PRIMARY PHOTOPROCESSES IN THE PHOTOSYNTHESIS OF ALGAE

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY TED MAR

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I. INTRODUCTION

A. FOREWORD

This investigation is concerned with the detailed study of the primary processes in photosynthesis, in particular that of photosystem II. Photosystem II, according to the presently accepted hypothesis, has a bulk pigment system consisting of chlorophyll a (Chl a) and accessory pigment molecules which acts as a light antenna system and a reaction center which traps the energy collected by the bulk pigments and uses it to perform a chemical reaction which leads to the production of oxygen. The primary events in photosystem II can be described as follows. After a photon is absorbed by the bulk pigment in photosystem II, the excitation energy migrates among the pigment molecules until it is finally trapped in the reaction center. The excitation energy can also be lost by radiative decay, known as fluorescence, or by non-radiative decay. After the excitation energy is trapped at the reaction center, this energy is used to drive an oxidation-reduction reaction or it may be lost by radiative or non-radiative decay. The reaction center can also act as a center for the recombination reaction of the primary oxidant and the primary reductant. These then are the primary processes we are concerned with. Our goal is to understand these processes in greater detail.

To study the primary events in the bulk pigment system, we have determined the rates and efficiencies of the three possible processes of the excitation energy following the absorption of a photon, namely, fluorescence, radiationless loss and trapping, by measurement of the

fluorescence lifetime (see Chapter III). We have shown that the mechanism of energy transfer between Chl a in vivo is due to the Förster mechanism by the measurement of the polarization of fluorescence. The polarization of fluorescence measured is assumed not to be due to loose chlorophyll-protein complexes within the chloroplasts (see Chapter IV). To study the primary events in the reaction center, we use the information obtained from delayed emission measurements. The relationship of the rate of delayed emission to the rate of photochemical reaction has led us to postulate that there exists another process that can occur in the reaction center. We postulate that intersystem crossing to the excited triplet state occurs within the reaction center and its rate competes with the rate of the oxidation-reduction reaction. Theoretical curves based on this postulate agree with the experimental decay of delayed emission (see Chapter I (E) and Chapter V). By using fluorescence transient, thermoluminescence and chemiluminescence measurements, we have further experimental proof that chemical recombination reaction does occur at the reaction center (see Chapter V). The details of the chemical reaction, at reaction center II, leading to oxygen evolution are also discussed (Appendix I).

To put this investigation in its proper framework, a brief outline of the currently accepted model for photosynthesis and then a review of the available research on the primary processes of photosynthesis will be presented below.

B. PHOTOSYNTHESIS -- A BRIEF REVIEW

The modern dogma of photosynthesis[1-4] of green plants is based mainly on two concepts, the photosynthetic unit and two light reactions. A photosynthetic unit is now conceived to be a structural unit that will perform a one electron photochemical oxidation-reduction reaction upon the absorption of one quantum of light. The unit consists of 200 to 300 molecules of Chl a, which absorb the light and act cooperatively to transfer the absorbed energy to a site where this energy is converted to chemical free energy. This site is called a reaction center. In higher plants, other pigments such as Chlorophyll b (Chl b) and carotenoids are also found and they act as accessory pigments transferring their absorbed quanta to Chl a.[5] As will be discussed later, there are two types of units and reaction centers -- one responsible for oxygen evolution and the other for the ultimate reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$). The reaction center for the oxygen evolving unit has not been isolated chemically (see Refs. 6 and 7) An as yet unknown molecule called Q[8] is postulated to act as the primary oxidant, and a hypothetical molecule called Z[9] acts as the primary reductant. The concept of the photosynthetic unit is based on the flashing light experiments of Emerson and Arnold.[10] They found, by using a series of short (10^{-5} sec) intense light flashes spaced 10^{-2} secs apart, that the maximum yield is one molecule of oxygen evolved for approximately two thousand four hundred chlorophyll molecules. Experiments with different dark times insured that the enzymatic dark reaction did not limit the oxygen evolving process. (The light intensity used for the experiment was not intense enough to

excite each and every individual molecule of Chl a in the unit, but enough to excite each unit.) Emerson and Lewis[11] showed that the quantum requirement was no less than eight quanta for every oxygen molecule evolved. These experiments imply that eight quanta absorbed by any eight of the 2400 chlorophyll molecules will evolve one molecule of oxygen. Assuming that each quantum absorbed is processed by one reaction center, it follows that 300 chlorophyll molecules are associated with one reaction center. This then is the concept of the photosynthetic unit. Numerous other experiments, for example, the study of the Hill reaction as a function of the size of the particles prepared by fragmentation of the chloroplasts,[12] the ratio of total number of chlorophyll molecules to enzymes like cytochromes which participate in dark reactions,[13] and the study of energy transfer,[3,5] have supported the existence of the photosynthetic unit.

The second important concept is that of the two light reactions. In 1957 Emerson, Chalmers and Cederstrand[14] discovered that the rate of oxygen evolution with a combination of two wavelengths of light, a wavelength smaller than 685 nm and a wavelength longer than 685 nm, is greater than the sum of the rates of oxygen evolved as measured separately with each wavelength. This "enhancement" in oxygen evolution led to the necessity of postulating another photochemical reaction (cf. Emerson and Rabinowitch[15]). Detailed action spectra for the "enhancement" of oxygen evolution (the Emerson effect),[15,16] the decline in fluorescence yield,[8,17,18] on combining far red light (710 nm) with red light (650 nm), the oxidation of P700[19] and cytochromes[20] with far red light and their reduction with red light led to the proposal that the two photoreactions have two different

pigment systems (cf. Duysens and Amesz[21]). Pigment system II contains a large proportion of the accessory pigment, Chl a 673, some Chl a 683 and the active chlorophyll a_{II} (P680), while pigment system I contains some accessory pigments, some Chl a 673, and a large proportion of Chl a 683, Chl a 693 and the reaction center P700 (see ref. 1). These and numerous other experiments[1-4] led to the acceptance of a model for green plant photosynthesis, formulated by Hill and Bendall,[22] based on the cooperative interaction of two light reactions. One light reaction, known as photoreaction II (because the photoreaction is associated with pigment system II), oxidizes an intermediate Z and reduces a substance called Q. Through a dark reaction, oxidized Z reacts with water to evolve oxygen (see Appendix I), the reduced Q, also through dark reactions, transports its electron through a series of intermediates[23] to P700, the reaction center for the light reaction, known as photoreaction I (because it is associated with pigment system I). Part of the energy of the photoreaction is stored in adenosine triphosphate (ATP)[24] which is made by the dark reactions. Light reaction I oxidizes P700 and reduces a hypothetical substance called X[25]. Again through a series of dark reactions, reduced X transfers its electron to $NADP^+$.[26] ATP and reduced NADP are then used to fix CO_2 into simple sugars either by the Calvin Cycle[27] or the C_4 dicarboxylic acid cycle.[28] This then is the general framework on which photosynthesis is based.

Although the Hill-Bendall model for photosynthesis is well established, alternate models have been recently proposed.[29,30] Also,

important questions, such as how does the energy migrate to the reaction center, what are the properties of the reaction centers that trap and store energy so efficiently, how is oxygen evolved, how is ATP made and many others, remain to be answered. Finding the answers to these questions is complicated by the fact that the molecular architecture of the photosynthetic unit is not fully known and by the fact that the measurements one can make on the system are restricted by the limitations of the instruments used. For this thesis, the primary processes in photosystem II have been studied. Information was obtained from measurements of fluorescence lifetime, delayed emission, fluorescence transient and of chemiluminescence. Information concerning photoreaction II that was gained from such measurements in this thesis and that found by earlier investigators will be discussed in the following sections.

C. FLUORESCENCE LIFETIME

After a photon is absorbed by a Chl a molecule in 10^{-15} seconds, the energy can remain in the excited state, ($\pi\pi^*$), for only a limited time before it relaxes back to the ground state. To be useful in photosynthesis, the energy must migrate to a reaction center (within the lifetime of the excited state). One of the methods of deactivation of the excited state is by the emission of a photon. This emission is commonly known as fluorescence. By measuring the decay rate of fluorescence one can determine the lifetime of the excited state.

The rate of decay of the excited state of the bulk chlorophyll is governed by α_h, the rate of radiationless transition, α_f, the rate of

fluorescence and α_t, the rate of trapping. The rate of change of N_s^* (the concentration of the excited singlet state)with time (t) , is given by

$$\frac{dN_s^*(t)}{dt} = G(t) - (\alpha_h + \alpha_f + \alpha_t)\, N_s^*(t) \qquad (1)$$

where G(t) is the rate of generation of the excited singlet state. Assuming that

$$G(t) = N_s^*(0)\, \delta(t)$$

and α_t, α_h and α_f are independent of time and of N_s^*, the solution to equation 1 can be written as

$$N_s^*(t) = N_s^*(0) \exp(-t/\tau) \qquad (2)$$

where

$$\tau = \frac{1}{\alpha_h + \alpha_f + \alpha_t} \qquad (3)$$

The excited state is shown to decay exponentially with an average lifetime of τ. If all the traps are closed, so that there is no transfer to the trap, the excited state will decay exponentially but with a lifetime τ_1 where

$$\tau_1 = \frac{1}{\alpha_f + \alpha_h} \qquad (4)$$

Two basic methods have been used to measure the fluorescence lifetime in photosynthesis -- the flash technique[31-35] and the phase delay[36-41] method. The flash technique employs a flash of light with a cut-off time shorter than that of the lifetime of fluorescence one wishes to measure. This flash of light is used to excite the fluorescent sample and the resultant fluorescence is detected with a photomultiplier. The signal from

the photomultiplier is displayed on a fast-time resolution oscilloscope. This method has the advantage that one can directly obtain information concerning the decay kinetics; one can see whether the decay is exponential or non-exponential and whether there is more than one decay component. In photosynthetic measurements, however, the fluorescence lifetime is so short that the measurements are limited both by the decay time of the exciting flash and by the response of the photomultiplier. The true time course of the fluorescence decay is modified by both the flash decay and the lag in the response of the photomultiplier. To obtain the true lifetime of the fluorescence, one must use the convolution integral.[31, 32]

$$F(t) = \int_0^t E(t-u)f(u)du \qquad (5)$$

where F(t) is the experimentally measured fluorescence decay, E(t) is the experimentally measured time response of the system (lamp + photomultiplier) and f(t) is the true time course of fluorescence. A modification of the convolution integral, "the method of moments,"[31] can also be used to eliminate the influence of the instrument response time from the observed decay time.

The second method of measuring fluorescence lifetime, the phase delay method, is a more sensitive technique in that the phase and hence the lifetime can be measured to a greater precision than the direct flash technique with present technology. Its drawbacks are that there are numerous possibilities of systematic errors and that it is an indirect method. The phase delay technique is based on the fact that if light with

amplitude M and modulated with frequency f, such that its intensity I(t) is described by the following equation,

$$I(t) = M \cos 2\pi ft \tag{6}$$

excites a species, which decays exponentially as $e^{-t/\tau}$, the fluorescent light (F) is described by the following equation.

$$F(t) = M \cos \phi \cos (2\pi ft - \phi) \tag{7}$$

where $\tan \phi = 2\pi f\tau$

Hence the fluorescence lifetime can be obtained by measuring the phase lag between the incident light and that of the fluorescence light.

The first measurements by Brody,[42] using the flash technique, in 1956 clearly showed that the fluorescence lifetime of Chl a in vivo is in the one nanosecond region. Since then, these measurements have been repeated many times both by the phase technique and flash technique; τ values ranging from 0.6 to 1.6 nsec for Chlorella have been found.[31,34] Müller, Lumry and Walker[41] partly resolved these differences by showing, with the phase technique, that the fluorescence lifetime varies with the intensity of the incident light. At low light intensity, when most of the reaction centers are open, the fluorescence lifetime is short (0.6 nsec). At high light intensity, when all the reaction centers are closed, the fluorescence lifetime increases to approximately 2 nsecs.

In green plants, the fluorescence may have more than one decay lifetime. One reason is that the decay constants may depend on time or the concentration of the excited state. The other reason is that fluorescence

can originate from different species. It can come from "dead" Chl a molecules which are not related to a reaction center or from Chl a molecules in photosystem II or photosystem I units. Fluorescence from photosystem II units may have two decay lifetimes. If the units are separated from each other, the lifetime from units with their reaction centers closed may be different from units with their reaction centers open. From experiments with algae, Joliot et al.[43] discussed a model in which the photosynthetic units in photosystem II are separated from each other, each unit having a single reaction center with its own harvesting light pigments. If the reaction center is open, a quantum absorbed by the unit will be trapped with a 100% efficiency at the reaction center. The fluorescence yield will be zero if there is no fluorescence from "dead" Chl a molecules. If the reaction center is closed, a quantum absorbed in that unit has a probability of 0.55 to 0.60 of being transferred to another unit of type II. The quantum is believed to move from one unit to another until it is trapped or is dissipated. If there is no fluorescence from "dead" Chl a molecules, fluorescence is believed to come only from units in photosystem II with closed reaction centers. However, when all the traps are open, fluorescence is still found to be present. This fluorescence is attributed by Lavorel[44] to Chl a molecules that are not associated with photosynthetic units containing reaction centers and to system I. Joliot's model, then, would predict two fluorescence lifetimes -- one component associated with Chl a in photosystem II with its reaction center closed and one associated with "dead" Chl a that is not in a photosynthetic unit and Chl a in photosystem I.

Clayton,[45] in a more general formulation, found that if one removes the assumption that there is no fluorescence when all the traps are open, it is possible to interpret Joliot et al.'s result by another model. This model assumes that many reaction centers are embedded in a large pool of light harvesting chlorophyll and that the quantum absorbed can wander freely among the chlorophyll (cf. Lavorel[46]). All or part of the fluorescence from "dead" chlorophyll or from photosystem I in Joliot's model would be from fluorescence of units with open traps. Theoretical calculations by Pearlstein[47] indicate that a finite time is needed for the reaction center to trap an excitation quantum if it is transferred by the weak resonance interaction between Chl a molecules. This model then would predict that the fluorescence will have predominantly one fluorescence decay lifetime because the model is analogous to the in vitro situation in which a high concentration of pigment molecules is mixed with a low concentration of quencher molecules.

All or part of the fluorescence that is not attributed to chlorophyll molecules in photosystem II may be fluorescence from photosystem I.[44] From the action spectra of fluorescence of red algae, Duysens[5] and French and Young[48] found that light absorbed by the phycobilins is more efficient in exciting Chl a fluorescence than light absorbed by Chl a itself. This led Duysens[5] to postulate that there exist two forms of Chl a in vivo in red algae, one weakly fluorescent and the other more strongly fluorescent. Since the phycobilins are now shown to be in photosystem II,[21] the strongly fluorescent Chl a which is associated with the phycobilins must be in photosystem II. The weakly fluorescent species is postulated to be in

photosystem I. It is, however, not yet possible to decide whether the weak fluorescence postulated to come from photosystem I comes from the photosynthetic unit itself or from "dead" chlorophyll, with no fluorescence from the unit itself.

It is generally accepted that most of the chlorophyll fluorescence *in vivo* comes predominantly from photosystem II when all the reaction centers are closed. In the case when all the reaction centers are open, there is no available experimental data to show the percentage of the fluorescence that is contributed by photosystem I, "dead" chlorophyll, or from the bulk of photosystem II.

Fluorescence lifetime of Chl *a* *in vivo* has been reported, by Murty and Rabinowitch,[33] to have two components. In Chlorella, the two values they obtain are 1.7 and 5.4 nsecs. These measurements, however, could not be repeated.[34, 35] Singhal and Rabinowitch[35] indicate that Murty's measurements may be erroneous due to the change in the incident pulse shape with time and the long time decay component may be due to the lamp decay and not to real fluorescence. Careful experiments by Nicholson and Fortoul[34] and by Singhal and Rabonowitch[35] with long time averaging techniques indicate that fluorescence decays with a single exponential lifetime at low light intensities when all the reaction centers are open. These measurements, however, cannot rule out the possibility that there are two fluorescing species having exponential decays differing by 0.2 to 0.3 nsec. Müller *et al.*,[41] using the phase technique, justify their assumption of one exponential decay by showing that the fluorescence lifetime is independent of the wavelength in the emission spectrum and by showing that the

fluorescence lifetime does not change when measured with different modulating frequencies. These are not good justifications because Krey and Govindjee[49] have shown that there is very little difference in the emission spectrum when all the reaction centers are open as compared with when they are closed. The change in frequency Müller et al.[41] used was not large enough to decide between a one component decay and a two component decay. Tumerman and Sorokin,[50] using the phase technique, found that the fluorescence yield varies linearly with the lifetime of the fluorescence under the assumption of only one lifetime. Briantais, Govindjee and Merkelo[51] have since confirmed these findings. Calculations done by Tumerman and Sorokin indicate that the assumption of only one lifetime can explain their experimental results and that two lifetimes cannot. A similar calculation has been done to confirm this (see Chapter III). To try to show that fluorescence has predominantly one decay, we have viewed directly the fluorescence decay of Chlorella when all the reaction centers are closed and the fluorescence decay when some of the reaction centers are closed. We were, however, unsuccessful due to the slow response of the photomultiplier.

When all the traps are closed, the fluorescence lifetime gives a measure of the rate of dissipation of the singlet excited state within the photosynthetic unit. We will show that the rate of dissipation is different for Chlorella, Porphyridium and Anacystis. Knowing the rate of dissipation and the rate of fluorescence, we have calculated the rate of trapping for the three algae. We will also show that the fluorescence lifetime for F685 (fluorescence band with a peak at 685 nm) at 77°K is the same as that at

room temperature (see Chapter III, B). This indicates that temperature has little effect on the rate of dissipation up to 77°K. Butler and Norris[37] have shown that the 720 nm fluorescence band (F720) in bean leaves at 77°K has a lifetime of 3.1 nsec which indicates that F720 is a π -π* transition. (They did not, however, report any lifetime measurements for the F685 fluorescence band at 77°K.)

D. POLARIZATION OF FLUORESCENCE AND ENERGY MIGRATION

When the reaction center is closed, the lifetime of the excited state is longer than when the reaction center is open and it will be shown (Chapter III, A) that energy can migrate from one unit to another. Therefore, the extent of energy migration is greater when the reaction center is closed. Although methods have been devised to measure directly the extent of exciton migration in solids,[52] these methods are inapplicable to biological systems. The only method available at present is an indirect one. This method is by the measurement of the polarization of fluorescence. Polarization of fluorescence, p, is defined by the following formula[53]

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad (8)$$

where $I_{\parallel}$ is the intensity of the fluorescence that has the same linear polarization as the incident light and $I_{\perp}$ is the intensity of the fluorescence that has polarization perpendicular to that of the incident light. The fluorescence is measured in a direction 90° to that of the incident light. A single molecule of Chl a in viscous medium, having no Brownian motion, has a

high polarization of fluorescence if it is excited in the red band. The reason for this high value is that the excited oscillator, within the lifetime of the excited state, cannot change its polarization either by Brownian motion or by transferring its energy to another oscillator. The emitted quantum will have the same polarization as the excitation quantum.

If polarized light were used to excite the Chl a molecules in the photosynthetic unit, only those Chl a molecules with absorption dipoles that are parallel to the polarization of the incident light will be excited. As energy is transferred from one Chl a molecule to another Chl a molecule, the "memory" of the initial polarization will be lost if the Chl a molecules are arranged in a random fashion within the chloroplast. This loss of memory is reflected in the decrease of the measured polarization of fluorescence of Chl a *in vivo*.[54, 55] From the decrease in the polarization of fluorescence and the lifetime of the fluorescence, one can calculate the extent of energy migration.[56] In these calculations, however, a specific energy transfer mechanism and the random orientation of the Chl a molecule must be assumed.

The first measurement of the polarization of fluorescence of Chlorella and the calculation of the extent of energy migration were made by Arnold and Meek.[55] Using an exciting light of 630 nm, they found that dilute Chl a in castor oil has a p value of 0.24 and a suspension of Chlorella 0.033. From their calculations they estimated a minimum number of 15-20 transfers before the energy is used. Weber,[57] using 366 nm Hg light, found that p for isolated cabbage chloroplasts has a value of 0.01 - 0.03. He

calculated the minimum value for the number of transfers to be 20 to 40. Teale[58] measured a p value of 0.06 in Chlorella with 660 nm light. His calculations show that there is a minimum of 140 transfers. In his calculations, he multiplied the number of transfers that are calculated by Weber's method by ϕ_o / ϕ where ϕ_o is the fluorescence yield of unquenched system and ϕ is the reduced yield by transfer quenching. Hence his value is the number of transfers that is possible if there is no energy quenching. If the calculations were done using Weber's formulation, the number of minimum transfers is only 12. Lavorel[59] measured a p value of 0.08 in Chlorella for fluorescence at 685 nm and a p value of 0.14 for 720 nm fluorescence (λ excitation, 650 nm). Their high values may be an artifact because their measurement of p for Chl a fluorescence at 720 nm in vivo, when excited by 550 nm, shows a value of 0.30; the intrinsic polarization of the Chl a molecule, in vitro, however, has a p_o value of only 0.10 at 550 nm. We speculate that their error may be due to the failure to correct for the polarization of the emission monochromator. Goedheer[60] attributed the high p values to the fact that Lavorel flows his Chlorella samples continuously through a thin tube, Goedheer feels that the flow may orient the Chlorella cells. On measuring a random stationary sample of Chlorella, Goedheer could not detect any noticeable increase in the polarization of fluorescence at 720 nm. R. A. Olson[61] also measured p for the emission band at 720 nm by using a ruby laser as the excitation source. His data indicates a p value of 0.68. Since the highest theoretical value for p is 0.5, one must conclude that his measurements were in error and most probably the scattered light from the laser was not taken into account. Using

Weber's polarization instrument, Govindjee[62] measured p at 720 nm for a randomly oriented sample of Porphyridium. He found that p was 0.03 when excited with 540 nm (absorbed preferentially by phycoerythrin) and 0.06 when excited by 440 nm (absorbed preferentially by Chl a). Cederstrand and Govindjee[63] found that p at 720 nm was 0.03 in randomly oriented chloroplast fragments from spinach which were enriched in pigment system II and 0.054 in fragments enriched in pigment system I. It has been amply shown then that the measured value of p of Chl a *in vivo* is definitely lower than that of a single Chl a molecule.

It has been well established that the lower polarization of fluorescence of Chl a *in vivo* is caused by energy migration. Arnold and Meek[55] have shown that the decrease is not due to scattering. They illuminated with polarized light a 10 cm suspension of Chlorella so dense that only multiplied scattered light was transmitted; in this condition, they found that 99% of polarization remained. Weber[57] has shown that the polarization *in vivo* is not due to Brownian motion. He measured the polarization of cabbage chloroplasts from 3° to 35°C and found that the polarization was constant within the experimental errors ($p = 0.001$). However, if the lowering of p is due only to energy migration, the calculated value of 20 to 40 transfers from the measured p value seems to be too small compared with other experimental and theoretical findings. Teale,[58] by adding fluorescence quencher m-dinitrobenzene to Chlorella and measuring its effect on the fluorescence excitation spectra and yield, calculated the average number of energy transfers between Chl a molecules, before the energy is quenched, to be 275. This calculation is done without the assumption regarding the

mutual orientation of the pigment molecules. Bay and Pearlstein,[64] using as a model for the photosynthetic unit a sphere of radius 74 Å with 400 Chl a molecules arranged randomly and a trap in the center of the sphere, calculated the mean number of jumps in a random walk, before it reaches the trap, to be 130. In both calculations, the Förster mechanism[65] of energy transfer was assumed. If a stronger interaction between the Chl molecules is assumed, then the number of jumps will be much larger. Hence, if the chlorophyll molecules are randomly oriented within the chloroplast, the measured polarization of fluorescence should be much lower than that observed. Weber[57] explains this discrepancy by pointing out that the chlorophyll in the chloroplast may be arranged in a partially random fashion and the calculated value for energy migration is the minimum value. Lavorel,[59] however, believes that the discrepancy may be due to Chl a molecules in system I being oriented with respect to one another so that the fluorescence for system I is not depolarized. Although he reported that p at 720 nm is higher than that of p at 685 nm, his measurements may not be correct as mentioned before Goedheer,[60] on measuring a randomly oriented sample of Chlorella, could not find any increased p at 720 nm. Cederstrand and Govindjee[63] showed that p has a value of 0.054 in isolated photosystem I from spinach; this value is close to the p value found for whole cells. It does not seem that the chlorophyll molecules in system I are highly oriented with respect to each other. However, this does not prove that there are no oriented pigment systems within the chloroplast.

The most widely quoted evidence that there are oriented pigments within the chloroplast are the experiments of Olson, Jennings and Butler.[66-70] Using a polarizing microscope, they found that on viewing a single chloroplast on its edge with unpolarized 436 Hg light, the fluorescence intensity is greater in one polarization than in the other. The fluorescence emission spectrum has a peak of 720 nm. These results do not prove that the pigments are oriented with respect to each other within the photosynthetic units but only that the pigments are oriented with respect to the chloroplast. The edge view but not the face view of the chloroplast shows difference in fluorescence intensity in either polarization. These experiments can be interpreted to mean that there are pigments on the edge of the chloroplast which can accept energy from other pigments and are spaced far apart though oriented parallel to each other due to some structural regularity of the chloroplast. The claim by Butler et al.[70] that the Chl a in vivo which absorbs at 680 nm is oriented and is responsible for the fluorescence band at F720 nm has three weaknesses. First, Cho and Govindjee[71] have shown that Chl a which absorbs at 680 nm fluoresces mainly at 685 nm not at 720 nm. Second, no dichroism is found when the face view of the chloroplast is examined even though Chl a 680 is present. Third, Chl a 680 constitutes about half of the Chl a in the chloroplast; if a high dichroic ratio is observed at 695 nm, one should also see a high dichroic ratio at the Soret band but this is not observed. In conclusion, the experiments of Olson, Jennings and Butler show that there are pigments located at the edge of the chloroplast which are oriented in one position relative to the chloroplast structure, but this is not a proof that neighboring

chlorophyll molecules within the photosynthetic unit are oriented with respect to each other.

