from system II to I is necessary to promote a maximum rate of cyclic electron flow. In other words, the light absorbed directly by system I is insufficient to saturate the in vivo cyclic electron flow. This may not be true. Also, it makes no sense! A greater difficulty arises from the observation that DCMU, which is not expected to inhibit cyclic electron transport, inhibits, all slow changes in fluorescence yield in Chlorella and Porphyridium as stated earlier. Direct measurements of ATP levels by Bedell (145) in the presence of DCMU suggest that the extent of cyclic phosphorylation in vivo may be low in Chlorella. (This conclusion, however, may not be correct if it is assumed that the rate of respiration decreases during illumination.)

On the other hand, there appears to be a larger amount of cyclic photophosphorylation in Anacystis (Table V.3). In Anacystis, the slow changes in the fluorescence yield are enhanced by DCMU. In this case, these changes appear to be supported by cyclic photophosphorylation. Uncouplers like S_{13} and S_6 and high concentrations of FCCP and atebrin inhibit these slow changes in fluorescence yield (Chapter VI). At the moment, it appears reasonable to assume that accumulation of high energy intermediate whether from cyclic or non-cyclic electron flow can lead to an energy dependent of structural changes, resulting in a shift in the extent of energy transfer from PS II to PS I.

9. Possible Relation of Chlorophyll Fluorescence to Ion Movements

Vredenberg (152, 198) proposed that ion exchange through the chloroplast membrane plays an important role in the "state" transitions. Simultaneous measurements of membrane potential across the plasmalemma and tonoplast and the slow MT fluorescence yield change in Nitella translusence showed that the decrease in fluorescence yield was accompanied by a decrease in potential (more negative). Although these experiments show a correlation between slow fluorescence yield changes and membrane potential changes, it is difficult to develop a clear relationship between the two processes without a quantitative knowledge of the direction and the magnitude of the potential as well as of the membranes and the ions involved.

One can assume that proton transport across the thylakoid membranes causes the slow changes in the fluorescence yield. Rumberg and Siggel (237) suggested that the development of a steep proton gradient in chloroplasts restricts the dark reduction of P700 by Cyt f. Govindjee and Papageorgiou (1) extended this idea and made the following speculations.

At P, according to their hypothesis, there develops a steep gradient which suppresses electron flow through system I. This gradient collapses as energy is harnessed in the form of some high energy intermediate. This causes an increase in the electron flow and the PS decline. During the P to S decline, the gradient is collapsing, the inhibitory effect of the gradient is being slowly removed, leading to an acceleration of O_2 evolution that continues beyond S. During the SM rise, the accelerated electron transport facilitates the build-up of the proton gradient and this leads to the increase in fluorescence yield. According to this view, the formation of the gradient enhances the fluorescence yield, and collapse of the potential decreases the yield. Uncouplers or ionophores do not permit the development of the proton gradient and thus inhibit the slow changes in fluorescence yield. This suggestion, apparently explains some data, but it is not free from difficulties. According to this view, a faster rate of electron transport would lead to a faster development of the gradient and this would lead to a fast SM rise in the fluorescence yield. Addition of methyl viologen, which is expected to accelerate the electron transport, does not seem to cause fast SM rise. Secondly, since the development of the gradient is assumed to cause the fluorescence rise and the formation of proton gradient is dependent on electron transport, it may be expected that the slow fluorescence yield changes have similar light saturation and intensity dependence as that of photosynthesis. This is, however, not true. Admittedly, more carefully thought out experiments are needed to fully elucidate the relation between the ion transport, the development of the gradient and the fluorescence yield changes. In the absence of such experimental results, we either assume that, either

due to the changes in the H^+ gradient or in the high energy intermediate, the thylakoid membrane undergoes a change in its structural organization such that energy transfer between two photosystems is either facilitated or prevented.

10. Possible Relation of Chlorophyll Fluorescence to the Structural Changes

The following results support the suggestion that an actual structural change of the thylakoid membrane accompanies the change in "states".