Although a partially random orientation of the chlorophyll molecules within the chloroplast could explain the discrepancy between the number of energy transfers before the energy is trapped calculated from p and that calculated from other experimental and theoretical findings, the existence of few isolated chlorophyll-protein complexes in the chloroplasts could also explain the discrepancy, this possibility has never been considered in the past. Let us first assume that for every one hundred chlorophyll molecules in the photosynthetic unit, three or four chlorophyll-protein molecules are isolated from the photosynthetic unit and they do not do any useful photochemistry. If one also assumes that these chlorophyll-protein molecules have the same fluorescence yield as the chlorophyll in the photosynthetic unit and that each chlorophyll-protein complex contains only one or two chlorophyll molecules so that there is very little energy migration within the complex, the polarization of fluorescence from these complexes will be high. Although their absorption crossection is assumed to be only 4% of that of the photosynthetic unit, they will still contribute a value of $0.04p_o$ to the overall polarization measurement (p_o is the intrinsic polarization of fluorescence of an isolated molecule). For Chl *a* molecules, p_o is measured to be 0 20 when excited at 630 nm. The p due to the assumed 4% loose chlorophyll-protein complexes would be 0.01. It is probable that the polarization of fluorescence of Chl *a* *in vivo* is due to these isolated chlorophyll-protein complexes if they exist in the chloroplast.

Experiments by Arnold and Meek[55] show that extracted chlorophyll-protein complexes have a p value of 0.16, a value much higher than that measured in vivo. Weber[56] also showed that micellar extracts of cabbage chloroplasts have a tenfold increase in the polarization of fluorescence. However, whether there exist isolated chlorophyll-protein complexes in the chloroplast is an open question. One would expect a plant to maximize its photosynthetic efficiency, and thata four or greater percent "loss" is unlikely. At any rate, there is no experimental evidence that can answer this question.

If we assume that there are no isolated chlorophyll-protein complexes within the chloroplasts, then the measured p is due to the retention of the "memory" of the initial polarization as energy migrates about a partially random system of chlorophyll molecules. Since the extent of energy migration is greater when all the reaction centers are closed than when they are open, we ask how is the polarization affected? The polarization of fluorescence will or will not be affected depending on the mechanism of energy transfer from a chlorophyll molecule to another chlorophyll molecule.

As yet, the mechanism by which excitation energy is transferred within the pigment system to the chemical reaction center is still controversial. It is generally agreed, however, that the mechanism of energy transfer from accessory pigments like phycocyanin to Chl a is that of resonance energy migration, a theoretical mechanism of energy transfer developed by Förster,[65] this is supported by the temperature dependence of the energy transfer from phycocyanin to Chl a in vivo (Cho and Govindjee[72]).

The exact mechanism of energy transfer between Chl a molecules in vivo is not yet known. Energy migration by means of diffusion of molecules can be ruled out because energy can reach the reaction center even at 1°K.[73] Energy migration can occur by means of electrons and holes in conductance bands as in photoconducting crystals. However, as pointed out by Rabinowitch,[74] the absorption spectrum of the chloroplasts does not show an absorption band which supports the existence of a conductance band; the absorption band in vivo is closer to that of Chl a in solution than that of crystalline Chl a. It has been suggested that the electrons and holes could migrate by "tunneling" from one chlorophyll molecule to another to the reaction center. (Recently Floyd, Chance and DeVault[75] have found evidence for the "tunneling" of electrons in the chloroplasts. At 77°K, they found that electrons can "tunnel" from a cytochrome to the reaction center of system II.) Experiments by Arnold and Maclay[76] on dried chloroplasts and experiments by McCree[77] on the photoconductivity of chlorophyll on monolayers show that the charge transfer process is not efficient enough to be the main mechanism for energy transfer within the photosynthetic units in the chloroplast.

The only remaining possibility is that it is the excitation energy that is transferred. Excitation energy can be transferred via the triplet state or the singlet state. The triplet migration can be ruled out from the flash photolysis experiments by Porter and Strauss[78] They could not find absorbance change down to 0.5% due to triplet-triplet absorption in vivo. Hence the fraction of chlorophyll molecules in the triplet state is too small to implicate the excited triplet state as the mechanism of energy transfer to the reaction center. The most reasonable mechanism for energy transfer

within the photosynthetic unit seems to be the migration of the excited singlet state (also see Rabinowitch[74, 79]).

For energy to migrate, some interaction must occur between the excited and unexcited molecules. The interaction energy arises from coulombic interaction[80] between electrons and nuclei of one molecule with those of another and can be represented by a sum of multiple interactions. Because the electric dipole interaction is much stronger than the higher multipole interaction, the higher multipole contribution to energy transfer is generally ignored. However, Robinson[3] points out that these higher pole transitions may be important in cases in which two dipoles are mutually perpendicular to each other. Assuming electric dipole interactions only, three cases of energy transfer, which Förster[81] has designated as "strong coupling," intermediate coupling" and "weak coupling", can occur. A detailed quantum mechanical derivation of the three cases has been done by Förster.[82] A classical derivation of the "weak coupling" case has been done by Hoch and Knox[83] Hence, only the qualitative results will be discussed here. The distinction between the three types of energy transfer is the strength of the interaction of neighboring molecules. The transfer is designated as "strong coupling" if the interaction energy between two molecules is greater than that between the electronic and the nuclear motions within the individual molecules. The transfer of excitation between the two molecules is so fast compared to the nuclear vibrations that the excitation may be regarded as delocalized over the two molecules. In crystals where this type of energy transfer is known to occur, the absorption spectrum is considerably different from that of an individual molecule. This difference

is due to the splitting of the electronic levels due to the strong interaction energy. The absorption spectrum of Chl a in vitro is very similar to the absorption spectrum of Chl a in vivo. This indicates that the energy transfer in the chloroplast is not of the "strong coupling" type. Hence the problem is to decide whether the transfer is of the "intermediate coupling" or the "weak coupling" type.

In the "intermediate coupling" case, the intermolecular interaction is less than the intramolecular interaction between electronic and nuclear motion. The interaction energy is not so great as to cause a distortion in the absorption spectra but it is great enough so that the excitation is still delocalized and the system can be described by stationary vibronic exciton states. Robinson[84] assumed that energy transfer is by delocalized excitons and calculated that the excitation energy can spread over a two dimensional aggregate the size of the photosynthetic unit in a time that is about 1,000 times faster than the lifetime of the singlet state of chlorophyll in the photosynthetic unit. To account for the fluorescence yield at low intensities when all the reaction centers are open, Robinson calculated that the trapping efficiency per encounter with the reaction center is about 1%. In this case, the excitation energy will have an equal probability of being in each chlorophyll molecule in the photosynthetic unit. This probability will be the same independent of whether the reaction center is open or closed. It follows that each molecule in the photosynthetic unit will have an equal probability of emitting a photon regardless of the state of the reaction center.

In the "weak coupling" case, the interaction between two molecules is much less than the intramolecular interaction between electronic and nuclear motion. Under these conditions, thermal equilibrium is established in the excited vibrational level before the excitation is transferred. Here we can regard the excitation as temporarily localized on a single molecule before it is transferred. In the photosynthetic unit, if the energy is transferred by the "weak coupling" case, the excitation can be visualized as a sequence of uncorrelated individual transfer processes ("random walk" or "Brownian motion"). Because the time needed for an excitation to reach the trap is much longer than that of the "intermediate coupling" case, the reaction center is assumed to be irreversible. In this "weak coupling" case, the number of molecules that the localized exciton visits will depend upon the lifetime of the localized exciton. If the reaction center is open, the number of molecules that the localized exciton visits is smaller than if the reaction center is closed.

Polarization of fluorescence can be used to distinguish between these two possible mechanisms of energy transfer among the chlorophyll molecules in the photosynthetic unit. As mentioned before, in the mechanism in which the excitation is delocalized over the photosynthetic unit, the excitation has an equal probability of being in each molecule within the unit regardless of whether the reaction center is open or closed. Hence, fluorescence will come from the same chlorophyll molecules regardless of the state of the reaction center. The polarization of fluorescence will be the same with the reaction center open or closed. In the mechanism in which the excitation is localized and energy migrates by means of random

hopping, the number of molecules that are visited by the excitation when the reaction center is closed is greater than when the reaction center is open. It follows that more molecules are involved in fluorescence when the reaction center is closed. If the molecules are arranged in a random fashion, the fluorescence will be more depolarized if more molecules are involved. The polarization of fluorescence of units with closed reaction centers should be lower than those units with open reaction centers. If the polarization of fluorescence changes on changing the state of the reaction center, then the excitation could be said to be transferred by a hopping process. If the polarization of fluorescence does not change on changing the state of the reaction center, then the excitation is transferred by means of a wave packet. To resolve this question, we have very carefully measured the polarization of fluorescence when most of the reaction centers are open and when all the reaction centers are closed. Our results (Chap. IV) indicate that the excitation energy is transferred by the hopping process.

E. DELAYED LIGHT EMISSION (DLE) AND THE REACTION CENTER

The state of the reaction center, whether it is open or closed, not only changes the fluorescence yield but also changes the yield of DLE. Hence the study of delayed light is important because it is another measurable parameter one can use to study the nature of the reaction center.

Delayed emission was discovered by Strehler and Arnold[85] in 1951 when they found that Chlorella gave off a very dim light minutes after the incident light was turned off. Further experiments showed that this delayed emission had the same emission spectrum as that of fluorescence.[86, 87, 88]

This proves that delayed emission comes from the singlet excited state and not the triplet excited state. Subsequent experiments by other workers have revealed many other properties of this delayed emission. Delayed light originates mainly from photosystem II. Proof of this comes from the action spectra of delayed light[87, 88] and from the fact that Scenedemus or Chlamydomonas mutants which lack photosystem II have negligible delayed emission and those lacking in photosystem I have a delayed emission comparable to that of normal cells.[88, 89] Delayed light may be a two-quanta phenomena. Jones[90] has shown that in the time range of 140-250 milliseconds after the excitation flash, the integrated signal is proportional to the square of the excitation intensity if the algae are kept in the dark for a long time. Delayed emission persists even at liquid nitrogen temperature.[91] Delayed emission has a very complex time decay and is affected by the addition of chemical poisons which affect the reaction centers.[92-99] The time decay does not follow first order kinetics. Qualitatively, the time decay has a fast decaying component in the millisecond region[98] and slow components lasting up to minutes. No quantitative explanation has been published for time course of this phenomenon.

Many theories have been evolved to explain the cause of delayed light emission. In Arnold and Azzi's solid state model,[100] photosystem II is divided into two regions, one containing Chl a only and one containing Chl b only. Each region has its own reaction center. Energy absorbed by Chl b is transferred to a reaction center where a photochemical reaction taken an electron from water and transfers it to the semiconductor band of Chl b in the bulk. Energy that is absorbed by Chl a is transferred to another

reaction center where a photochemical reaction takes an electron from the chlorophyll and transfers it to an electron transport chain. This reaction leaves a hole in the semiconductor band of Chl a which can migrate about the Chl a pigment system. There is a small probability that the electron from the Chl b pigment system can recombine with the hole in the Chl a pigment system at the interface. The recombination of the electron and hole will produce an excited chlorophyll molecule which then gives off a photon which is seen as delayed light. There are many objections to this particular model of Arnold and Azzi. The blue-green alga Anacystis, which lacks Chl b whose existence is vital to the above model, has measurable delayed emission.[101] This objection can be circumvented by giving phycocyanin the role played by Chl b. The existence of two reaction centers for system II plus the reaction center for system I in Arnold and Azzi's model implies that three quanta are needed to transfer an electron from water to $NADP^+$; this implies a minimum quantum requirement of 12 per oxygen evolved. New measurements have confirmed that young Chlorella cells can evolve oxygen with only 8 quanta of light.[102]

In a model similar to that of Arnold's, Bertsch[103] postulates that photosystem II consists of a pigment system with no separation of Chl a and Chl b and of a single reaction center but one with two distinct trapping sites. The trapping sites are similar to Arnold's two reaction centers in that one site oxidizes water leaving an electron in the reaction center and the other site reduces the first intermediate in the electron transport chain leaving a hole in the reaction center. The resultant electron and hole in the reaction center are free to migrate. A collision of an electron and hole

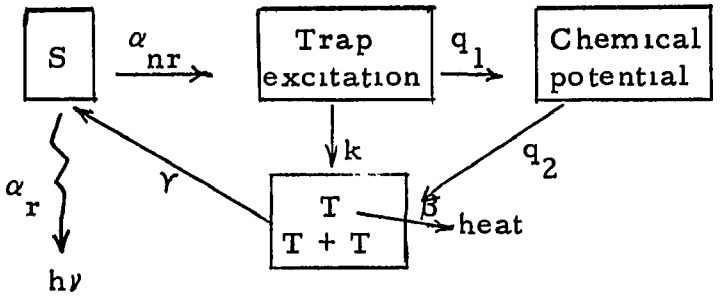
which results in an excited chlorophyll molecule, will give rise to delayed emission. The same objection applies to this model in that the minimum predicted quantum requirement for a molecule of oxygen in this model is twelve while the measured quantum yield is eight.[102]

Other investigators, notably Clayton,[45] have postulated that delayed light is due to a recombination of the first oxidized product (Z^+) and the first reduced product (Q^-) following a photochemical reaction in photosystem II. The energy from the recombination of Z^+ and Q^- can be used to excite a chlorophyll molecule. The resultant fluorescence from the excited singlet chlorophyll molecule is the detected delayed light. As pointed out by Lavorel,[88] this theory has a serious weakness in that the excited state of a chlorophyll molecule requires 1.8 ev. From the measured oxidation-reduction potential of Q^-, Lavorel calculated that only 1.0 ev may be available for such a back reaction. This model cannot explain why the delayed light emission should vary with the square of the incident light as measured by Jones.[90] Lavorel[88] has proposed a model in which triplet state is an intermediate state. It can be either formed directly from the singlet at the trap, or from the back reaction of Q^- with Z^+. The two triplets can then combine to give one singlet. Lavorel did not make quantitative predictions.

Independently of Lavorel and in collaboration with W. Stacy, C. Swenberg and Govindjee,[101] the author has formulated a detailed model which also removes the difficulties faced by other models. We feel the overall process resulting in delayed light emission is as follows. After a photon is absorbed by the chlorophyll in photosystem II, the energy can be lost by radiationless decay or by fluorescence. Most of the energy, however,

can migrate to a reaction center. At the reaction center, the excitation energy may either be used for an oxidation-reduction reaction or it may be lost by fluorescence or internal conversion, or it may undergo intersystem crossing and populate a triplet state of the trap. This triplet exciton can then migrate into the chlorophyll bulk pigment where it can either decay by radiationless transition or it can undergo fusion, and produce an excited singlet state. The resultant emission from the singlet state is the observed delayed light emission. We also assume that the triplets can be produced at the reaction center by a chemical back reaction between the primary oxidant (Z^+) and theprimary reductant (Q^-). We feel that although this chemical back reaction does not have enough energy to populate the singlet state, it does have enough to populate the triplet state. This model does not require two trapping centers as do models which involve electron and hole recombinations and hence is more consistent with quantum yield measurements of oxygen.

Our model can also explain the complex time decay kinetics of the delayed light. Schematically, our model can be seen as:



where S = the excited singlet state

T = the excited triplet state

α_r = radiative rate constant for the singlet state

α_{nr} = non-radiative rate constant

q_1 = rate of energy stored as chemical potential

k = rate of intersystem crossing

q_2 = rate of chemical back reaction multiplied by the concentration of the primary oxidized product and the primary reduced product

γ = rate of recombination of the triplet

β = rate of radiationless decay of the triplet

To facilitate the solving of the rate equations, we will first assume that $q_2 = 0$; this assumption is valid at low light intensities and at times shortly after the incident light is turned off. The three rate equations for our model can be written as:

$$\frac{dn_s}{dt} = -(\alpha_r + \alpha_{nr})n_s + \frac{1}{2}\gamma n_T^2 + G(t) \tag{9}$$

$$\frac{dx}{dt} = \alpha_{nr} n_s - (q_1 + k)x, \quad \alpha_{nr} = C(N-x) \tag{10}$$

$$\frac{dn_T}{dt} = kx - \beta n_T - \gamma n_T^2 \tag{11}$$

where $G(t)$ = rate of populating the singlet

n_s = singlet exciton population density

n_T = triplet exciton population density

X = density of excited trap molecules

N = density of trap molecules

C = constant

The three coupled differential equations cannot be solved in a closed form. At time t, approximately 10^{-3} to 10^{-5} secs. which is long compared with normal fluorescence decay time of about 10^{-9} seconds ($t^{-1} \ll \alpha_r + \alpha_{nr}, k + q_1$), the faster response times of n_s and X compared to that of n_T allow us to make the following assumptions:

$$\frac{dn_s}{dt} = 0 \tag{12}$$

$$\frac{dx}{dt} = 0 \tag{13}$$

$$G(t) = n_s(0)\delta(t) \tag{14}$$

where t = 0 is set at the instant when the excitation light is shut off. Under conditions which allow us to assume $q_2 = 0$, $N \gg X$

$$\frac{dn_T}{dt} = -\xi\gamma n_T^2 - \beta n_T \tag{15}$$

where $$\xi = 1 - \frac{1}{2}(1 - \phi)\left(\frac{k}{k+q_1}\right)$$

$$\phi = \frac{\alpha_r}{\alpha_r + \alpha_{nr}}$$

Equation 15 can be solved with the initial condition $n_T(0) = n_{To}$ = constant. The solution is

$$n_T(t) = \frac{\beta n_{To}}{(\xi\gamma n_{To} + \beta)\exp(\beta t) - \xi\gamma n_{To}} \tag{16}$$

The DLE produced by triplet exciton fusion is:

$$F(t) = \frac{1}{2} \gamma \phi n_T^2 \quad (t) \tag{17}$$

Hence we get:

$$F(t) = \frac{\beta^2 F_o}{\left\{\left[\left(\frac{2\xi^2 \gamma F_o}{\phi}\right)^{1/2} + \beta\right] \exp(\beta t) - \left(\frac{2\xi^2 \gamma F_o}{\phi}\right)^{1/2}\right\}^2} \tag{18}$$

where $F_o = F(0)$.

Note that the above equation is derived for delta function light excitation. For excitation with rectangular pulses of light, we have:

$$n_T(t) = \frac{1}{T} \int_{-T}^{o} \frac{\beta\, n_{To}\, du}{(\xi \gamma n_{To} + \beta) \exp(\beta t - \beta u) - \xi \gamma n_{To}} \tag{19}$$

where $t = 0$ is the instant that the excitation pulse is shut off and $t = T$ is the pulse length. Integrating equation 19 and using equation 17, we have:

$$F(t) = \frac{F_o}{(\lambda T)^2} \left[-\beta T + \ell n \frac{(\lambda+\beta)\exp(\beta t+\beta T) - \lambda}{(\lambda+\beta)\exp(\beta t) - \lambda}\right]^2 \tag{20}$$

where $\lambda^2 = \frac{2\xi^2 \gamma F_o}{\phi}$

At incident light intensities so low that

$$\left(\frac{2\xi^2 \gamma F_o}{\phi}\right)^{1/2} << \beta$$

is satisfied,

$$n_T(t) = \frac{1}{T} \int_{-T}^{o} \frac{\beta n_{To}\, du}{\beta \exp(\beta t - \beta u)}$$

$$= n_{To} \exp(-t) + \text{constant} \tag{21}$$

so that

$$F(t) = F_o \exp(-2\beta t) + \text{constant} \qquad (22)$$

At low light intensities, the DLE is a simple exponential decay with a decay lifetime of $1/2\beta$.

We have numerically fitted these time dependence of the DLE predicted from our model with the experimental data. The DLE in the green alga Chlorella pyrenoidosa has been measured in the range of 1 to 12 milliseconds after excitation with light pulses ($\lambda = 6328\text{Å}$) of 100 microsecond and 4.5 millisecond duration. Figure 1 shows the theoretical and experimental time dependence of DLE with 100 microsecond flash excitation. Note that the addition of chemicals, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and hydroxylamine which affects in a region close to the reaction center, changes the rate constant ζ. From equation 18 it can be seen that an increase in ζ implies an increase in q_1. Thus the data indicates that since cells with DCMU decrease ζ over normal cells, the addition of DCMU causes a decrease in q_1, the rate at which the trap molecule is chemically deactivated. The addition of hydroxylamine to the cell has the opposite effect. Figure 2 shows the theoretical and experimental time dependence of DLE with light pulses of 4.5 millisecond duration. Only in the cases of the normal cell and cells with hydroxylamine could the theoretical curve match with the experimental curve. The failure to fit the DCMU case is due to the presence of the slow build-up component in the decay curves. This was explicitly left out of the theoretical rate equation by setting q_2 equal to zero. Methyl viologen is a strong electron acceptor just above the

level of ferredoxin.[104] It does not affect the region close to the reaction center of photosystem II. Hence the addition of methyl viologen to cells with hydroxylamine or with DCMU should not affect the kinetics of the delayed light.

The present theory also predicts that the DLE should vary with the square of the intensity of the exciting light at low light intensity. Figure 3 shows our measurement of the dependence of the delayed light intensity on the intensity of the excitation light in the 1.5 to 5.0 msec range. The square law dependence reported by Jones[90] in the 140 to 250 msec range is confirmed in the 1.5 to 5.0 msec range at very low light levels.

It seems that the triplet fusion model offers a valid alternate explanation to that of the electron hole recombination for the origin of DLE. However, the existence of the chlorophyll triplet state *in vivo* has not been found experimentally. Flash photolysis measurements[78] have failed to show any triplet-triplet absorption. Phosphorescence from Chl *a in vivo* has not been reported.[105] The latter is not a valid objection against the existence of the triplet state because the rate of internal conversion could be greater than the rate of intersystem crossing inside the cell. Flash photolysis measurements, as used by Porter and Strauss[78] to detect chlorophyll triplets in the chloroplast, are only sensitive enough to resolve absorbance changes down to 0.5%. A calculation of the triplet exciton density will show that this is not sensitive enough to detect the triplet state (see Chapter V). At one millisecond after the excitation light was turned off a typical emission rate (F) for Chlorella was approximately 10^8 photons/cm^2 sec. With $\gamma \sim 10^{-4}$ cm^2/sec and $\phi \sim 2 \times 10^{-2}$, the triplet density at one millisecond is

Figure 1. Intensity of flash excited delayed emission (labeled fluorescence) versus time for Chlorella pyrenoidosa (normal), for Chlorella with 10^{-5}M DCMU and for Chlorella with 10^{-3}M hydroxylamine. Excitation pulse is 100 μsec wide with 50 μsec rise and fall time. Solid dots are experimental points. Solid lines are theoretical curves calculated from the kinetic model of triplet-triplet fusion (see text). (After Stacy, Mar, Swenberg and Govindjee.[101])

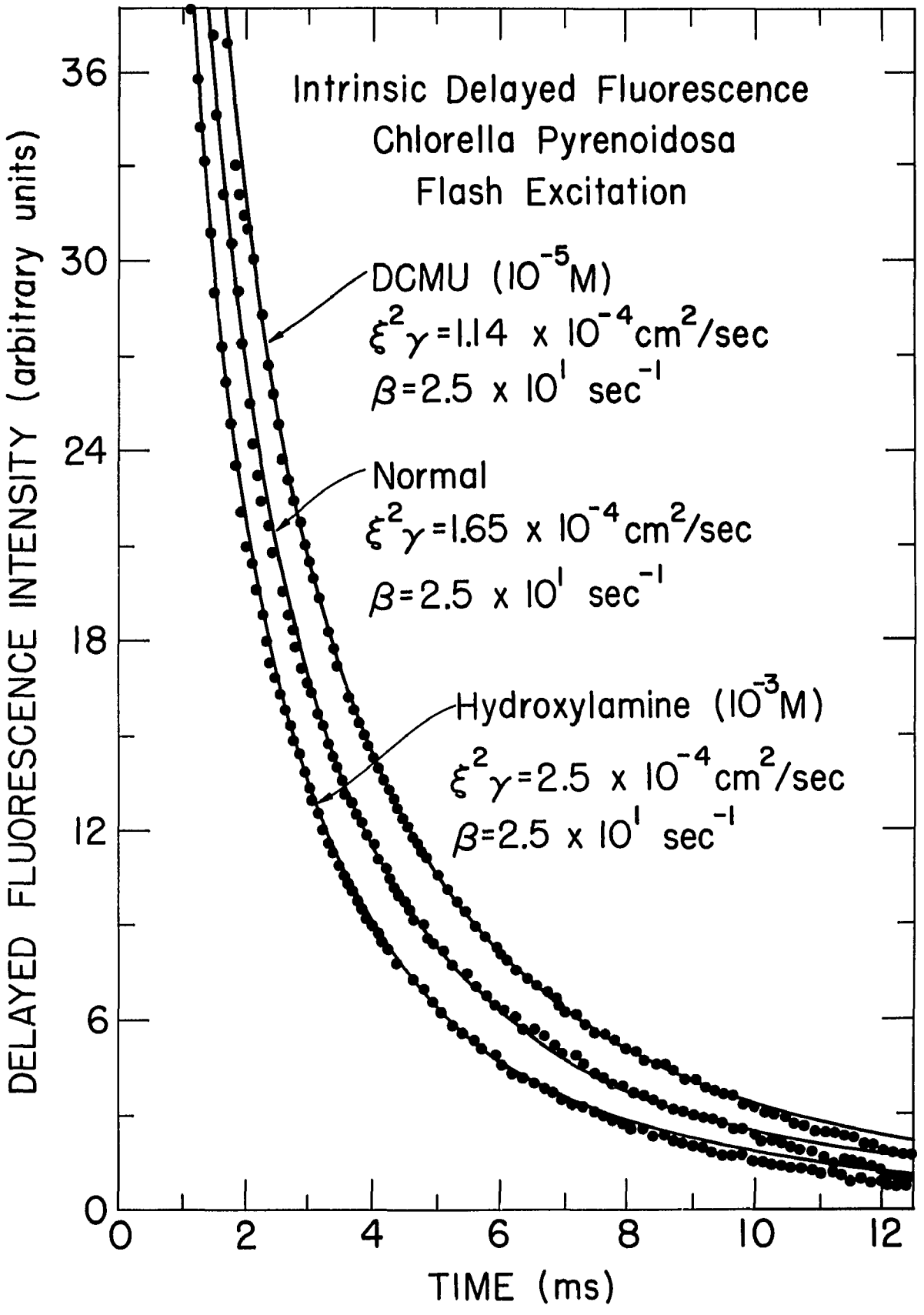

Intrinsic Delayed Fluorescence
Chlorella Pyrenoidosa
Flash Excitation
DCMU (10^{-5}M)
$\xi^2\gamma = 1.14 \times 10^{-4}$ cm^2/sec
$\beta = 2.5 \times 10^{1}$ sec^{-1}
Normal
$\xi^2\gamma = 1.65 \times 10^{-4}$ cm^2/sec
$\beta = 2.5 \times 10^{1}$ sec^{-1}
Hydroxylamine (10^{-3}M)
$\xi^2\gamma = 2.5 \times 10^{-4}$ cm^2/sec
$\beta = 2.5 \times 10^{1}$ sec^{-1}
DELAYED FLUORESCENCE INTENSITY (arbitrary units)
0
6
12
18
24
30
36
0
2
4
6
8
10
12
TIME (ms)

Figure 2A. Intensity of square wave excited delayed light emission (labelled fluorescence) versus time for Chlorella pyrenoidosa (normal), for Chlorella with 10^{-3}M methyl viologen and for Chlorella with 10^{-5}M DCMU. Excitation pulse is 4.5 msec with a rise time of 50 μsec. Repetition rate is 25 hz. Solid dots are experimental points. Solid line is theoretical curve calculated from the kinetic model of triplet-triplet fusion (see text). (After Stacy, Mar, Swenberg and Govindjee.[101])

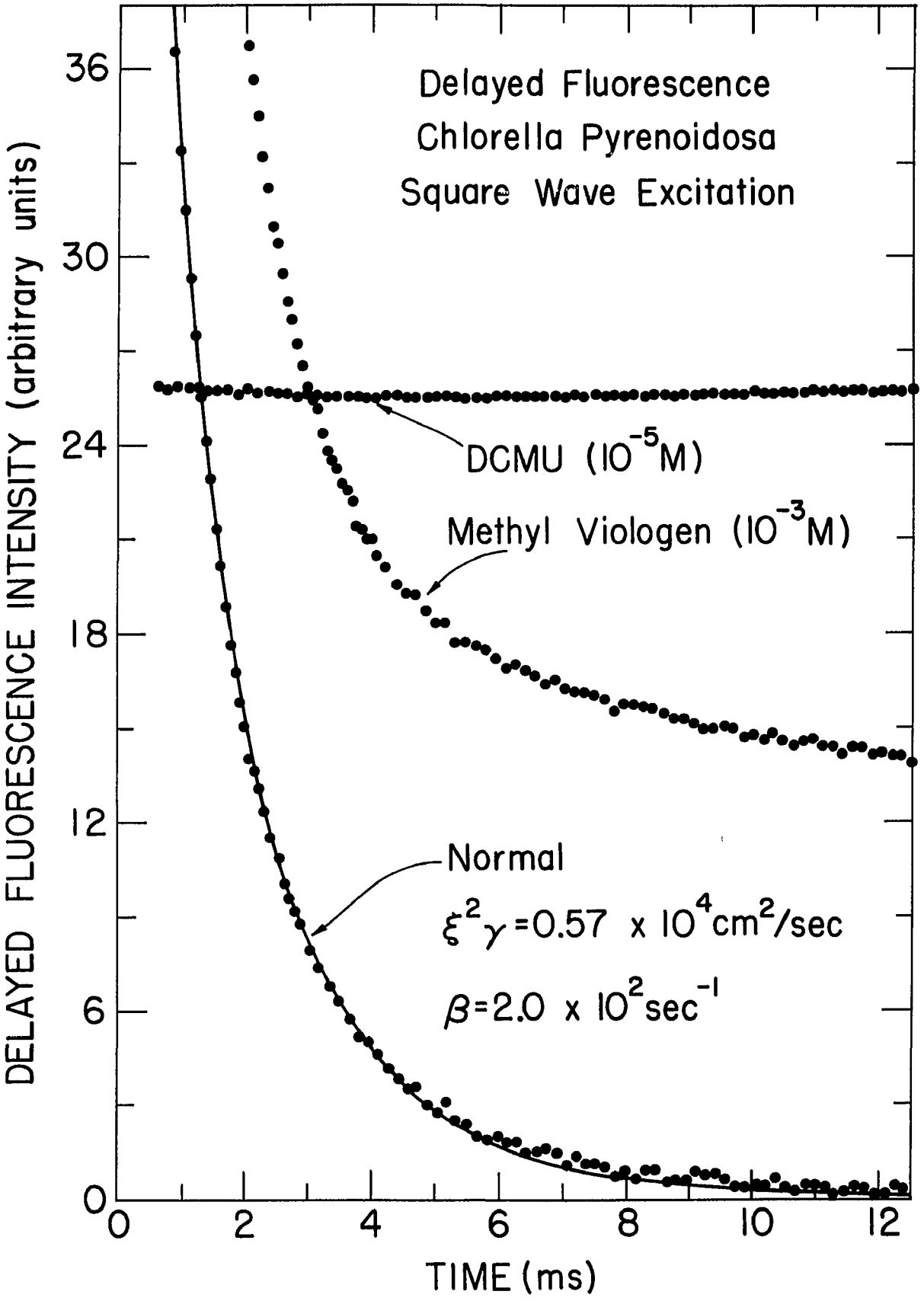
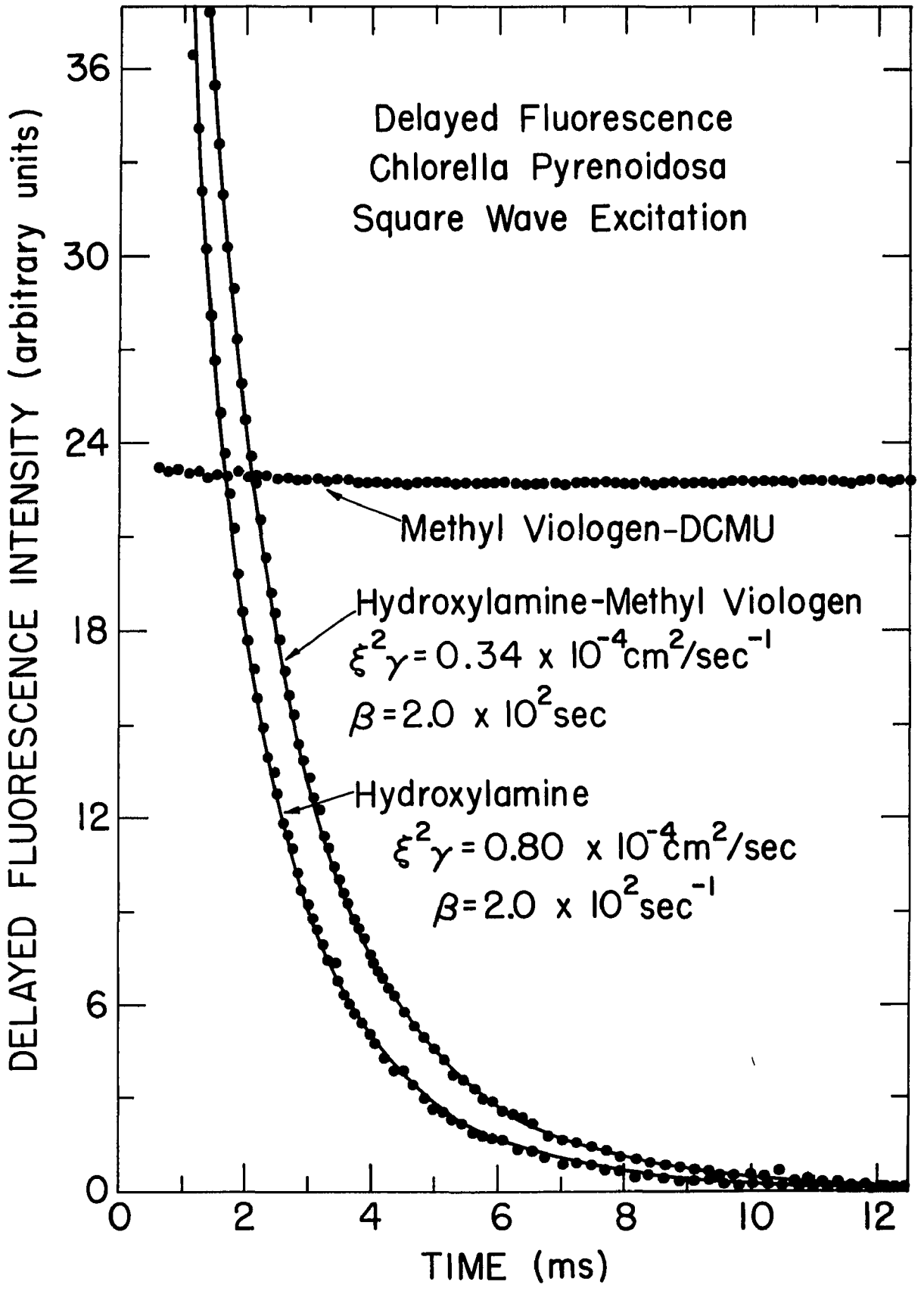

Delayed Fluorescence
Chlorella Pyrenoidosa
Square Wave Excitation
DCMU (10^{-5}M)
Methyl Viologen (10^{-3}M)
Normal
$\xi^2\gamma = 0.57 \times 10^4 cm^2/sec$
$\beta = 2.0 \times 10^2 sec^{-1}$
DELAYED FLUORESCENCE INTENSITY (arbitrary units)
TIME (ms)
0
6
12
18
24
30
36
0
2
4
6
8
10
12

Figure 2B. Intensity of square wave excited delayed light emission (labelled fluorescence) intensity versus time for Chlorella pyrenoidosa with 10^{-3}M hydroxylamine, for Chlorella with both 10^{-3}M methyl viologen and 10^{-5}M DCMU and for Chlorella with both 10^{-3}M hydroxylamine and 10^{-3}M methyl viologen. (After Stacy, Mar, Swenberg and Govindjee.[101])

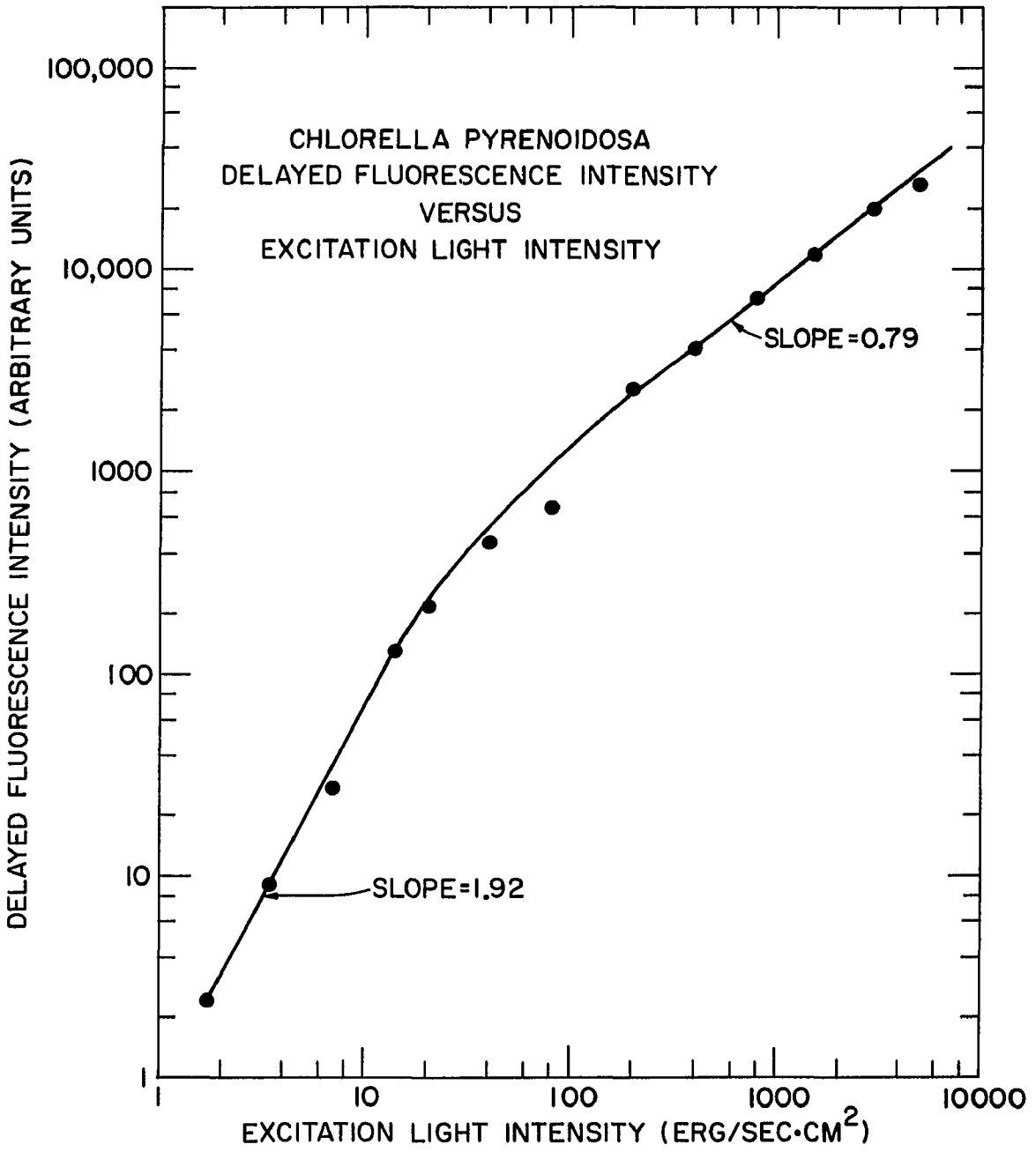

Delayed Fluorescence
Chlorella Pyrenoidosa
Square Wave Excitation
Methyl Viologen-DCMU
Hydroxylamine-Methyl Viologen
$\xi^2\gamma = 0.34 \times 10^{-4} cm^2/sec^{-1}$
$\beta = 2.0 \times 10^2 sec$
Hydroxylamine
$\xi^2\gamma = 0.80 \times 10^{-4} cm^2/sec$
$\beta = 2.0 \times 10^2 sec^{-1}$
DELAYED FLUORESCENCE INTENSITY (arbitrary units)
TIME (ms)
0
6
12
18
24
30
36
0
2
4
6
8
10
12

$n_T = \left(\frac{2F}{\gamma\phi}\right)^{1/2} \sim 10^7$ cm^2. If the lamellar area per chlorophyll molecule is 2×10^{-14} cm^2, then the proportion of the triplet state is approximately 2×10^{-7}. The expected change in the absorption spectrum corresponding to the above triplet density is less than 10^{-6}. Detailed calculations are presented in Chapter V.

We have tried[101] -- although unsuccessfully -- to directly prove the existence of the triplet state by measuring the effect of a magnetic field on delayed light. It has been shown that in crystalline anthracene and tetracene, the delayed fluorescence produced by triplet exciton fusion is sensitive to a magnetic field.[106] Within the limits of experimental accuracy (2%), we found no changes in the intensity of DLE with fields as high as 18 kilogauss. It has been theoretically shown by Merrifield[107] that the reason why the magnetic field affects the intensity of the DLE in anthracene and tetracene crystals is that the magnetic field affects the probability that a particular collision between two triplet excitons will result in a singlet exciton. Therefore, the reason we did not observe any magnetic effect may be due to the low annihilation probability of the triplet to singlet within the chloroplast.[108] The direct proof of the triplet state would require a more sensitive search for a magnetic field dependence of the DLE or a more sensitive flash photolysis measurement.

In this investigation, we have extended the DLE measurements to a red alga, Porphyridium cruentum (see Chapter V). We have also explored more fully the assumption that the long time delayed light emission is due to a back reaction of the first oxidized and reduced products to populate a triplet

Figure 3. Logarithmic plot of the intensity of delayed emission (labelled fluorescence) intensity versus excitation light intensity for Chlorella pyrenoidosa. (After Stacy, Mar, Swenberg and Govindjee [101]).


CHLORELLA PYRENOIDOSA
DELAYED FLUORESCENCE INTENSITY
VERSUS
EXCITATION LIGHT INTENSITY
SLOPE=0.79
SLOPE=1.92
DELAYED FLUORESCENCE INTENSITY (ARBITRARY UNITS)
EXCITATION LIGHT INTENSITY (ERG/SEC·CM²)
100,000
10,000
1000
100
10
1
10
100
1000
10000

state in the reaction center. Confirmation of these ideas was obtained by measurements of fluorescence induction and of chemiluminescence and thermoluminescence (see Chapter V).

The details of the chemical reactions, at the reaction center II, leading to oxygen evolution are discussed in Appendix I. Two new models as alternatives of the models of Joliot et al.[109] and of Kok et al.[110] are presented. These models explain all the existing data on kinetics of oxygen evolution.

II. MATERIALS AND METHODS

A. BIOLOGICAL SPECIMENS AND PREPARATION OF SAMPLES

Unicellular algae were used in all the experiments performed in this study. These algae were chosen because they are well characterized and are convenient to prepare for experimentation. They can be grown under controlled conditions at all times. Their population per unit volume is large so that the measured value is an average value of thousands of cells. Also they do not lose their activity within the time of experimentation as chloroplasts extracted from leaves do.

The three algae used were the green alga Chlorella pyrenoidosa, the red alga Porphyridium cruentum, and the blue-green alga Anacystis nidulans. Chlorella pyrenoidosa is spherical in shape with a diameter of 5 microns. The chloroplast is cup shaped and occupies more than half the cell. The pigments Chl a, Chl b and carotenoids are found within the chloroplast. Each cell of Porphyridium cruentum is enclosed in a gelatinous sheath varying in thickness from a thin membrane to an envelope half as thick as the diameter of the cell. The cell diameter has been measured to be 9.68 to 12.6 microns including the outer sheath.[111] Chloroplasts of Porphyridium cruentum contain phycoerythrin and also phycocyanin instead of Chl b as accessory pigments. Anacystis nidulans is different from Chlorella and Porphyridium in that it does not have a chloroplast membrane. It has phycocyanin and allophycocyanin instead of Chl b as accessory pigments Anacystis[112] has been shown to be cylindrical in shape with a mean diameter of 0.92 ($\pm$ 0.01)μ and length of 2.67 ($\pm$ 0.82)μ.

All the algae used in this study were cultured by Mr. Alan Stemler. The growth conditions and the nutrient media used were patterned after those described by Govindjee and Rabinowitch.[113] A continuous flow of 5% CO_2 mixed with air was used for the growth of Chlorella and Porphyridium while only air was used for Anacystis. The temperature was held at 18°C for the growth of Chlorella and Porphyridium and at 23°C for Anacystis. Light for the growth of Chlorella and Anacystis was obtained from a 60 watt tungsten bulb placed 12 inches away from the algae, while light used for the growth of Porphyridium was obtained from two 15 watt cool-white fluorescent lamps which were placed 12 inches away from the algae.

To prepare the algae for experimentation, they were spun down in a centrifuge at 5000 x g for five minutes, the growth medium was discarded and the cells were resuspended in carbonate-bicarbonate buffer. The buffer is used to maintain the CO_2 concentration above the value which would limit photosynthesis and to stop the growth of the algae since the buffer lacks many inorganic nutrients necessary for growth. For Chlorella and Anacystis, an 85 to 15 mixture of 0.1 molar solution of $NaHCO_3$ and K_2CO_3 (pH, 9.2) was used. For Porphyridium, the buffer consisted of 16 g. of $NaHCO_3$, 1.0 g of Na_2CO_3, and 15.2 g. of NaCl per liter of water (pH, 8.5).

B. ABSORPTION MEASUREMENTS

Absorbance measurements were made on each algae sample used to check the pigment composition of the algae and to adjust the concentration of the algae to the particular absorbance needed for the experiment. Absorbances

were measured on a Bausch and Lomb spectrophotometer, Spectronic 505, equipped with an integrating sphere to correct for errors due to scattering. The measuring half band width was 5 nm.

C. FLUORESCENCE MEASUREMENTS

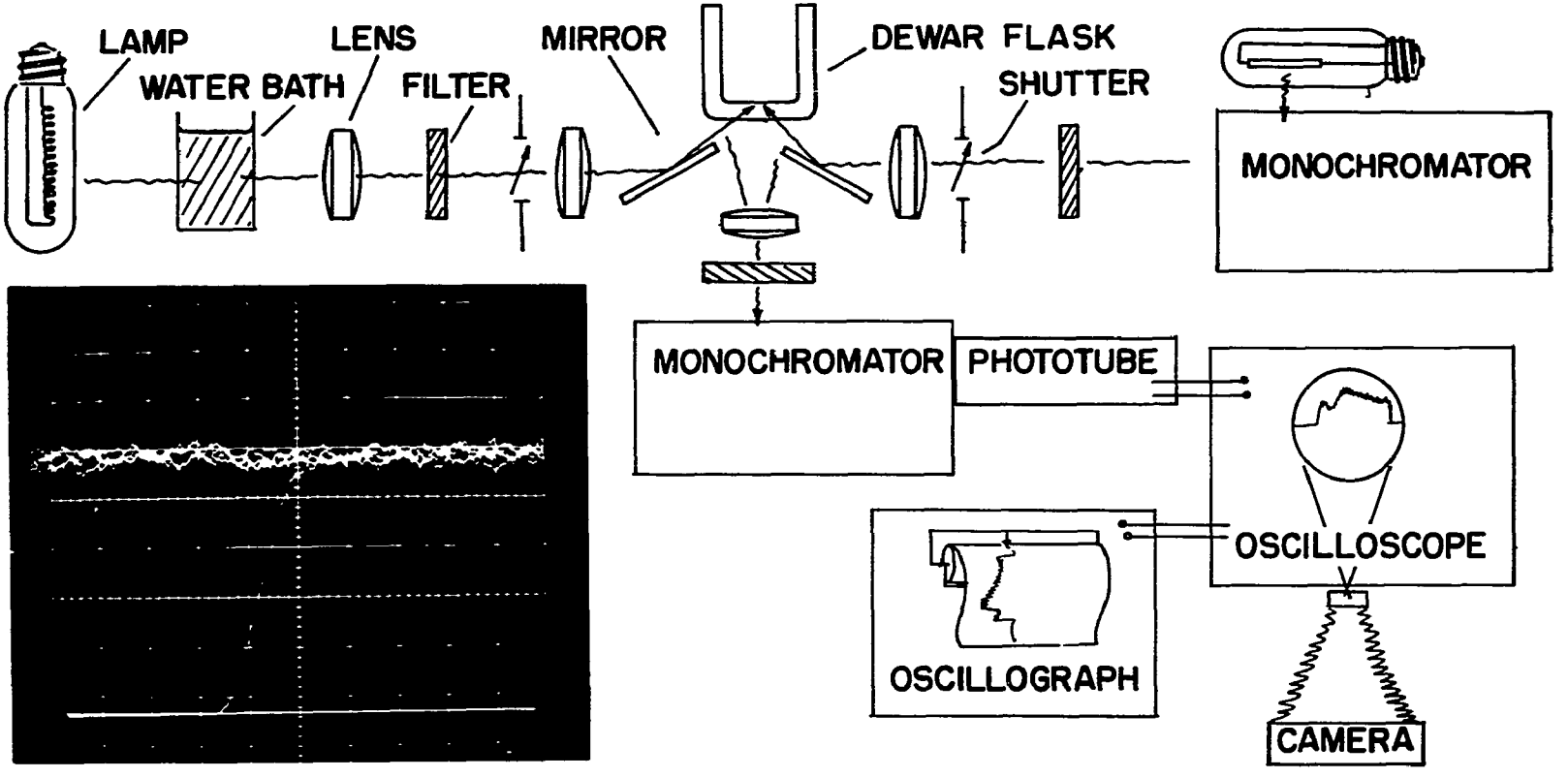
Fluorescence measurements were carried out with a spectrofluorometer designed and built by Govindjee and Spencer (see ref. 114). The instrument is shown in Figure 4. The procedure for measuring fluorescence at liquid nitrogen temperature was the same as that described by Cho and Govindjee.[71] The procedure for measuring fluorescence transients was the same as that outlined in detail by Munday and Govindjee.[115]

D. FLUORESCENCE LIFETIME MEASUREMENTS

The instrumentation for the measurement of fluorescence lifetime centers around the mode-locked He-Ne laser.[116] The laser was designed and built by Dr. H. Merkelo and Mr S. R. Hartmann of the Electrical Engineering Department at the U. of I.

The mode-locked He-Ne laser[40] emits pulses of 6328 Å light with a half band width of 1.2 nsec, every 10.22 nsec, as measured by a "PIN" diode. A picture of the nanosecond pulse is shown in Figure 5. The average intensity is 10^5 ergs per cm^2 per second. Measurement of the pulse with a photomultiplier shows the pulse as having a wider half band width. This is shown in Figure 6. This shows that the limiting factor in measuring fluorescence lifetime is not due to the light source but is due to the poor time response of the photomultiplier.

Figure 4. Block diagram of the spectrofluorometer.[115] The oscilloscope picture of the sloping vertical line on the bottom left of the diagram shows the phototube signal when the shutter is opened. Horizontal scale is one msec/cm.


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

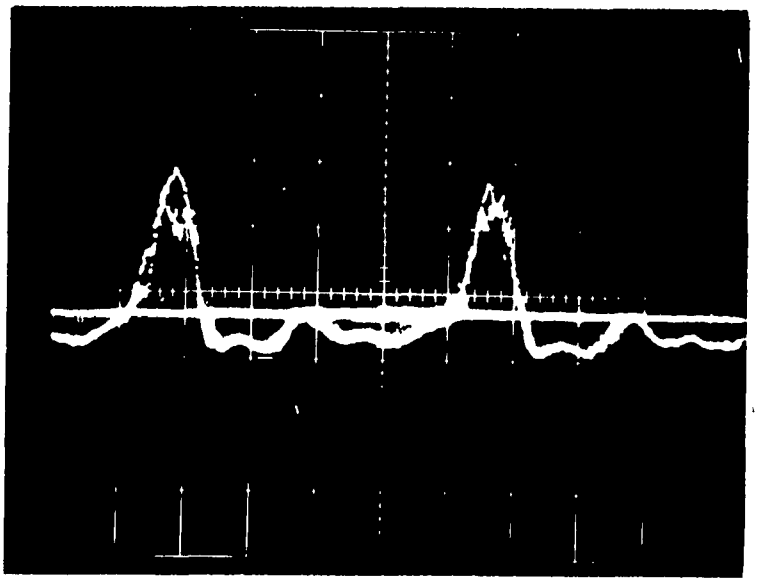


Figure 5. A photograph of a sampling oscilloscope trace showing a mode-locked laser pulse as monitored by a "PIN" diode. Horizontal scale is two nsec/division.