(1) In isolated broken chloroplasts, addition of cations induces a shrinkage in light as well as an enhancement in fluorescence yield (Figures 106, 107; also see 57). Murata (238) has shown that the kinetics of scattering change are similar to that of slow change in fluorescence yield. In the presence of salts, chloroplasts are known to shrink. We observed a slow increase in the yield of Chl a by the addition of Mg^{2+} in both spinach and lettuce chloroplasts; these results are similar to those of Murata (57). Cations only change the variable yield not the constant Fo level (our results). This increase in yield by Mg^{2+} occurs both in the presence and in the absence of DCMU. Thus both cyclic and non-cyclic electron flow supports the slow enhancement of the fluorescence yield. This ion-induced enhancement of fluorescence yield accompanies a change in the emission characteristics both at room (our results) and at liquid nitrogen temperatures (Figures 108, 109 and 110); there is an increase of system II fluorescence. We have also observed that the addition of Mg^{2+} to system II particles does not cause any change in the emission spectrum or in the fluorescence yield (our results). Thus the enhancement of fluorescence yield as well as an increase in life time of Chl a fluorescence (our results) are due to a redistribution of

absorbed quanta between the two interacting photosystems--there is a decrease in the spill over of energy from system II to system I.

We observed light induced swelling in intact cells of blue-green alga Anacystis (Figure 96). Unlike the case of isolated chloroplasts, the kinetics of volume change, as measured by the change in the absorbance at 540 nm, are slower than that of the fluorescence yield change (SM rise). As stated earlier, we do not expect an exact correspondence between the two events, as the volume change reflects the gross configurational change of the thylakoid membrane, while fluorescence yield ought to be affected by changes in the tertiary and quaternary structure of the membrane proteins.

(2) Fixation of cells or chloroplasts with glutaraldehyde, which immobilizes the protein structure by the formation of methylene bridges (175), eliminates all slow fluorescence yield changes, although these fixed cells do carry out electron transport (system I and II reactions); it is, however, known that fixation with glutaraldehyde brings about a loss in the synthesis of ATP (173). The lack of ATP synthesis is probably, responsible for the inability of such cells to fix CO_2 (Figure 34). We believe that loss of slow fluorescence yield changes in fixed cells of Porphyridium (Figure 35) and Anacystis (Figures 98, 99) is due to their lack of ability for structural alterations. Thus, changes in the structure of the thylakoid membranes accompany the fluorescence changes and the loss of ability for structural changes causes a loss of slow fluorescence yield changes both in algae as well as in isolated chloroplasts. It appears that an actual change in the membrane orientation leads to an acceleration or a suppression of electronic excitation transfer between the two photosystems.

The remarkable effects of cations in inducing fluorescence yield changes in isolated chloroplasts prompted us to examine the effect of ions on the fluorescence yield changes in Chlorella. Cations indeed caused redistribution of absorbed light, but unlike isolated chloroplasts, they favored a large spill over to photosystem I (Figures 60, 65). Cation-induced shrinkage, as well as shrinkage caused by the salts of weak acids, caused a lowering of the fluorescence yield both in the presence and in the absence of DCMU (Figure 62, Table IV.8). Addition of cations caused a shift towards state II as evidenced by enrichment of the long wavelength fluorescence band. Only at relatively high (external) concentrations, did salts induce a decrease in the yield at P and a loss of the PS decline. Unlike chloroplasts, we have never observed any enhancement of fluorescence yield at any concentration of the ions.

We have no satisfactory explanation for the contradictory results obtained with intact algal cells (Figure 62) and broken chloroplasts (Figure 106) although in both cases thylakoid membranes shrink in the presence of Mg^{2+} ions. We imagine that the presence of an outer envelope causes the two photosystems to remain in close proximity with each other, when the membranes shrink. In isolated broken chloroplasts, the contraction and folding of membranes by ions must separate the two systems away from each other. Although the gross macroscopic structural changes appear to be the same in isolated chloroplasts and in intact algal cells, the actual spatial conformation of both photosystems must have been different in both cases to cause opposite results. This leads to the suggestion that the actual conformation of the membrane, and not the gross structure of the chloroplasts, is important for the understanding of the fluorescence phenomena.