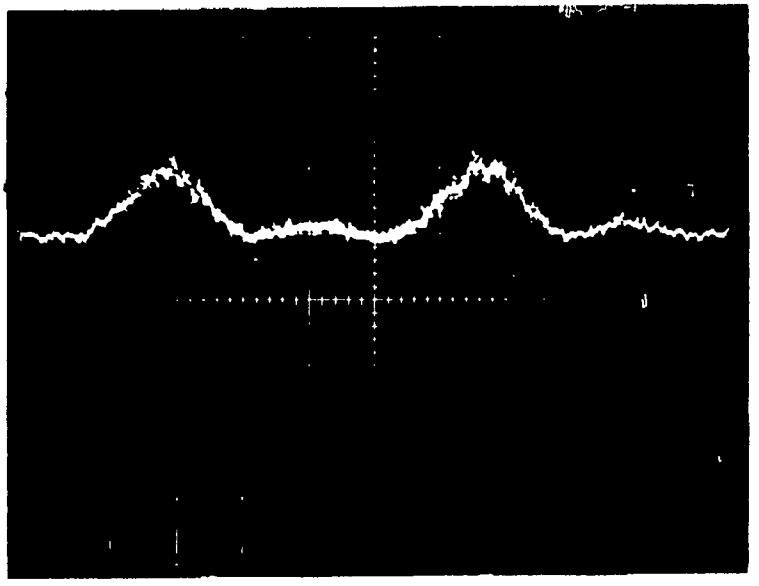


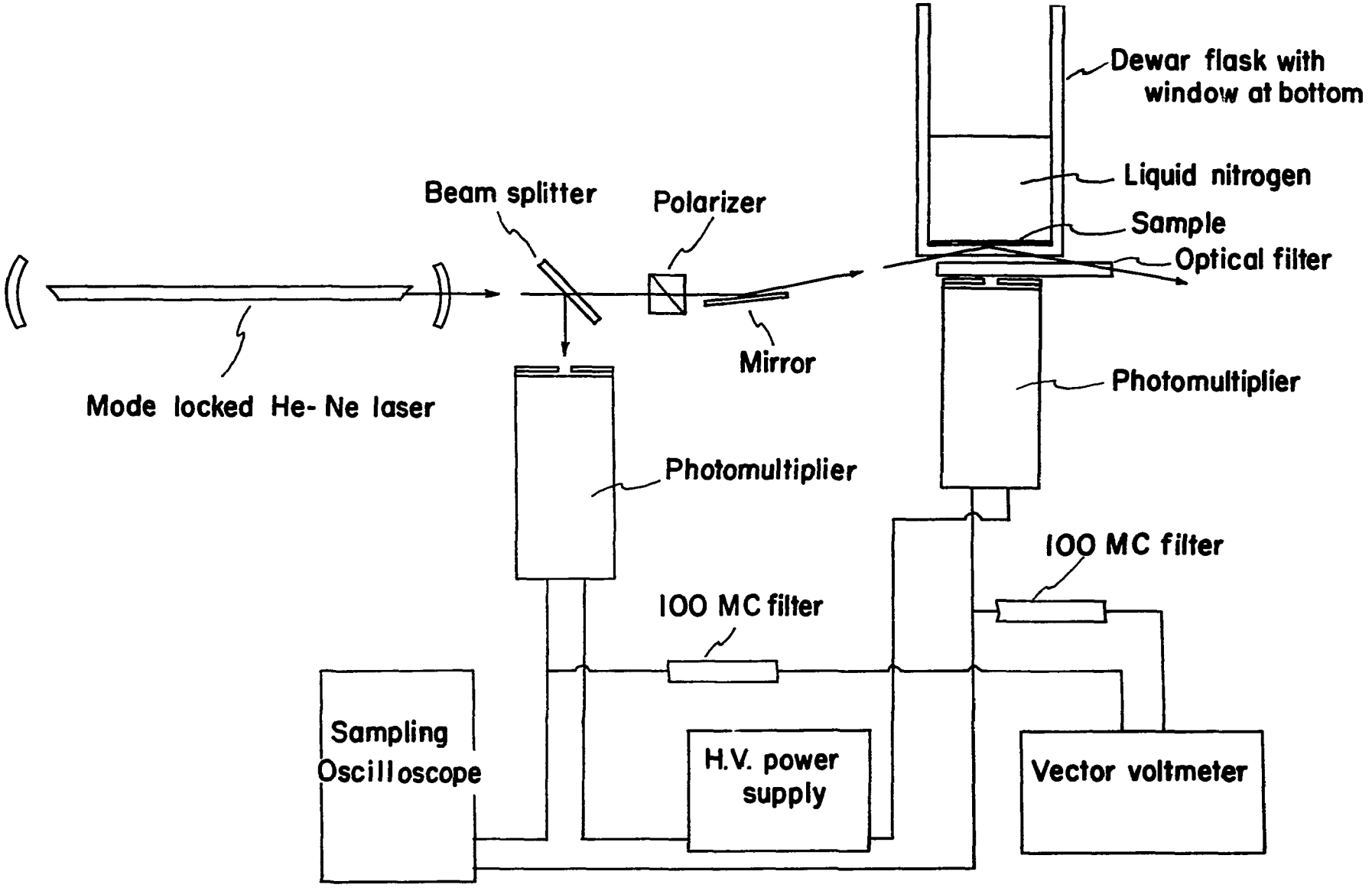
Figure 6. A photograph of a sampling oscilloscope trace showing a mode-locked laser pulse as monitored by a photomultiplier (RCA 7102). Horizontal scale is two nsec/division.

The photomultiplier used was an RCA 7102. Its photocathode has an S-1 response which is ideal for measuring fluorescence from photosynthetic systems. The biasing voltage for the photomultiplier was generally 1500-1600 volts. The photomultiplier is shielded from stray magnetic fields by a mu-metal cylinder. To minimize the transit time spread of the electrons in the photomultiplier,[32, 38] the photocathode was covered except for an aperture of 5 mm in diameter at the center.

For direct observation of the fluorescence decay with time, the arrangement of the instrument was similar to the one shown in Figure 7 (modified after ref. 40). The incident light from the He-Ne laser is partially reflected by a microscope glass slide which acts as a beam splitter This reflected light excites a photomultiplier, and the resulting signal from the photomultiplier acts as a trigger for the sampling oscilloscope. The light beam passes through a one cm cuvette, the surface of the cuvette facing the incident light is kept perpendicular to the direction of that beam. The position of the cuvette is adjusted so that the beam is about one mm from the side that is parallel with the beam. The measuring photomultiplier is placed perpendicular to the exciting beam. It is placed as close as possible to the cuvette and is separated only by a red cut-off light filter (Corning CS 2-64) which is used to block the scattered exciting light from reaching the photomultiplier. The signal from the photomultiplier is amplified by a Tektronic 661 Sampling oscilloscope. The signal displayed on the oscilloscope screen was recorded on Polaroid 3000 film.

The measurement is made in the following way. The picture for the fluorescence decay of the sample is first taken. Then without disturbing

Figure 7. Block diagram of the apparatus for the measurement of fluorescence lifetimes at 77°K (modified after ref. 40).


Dewar flask with window at bottom
Liquid nitrogen
Sample
Optical filter
Beam splitter
Polarizer
Mirror
Mode locked He- Ne laser
Photomultiplier
Photomultiplier
100 MC filter
100 MC filter
Sampling Oscilloscope
H.V. power supply
Vector voltmeter

the biasing voltage in the photomultiplier, a scattering suspension of "chalk" is substituted for the biological sample, the red cut-off filter is taken out and the intensity of the scattered light is adjusted so that its signal detected by the photomultiplier is nearly equal to that of the signal due to fluorescence. This is done by using a polarizer placed between the cuvette and the laser. Since the beam is polarized, the polarizer acts as a variable intensity filter. The signal from the scattered sample is recorded and this is used as the data for the response of the whole instrument.

For direct measurement of fluorescence lifetime at liquid nitrogen temperature, the arrangement of Dr. Merkelo's apparatus is as shown in Figure 7. The dewar flask and the procedure for freezing the cell are the same as that of Cho and Govindjee.[71, 117] Instead of using a scattering sample of "chalk" to monitor the photomultiplier response, the scattered light from the sample itself was used. The wavelength of excitation was 6328 Å as in other experiments.

The optical arrangement of the instrument for the phase shift measurements is exactly the same as that for direct decay measurements. Since the mode-locked iHe-Ne laser emits pulses of light every 10.22 nsec, no external modulator is needed to modulate the light. The signals from the reference and the signal photomultiplier are passed through a 100 Mhz electrical filter into a phasemeter (Hewlett Packard Model 8405A Vector Voltmeter). The phasemeter compares the phase difference between the reference signal and the fluorescence signal and produces a voltage output that is proportional to the phase difference. The voltage output is then fed into a chart recorder and the phase difference is recorded. The phasemeter

can measure voltage of either the reference signal or the fluorescence signal at the same time as it measures the phase difference. First, the sample fluorescence is measured, then the sample and the red cut-off filter are removed. A chalk suspension is then placed in the same position as the sample without changing any adjustment of the instrument. The polarizer is used to adjust the intensity of the laser so that the scattered light intensity equals that of the fluorescence light intensity measured previously. The phase difference as measured by the phasemeter is set near zero. The signal recorded on the chart recorder will act as the baseline. The chalk suspension is then removed and the sample suspension and the filter are replaced. The resulting phase difference signal is then recorded by the chart recorder.

The calibration of the phase shift apparatus was checked in two ways. The first method was by using the apparatus to measure the velocity of light. First the phasemeter was set at zero by using a scattering suspension. The distance between the scattering suspension and the laser was marked. This distance was then varied and the resultant change in the phase was recorded. Since the change in phase is related to the change in time by

$$\Delta \phi = 360^{o} f \Delta t \qquad (23)$$

where f is the pulse frequency

Δt is the change in time

$\Delta \phi$ is the change in phase in degrees

$$\Delta t = \frac{\Delta d}{c} \qquad (24)$$

where Δd is the change in distance

c is the velocity of light

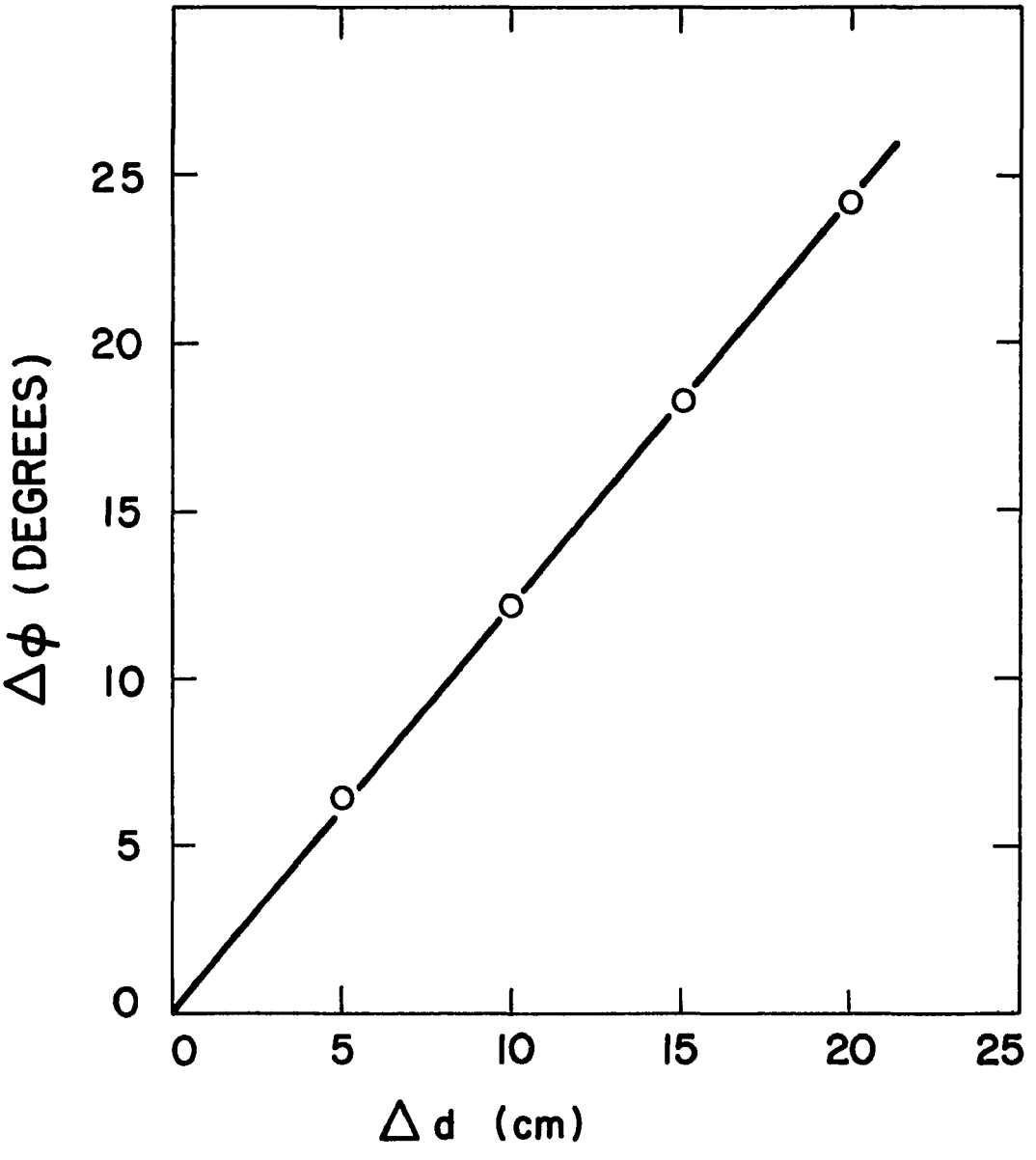
Combining equations 23 and 24, we get:

$$c = 360^{o} f \frac{\Delta d}{\Delta \phi} \qquad (25)$$

where $\frac{\Delta d}{\Delta \phi}$ is the slope calculated from the experimental values plotted in Figure 8. Since $\frac{\Delta d}{\Delta \phi}$ is calculated to be .8210 cm/degree and f is measured to be 102.207 Mhz, the velocity of light is measured to be 3.02×10^{10} cm/sec This value differs from the standard value for the velocity of light (2.997×10^{10} cm/sec.) by less than 1%. The second method of checking the calibration of the phase shift apparatus is by measuring the fluorescence of Chl b in ethyl ether with values obtained by flash excitation. The lifetime of Chl b measured by the phase method is 3.87 ± 0.005 nsec. This is in good agreement with Brody's[42] value of 3.9 ± 0.4 nsec.

One cause of systematic error that we cannot eliminate completely is the phase dependence on the wavelength at which the fluorescence emission is detected. Müller et al.[38] have reported differences of 1.5 nsec in fluorescence lifetimes of Chl a in vitro when measured with 682 nm scattered light and 450 nm scattered light as the reference. Their measurements show that the error becomes larger as the spectral difference between the wavelengths of the scattering light used as reference and the wavelength of fluorescence increases. Since the He-Ne laser emits only one frequency of light, we cannot correct exactly for this type of error by using the same wavelength of the scattered light as the fluorescence. Assuming that methylene blue has a constant lifetime over its whole fluorescence spectrum, the difference in phase when measured at different fluorescent wavelengths was used as the instrumental error.

Figure 8. The change in phase versus the change in the distance of the scattering sample from the laser.


25
20
15
10
5
0
Δφ (DEGREES)
0
5
10
15
20
25
Δ d (cm)

E. POLARIZATION OF FLUORESCENCE MEASUREMENTS

The apparatus to measure the polarization of fluorescence was assembled by the author; it was designed to eliminate systematic errors in the measurement. Theoretically only two measurements are needed to obtain the polarization -- one is the fluorescence ($_{\parallel}F_{\parallel}$) that is polarized in the same direction as the incident light and that is given off in a direction perpendicular to the direction of the incident light; the other is the fluorescence ($_{\parallel}F_{\perp}$) that is polarized perpendicular to the polarization of the incident light and that is also given off perpendicular to the direction of the incident light. As shown in Figure 9, the polarization of the incident light is perpendicular both to the direction of observation and to the direction in which the beam is travelling. The polarization of fluorescence, as mentioned in the introduction, is defined as:

$$p = \frac{{}_{\parallel}F_{\parallel} - {}_{\parallel}F_{\perp}}{{}_{\parallel}F_{\parallel} + {}_{\parallel}F_{\perp}} \qquad (8')$$

The systematic errors in measuring p come mainly from instrumental factors which may selectively transmit one polarization more than another. If the polarizer is not mounted perfectly, a rotation by 90^{o} may either tilt the polarizer very slightly to one side or may offset the polarizer from the center of the light path. These errors can easily be detected by watching the reflection of a laser beam from the face of a polarizer rotate in a small circle as one rotates the polarizer. This will cause light of one polarization to be transmitted more than another. The cut-off colored filters transmit the light of one polarization more than the other if they

are not perfectly perpendicular to the incident light. The photomultiplier has been found to respond differently to the light of different polarizations.[118] Other systematic errors in measuring p have been discussed in detail by Weber.[119]

To overcome these systematic errors, we made two other measurements in addition to $_{\parallel}F_{\parallel}$ and $_{\parallel}F_{\perp}$. We measured the intensity of the fluorescence with polarization parallel ($_{\perp}F_{\parallel}$) and perpendicular ($_{\perp}F_{\perp}$) to that of the incident light except that the polarization of the incident light was now rotated by 90° so that it was parallel to the direction of observation of the fluorescence but still perpendicular to its own path. This is shown in Figure 9. Geometrically, one can easily see that $_{\perp}F_{\parallel}$ must be equal to $_{\perp}F_{\perp}$ because the polarization of the incident light is perpendicular to both $_{\perp}F_{\parallel}$ and $_{\perp}F_{\perp}$, and there is no reason why fluorescence polarized in one direction should be different from fluorescence polarized in another direction. Any measured differences then must be due to instrumental factors The ratio $_{\perp}F_{\parallel}/_{\perp}F_{\perp}$ can be used to correct for the error in measuring $_{\parallel}F_{\perp}$. Applying this correction factor to the definition of p, we have:

$$p = \frac{{}_{\parallel}F_{\parallel} - {}_{\parallel}F_{\perp}\left(\frac{{}_{\perp}F_{\parallel}}{{}_{\perp}F_{\perp}}\right)}{{}_{\parallel}F_{\parallel} + {}_{\parallel}F_{\perp}\left(\frac{{}_{\perp}F_{\parallel}}{{}_{\perp}F_{\perp}}\right)} \tag{25}$$

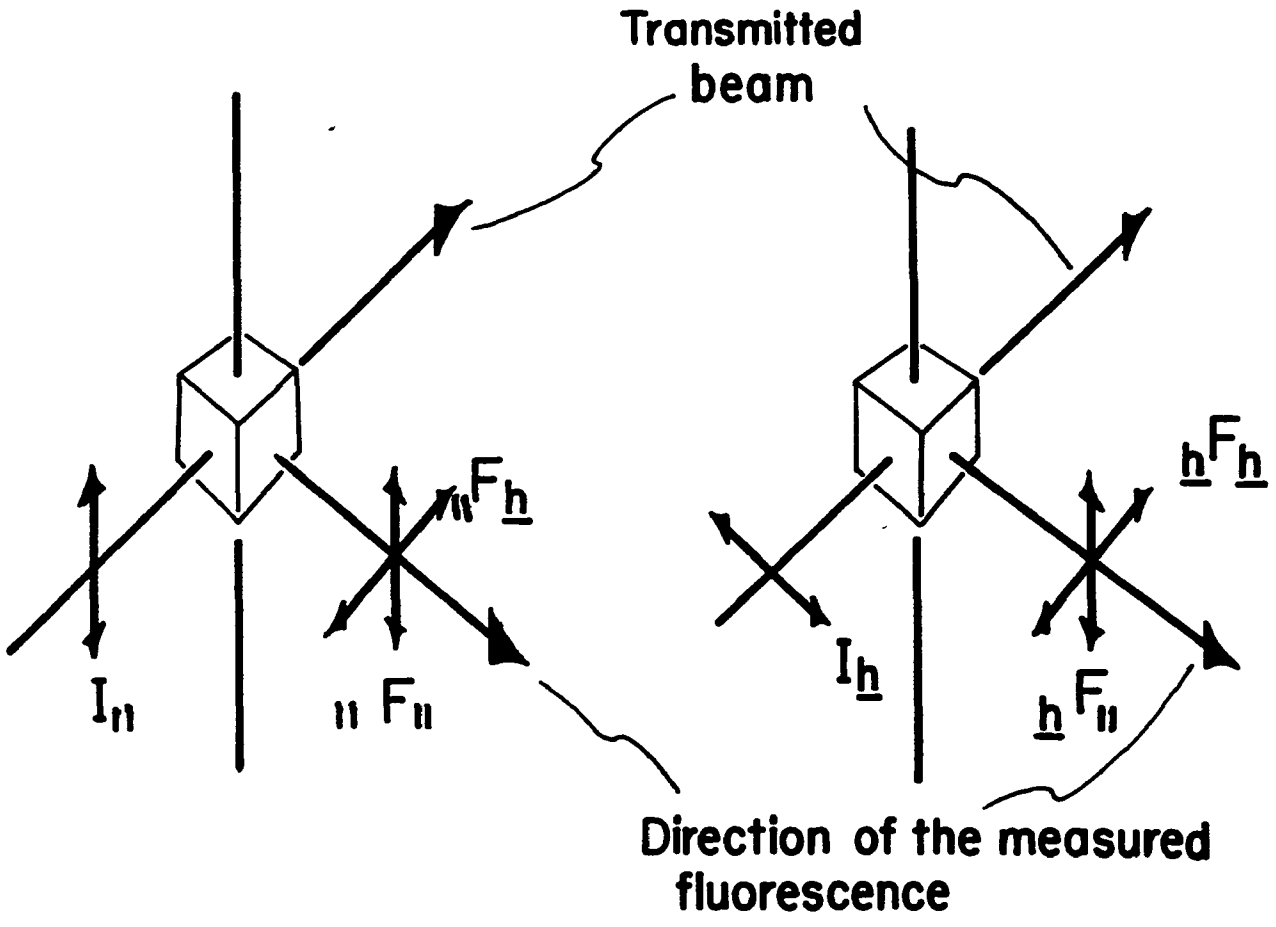
Rearranging the equation, we have:

$$p = \frac{1 - \left(\frac{{}_{\parallel}F_{\perp}}{{}_{\parallel}F_{\parallel}}\right)\left(\frac{{}_{\perp}F_{\parallel}}{{}_{\perp}F_{\perp}}\right)}{1 + \left(\frac{{}_{\parallel}F_{\perp}}{{}_{\parallel}F_{\parallel}}\right)\left(\frac{{}_{\perp}F_{\parallel}}{{}_{\perp}F_{\perp}}\right)} \tag{26}$$

We designed the instrument so that it could measure ${}_{\perp}F_{\parallel}/{}_{\parallel}F_{\parallel}$ and ${}_{\parallel}F_{\perp}/{}_{\perp}F_{\perp}$. To measure ${}_{\perp}F_{\parallel}/{}_{\parallel}F_{\parallel}$, we changed the incident light every few milliseconds from one with one polarization ($I_{\parallel}$) to another with the perpendicular polarization ($I_{\perp}$). The resultant fluorescence signal ${}_{\parallel}F_{\parallel}$ and ${}_{\perp}F_{\parallel}$ was measured with its polarizer fixed at one polarization. Since both fluorescence signals are measured exactly the same way, systematic error in the measurement will be exactly the same in both cases. By measuring ${}_{\parallel}F_{\parallel}$ and ${}_{\perp}F_{\parallel}$ within a short time, we eliminated long time errors such as light intensity fluctuations and changes in fluorescence. To reduce the random noise of the photomultiplier, the fluorescence signal was stored in a computer of average transients (CAT), Model 400 C manufactured by Mnemetron Division of Technical Measurements Corp. The CAT was borrowed from the laboratory of Prof. C. L. Prosser. As continually successive signals are added, the CAT averages out the random noise. By turning the fluorescence polarizer 90° to that of the above measurements, we measured ${}_{\parallel}F_{\perp}$ and ${}_{\perp}F_{\perp}$ exactly as before Again, since both fluorescence are measured exactly the same way, the systematic error will be exactly the same in both measurements. By taking the ratio of ${}_{\parallel}F_{\perp}$ and ${}_{\perp}F_{\perp}$, the systematic error should cancel. Hence, having the ratio ${}_{\perp}F_{\parallel}/{}_{\parallel}F_{\parallel}$ and ${}_{\parallel}F_{\perp}/{}_{\perp}F_{\perp}$, we can calculate p from a slight rearrangement of equation 26:

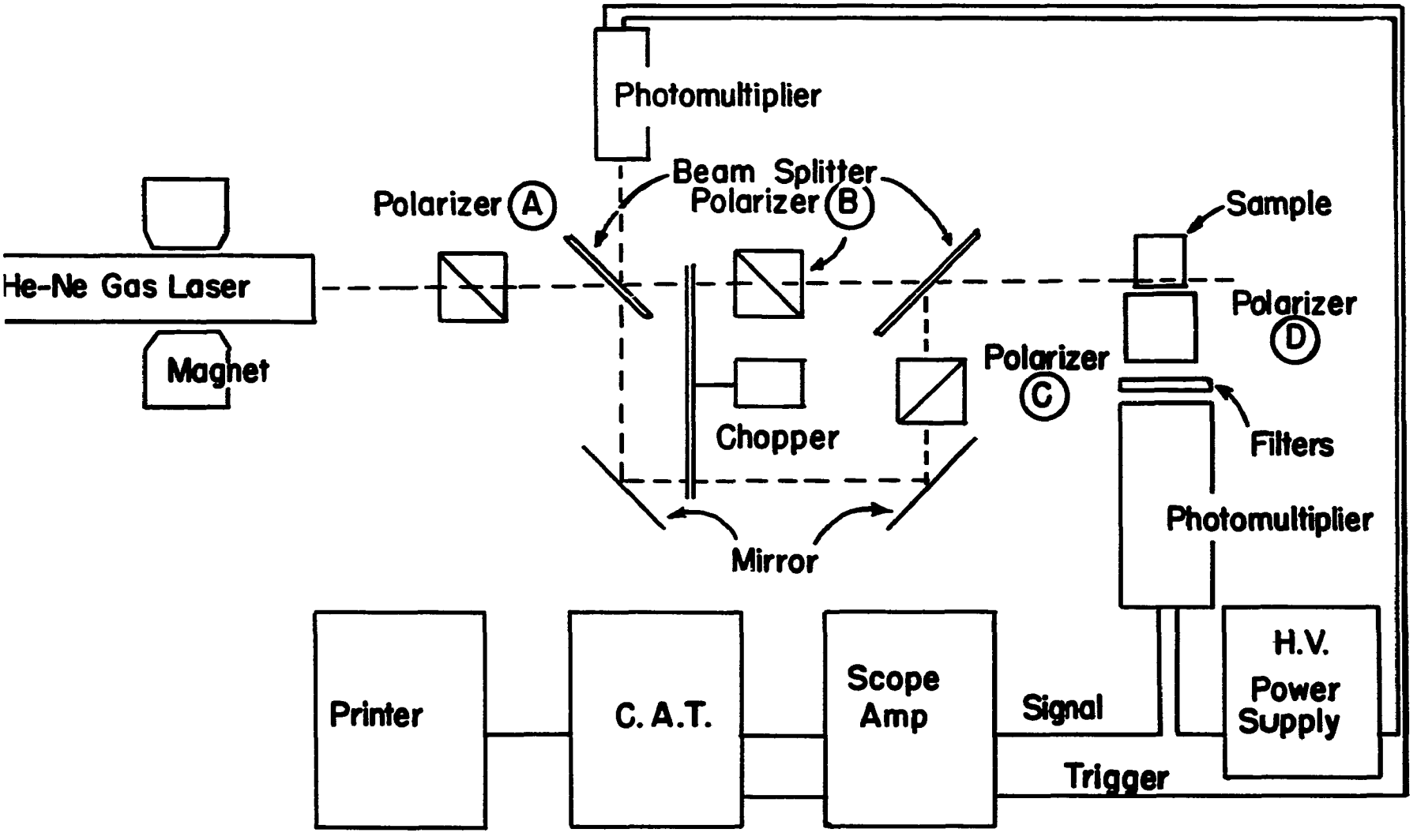
$$p = \frac{1 - \left(\frac{{}_{\perp}F_{\parallel}}{{}_{\parallel}F_{\parallel}}\right)\left(\frac{{}_{\parallel}F_{\perp}}{{}_{\perp}F_{\perp}}\right)}{1 + \left(\frac{{}_{\perp}F_{\parallel}}{{}_{\parallel}F_{\parallel}}\right)\left(\frac{{}_{\parallel}F_{\perp}}{{}_{\perp}F_{\perp}}\right)} \qquad (27)$$

Figure 9. Definition of F.


Transmitted
beam
F_h
$I_{||}$
$F_{||}$
$_hF_h$
I_h
$_hF_{||}$
Direction of the measured
fluorescence

The instrument assembled to measure the polarization of fluorescence is shown in Figure 10. Polarizer A is a nicol prism, 5 mm in crossection obtained from a polarizing microscope. The polarizers B, C and D are Glan Thompson prisms, 12 mm in crossection obtained from Karl Lambrecht Crystal Optics Co. The excitation source is a Coleman Model 75 He-Ne laser. The laser was placed between a 5000 gauss permanent magnet to stabilize its intensity and polarization direction.[120] The laser beam passes through a rotatable polarizer A to a beam splitter. One beam passes through polarizer B which is set at a fixed polarization. The other beam is deflected by two mirrors to polarizer C which is set at a polarization angle 90^{o} to that of polarizer B. The two beams are recombined by another beam splitter and then are directed to the sample. A mechanical chopper is used to alternate the two beams 70 times per second. The sample sees first one beam, then a period of darkness, then the other beam, then another period of darkness, and then the first beam again and so on. The beam intensity is 550 ergs/cm^{2} sec. This was measured with Yellowspring radiometer Model 65. Polarizer D is used as the observation polarizer. It is rotatable and it is in one of two positions. Its polarizing angle is either the same as that of polarizer B or it is the same as that of polarizer C. A cut-off colored filter (usually Corning CS 2-64 unless otherwise specified) is placed in front of the photomultiplier to separate the fluorescent light from the scattered incident light. The photomultiplier used to detect the fluorescence was an EMI 9558B. It is generally biased at 900 volts for low noise operation. The signal

Figure 10. Block diagram of the apparatus for the measurement of polarization of fluorescence.

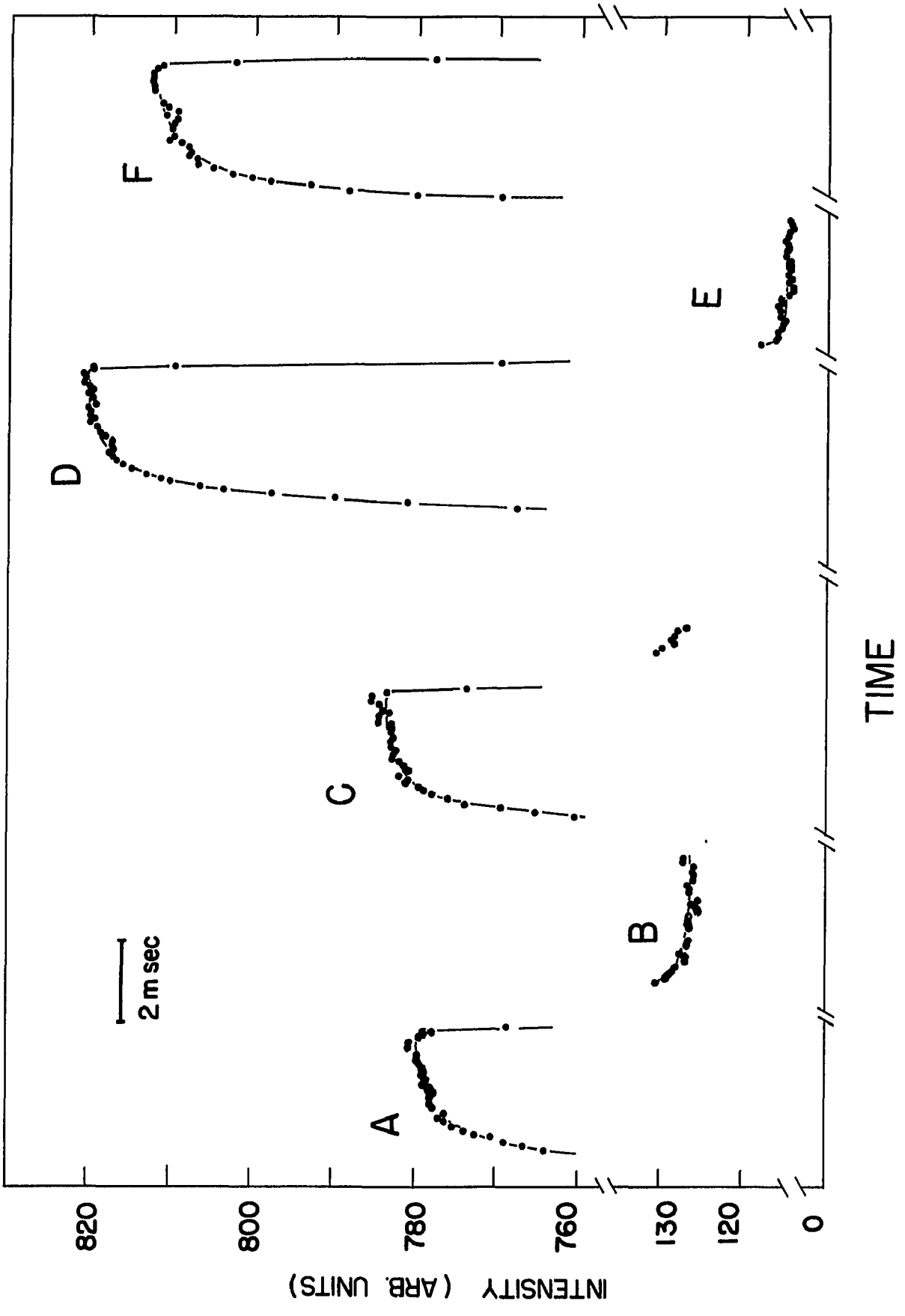

Photomultiplier
Beam Splitter
Polarizer (A)
Polarizer (B)
Sample
He-Ne Gas Laser
Polarizer (D)
Polarizer (C)
Magnet
Chopper
Filters
Photomultiplier
Mirror
Printer
C. A. T.
Scope Amp
Signal
H.V. Power Supply
Trigger

from the photomultiplier is fed to channel A of a Tektronic 502 oscilloscope where it is amplified. The amplified signal is then fed into the CAT. After an averaging time of five minutes, the signal is recorded on a printer. The recorded signal then was analyzed. Figure 11 shows a typical signal recorded by the CAT. Another photomultiplier (RCA 7102) is used to monitor the light that is reflected from the front face of polarizer B. This signal is passed through a high pass filter into the Channel B of the oscilloscope. The high pass filter consists of a 25 $m\mu f$ capacitor in series with the photomultiplier output and a $10^6 \Omega$ resistor across the photomultiplier output to ground. The amplified signal from the oscilloscope is used as a trigger for the CAT.

F. DELAYED LIGHT EMISSION (DLE) MEASUREMENTS

The instrument assembled to measure the kinetics of delayed emission is shown in Figure 12. (This instrument was assembled by W. T. Stacy and the author in the Materials Research Laboratory of the U. of I.) A He-Ne gas laser (Spectra Physics Model 115 or Model 130) was used as the excitation source. The laser provides a continuous beam of 6328 $\mathring{A}$ light with peak intensity of 10^{15} photons/cm^2 sec. A mechanical chopper was used to obtain light pulses of 4.5 msec durations. The algae were contained in a thin-wall plastic cuvette to avoid long time decay emitted by most glass cuvettes. For the study of decay kinetics it was placed between the pole faces of a four inch water cooled electro magnet (Varian Model V4004). A four foot quartz light pipe (1/2 inch diameter)

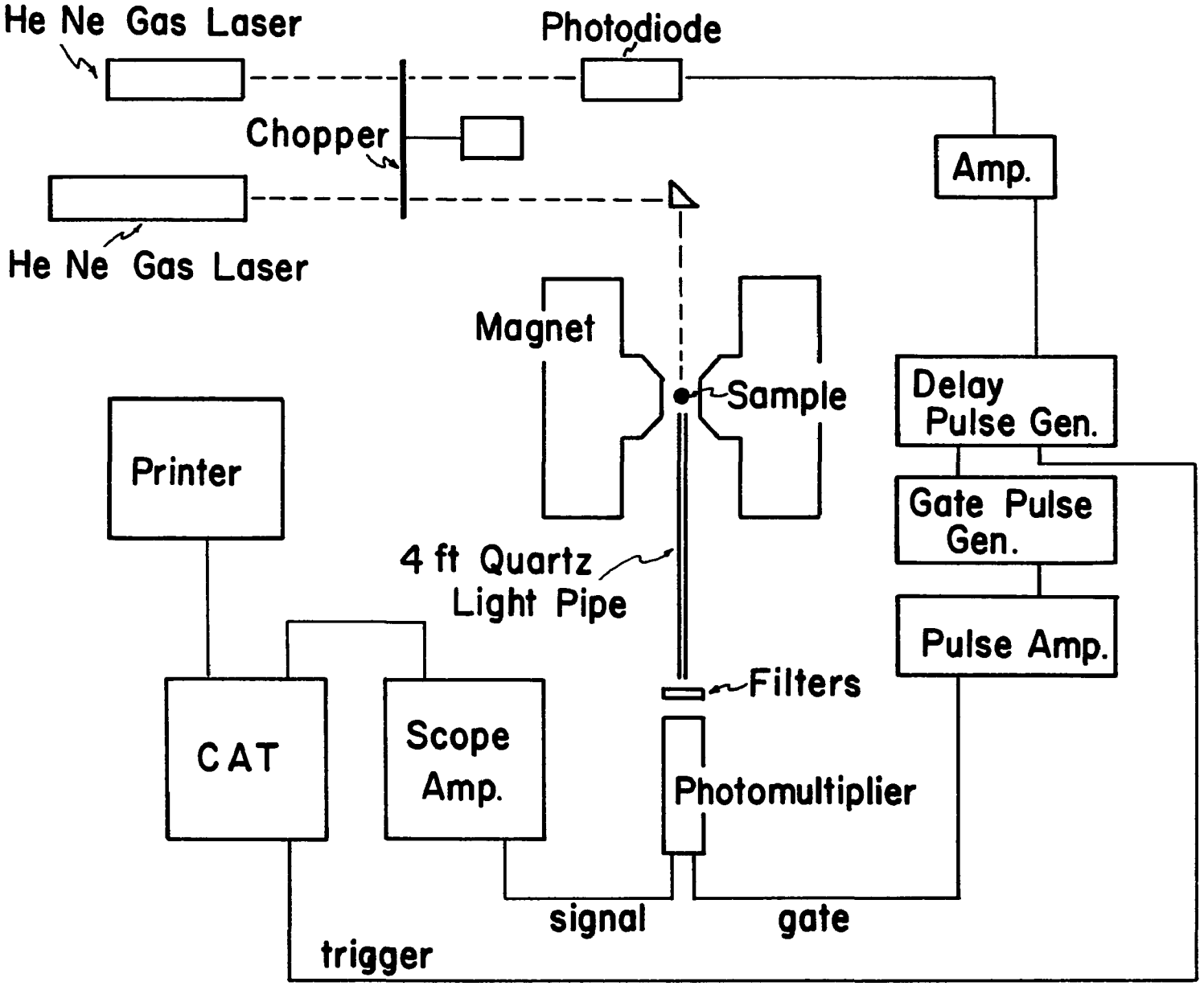
Figure 11. Typical fluorescence signals from the output of the CAT used to calculate the polarization of fluorescence. A, B and C are signals that have been averaged for five minutes. D, E and F are another set of signals. The fluorescence is measured in a direction perpendicular to the direction of the incident light. A is the fluorescence intensity of ${}_{\perp}F_{\parallel}$. B is the dark baseline. C is the fluorescence intensity of ${}_{\perp}F_{\perp}$. D is the fluorescence intensity of ${}_{\parallel}F_{\parallel}$. E is the dark baseline. F is the fluorescence intensity of ${}_{\parallel}F_{\perp}$. (See Fig. 9 for definition of F.)


820
800
780
760
130
120
0
INTENSITY (ARB. UNITS)
TIME
A
B
C
D
E
F
2 m sec

conducts the emitted light from the sample to the photomultiplier. The long quartz light pipe was used to place the photomultiplier out of range of the stray magnetic fields from the magnet. Magnetic fields greatly distort the photomultiplier signal. To further eliminate the stray magnetic field, the photomultiplier is covered with many layers of mu-metal. Two Corning CS 2-64 filters were placed in front of the photomultiplier to cut down stray incident light. Luminescence from the quartz light pipe and the cuvette was taken into account by measuring the luminescence without any algae in the cuvette and subtracting it from the luminescence measured with algae.

The photomultiplier used was an RCA 7265. The method of gating the photomultiplier was that used by De Martini and Wacks.[121] The photomultiplier is cut off by holding its first dynode voltage negative with respect to the cathode. The photomultiplier can be quickly turned on by applying a positive square pulse to the first dynode This positive square pulse was provided by an externally-triggered pulse generator, General Radio Model 1217-C. The pulse was amplified and fed into the photomultiplier dynode circuit by a low output impedance cathode follower. The typical pulse used has a rise time of 10 microseconds, a pulse height of about 200 volts and a pulse length of 15 milliseconds. This allows the photomultiplier to turn on in 0.1 milliseconds. To provide proper synchronization, a second He-Ne laser beam passes through the same light chopper as that of the excitation beam. The resultant pulses excite a photodiode, whose amplified signal, in turn, triggers a delay pulse generator

Figure 12. Block diagram of the apparatus for the measurement of the time dependence and the magnetic field dependence of delayed light emission.[101]


He Ne Gas Laser
Photodiode
Chopper
Amp.
He Ne Gas Laser
Magnet
Sample
Delay
Pulse Gen.
Printer
Gate Pulse
Gen.
4 ft Quartz
Light Pipe
Pulse Amp.
Filters
CAT
Scope
Amp.
Photomultiplier
signal
gate
trigger

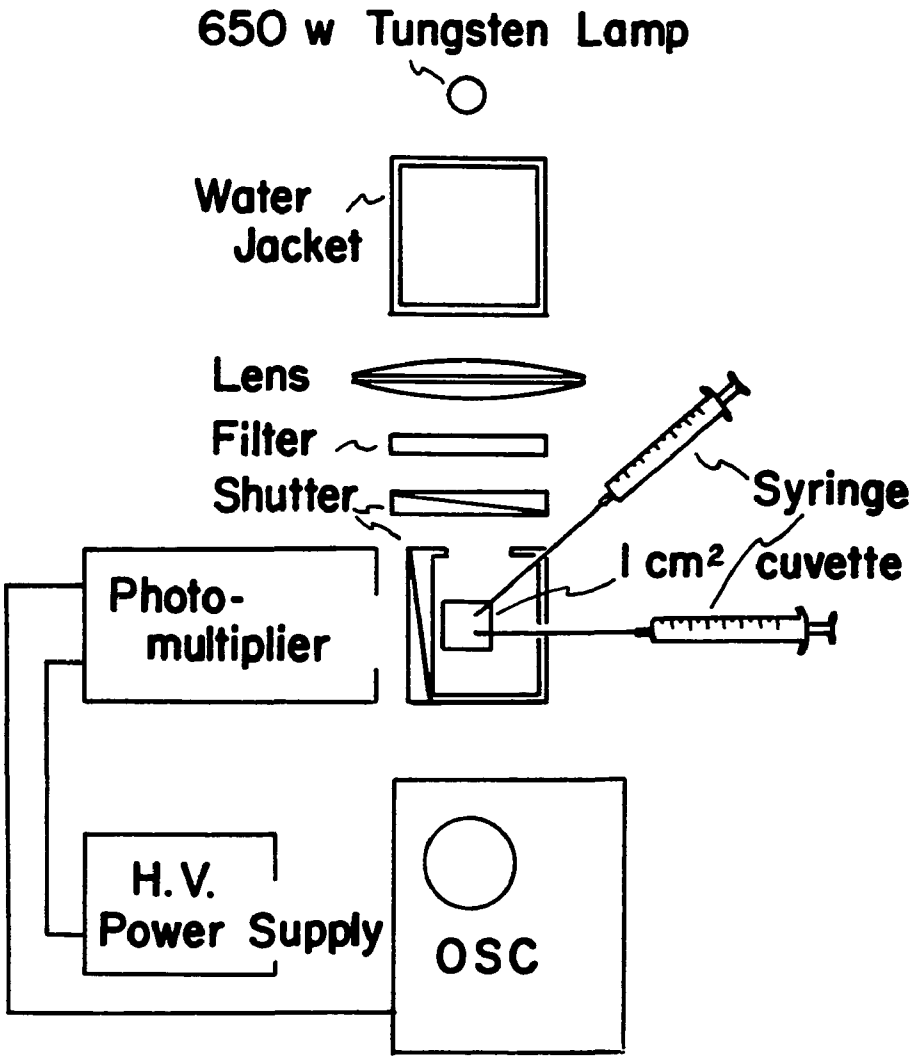
identical to the gate pulse generator. A variable delay was provided by triggering the gate pulse generator with the second spike of the differentiated output of the delay pulse generator The delay was adjusted so that the gate pulse generator was turned on in 0.1 milliseconds after the incident beam which excites the sample was cut off.

The resultant photomutliplier signal was amplified by an oscilloscope (Tektronic 502). The output was fed into the CAT. Using 400 storage units in 31.25 millisecond sweep, the CAT averages out the random noise by successive signals being continually added. The accumulated contents of the memory units were recorded with a printer.

G. CHEMILUMINESCENCE AND THERMOLUMINESCENCE MEASUREMENTS

The method of measuring chemiluminescence was the same as that first used by Mayne and Clayton.[122] The instrument that was assembled by the author to measure both chemiluminescence and thermoluminescence is shown in Figure 13. The illumination light was provided by a Sylvania lamp type DWY. The intensity used for all experiments was 3.8×10^5 ergs/cm^2 sec. This was measured by Yellowspring radiometer Model 65. A one centimeter square glass cuvette was used to hold the sample. The cuvette was placed in a light tight box in front of the photomultiplier. Two small holes were made on top of the box to allow two hypodermic needles to extend to the bottom of the cuvette. The photomultiplier used was an EMI 9558B. The signal from the photomultiplier was displayed on a Tektronic 502 oscilloscope and was recorded on Polaroid 3000 film.

Figure 13. Block diagram of the apparatus for the measurement of chemiluminescence and thermoluminescence.


650 w Tungsten Lamp
Water Jacket
Lens
Filter
Shutter
Syringe
1 cm²
cuvette
Photo-multiplier
H.V. Power Supply
OSC

The chemiluminescence measurements were made as follows. One ml of the sample was placed in the cuvette inside the light tight box. The sample was illuminated for ten seconds. Then ten seconds after the illumination, 0.5 ml of 0.2 M succinic acid was injected into the cuvette. The shutter between the cuvette and the photomultiplier was open. Ten seconds after the injection of the acid, one ml of 0.1 M Tris (pH, 8.5) was injected. The resultant luminescence signal was recorded.

The thermoluminescence measurements were made in a similar way. One ml of the sample was placed in the cuvette. The sample was illuminated for ten seconds. After a dark time of ten seconds, one ml of hot water was injected into the cuvette. The resultant luminescence signal was then recorded. Temperature was measured with a TRI-R electronic thermometer For measurements with Chlorella cells, the temperature before injection of hot water was 26°C. With chloroplasts, the temperature was 16°C. The temperature of the hot water injected was measured to be 53°C. After 1 ml of the hot water was injected into 1 ml of Chlorella cells, the temperature was recorded to be 38°C. After 1 ml of hot water was injected into 1 ml of chloroplasts, the temperature was recorded to be 33°C.

III. FLUORESCENCE LIFETIME: RESULTS AND DISCUSSION

A. MEASUREMENTS AT ROOM TEMPERATURE

The purpose of measuring fluorescence lifetime is to obtain information concerning the fate of the excitation energy after a photon is absorbed by the bulk pigment system (also see Chapter I, section C, for introduction to this problem). We will show that from the measurements of fluorescence lifetime we can calculate the rates and efficiencies of the three major ways of deactivation of the excitation energy. First we will discuss the results obtained by flash excitation method and then by the phase shift method.

An oscilloscope picture of the excitation pulse from the mode-locked He-Ne laser, as monitored by the photomultiplier, is seen in Figure 14. This time response curve is used as the time response for the whole instrument. Figure 15 shows the fluorescence decay curve of Chlorella with 10^{-5}M DCMU added. The fluorescence decay curve of normal Chlorella is shown in Figure 16. The fluorescence decay curve of normal Anacystis and Anacystis with 10^{-5}M DCMU added were almost identical. Figure 16 shows the decay curve of Anacystis with 10^{-5}M DCMU added.

Since the decay time of the exciting pulse is close to the fluorescence decay time of the algae, it is necessary to take into account the time response of the instrument to obtain the true fluorescence lifetime. As mentioned in the introduction, the convolution integral[32] method can be used to deduce the true emission kinetics. Rewriting the convolution integral for convenience, we have:

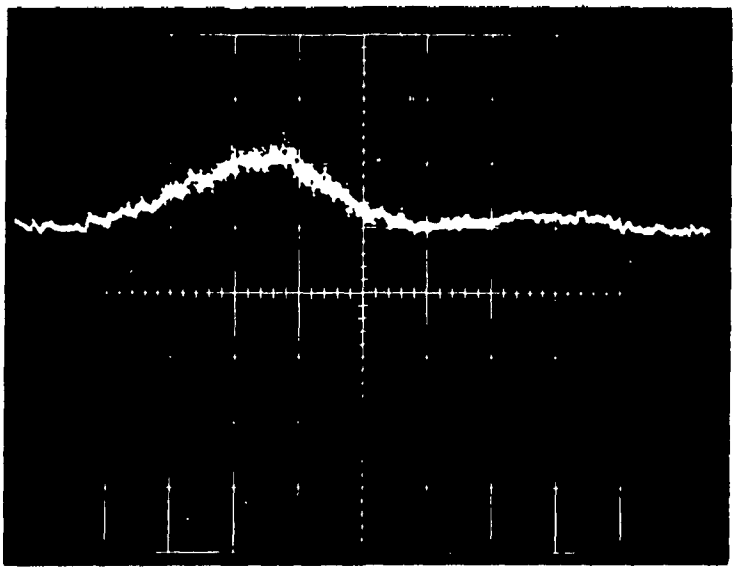


Figure 14. A photograph of a sampling oscilloscope trace showing a mode-locked laser pulse as monitored by a photomultiplier. Horizontal scale is one nsec/division.