Like cations, many protonophorous uncouplers (e.g., FCCP, CCCP and DNP) induce slow changes in the fluorescence yield. Both in algae as well as in chloroplasts, FCCP causes a lowering of the fluorescence yield in the presence of DCMU. This quenching of fluorescence yield, we believe, is also due to a redistribution of absorbed light in favor of system I (Figures 52, 53, 54). Uncouplers like FCCP seem to have multiple functions besides uncoupling. FCCP seems to inhibit back recombination of Z^+ and Q^- and it induces, at high concentrations, permeability changes facilitating energy transfer to system I. Atebrin, which seems to behave like FCCP in its effect on the fast fluorescence yield changes, is, however, more effective at alkaline pH. As suggested by Schuldiner and Avron (234) atebrin may behave like amines and NH_4Cl (see Figure 6), and uncharged molecules of atebrin may enter into the membrane and cause some screening effect, and thus, cause a lowering of fluorescence intensity.

11. Quenching of Chlorophyll a Fluorescence by PMS

In isolated broken chloroplasts, the slow changes in the fluorescence yield can be induced by the addition of cations or by exogenous proton permeating agents. In recent years the quenching effect of cofactors of cyclic electron transport like PMS and DAD (diaminodurene) has been reinvestigated in detail (193, 194). PMS causes a light-induced energy dependent quenching of Chl a fluorescence in the presence of DCMU which is sensitive to a large number of uncouplers and ionophorous antibiotics. Because of the sensitivity to uncouplers, it has been assumed that this quenching reflects the high energy state associated with proton translocation. We have observed that PMS, an activator of cyclic electron transport, induces slow decline in fluorescence yield in the presence of

DCMU in isolated chloroplasts (Figure 113) as well as in Chlorella (Figure 55). In Chlorella and in Porphyridium reduced DCPIP also induces a quenching. Both $DCPIPH_2$ induced and $PMSH_2$ induced quenching of fluorescence are reversed by uncouplers. However, inhibitors of terminal phosphorylation are less effective (Figure 58) in suppressing the quenching of fluorescence yield.

PMS induced lowering of fluorescence yield, in the presence of DCMU, is not due to an increase in energy transfer from strongly fluorescent system II to weakly fluorescent system I as no change in the emission spectra was observed (Figure 57, Table VI.6). Unlike cations, PMS causes a lowering in the yield of fluorescence in chloroplasts as well as in Chlorella. Again, fixation of chloroplasts with glutaraldehyde eliminates the PMS activated quenching of fluorescence. This suggests that an energy dependent alteration of structural states of the chloroplasts induces the lowering of fluorescence yield by PMS. Preliminary measurements of the lifetime of chlorophyll fluorescence (τ) shows that the PMS induced fluorescence yield lowering is indeed associated with a decrease in the lifetime of Chl *a* fluorescence. This suggests that quenching by PMS and also perhaps by DAD, is due to an accelerated conversion to heat (internal conversion). It is usually assumed that the rate constant for radationless heat loss (k_h) does not change under changing physiological conditions. But these results indicate that an energy dependent structural alteration may, under some conditions, alter the rate of dissipating absorbed photon as heat (also see ref. 1).

Murata (238) recently compared the time course of the scattering change with that of the slow lowering of fluorescence yield induced by PMS and could not find any positive correlation between the two. This led