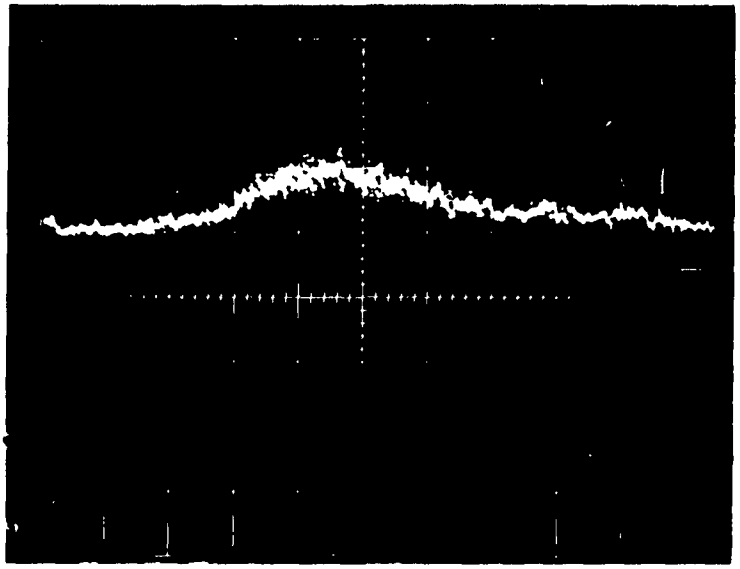


Figure 15. A photograph of a sampling oscilloscope trace showing the fluorescence decay of Chlorella pyrenoidosa with 10^{-5}M DCMU. Horizontal scale is one nsec/division.

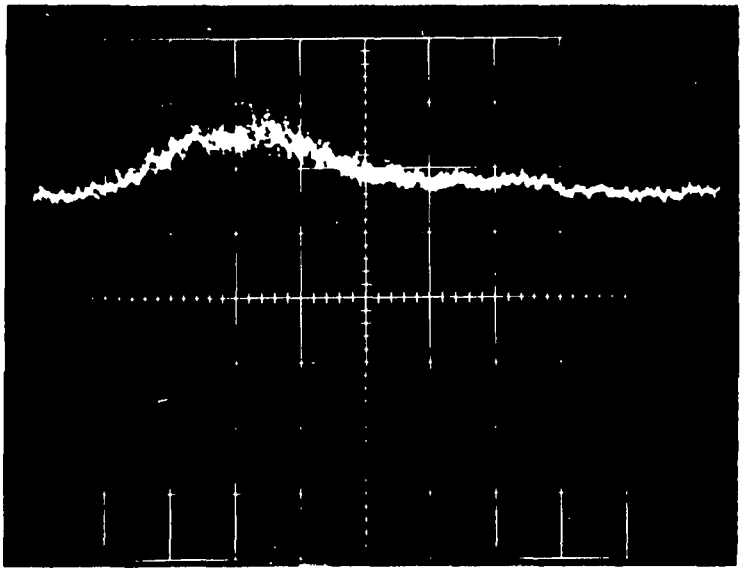


Figure 16. A photograph of a sampling oscilloscope trace showing the fluorescence decay of normal Chlorella pyrenoidosa. Horizontal scale is one nsec/division.

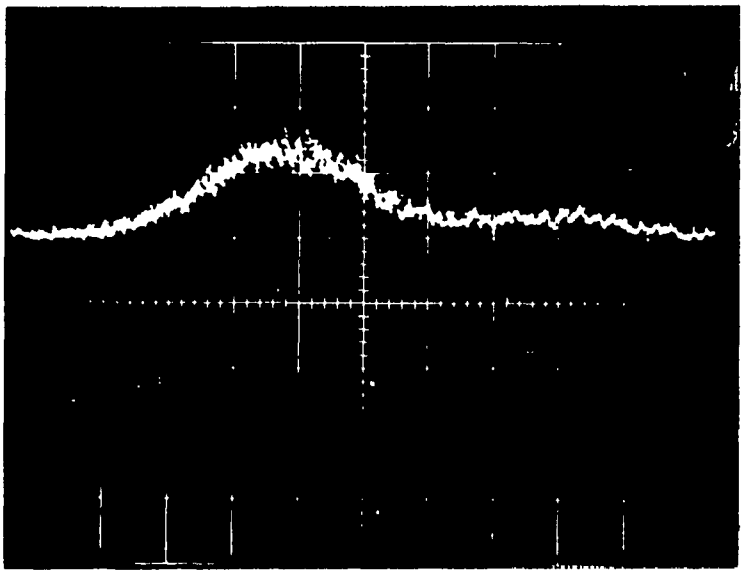


Figure 17. A photograph of a sampling oscilloscope trace showing the fluorescence decay of Anacystis nidulans with 10^{-5}M DCMU. Horizontal scale is one nsec/division.

$$F(t) = \int_0^t G(u)\, H(t-u)\, du$$

where F(t) is the fluorescence decay curve as viewed by the same detecting system as the incident pulse

G(t) is the true time course of fluorescence

H(t) is the exciting light as viewed by the detecting system

The data points for H(t) are taken directly from the photograph in Figure 14. G(t) is assumed, as a first possibility, to be a single exponential decay with lifetime ranging from 0.2 nsec to 2 nsec. With these values, the convolution integral is numerically evaluated to give a series of curves, F(t), one for each postulated lifetime. These calculated decay curves, F(t), are shown in Figures 18 and 19.

The experimental fluorescence decay curves, shown in Figures 14-16, are normalized at their highest intensity of fluorescence and are plotted in Figures 18 and 19 and compared with the calculated F(t) curves. For Chlorella with DCMU added, as shown in Figure 19, the experimental decay curve best fits the F(t) decay curve calculated by assuming the true fluorescence decay has a single exponential lifetime of 1.8 nsecs. The fit is not very good at the rising part of the curve and at the end of the decay. This can be attributed to the distortion of the signal by the secondary electron emission at the photomultiplier anode. This distortion is clearly shown in the tail of the decay curve for the flash which showed a small "second wave" of emission. Because of this distortion, we cannot tell whether or not there is a longer lived fluorescence component whose intensity is about one third that of the main fluorescence component. For

Figure 18. Time course curves of the scattered laser pulse and of the fluorescence of Anacystis nidulans. The dark line curves are experimental curves. The light line curves are predicted fluorescence curves -- calculated numerically by assuming the true fluorescence decay to have one exponential lifetime whose values are indicated in the figure

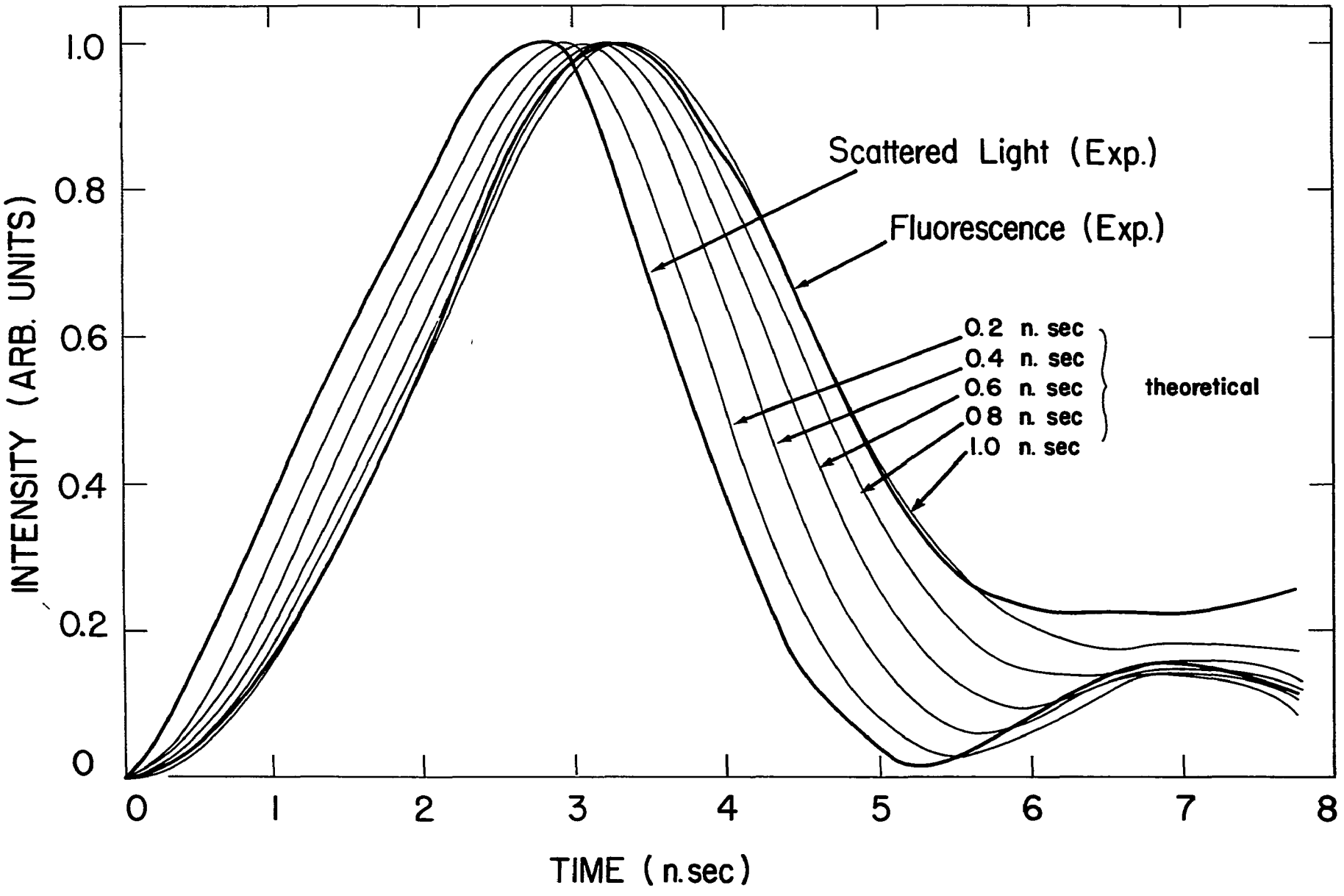
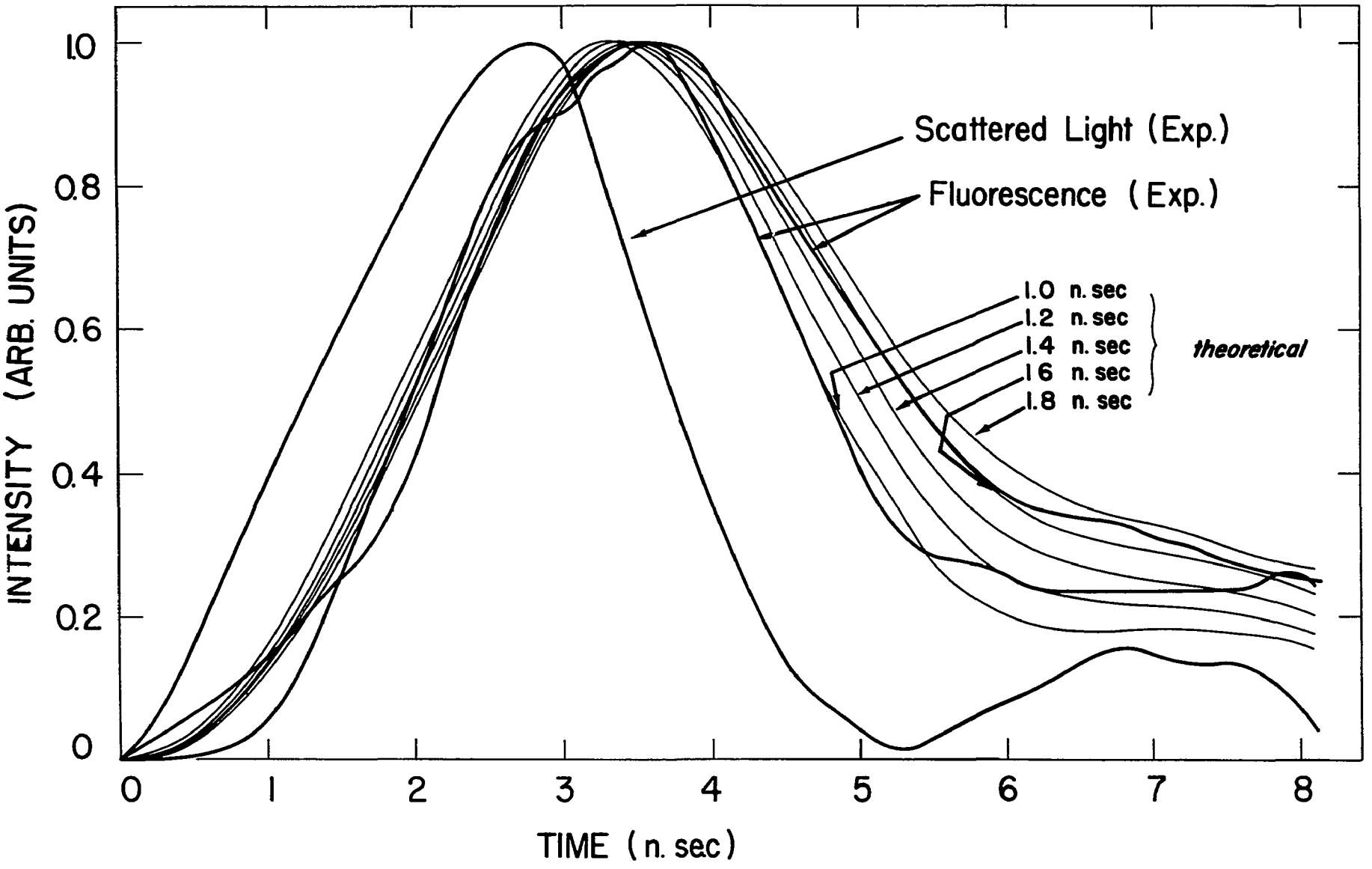

INTENSITY (ARB. UNITS)
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
8
TIME (n.sec)
Scattered Light (Exp.)
Fluorescence (Exp.)
0.2 n. sec
0.4 n. sec
0.6 n. sec
0.8 n. sec
1.0 n. sec
theoretical

Figure 19. Time course curves of the scattered laser light and of the fluorescence of Chlorella pyrenoidosa. The dark lines are experimental curves. The one which matches the thoeretical 1.0 nsec curve is the fluorescence curve of normal Chlorella. The other which matches the theoretical 1.8 nsec curve is the fluorescence curve of Chlorella with 10^{-5}M DCMU. The light line curves are fluorescence curves calculated numerically by assuming the true fluorescence decay to have one exponential lifetime whose values are indicated on the figure.


INTENSITY (ARB. UNITS)
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
8
TIME (n. sec)
Scattered Light (Exp.)
Fluorescence (Exp.)
1.0 n. sec
1.2 n. sec
1.4 n. sec
1.6 n. sec
1.8 n. sec
theoretical

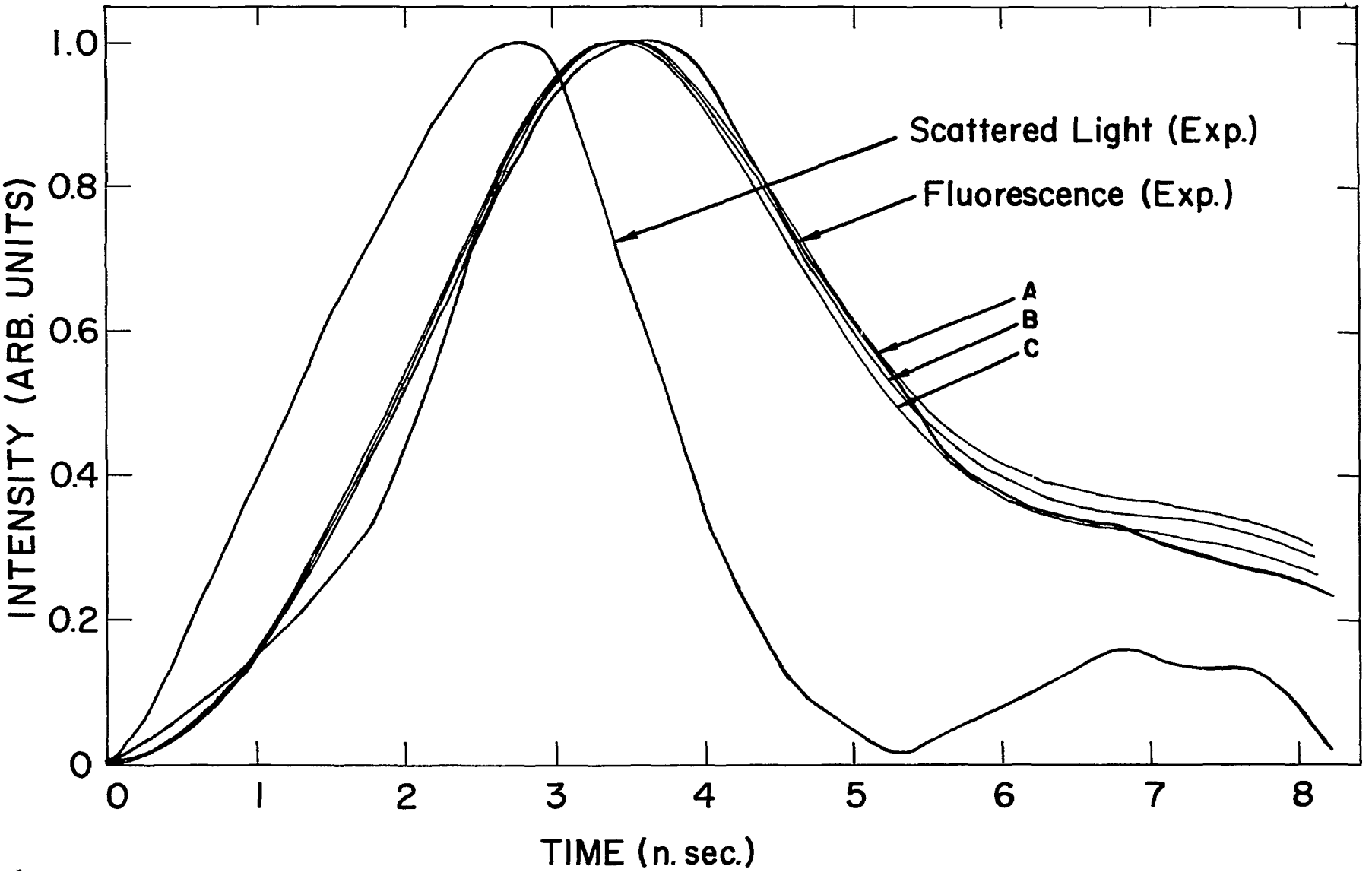
normal Chlorella, also shown in Figure 19, the best fit for the experimental decay curve is the F(t) decay curve calculated by assuming the true fluorescence decay to be a single exponential lifetime of 1.0 nsec. As shown in Figure 18, the best fit for Anacystis with DCMU added is the curve calculated from a single exponential decay with a lifetime of 1.0 nsec. These results show that the main fluorescence band in algae may have a single exponential decay.

Although the experimental fluorescence decay kinetics of Chlorella with DCMU matches the decay curve calculated with the true fluorescence decay having only one exponential decay component, we want to test whether it will also match the calculated decay curve if we assume that the true fluorescence decay has two exponential decay components. Let us assume that when all the reaction centers are open, there is no fluorescence from the active system II photosynthetic units. In fluorescence transient studies, the "0" level of fluorescence corresponds to the condition in which all the reaction centers are open. This level of fluorescence, if it does not come from the photosynthetic unit II, may come from "dead" chlorophyll molecules that are not associated with photochemistry or from photosystem I. The "0" level fluorescence yield in Chlorella has been measured to be about one third of the fluorescence yield when all the reaction centers are closed. The fluorescence lifetime of "0" level has been measured by Briantais, Govindjee and Merkelo,[51] using the phase shift method, to be approximately 0.6 nsec. This agrees well with the lifetime values at steady state obtained by Nicholson and Fortoul,[34] and Singhal and Rabinowitch[35] using such low light intensities that all the reaction centers remain open (the reaction centers that

are closed by a photon of light are re-opened by a dark reaction faster than another photon can reach the closed reaction center). When all the reaction centers in photosystem II are closed, then there should be two main fluorescence decay times -- one due to the photosystem II units when their reaction centers are closed and the other due to the composite lifetime of fluorescence from "dead" chlorophyll molecules or photosystem I units. We assume that one decay has an exponential lifetime of 0.6 nsec which is the composite lifetime of the "dead" chlorophyll molecule, if it exists, and photosystem I units. Its peak intensity is assumed to be 33% of the total fluorescence intensity. We then allow the other fluorescent lifetime due to closed photosystem II units to vary from 1.6 nsec to 2.4 nsecs. Curves calculated with two fluorescence lifetime components, one with an intensity 33% of the fluorescence with an exponential decay of 0.6 nsec and the other with an intensity 67% of the fluorescence with an exponential decay of 2.2 nsec, 2.4 nsec and 2 6 nsec, are shown in Figure 20. The experimental curve for Chlorella with DCMU added does not fit these calculated curves as well as the calculated curve with one exponential lifetime does. Although this result does not prove that the fluorescence has only one exponential lifetime, it suggests that one exponential lifetime at 1.8 nsec is a better assumption than the two exponential lifetimes at 0.6 nsec and 2.4 nsecs.

Although the experimental fluorescence decay curve of normal Chlorella matches the calculated decay curve with the assumption of only one exponential lifetime, we want to further test whether it will also match the calculated decay curve with the assumption of two exponential lifetimes of 0.6 nsec which corresponds to the lifetime when all the reaction centers are

Figure 20. Time course curves of the scattered laser light and of the fluorescence of Chlorella pyrenoidosa with 10^{-5}M DCMU. The dark line curves are the experimental curves. The light line curves are the fluorescence curves calculated numerically by assuming the true fluorescence decay to have two exponential lifetimes. One exponential lifetime is assumed to be 0.6 nsec and its fluorescence intensity is one third of the total fluorescence intensity. The other fluorescence lifetime is assumed for Curve A to be 2.6 nsec, for B to be 2.4 nsec and for C to be 2.2 nsec.

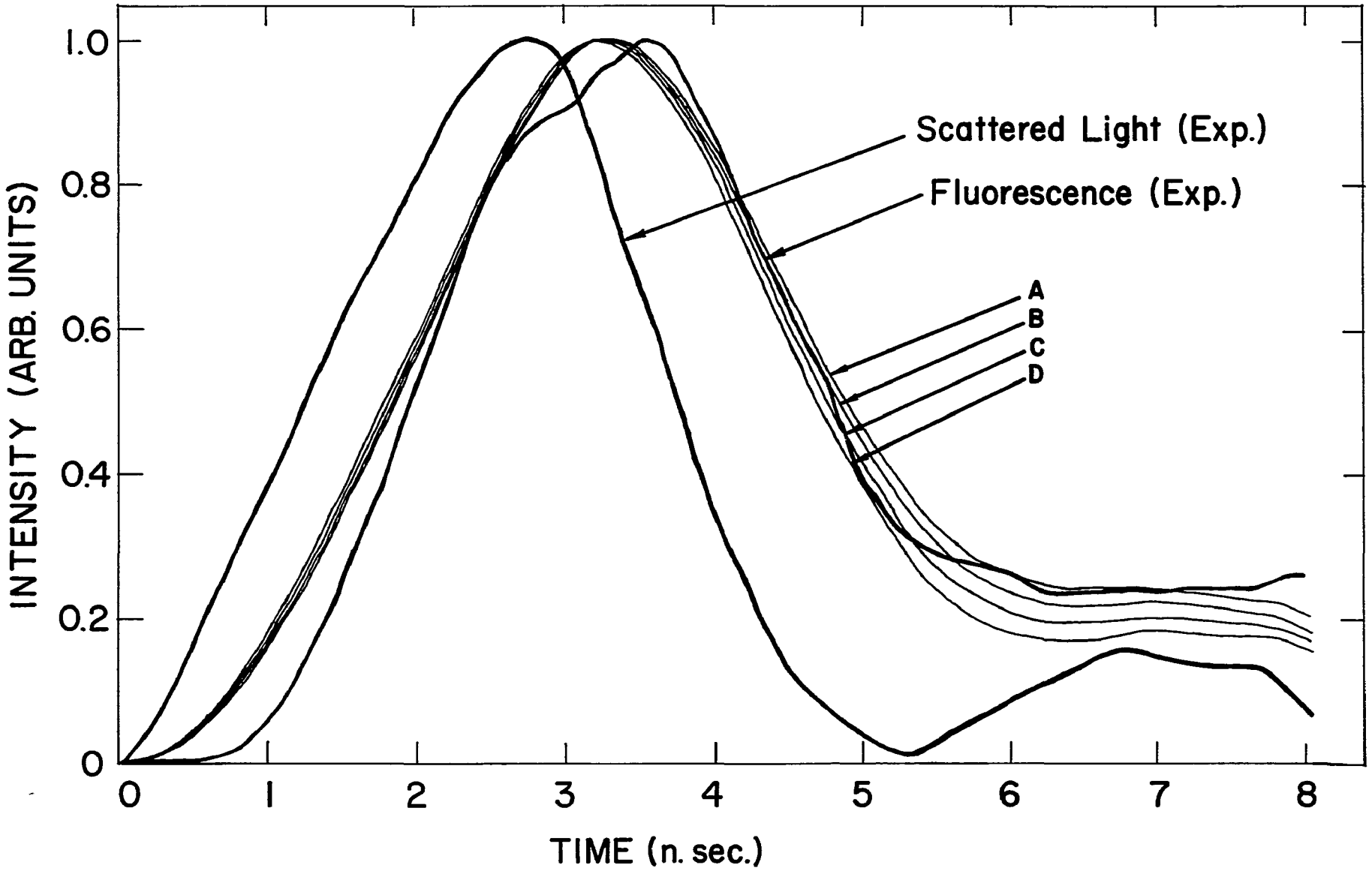

1.0
0.8
0.6
0.4
0.2
0
INTENSITY (ARB. UNITS)
0
1
2
3
4
5
6
7
8
TIME (n. sec.)
Scattered Light (Exp.)
Fluorescence (Exp.)
A
B
C

and 1.8 nsec which corresponds to the lifetime when all the reaction centers are closed. Normal Chlorella cells, illuminated by light of intensity below that of saturation of photosynthesis, will have some reaction centers open and some reaction centers closed. If the units are arranged so that the energy from one unit can migrate to its neighboring units, then in normal cells the fluorescence would have only one exponential decay. If the units are arranged so that energy transfer from one unit to another is impossible, then the fluorescence would have two exponential decays, one corresponding to units with open reaction centers and one corresponding to units with closed reaction centers.