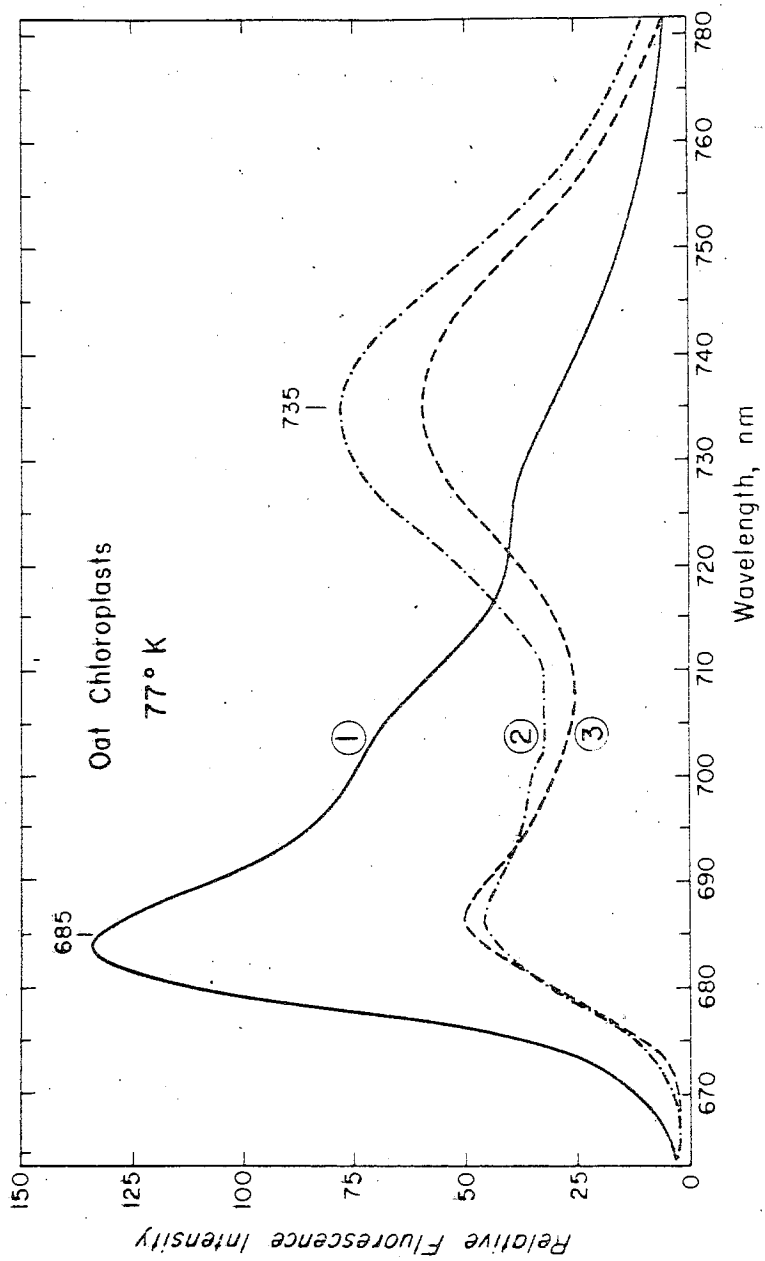
him to conclude that a structural alteration of the chloroplast is not the cause of PMS-induced fluorescence yield changes. It is true that a gross structural change (volume alteration) of chloroplast is not related to the fluorescence yield change. However, the microscopic conformational alteration of the thylakoid membranes must alter the spatial orientation of chlorophyll molecules and other embedded carriers and chromophores such that either an enhancement or suppression of the energy transfer between various forms of chlorophyll a molecules occurs. This could lead to an enhancement or suppression of fluorescence yield. The microscopic conformational change of the membrane structure is one of the earlier events in the hierarchy of events leading to macroscopic volume changes of the chloroplasts (see Figure 5). Hence, in all likelihood, the kinetics of volume change would not correlate with the fluorescence changes. Tamai (239) has shown that broken chloroplasts incubated in a phosphate buffer exhibit a photoshrinkage under non-phosphorylating conditions. Addition of PMS causes a swelling and these chloroplasts assume a maximal volume after 3-5 minutes of illumination. However, longer periods of illumination induces a shrinkage. It appears that PMS-induced quenching is probably related to the energy dependent swelling aspect of the conformational alteration of the membrane.

12. Chlorophyll Fluorescence and Ionic Interactions

Changes in membrane properties which are identified as conformational changes are believed to be linked to the electron transport via ion transport. Some investigators (121, 122) believe that proton extrusion or uptake changes the environment of the membrane. This results in the alteration (decrease) in membrane thickness and interloculus distance. However,

other investigators (126) consider the membrane of chloroplasts and mitochondria, within certain limits, to behave as flexible polyanionic macromolecules would. Thus, the charge distribution on the surface of the membrane would regulate the conformation. Electron transport-induced ion transport would alter the charge distribution and, hence, the conformation of the membrane. Deionization of chloroplasts would lead to the removal of loosely bound counter ions and this would result in a repulsion between the charges. As a result, the membrane would uncoil and the interloculus space would tend to enlarge. Addition of cations to the medium would induce cation-anion interaction and would reduce the repulsive interaction. This would lead to a folding and contraction of the membrane. The two interacting photosystems are either moved apart or come close to each other and this would cause either a suppression or an enhancement of energy transfer between the two photosystems. According to this picture, increasing the pH of the medium would enhance the repulsion of anions and lowering the pH would reduce this repulsive interaction due to protonation of anions. Figure 119 shows changes in emission spectra (measured at 77^{o} K) of oat chloroplasts suspended in neutral, acidic and alkaline medium before freezing. We observed that in acidic pH the main system II fluorescence band is enhanced and the longwave length band is suppressed extensively over the control at neutral pH. This emission spectrum looks similar to the emission spectra of chloroplasts treated with Mg^{2+} or other cations (Figure 110). On increasing the pH of the medium, by adding NaOH, the emission at 685 nm is progressively lowered and the long wave length fluorescence band (735 nm band) is enhanced. Unlike Mg^{2+} treatment, acidification did not cause a large increase in yield of fluorescence at room temperature. This is because, at this low pH, there is

Figure 119. Emission spectra of Chl *a* fluorescence in oat chloroplasts suspended in acidic, alkaline and neutral medium. Curve #3 (dashed line), suspended in 0.005 M phosphate buffer pH 7.0; curve 1 (solid line), sample was acidified to pH 3.8; curve 2 (dashed and dotted line), same sample was titrated to pH 9.5 by adding 0.2 M NaOH. These spectra are not corrected for the spectral sensitivity of the spectrofluorometer (Bausch and Lamb monochromator blazed at 750 nm, and EMI 9558B photomultiplier).


Oat Chloroplasts
77° K
685
735
①
②
③
Relative Fluorescence Intensity
Wavelength, nm
0
25
50
75
100
125
150
670
680
690
700
710
720
730
740
750
760
780

some loss of chlorophyll from the membrane. A detailed titration of chloroplasts with acid and alkali, which we have not done, will resolve the picture. However, our results indicate that the spectra are reversible; therefore, any specific loss of any particular form of Chl a is not the cause for the change in fluorescence emission characteristics. Thus, the shift in emission characteristics may be due to alteration in the membrane structure. It seems that ionic forces play an equally important role compared to hydrophobic interactions in the organization of the membrane. It is however, difficult to decide if hydrophobic interaction or electrostatic attraction is the dominant force in organization of membranes.

13. Summary and Conclusions

In summary, there are at least three factors (redox state of Q, excitation energy transfer and heat losses) that regulate the chlorophyll a_2 fluorescence yield during photosynthesis. Since most of the variable fluorescence is emitted by oxygen evolving system II, these factors regulate its yield. As has been documented by many workers, the yield of fluorescence during the first few seconds of bright illumination reflects the state of the trap. Closure of the trap results in the formation of polarized donor (Z^+) and acceptor (Q^-). As proposed by Duysens and Sweers (10), it is the redox state of Q that determines the fluorescence yield. During the OIDP transient, the redox state of Q, (that is, the conversion of Q to Q^-) is the primary determinant of the fluorescence yield. We have shown that in certain cases (such as tris-washed chloroplasts) when the feeding of H_2O to PS II is inhibited, an accumulation of oxidized donor (Z^+) occurs. The state of the donor does not seem to influence

the yield of fluorescence. In this specific case (tris-washed) there does not seem to be an increased cycling of electrons via system II (Chapter VI). Under other conditions, where flow of electrons from water is blocked, a rapid cycling of electrons may be possible causing a lowering in the fluorescence yield. But in such cases, it is the Q/Q^- ratio that determines the yield and not the ratio of Z^+/Z. We cannot say from our experiments if there is more than one form of Q. But if there are two forms of Q, as has been suggested (164), their quenching affinity must not be too different.

The distribution of absorbed quanta in favor of one photosystem or the other is the second important factor that alters the yield. The interconversion of states I and II is the predominant factor in the PSMT phase of the fluorescence transient. Thus, the yield at P is determined both by the redox level of Q and the state of the membrane. In Chlorella and in Porphyridium, the potent photosynthetic inhibitor DCMU, which blocks the electron transport after Q, seems to suppress the spill over of energy to system I. In these algae, in the presence of DCMU there is no interconversion of states; that is, state I persists both in the light and in the dark. In Anacystis, however, there is a "state conversion" from the less fluorescent state 2 to state 1 in light in the presence of DCMU. In broken chloroplasts, DCMU does not seem to affect the extent of energy transfer (state interconversion). Isolated chloroplasts mostly remain in 'state 2'. We do not know the cause of these differences, but we speculate that in Chlorella (and in Porphyridium), inhibition of electron flow by DCMU prompts a rapid (with a higher probability) energy migration among system II units. Such a high probability of intra-unit

excitation transfer may limit inter-system excitation energy spill over. In the presence of DCMU, Mar and Govindjee (240) observed a greater depolarization of fluorescence in Chlorella and Porphyridium.