Figure 21 shows the decay curves calculated by assuming the fluorescence decay has two exponential lifetimes and is equal in intensity. One lifetime is fixed at 0.6 nsec, the other is varied from 1.4 to 2.0 nsec. The measured fluorescence decay curve of normal Chlorella fits the calculated curve with two fluorescence lifetimes of 0.6 nsec and 1.8 nsec. Hence from our data it is impossible to decide whether the fluorescence has one or two lifetimes.

The above experiments were designed and done before we were aware of the results of Tumerman and Sorokin.[50] Their results clearly indicate that the fluorescence observed has predominantly one lifetime. They showed that throughout the fluorescence induction curve of Chlorella, the fluorescence yield changes linearly with the fluorescence lifetime as measured by the phase shift method. These results were confirmed by more precise experiments of Briantais, Govindjee and Merkelo.[51] Tumerman and Sorokin[50] have concluded that their results are incompatible

Figure 21. Time course curves of the scattered laser light and of the fluorescence of normal Chlorella pyrenoidosa. The dark line curves are the experimental curves. The light line curves are the fluorescence curves calculated numerically by assuming the true fluorescence decay to have two exponential lifetimes. One exponential lifetime is assumed to be 0.6 nsec and its fluorescence intensity is one half of the total fluorescence intensity. The other fluorescence lifetime is assumed for Curve A to be 2.0 nsec, for B to be 1.8 nsec, for C to be 1.6 nsec and for D to be 1.4 nsec.


1.0
0.8
0.6
0.4
0.2
0
INTENSITY (ARB. UNITS)
0
1
2
3
4
5
6
7
8
TIME (n. sec.)
Scattered Light (Exp.)
Fluorescence (Exp.)
A
B
C
D

with the assumption of two fluorescence lifetimes, 1.0 nsec for the open reaction center and 5.0 nsec for the closed reaction center. We have calculated some predicted results using different assumptions. Since the data we wish to compare are made with the phase shift method, we must first calculate the fluorescence lifetime that would be obtained from the phase shift measurements due to two fluorescence components with different lifetimes and intensities.

It has been shown by Bailey and Rollefson[123] that if the fluorescence has only one exponential decay component, then the phase shift measured (θ) is related to the fluorescence lifetime (τ) by the equation

$$\tan \theta = \omega \tau \tag{28}$$

where ω is 2π multiplied by the modulating frequency f.

Let us assume that the fluorescence has two exponential decay components with lifetimes of τ_1 and τ_2. Each lifetime will have its corresponding phase shift θ_1 and θ_2. Since the incident intensity is sinusoidally modulated, then the fluorescence that is emitted will have the wave form

$$A \cos \theta_1 \sin (\omega t - \theta_1) + B \cos \theta_2 \sin (\omega t - \theta_2) = 0 \tag{29}$$

where A and B are the relative amplitudes of the two fluorescence components.

Let ϕ be the phase angle such that this waveform has its peak intensity at times $t_f = (\frac{\phi}{\omega} + \frac{n}{\omega} \frac{\pi}{2})$, where n = 0, 1, 2, 3, . . . Since the incident light has its peak intensity at time $t_i = \frac{n}{\omega} \frac{\pi}{2}$, then the lag in time between the peak intensity of fluorescence and the peak intensity of the incident light is $\frac{1}{\omega} (\phi)$. ϕ then is the resultant phase shift one measures

when the fluorescence has two lifetime components. By differentiating the fluorescence waveform with respect to t, and setting it equal to zero, the result we obtain is

$$\frac{A \cos \theta_1}{\omega} \cos (\omega t_f - \theta_1) + \frac{B}{\omega} \cos \theta_2 \cos (\omega t_f - \theta_2) = 0 \qquad (30)$$

Substituting for t_f,

$$\frac{A \cos \theta_1}{\omega} \cos (\phi - \theta_1) + \frac{B \cos \theta_2}{\omega} \cos (\phi - \theta_2) = 0 \qquad (31)$$

If one knows the relative amplitudes of the two fluorescence components, and the phase shift ϕ, one can calculate the fluorescence lifetime of each of the two components.

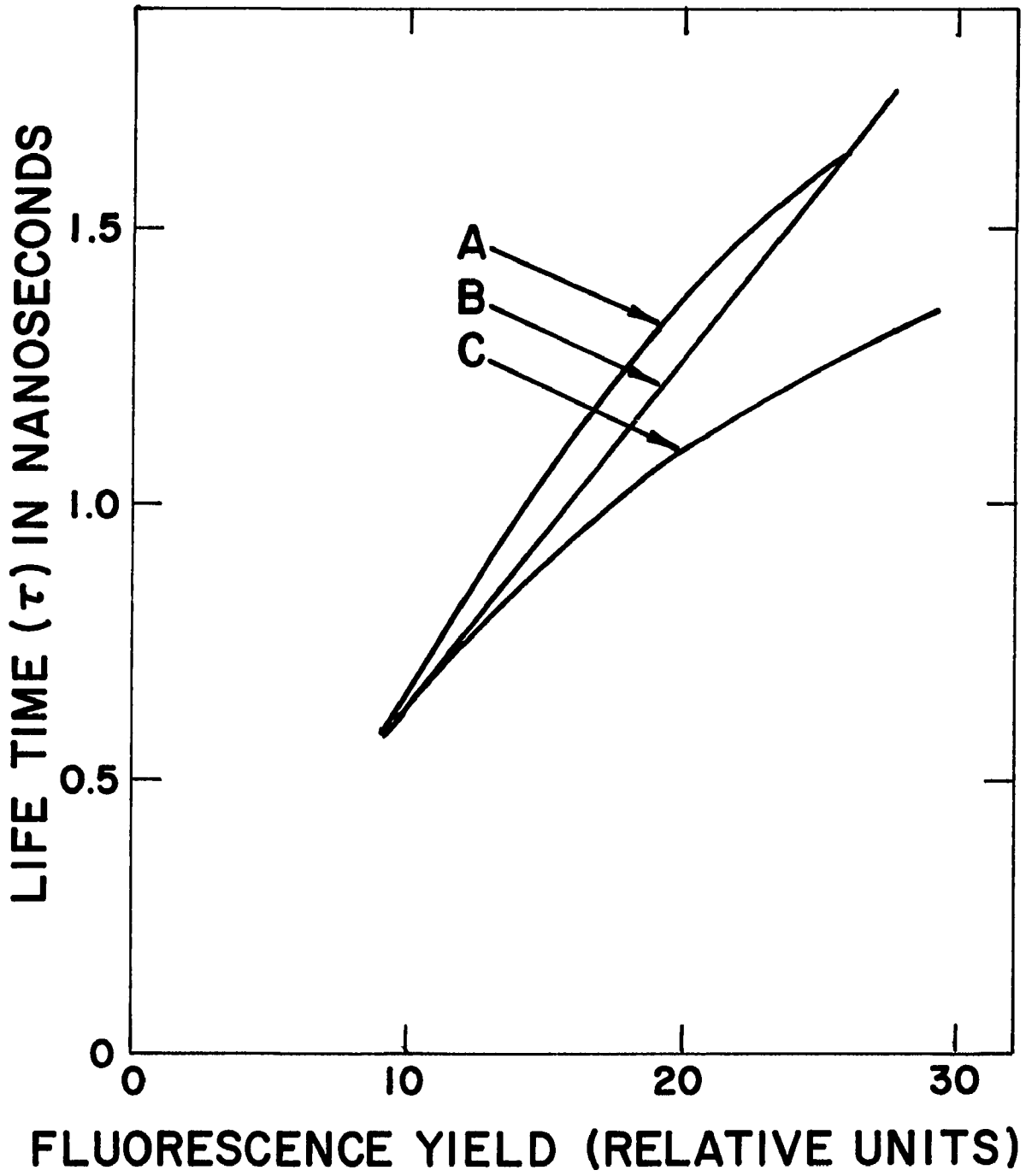
We will first consider the case in which the photosynthetic units in photosystem II are independent of each other. We will assume that the fluorescence lifetime of the unit with open reaction center is 0.57 nsec and that with closed reaction center is 1.67 nsec. The value for these two lifetimes is obtained from the experimental results of Briantais et al.[51] The lifetime they obtained for the "O" level is 0.57 nsec and for the "p" level is 1.67 nsec. Using equation 28, and f as 102.2 Mhz, we can calculate the phase shift θ corresponding to the two fluorescence lifetimes. The two θ's are substituted into equation 31. ϕ and the corresponding fluorescence yield are then calculated for different ratios of the two fluorescence amplitudes A and B. A is the fluorescence intensity due to units with open reaction centers and B is the fluorescence intensity due to

units with closed reaction centers. The phase angle ϕ is converted to fluorescence lifetime by equation 28. These calculated lifetimes are plotted with the corresponding fluorescence yields. The result is shown in curve A of Figure 22.

We will now consider the next possible case in which the photosynthetic unit in photosystem II does not fluoresce when its reaction center is open. The fluorescence lifetime of the unit with its reaction center closed is assumed to be 2.4 nsec. This value is obtained from the flash decay experiments discussed earlier (Figure 20). The fluorescence that is detected when all the reaction centers are open is assumed to be due to "dead" chlorophyll and photosystem I fluorescence. The lifetime of this fluorescence component is assumed to be 0.57 nsec. The two lifetimes are converted to θ by using equation 28 and f as 102.2 Mhz. The two calculated θ's are substituted into equation 31. In this case, A is held constant and B is varied. A is the fluorescence intensity that is due to "dead" chlorophyll and photosystem I. B is the fluorescence intensity due to units with closed reaction centers. The corresponding values of ϕ and fluorescence yield to different values of B are calculated. ϕ is converted to fluorescence lifetime by equation 28. The calculated lifetimes from the different values of ϕ are plotted with the corresponding fluorescence yield. The result is shown in curve C of Figure 22.

The last case we will consider is the one in which the photosystem II units are interconnected. The fluorescence lifetime with all the reaction centers open is assumed to be 0.57 nsec and with reaction centers closed

Figure 22. Theoretical curves of fluorescence lifetime versus the fluorescence yield. The fluorescence lifetime (τ) is calculated from the phase angle (θ) measured by the phase shift method by the formula $\tan \theta = 2\pi f \tau$ where $f = 102.207$ Mhz. Curve A is the predicted curve if there exist two fixed fluorescence decays, one at 0.57 nsec and the other at 1.67 nsec, the τ and the fluorescence yield change on changing the ratio of the two fluorescing components. Curve B is the predicted curve if there exists only one fluorescence lifetime. Curve C is the predicted curve if there exist two fluorescent components, one component with a fixed lifetime of 0.57 nsec and a fixed fluorescent yield, the lifetime of the other component fixed at 2.4 nsec. The fluorescence yield and the phase shift vary with the ratio of the intensity of the two components (cf. with ref. 51).


LIFE TIME (τ) IN NANOSECONDS
1.5
1.0
0.5
0
A
B
C
0
10
20
30
FLUORESCENCE YIELD (RELATIVE UNITS)

to be 1.67 nsec. Since the units are interconnected, there should be only one fluorescence lifetime. Assuming there is no static fluorescence quenching, the fluorescence lifetime is linearly proportional to the fluorescence yield. This is shown in curve B of Figure 22.

Of the three theoretical curves calculated, only curve B matches with the experimental results.[50, 51] Hence fluorescence decay has predominantly one lifetime. This implies that the photosystem II units are interconnected and that energy from one unit can migrate to all other units.

Using the phase shift method, we obtain more precise measurements of the fluorescence lifetime. Since the fluorescence has predominantly one lifetime, the lifetime is simply related to the phase shift by equation 28. Table I shows the phase angle measured and the calculated lifetime for a number of experiments with three different algae with 10^{-5}M DCMU added. The cause of the variation in the same species is not due to instrumental variation but may be due to slight differences in the growth condition of the algae. Taking an average of the results of all the experiments with DCMU added, Chlorella is shown to have a fluorescence lifetime of 1.74 nsec, Anacystis 0.74 nsec and Porphyridium 1.03 nsec. Müller et al.,[41] using the phase method have found that the fluorescence lifetime of Chlorella with 10^{-5}M DCMU added is 1.92 nsec. No lifetime measurements of Anacystis and Porphyridium with DCMU added had been made before the present work.

The phase angle measured with normal cells and the lifetime calculated from the phase in a number of experiments are shown in Table II. The average lifetime of Chlorella is 1.31 nsec, for Anacystis 0.66 nsec

TABLE I

FLUORESCENCE LIFETIME OF ALGAE WITH 10^{-5}M DCMU ADDED

θ is the measured phase shift. τ is the lifetime calculated from $\tan \theta / 2\pi f$. Exciting wavelength is 632.8 nm. Frequency of modulation, f, is 102.207 Mhz. Filter used for fluorescence measurement is Corning CS-2-64.

Chlorella		Anacystis		Porphyridium	
θ in degrees	τ in 10^{-9} sec	θ in degrees	τ in 10^{-9} sec	θ in degrees	τ in 10^{-9} sec
51.3	1.94	29.6	0.89	37.6	1.20
49.4	1.82	23.8	0.69	38.1	1.22
48.6	1.77	25.5	0.74	31.3	0.95
48.2	1.74	23.4	0.68	31.1	0.94
50.4	1.88	25.3	0.74	34.7	1.08
46.7	1.65			29.4	0.88
47.0	1.67			30.8	0.93
46.5	1.64				
44.6	1.54				
47.7	1.71				
average	1.74	average	0.74	average	1.03

TABLE II

FLUORESCENCE LIFETIME OF NORMAL ALGAE

θ is the measured phase shift. τ is the lifetime calculated from $\tan \theta / 2\pi f$. Exciting wavelength is 632.8 nm. Frequency of modulation, f, is 102.207 Mhz. Filter used for fluorescence measurement is Corning CS-2-64.

Chlorella		Anacystis		Porphyridium	
θ in degrees	τ in 10^{-9} sec	θ in degrees	τ in 10^{-9} sec	θ in degrees	τ in 10^{-9} sec
46.9	1.66	19.2	0.54	12.0	0.33
42.8	1.44	23.4	0.67	9.4	0.26
46.3	1.63	28.2	0.83	9.4	0.26
35.0	1.09	27.5	0.81	11.9	0.33
35.2	1.10	15.6	0.43	9.4	0.26
34.7	1.08	23.1	0.66	12.0	0.33
36.1	1.14	23.1	0.66	17.8	0.50
32.0	0.97	24.9	0.72	14.7	0.41
41.3	1.37	22.3	0.64		
41.6	1.38	22.2	0.64		
40.6	1.33				
41.3	1.37				
43.1	1.46				
average	1.31	average	0.66	average	0.34

and for Porphyridium 0.34 nsec. The lifetime for Chlorella is comparable with previous measurements. Our measurements of Anacystis and Porphyridium are lower than those previously reported.[32,42] For Anacystis, both Brody[42] and Tomita and Rabinowitch[32] reported a lifetime of 1.2 ± 0.2 nsec. However, Singhal and Rabinowitch[35] reported a value of 0.5 ± 0.2 nsec at low light intensities. For Porphyridium, Brody[42] and Tomita and Rabinowitch[32] reported a value of 1.5 nsec. Nicholson and Fortoul[34] measured 0.7 ± 0.2 nsec and Singhal and Rabinowitch[35] reported 0.5 ± 0.2 nsec at low light intensities. The reasons for different results are several: different intensities of exciting light, different wavelengths of excitation and the varied precision of the instruments used.

Fluorescence lifetime measurements of Anacystis both by the flash method and by the phase shift method show that the lifetime of normal cells after approximately five minutes of illumination is close to the lifetime of cells with DCMU added. This result is in agreement with fluorescence yield measurements of Anacystis[124] which show that the addition of DCMU does not significantly increase the fluorescence yield of normal cells after a long time of illumination.

From the fluorescence lifetime (τ_1) measured with cells with DCMU added, the rate of radiationless loss not due to trapping at the reaction center (α_h) can be calculated. From equation 4 in the introduction, one can show that

$$\alpha_h = \frac{1}{\tau_1} - \frac{1}{\tau^o} \qquad (32)$$

where $\tau^o = \frac{1}{\alpha_f}$

τ^o is calculated by Brody and Rabinowitch[31] to be 15.2 nsec. The τ^o value is obtained for Chl a in vitro and we will assume that this τ^o value is the same for Chl a in vivo. Substituting the measured τ_1 into equation 32 for Chlorella, Anacystis, and Porphyridium, we find that the rate of radiationless decay for Chlorella is 5.09×10^8 sec^{-1}, for Anacystis α_h is 12.86×10^8 sec^{-1} and for Porphyridium α_h is 9.09×10^8 sec^{-1}. These results are tabulated in Table III; they show that of the three algae, Anacystis has the highest rate of radiationless decay.

The rate of energy trapping (α_t) can also be calculated. From equation 3 in the introduction, we have

$$\alpha_t = \frac{1}{\tau} - \frac{1}{\tau_1} \qquad (33)$$

τ is the lifetime when all the reaction centers are open. This corresponds to the lifetime at the "0" level of the fluorescence induction curve. For Chlorella, the lifetime of the "0" level has been measured by Briantais, Govindjee and Merkelo[51] to be 0.6 nsec. Substituting this value for τ into equation 33, we calculate the rate of trapping for Chlorella to be 1.19×10^9 sec^{-1}. The efficiency of the trapping process is calculated to be 66%.

Müller et al.,[41] also using fluorescence lifetime measurements, calculated the efficiency of trapping in Chlorella to be 82%. The main difference with our calculations lies in the fact that they use 0.35 nsec as the fluorescence lifetime when all the traps are open. They obtain this value from extrapolating the fluorescence lifetime to zero incident intensity from their data of fluorescence lifetime at different light intensities. Weber,[125]

based upon fluorescence quantum yield, estimates the trapping efficiency in Chlorella to be 80%. This estimate is based upon the experiments of Teale[125] which showed that inhibition of photosynthesis by DCMU results in a fivefold increase in fluorescence yield, however, a threefold increase of fluorescence has been resported by many workers.[51, 126, 127] With a threefold increase, the trapping efficiency is calculated to be 66%. Hoch and Knox[83] have estimated the efficiency of trapping for photosynthetic bacteria to be 66% also from fluorescence yield. The differences in the values of the efficiency of trapping may be due to the different conditions of the cells used. For young Chlorella cells, Govindjee et al.[102] have reported a quantum yield of oxygen evolution (at 680 nm) of 0.12 and for mature cells a quantum yield of 0.07.

For Porphyridium, as shown in Figure 28 of Chapter V, fluorescence level at "0" is about a third of the fluorescence level when DCMU is added. Hence its lifetime should be one third that of the lifetime when DCMU is added, which has been measured to be 1.03 nsec. (see Table I). The lifetime of the "0" level is then 0.34 nsec. Substituting this value into equation 33, we calculate a trapping time for Porphyridium of 1.97×10^{9} sec^{-1}. The efficiency of the trapping process is calculated to be 68%. As can be seen in Table III, although the rate of radiationless loss for Porphyridium is larger than that of Chlorella, its trapping rate is also larger than that of Chlorella; hence the efficiency for trapping for both Chlorella and Porphyridium (as grown in our laboratory) are about the same. In the case of Anacystis, the "0" level fluorescence is about one half the fluorescence level when DCMU

TABLE III

RATE CONSTANTS FOR THE DECAY OF THE EXCITED FIRST SINGLET STATE OF CHL A IN VIVO

Sample	α_f in sec^{-1}	α_h in sec^{-1}	α_t in sec^{-1}
Chlorella	6.57×10^7	5.09×10^8	1.19×10^9
Anacystis	6.57×10^7	12.86×10^8	1.98×10^9
Porphyridium	6.57×10^7	9.09×10^8	1.97×10^9

Where

α_f = rate of fluorescence

α_h = rate of radiationless loss except trapping

α_t = rate of trapping

TABLE IV

THE EFFICIENCY OF EACH DECAY PROCESS OF THE EXCITED FIRST SINGLET STATE OF CHL A IN VIVO

Sample	ϕ_f	ϕ_h	ϕ_t
Chlorella	0.04	0.30	0.66
Anacystis	0.02	0.39	0.59
Porphyridium	0.02	0.30	0.68

Where $$\phi_f = \frac{\alpha_f}{\alpha_f + \alpha_h + \alpha_t}$$

$$\phi_h = \frac{\alpha_h}{\alpha_f + \alpha_h + \alpha_t}$$

$$\phi_t = \frac{\alpha_t}{\alpha_f + \alpha_h + \alpha_t}$$

is added.[128] Hence, the lifetime, when all the reaction centers are open, is about one half that of the measured lifetime when all the reaction centers are closed. Using the assumed lifetime of 0.3 nsec when all the reaction centers are open, we calculate the trapping rate for Anacystis to be 1.98×10^{9} sec^{-1}. The efficiency for the trapping process for Anacystis is calculated to be 59%. The efficiency for the radiationless loss process is calculated to be 39%. This high radiationless loss efficiency explains why the lifetime of the normal cell measured is almost the same as that of the poisoned cell.

Table III and IV summarize the results obtained. Chlorella and Porphyridium have higher quantum efficiency for trapping a photon in photosystem II than Anacystis. The reason for the lower efficiency in Anacystis is its high radiationless loss rate. The reason for the high radiationless loss rate in Anacystis is open for speculation. It may be due to energy transfer to weakly fluorescent photosystem I.

B. MEASUREMENTS AT LIQUID NITROGEN TEMPERATURE

Very little is known about the mechanism of the non-radiative decay process. As discussed in the previous section, the rate of non-radiative decay is related to the fluorescence lifetime when all the reaction centers are closed. Measurements of fluorescence lifetime at 77°K would give us some information concerning the temperature dependence of the non-radiative decay process.

The fluorescence lifetime of Chlorella measured at 77°K with the flash decay method is shown in Figures 23 and 24. Figure 25 shows

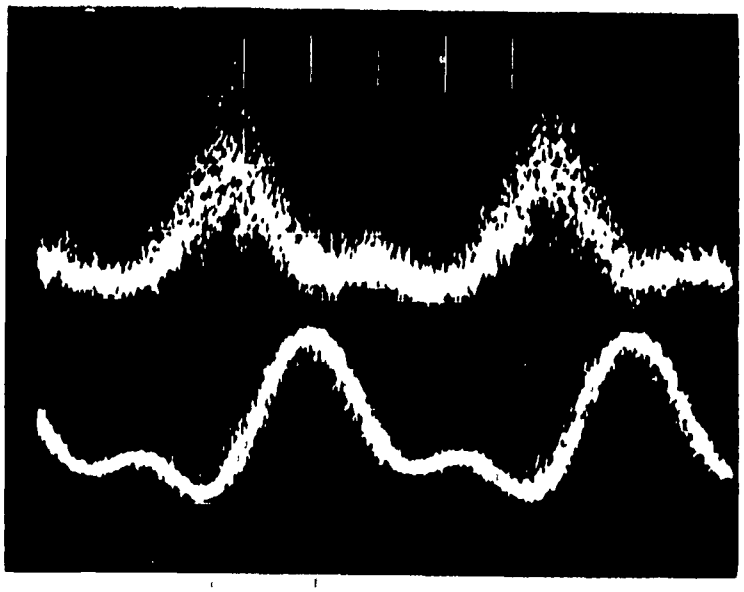


Figure 23. A photograph of a sampling oscilloscope trace showing the mode-locked laser pulse as monitored by the measuring photomultiplier (top curve); and as monitored by the trigger photomultiplier (bottom curve). Horizontal scale is two nsec/division.

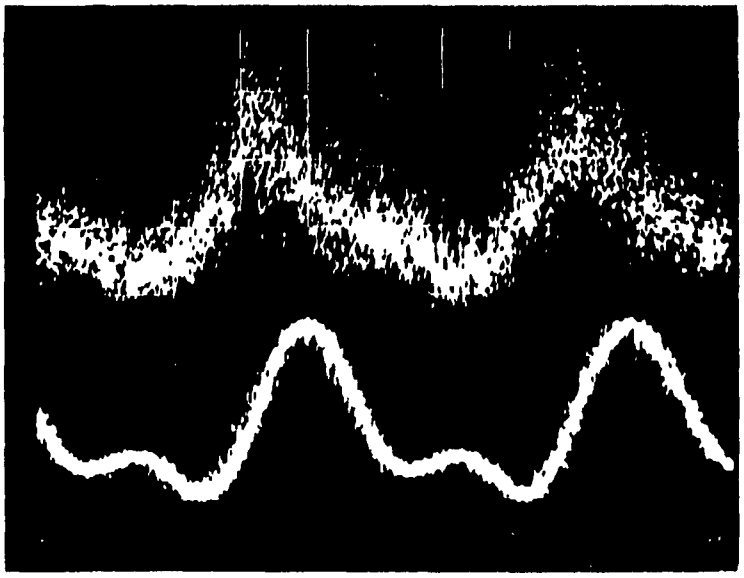


Figure 24. A photograph of a sampling oscilloscope trace showing the fluorescence decay of Chlorella pyrenoidosa at 77°K (top curve); bottom curve is the same as bottom curve of Figure 23. Horizontal scale is two nsec/division.

Figure 25. Time course curves of the scattered laser light and of the fluorescence of Chlorella pyrenoidosa at 77°K. The dark line curves are the experimental curves. The light line curves are the fluorescence curves calculated numerically by assuming the true fluorescence decay to have one lifetime whose values are indicated in the figure.