The control mechanism of the hypothetical state interconversion, as reflected by fluorescence yield changes, the changes in emission spectra, and the changes in the rate of electron transport, are related to the spatial orientation of the two interacting photosystems which in turn are regulated by structural changes of the thylakoid membrane. The structural alteration of thylakoid membrane is supported at the expense of high energy intermediates generated by electron transport. It is reasonable to assume, from the study of various cation effects on isolated chloroplasts and intact algal cells, that energy dependent structural alterations are caused by transport of alkali ions across the membranes. The exact relationship between structural alteration of the thylakoid membrane and photosynthesis-dependent ion transport is highly complex. We cannot predict the relationship without further elucidation of events. Both cyclic and non-cyclic electron transport provide energy for structural changes of this membrane. In blue-green algae, like Anacystis, it seems that the cyclic electron transport mostly powers the structural alteration of the membrane leading to the slow SM rise of the fluorescence yield (state II to state I interconversion).

A third kind of regulation of fluorescence yield is associated with the rate of conversion to heat (internal conversion). This is characterized by a change in the fluorescence yield without associated changes in the emission spectra. This type of regulation of fluorescence yield, in association with energy spill over from system II to system I (state-transition), seems to influence the slow MT decline part of the

fluorescence transient. Like state interconversions, this kind of variation in the fluorescence yield is also brought about by conformational alteration of the membrane and seems to be supported by system I-driven cyclic photophosphorylation. Recent studies of Wraight and Croft (194) indicate that this third type of control of fluorescence yield is related to the electrochemical state of the thylakoid membrane. It seems that the direction of the gradient (membrane potential), i.e., whether the inside of the thylakoid membrane is more positive or less positive, controls the conformation of the membrane such that the conversion to heat is either suppressed or enhanced. The exact physiological condition when this mode of regulation becomes predominant is uncertain at this time.

It is possible that system I-driven cyclic phosphorylation in vivo may facilitate a reductive dephosphorylation reaction that would cause a variation of fluorescence yield. But this will be reflected in the redox state of Q. The existence of such a possibility has been proposed (158) but has not yet been characterized.

The complete physiological significance of these phenomena, described here, is far from clear. But we can start to operate with the speculative model that such structural transitions insure a regulation of excitation migration among the units, a balanced distribution of absorbed energy between the two photosystems, and a protection against excessive photodestruction. All these regulative factors, in turn, probably insure controlled and optimal chemistry at the reaction centers. It is by changing the form of the membrane that photosynthetic apparatus regulates its function.

APPENDIX

EFFICIENCY OF ENERGY TRANSFER FROM SYSTEM II TO I

Recently Mar et al. (45) estimated the three modes of dissipation of excitation energy following the absorption of a photon, namely, trapping fluorescence and radiationless losses in three algae, Chlorella, Anacystis and Porphyridium. These estimations were derived from the lifetime of fluorescence (τ) measurements with and without DCMU. However, the radiation losses calculated from τ measurements included the energy transfer from PS II to PS I which is assumed to be weakly or non-fluorescent at room temperature. In this section, we have estimated the rates and efficiency of the energy transfer from PS II to PS I in isolated chloroplasts and in algae from the observed fluorescence yield changes.

From the definition of $\bar{\Phi}_f$ one obtains the following relation:

$$\phi_f = \frac{k_f}{k_H + k_f + k_p} \quad , \tag{1}$$

where k_f, k_H, and k_p are first order rate constants for fluorescence, radiationless losses and trapping of energy in photosynthesis. In the presence of DCMU, k_p is zero as all traps are closed. Thus,

$$\bar{\Phi}_{f_{DCMU}} = \frac{k_f}{k_H + k_f} \quad . \tag{2}$$

Equation (2) can be rearranged as follows:

$$k_H = k_f \left[\frac{1}{\Phi_{f_{DCMU}}} - 1 \right], \text{ and } K_p = k_f \left[\frac{1}{\Phi_f} - \frac{1}{\Phi_{f_{DCMU}}} \right]. \tag{3}$$

An increase in the yield of fluorescence by the addition of Mg^{2+} ions to the DCMU treated chloroplasts is observed. Thus the rate constant (k_H') for radiationless losses in the presence of DCMU and Mg^{2+} will be represented as

$$k_H' = k_f \left[\frac{1}{\Phi_{f_{(DCMU + Mg^{2+})}}} - 1\right]. \qquad (4)$$

If one assumes that the spill over of quanta from PS II to PS I is negligible when Mg^{2+} is present, the rate constant (k_t) of energy transfer from PS II to PS I can be written as follows:

$$k_t = (k_H - k_H') = k_f \left[\frac{1}{\Phi_{f_{(DCMU)}}} - \frac{1}{\Phi_{f_{(DCMU + Mg^{2+})}}}\right] \qquad (5)$$

From our measurements the relative ratio of $\phi_{f_{(DCMU + Mg^{2+})}} / \phi_{f_{DCMU}}$ is approximately 1.60. Substituting this value into Eq. (5), we obtain

$$k_t = 0.375 \times k_f / \phi_{f_{DCMU}} \qquad (6)$$

The value for k_f can be assumed to be the same as estimated by Brody and Rabinowitch (241) for Chl *a* *in vitro*. From their computation of τ_o for Chl *a* in solution as 15.2×10^{-9} sec, the k_f is 6.57×10^7 sec^{-1}. This assumption may not be accurate in view of the existence of various forms of Chl *a* (see French, 242). However, in the first approximation k_f *in vivo* is very close to that of k_f for Chl *a* in solution, *i.e.*, 6.57×10^7 sec^{-1}.

The $\phi_{f_{DCMU}}$ can also be estimated from the measurements of the ratio of variable to constant (F_o) fluorescence level and assuming that the lifetime of fluorescence at the F_o level is the same as measured by Müller *et al.* (243) in very weak intensity of excitation. We usually observe a

value of 3.0 for $(F_\infty - F_o)/F_o$ in isolated broken spinach (or oat) chloroplasts. If the lifetime at F_o is taken to be 0.3×10^{-9} sec (243) and hence, for F_∞ as 0.9×10^{-9} sec., then,

$$\phi_{f_{DCMU}} = \tau/\tau_o = \frac{0.9 \times 10^{-9} \text{ sec}}{15.2 \times 10^{-9} \text{ sec}} = 0.059 \qquad (7)$$

(If, however, we assume that the lifetime of Chl *a* fluorescence at F_o is equal to that of the lifetime of fluorescence at the '0' level in *Chlorella*, as measured by Briantais *et al.*, (48), to be 0.6×10^{-9} sec, then $\phi_{f_{DCMU}} = 0.118$.)

Inserting the values of k_f (6.57×10^{-7} sec^{-1}) and $\phi_{f_{DCMU}}$ = (.059) in Eq. (6) we get, $k_t = 4.2 \times 10^8$ sec^{-1}. Thus the efficiency of energy transfer from PS II to PS I can be estimated from the following equation,

$$\phi_t = \frac{k_t}{k_f + k_H + k_p} \qquad (8)$$

where ϕ_t represents the quantum yield of energy spill over from PS II to PS I.

Inserting the values of k_t, k_f, k_H, and k_p we estimate that ϕ_t to be 0.118. (The value of k_H and k_p were estimated--from Eq. (3)--to be 10.5 $\times 10^8$ sec^{-1} and 23.40 $\times 10^8$ sec^{-1} respectively. Assuming the spill over of photons are utilized in driving photochemical reactions at the reaction centers of PS I, one estimates the overall efficiency of photosynthesis to be approximately 70% to 78%.

From the above method of calculations, it is also possible to estimate the rate constant for the back reaction (k_b) between Z^+ and Q^-, assuming that in the presence of a saturating amount of NH_2OH this reaction is completely inhibited. From our data, we estimate a 6% increase in the

yield in the presence of DCMU and NH_2OH over DCMU. This value enables us to compute

$$k_b = \phi_f \left[\frac{1}{\phi_{f_{DCMU}}} - \frac{1}{\phi_{f_{(DCMU + \overline{NH}_2OH)}}} \right] \qquad (9)$$

Substituting

$$= \left(\frac{0.06}{1.06}\right) \frac{k_f}{\phi_{f_{DCMU}}} = 0.056\ k_f/\phi_{f_{DCMU}}$$

$$= 6.23 \times 10^7\ sec^{-1}.$$

The efficiency of this back reaction is in the neighborhood of 1.7 to 2%.

It is possible to extend the above calculations to intact cells. In the case of Anacystis, DCMU causes a slow rise in fluorescence yield similar to the effect of the addition of cations to washed broken chloroplasts. This increase in fluorescence yield is also accompanied by a spectral variation very similar to the effect of Mg^{2+} in broken chloroplasts (Figure 89). We observe that uncouplers like S_{13} suppress the yield as well as cause a shift in the emission spectra (Figure 94). We consistently observe a 1.4 to 1.6 times enhancement in fluorescence yield in the presence of DCMU compared to the initial OI rise phase. In other words, the ratio of Fi to F_M varies from 1.4 to 1.6. In the presence of DCMU the rapid rise of fluorescence yield to Fi level is due to the accumulation of QH and the slow rise of fluorescence yield is regulated by factor(s) other than the redox state of Q. (One also obtains a similar ratio between the yield at low light to that in very bright light in the presence of DCMU.) We wish to interpret that this slow enhancement in yield is also due to a suppression of energy transfer from PS II to PS I. Again, assume that the lifetime of Chl a fluorescence at "O" level, when all the traps are

open, is 0.3 nsec. From our measurements the ratio of relative yield at M/0 is approximately 3.25. Thus the lifetime of Chl *a* fluorescence at maximal level of fluorescence would be 0.97 nsec. (This value is slightly higher than that of Mar *et al.* (45) who obtained a value of 0.71 nsec. From the relation $\phi_{f_{DCMU}} = \tau_{DMCU}/\tau_o$, we obtain $\phi_{f_{DCMU}} = 0.0638$. Using Equation (6), we obtain

$$k_t = 0.375 \times \frac{6.57 \times 10^7}{0.0638} = 3.643 \times 10^8 \text{ sec}^{-1} \text{ and}$$

$$k_p = \left(\frac{1}{\tau_{\text{at "0"}}} - \frac{1}{\tau_{\text{at "M"}}}\right) = \left(\frac{1}{0.30} - \frac{1}{0.97}\right) \times 10^9 \text{ sec}^{-1}$$

$$= 2.303 \times 10^9 \text{ sec}^{-1} = 23.03 \times 10^8 \text{ sec}^{-1}.$$

Similarly, $k_H = \left(\frac{1}{\tau_{\text{at M}}} - \frac{1}{\tau_0}\right) = (1.030 - 0.065) \times 10^9 \text{ sec}^{-1}$

$$= 9.65 \times 10^8 \text{ sec}^{-1}.$$

Thus, $\phi_t = \frac{k_t}{k_f + k_H + k_p} = \frac{3.643 \times 10^8}{(0.657 \times 10^8) + (9.650 \times 10^8) + 23.03 \times 10^8}$

$$= \frac{3.643}{33.337} = 0.109 .$$

Thus the efficiency of energy transfer from PS II to PS I is approximately 11% in *Anacystis*.

For *Chlorella*, we can assume that the S to M rise is also due to the suppression spill over of energy from PS II to PS I as manifested by an accelerated rise of O_2 evolution; this suggests a greater availability of photons to PS II reaction centers. In our experiments the M/S ratio ranged from 1.3 to 1.45. Using the M/S ratio of 1.4, we obtain a k_t of 2.17×10^8 sec^{-1} and using the values of k_H and k_p as estimated by Mar

et al. (45) $\bar{\Phi}_t$ to be 0.122 or 12.2%. Bonaventura and Myers (155) have estimated a maximal variation by α, the fraction of quanta that appear in PS II somewhere between 9 and 10%. Thus, our estimate of 11% for the spill over of energy from PS II to PS I seems to be very close to their variability in α. We realize that in all these calculations we have assumed a total suppression of spill over of photons which is not necessarily true. In other words, that estimated k_t values are slightly lower than the actual values.

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