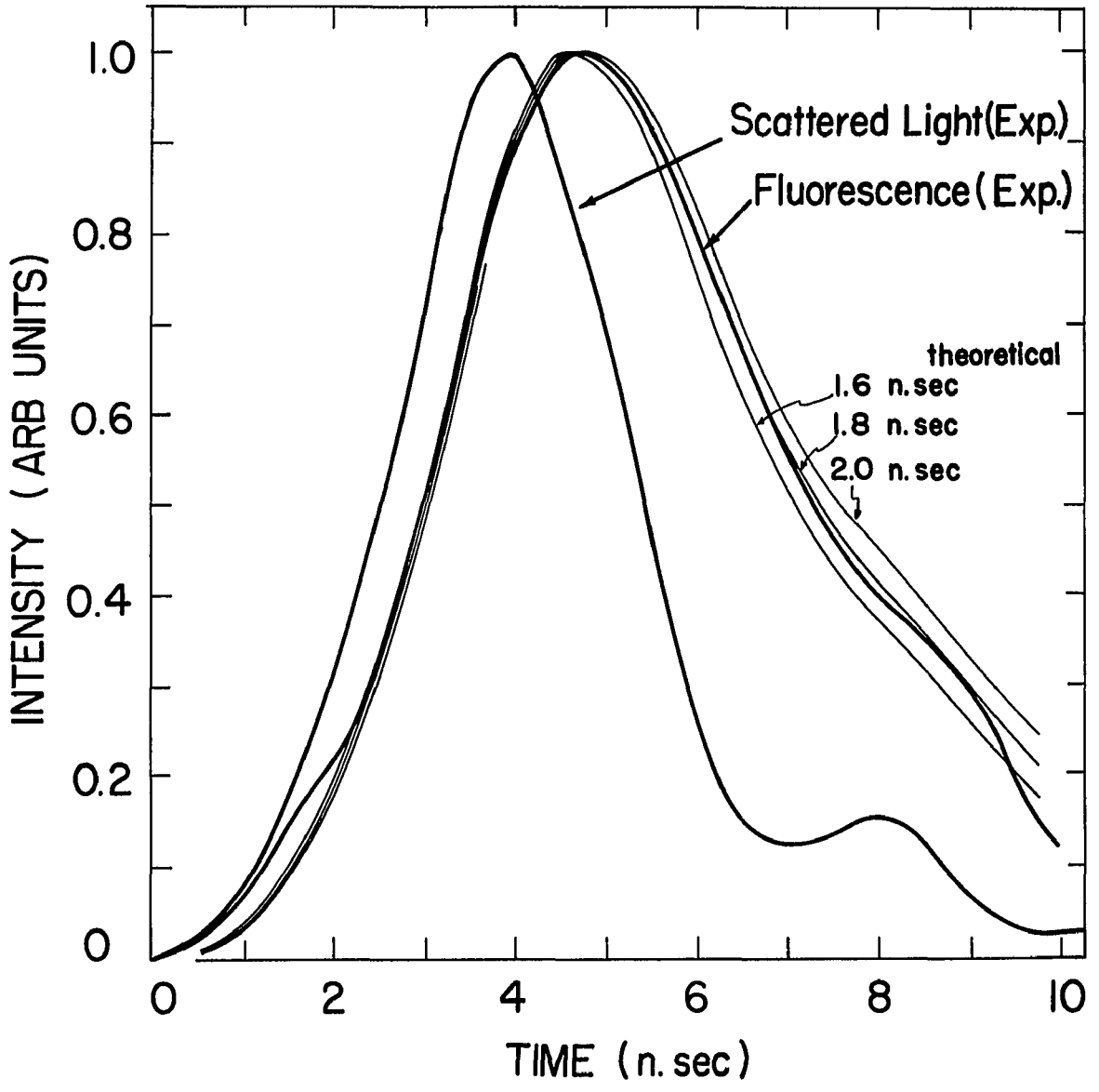
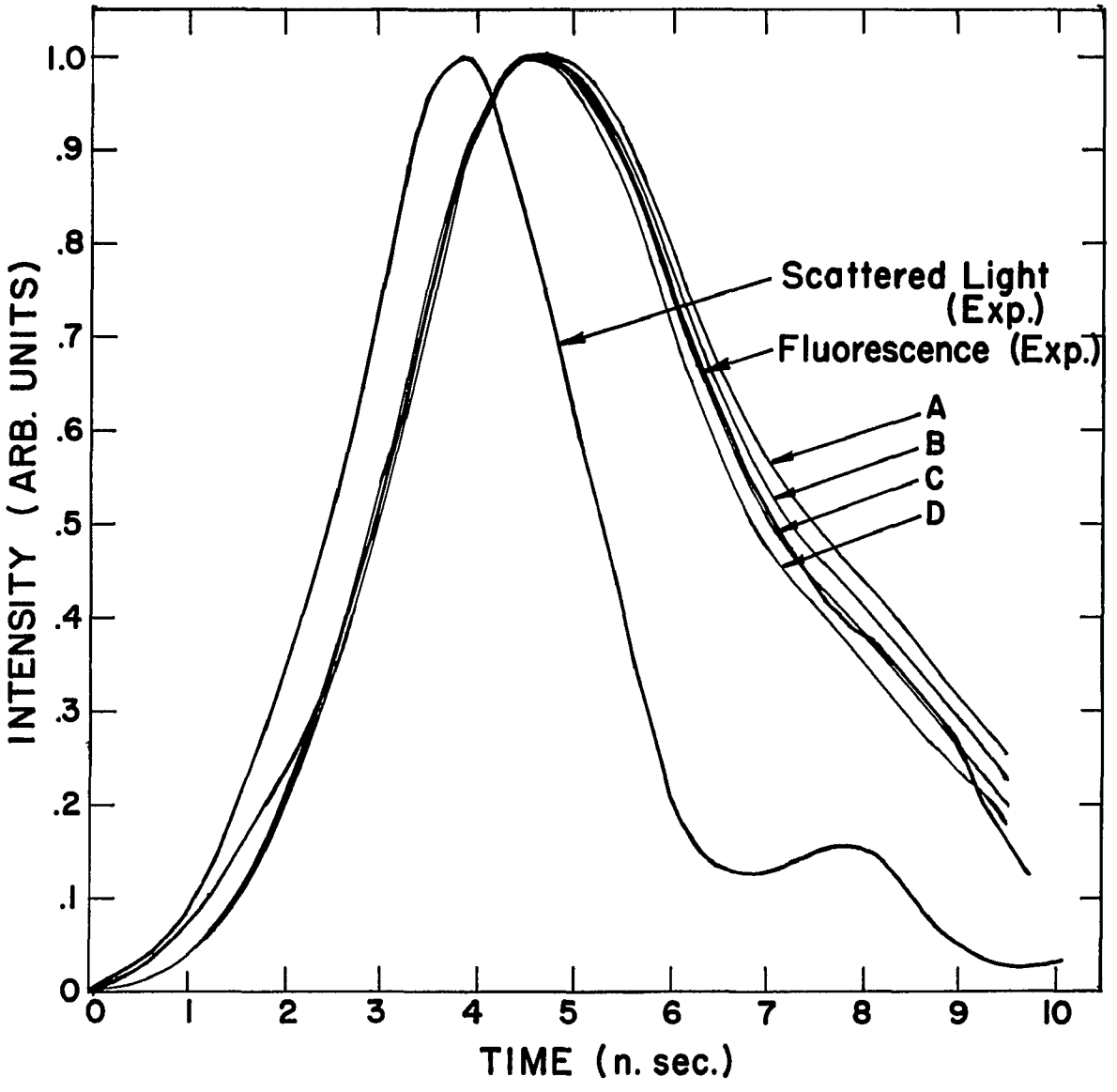

1.0
0.8
0.6
0.4
0.2
0
INTENSITY (ARB UNITS)
0
2
4
6
8
10
TIME (n. sec)
Scattered Light(Exp.)
Fluorescence(Exp.)
theoretical
1.6 n. sec
1.8 n. sec
2.0 n. sec

Figure 26. Time course curves of the scattered laser light and of the fluorescence of Chlorella pyrenoidosa at 77°K. The dark line curves are the experimental curves. The light line curves are the fluorescence curves calculated numerically by assuming the true fluorescence decay to have two lifetimes. One lifetime is assumed to be 1.6 nsec and its fluorescence intensity is one fourth that of the total fluorescence intensity. The other lifetime is assumed for Curve A to be 2.2 nsec, for B to be 2.0 nsec, for C to be 1.8 nsec and for D to be 1.6 nsec.


INTENSITY (ARB. UNITS)
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0
1
2
3
4
5
6
7
8
9
10
TIME (n. sec.)
Scattered Light (Exp.)
Fluorescence (Exp.)
A
B
C
D

that the measured fluorescence decay can be fitted with the calculated decay curve with a one exponential lifetime of 1.8 nsec. The fluorescence decay, however, has more than one decay as will be shown later with phase shift measurements. The second component could be due to the relatively strong emission band near 730 nm at 77°K, which does not exist at room temperature. Analyzing the fluorescence spectra of Chlorella at 77°K,[71,118] we obtain approximately one fourth of the fluorescence from the F685 and F698 bands and three fourths from the F720 band. Assuming that one fourth of the fluorescence has a lifetime of 1.6 nsec (the fluorescence lifetime at room temperature when all the reaction centers are closed) and varying the lifetime of the other three fourths of the fluorescence, we find that the experimental fluorescence decay curve can be fitted with the calculated decay curve with two exponential lifetimes as shown in Figure 26. The exponential lifetimes that fit best are 1.6 nsec and 1.8 nsec. These two lifetimes, however, are not unique and other pairs of lifetimes can be fitted to the experimental decay curve. Furthermore, the noise is too high for a precise measurement of the shape of the decay.

The fluorescence lifetime was measured more precisely with the phase shift method. Table V shows the phase shifts obtained from the three algae using two different glass cut-off filters. The first filter is a Corning CS 2-64, which has zero transmittance up to 640 nm, 50% transmittance at 670 nm and 85% transmittance at 720 nm and longer wavelengths. The second filter is a Corning CS 7-59, which has zero transmittance up to 685 nm, 50% transmittance at 710 nm and 85% transmittance at 750 nm and

longer wavelengths. The results for all three algae show that the phase shift increases on using the CS 7-59 filter which cuts off most of the F685 fluorescence band and allows mainly the F730 band to go through. As discussed in the previous chapter (see p. 57), the phase shift is corrected for the error due to the differences in the emission wavelength that the photomultiplier monitors by subtracting the differences in the phase shift as measured with methylene blue with the two cut-off filters. The corrected phase shifts are also shown in Table V.

The value of the fluorescence lifetime of the F685 band and the F730 band cannot be calculated exactly from this data. Detailed analysis was not done for several reasons. First, there may be more than two fluorescing components. The F730 band may have two lifetimes due to the fact that 62% of its fluorescence may come from photosystem I and 38% from photosystem II.[129] Also there may be more fluorescing components in the 740 nm to 800 nm region.[129] Second, as yet, it is impossible to know the exact contribution of each band. For example, F680 has a vibrational band at 740 nm at room temperature, but at 77°K this vibrational band cannot be distinguished from the F730 band. Third, the ratio of the fluorescence intensity of the F680 band and the F730 band changes depending on the method of cooling the sample.[118, 129] The fluorescence spectra must be made on the same frozen sample as that used on measuring the lifetime. Fourth, the error introduced in the phase shift measurement by the difference in the wavelength of the emitted light and the wavelength of the scattered reference light must be corrected for exactly. Hence, a detailed analysis with many assumptions and parameters was not done.

TABLE V

PHASE SHIFT MEASUREMENT OF ALGAE AT 77°K

θ is the phase shift in degrees. 2-64 and 7-59 are the Corning cut-off filter used. Exciting wavelength is 632.8 nm. The frequency of modulation is 102.207 Mhz.

Chlorella		Anacystis		Porphyridium		Methylene Blue (23°C)	
θ (2-64)	θ (7-59)	θ(2-64)	θ(7-59)	θ (2-64)	θ (7-59)	θ (2-64)	θ (7-59)
54.7	60.0	33.7	36.1	37.1	40.9	14.3	16.4
51.1	56.6	24.3	26.4	34.5	39.0		
52.0	58.0	25.6	27.8	33.7	38.8		
52.0	56.9	24.2	27.7	34.9	39.9		
51.1	55.4	28.9	30.5	31.5	36.4		
54.8	59.5	22.4	24.7				
51.1	58.2						
49.6	55.2						
49.1	54.5						
average θ		average θ		average θ			
51.7	57.1	26.5	28.8	34.3	39.0		
corrected θ		corrected θ		corrected θ			
51.7	55.0	26.5	26.7	34.3	36.9		

To find the approximate lifetimes of the excited species that are responsible for the F685 and F730 bands, a rough estimate must be made of the percentage of the fluorescence intensity that is contributed by each band. These estimates are made from fluorescence emission spectra of the algae at 77°K. Rough estimates are shown in Table VI. The excited species that are responsible for the F695 band are assumed to have the same fluorescence lifetime as those responsible for the F685 band. Then using equation 31 we can estimate the two lifetimes for Chlorella by solving the simultaneous equation

$$\begin{aligned} .25 \cos\theta_1 \sin(51.7 - \theta_1) + .75 \cos\theta_2 \sin(51.7 - \theta_2) &= 0 \\ .06 \cos\theta_1 \sin(55.0 - \theta_1) + .94 \cos\theta_2 \sin(55.0 - \theta_2) &= 0 \end{aligned} \qquad (34)$$

The approximate solution for θ_1 is 42.0° and for θ_2 is 56.0°. Calculating the fluorescence lifetime from the phase shift, we obtain 1.4 nsec for the excited species that are responsible for the F680 band and 2.31 nsec for those responsible for the F730 band. Similarly we find that the fluorescence lifetime for the excited species that are responsible for the F680 band for Anacystis is 0.77 nsec and the fluorescence lifetime for those responsible for the F730 band is 0.79 nsec. For Porphyridium, the excited species that are responsible for the F680 band were found to have a fluorescence lifetime of 0.91 nsec and those responsible for the F730 band a lifetime of 1.22 nsec. In each case the lifetime of the excited species that are responsible for the F730 band is longer than that of those responsible for the F680 band. This result is expected as the fluorescence yield of the F730 band increases at 77°K. Our τ values are smaller than the 3.1 nsec

TABLE VI

FLUORESCENCE LIFETIME OF ALGAE AT 77°K

F680 and F730 are the two predominant fluorescence bands at 77°K. 2-64 and 7-59 are the Corning cut-off filters used to monitor the fluorescence. The first two lines in the table show the percentage of the total fluorescence that is in each band as allowed by the cut-off filter. The third line is the phase angle calculated for each band and the last line shows the lifetime calculated for each band.

	Chlorella		Anacystis		Porphyridium	
	F680	F730	F680	F730	F680	F730
2-64	0.25	0.75	0.50	0.50	0.45	0.55
7-59	0.06	0.94	0.16	0.84	0.13	0.87
θ in degrees	42.0°	56.0°	26.2°	26.8°	30.2°	38.0°
τ in 10^{-9} sec	1.40	2.31	0.77	0.79	0.91	1.22

that Butler and Norris[37] found for the lifetime of the excited species that are responsible for the F730 band in bean leaves at 77°K. This difference may be attributed to the different biological specimens used. Note that the F730 lifetime of Anacystis is much lower than that of Chlorella. On comparing the fluorescence lifetime of the excited species that are responsible for the F730 band in the three algae, we found that they vary in the same way as the fluorescence lifetime of the chlorophyll molecule with fluorescence band at 685 nm. This result implies that the fluorescence lifetime of the chlorophyll molecule with fluorescence band at 730 nm may be controlled by the rate of energy transfer from Chl a with fluorescence at 685 nm to the Chl a with fluorescence at 730 nm as well as by the temperature. If the main cause for non-radiationless loss in the Chl a molecules with fluorescence at 685 nm is due to energy transfer to Chl a molecules which fluoresce at 730 nm, then the rate of energy transfer will be proportional to the rate of non-radiationless loss. Since Chl a 678 that fluoresces at 685 nm in Chlorella has the lowest rate of non-radiationless decay of the three algae used (as it has the longest fluorescence lifetime), it will have the lowest rate of energy transfer from Chl a 678 to the long wave form of Chl a that fluoresces at 730 nm.

In all three algae, the fluorescence lifetime for the excited species that are responsible for the F685 band at 77°K has a value similar to the fluorescence lifetime with DCMU added at room temperature. If one assumes that the rate of fluorescence does not change from room temperature to 77°K, then α_h, the rate of radiationless loss calculated from the fluorescence lifetime of algae with reaction centers closed by DCMU, at room temperature is equal to the fluorescence lifetime of algae whose

reaction centers are closed by low temperature at 77°K (cf. equation 29). α_h, the rate of radiationless loss, then, is temperature independent. This result agrees with the fact that α_h for Chl a *in vitro* is also temperature independent. Butler and Norris[37] found that 10^{-5}M Chl a dissolved in ethanol has the same lifetime at room temperature and at liquid nitrogen temperature. Since the mechanism of radiationless loss is not known, it is difficult to speculate on the importance of this result.

The experimental results at 77°K did point out two facts. First, the fluorescence lifetime of the F730 band does not have one unique fluorescence lifetime, but varies from one species to another. Second, the lifetime of F685 at 77°K is similar to that of F685 at room temperature with all the reaction centers closed. Hence one of the properties of the mechanism of radiationless loss is that its rate is temperature independent up to 77°K.

In summary, comparison of the existing experimental results[50, 51] which show fluorescence yield is linearly proportional to the fluorescence lifetime with the results predicted with the assumption of one and two lifetimes shows that the decay of Chl a *in vivo* has only one major lifetime. This result implies that the photosynthetic units are all connected and any one of a number of reaction centers act as a trap of excitation energy absorbed by any chlorophyll molecule. Direct experimental proof by the flash technique of the existence of only one lifetime failed because of the poor time response of the photomultiplier.

From the value of the fluorescence lifetime when the reaction center is closed and when it is open, we have calculated the rates and efficiencies of the primary processes of the excitation energy after a photon is absorbed,

namely fluorescence, radiationless loss and trapping. The values of the rates and efficiencies for the three algae are shown in Tables III and IV. The rate of fluorescence is calculated to be approximately 10^7 sec^{-1}, the rate of radiationless loss to be approximately 10^8 sec^{-1} and the rate of energy trapping to be approximately 10^9 sec^{-1}. The lifetime when the reaction center is closed is measured precisely in DCMU-treated algae by the phase shift technique, and the lifetime when the reaction center is open is calculated from the ratio of the fluorescence yield of the "0" level to that of the DCMU level. (This is possible since the yield is linearly proportional to the lifetime.) Measurements at $77^{\circ}K$ show that the rate of radiationless loss is approximately the same as that at room temperature.

IV. POLARIZATION OF FLUORESCENCE: RESULTS AND DISCUSSION

To resolve the question of whether the excitation energy is transferred by means of a random "hopping" process or by means of a "wave packet," we have measured the polarization of fluorescence when most of the reaction centers are open and when all the reaction centers are closed (also see Chapter I, section D, for introduction to this problem); the latter is achieved by the addition of DCMU.

Table VII shows the polarization of fluorescence measured (as discussed in Chapter II, section E) for Chlorella and Porphyridium with and without the addition of 10^{-5}M DCMU. A lower concentration of DCMU (10^{-6}M) gave similar results. The exciting wavelength was 632.8 nm and a Corning CS 2-64 filter was used to separate the fluorescence from the exciting light. The polarization of fluorescence of cells with DCMU added is smaller than the normal cells in both cases. The difference is clearly seen in the case of Porphyridium.

Table VIII shows the polarization of fluorescence of Chlorella and Porphyridium measured with different cut-off filters. The transmittance properties of CS 2-64 and CS 7-59 filters have been described in the previous chapter. The CS 7-69 is a filter that has zero transmittance up to 710 nm, 50% transmittance at 740 nm and a peak transmittance of 80% at 760 nm. The polarization of fluorescence using these cut-off filters does not seem to change. (For a discussion of results obtained by other investigators, see Chapter I, section D).

Two explanations can be given for the decrease in p when DCMU is added to the cell. First, a "trivial" possibility. The decrease in p may be due to loose (or "dead") chlorophyll-protein complexes with high p. We define loose as not being attached to any photosynthetic unit. These chlorophyll-protein complexes are individually isolated due to an imperfection in the synthesis of the structure of the chloroplast by the cell. These chlorophyll-protein complexes may have a high value of p because the extent of energy migration is small if the number of chlorophyll molecules in the complex is small. If the number of chlorophyll molecules is large, then they could be aligned with respect to each other. Although their p value may be large, their contribution to the total fluorescence is small compared with that coming from the photosynthetic unit. Assuming that the p from the photosynthetic unit due to extensive energy migration is zero, then the small p measured is due entirely to the chlorophyll-protein complex. Since the fluorescence yield increases when DCMU is added to the cell, the percentage of the total fluorescence that is due to the loose chlorophyll-protein complexes decreases. Hence p will also decrease. This can be easily seen from the definition of p in equation 8. An increase in the fluorescence yield from the completely depolarized component will only add to the denominator $I_{\parallel} + I_{\perp}$. Hence the ratio $\frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$ or p will decrease.

The number of loose chlorophyll-protein complexes can be calculated from the measured value of p. We will assume that the p for the complex is 0.20.[57] Using Weber's Summation Law,

$$\left(\frac{1}{p} - \frac{1}{3}\right)^{-1} = \sum_i f_i \left(\frac{1}{p_i} - \frac{1}{3}\right)^{-1} \qquad (35)$$

TABLE VII

POLARIZATION OF FLUORESCENCE

Excitation wavelength is 632.8 nm. Corning filter CS-2-64 was used.

Sample	Normal	10^{-5}M DCMU
Chlorella	0.008	0.005
	0.009	0.006
	0.009	0.006
	0.008	0.006*
Porphyridium	0.021	0.010
	0.021	0.013
	0.021	0.011

*10^{-6}M DCMU added

TABLE VIII

POLARIZATION OF FLUORESCENCE

Fluorescence is monitored with three Corning cut-off filters. Excitation wavelength is 632.8 nm.

Sample	CS 2-64	CS 7-59	CS 7-69
Chlorella + 10^{-5}M DCMU	0.005	0.004	0.004
Porphyridium +10^{-5}M DCMU	0.011	0.012	0.010

where p is the overall polarization

f_i is the fraction of the fluorescence intensity contributed by the i^{th} group of oscillators

p_i is the polarization of the i^{th} group of oscillators

and the measured value of p as 0.01 in Chlorella, then f, the probability that the fluorescence comes from the loose chlorophyll-protein complex, can be calculated. Substituting known values into equation 35, we have

$$\left(\frac{1}{.01} - \frac{1}{3}\right)^{-1} = f\left(\frac{1}{.20} - \frac{1}{3}\right)^{-1} + (1-f)\left(\frac{1}{0} - \frac{1}{3}\right)^{-1}.$$

Solving the above equation, f for Chlorella is 5%. Assuming the fluorescence yield of the loose chlorophyll-protein complex inside the cell is the same as the fluorescence yield of photosystem II and hence twice as large as the measured fluorescence yield, then the absorption crossection of the complex is 2.5% that of the photosynthetic unit in Chlorella. In Porphyridium a similar calculation shows that the absorption crossection of the loose chlorophyll-protein complex is 5% that of the photosynthetic unit. Since the chlorophyll-protein complex could have very similar emission spectra to that of the photosynthetic unit, it is not surprising that there is no change in p when different bands of the fluorescence spectra were measured. However, the experimental result does show that any highly oriented chlorophyll that fluoresces in the far-red region does not contribute significantly to the measured p.

The second explanation assumes that there are no loose chlorophyll-protein complexes. The small value of p measured is due to the partial

retention of the initial polarization as energy migrates about a partially random system of chlorophyll molecules. To see why p should decrease on the addition of DCMU to the cell, Weber's Summation Law as shown in equation 35 will again be used. We will define the variable h_i by the following equation

$$\left(\frac{1}{p_i} - \frac{1}{3}\right)^{-1} = h_i \left(\frac{1}{p_o} - \frac{1}{3}\right)^{-1} \tag{36}$$

where p_o is the polarization of the emitter at infinite dilution

Then combining equations 35 and 36, we have

$$\left(\frac{1}{p_i} - \frac{1}{3}\right)^{-1} = \left(\frac{1}{p_o} - \frac{1}{3}\right)^{-1} \sum_i f_i h_i \tag{37}$$

Since p and p_o are much less than 3, we can ignore the 1/3 in the above equation. Equation 37 becomes

$$p = p_o \sum_i f_i h_i \tag{38}$$

Let p_1 be the polarization of fluorescence measured in the normal cell and p_2 be the polarization of fluorescence measured in cells with DCMU added. Then,

$$\frac{p_1}{p_2} = \frac{\sum_{i=1}^{N_1} f_i^{\,1} h_i}{\sum_{i=1}^{N_2} f_i^{\,2} h_i} \tag{39}$$

where N_1 is the total number of chlorophyll molecules visited by the exciton before it is dissipated in the normal cell

N_2 is the total number of chlorophyll molecules visited by

the exciton before the energy is dissipated in the DCMU treated cell

Assuming that the chlorophyll molecules are oriented with respect to each other in a partially random way such that only the initial molecule that absorbed the polarized photon will emit light of high polarization, then h of the initial absorbing molecule (h_o) is larger than the h of all other molecules. Hence h_i for $i > 1$ in equation 39 can be neglected. Equation 39 becomes

$$\frac{p_1}{p_2} = \frac{f_o^1}{f_o^2} \tag{40}$$

where f_o^1 is the fraction of the total fluorescence that is contributed by the chlorophyll molecules that initially absorb the polarized photons in normal cells

f_o^2 is the fraction of the total fluorescence that is contributed by the chlorophyll molecules that initially absorb the polarized photons in cells with DCMU added

The measured ratio of p_1 and p_2 for Chlorella is 1.33 and for Porphyridium is approximately 2.0. From equation 40, this implies that the fraction of the total fluorescence that is emitted by the original absorber when the cells have most of their reaction centers open is greater than when the cells have all their reaction centers closed. If one assumes that the fluorescence yield of the initial absorber is the same in normal cells and in cells with DCMU added, then the ratio of the initial absorbing chlorophyll

molecule to the number of excited chlorophyll molecules that can fluoresce is smaller in cells with DCMU added. This implies that the total number of excited chlorophyll molecules that can fluoresce is greater in cells with DCMU added than in normal cells. Hence the number of molecules that are visited by the excitation energy when the reaction centers are closed is greater than when the reaction centers are open. As discussed in the introduction (p. 21), this result implies that the energy migrates in a hopping motion and the pairwise transfer is due to the Förster mechanism. If the energy migrates as a delocalized exciton, then, as mentioned in the introduction, the excitation has an equal probability of being in each molecule within the unit regardless of whether the reaction center is open or closed. Hence in this case, f_o^1 will be equal to f_o^2 and there should be no change in the measured p for normal cells and for cells with DCMU added. Hence the measured change in p, if not due to loose chlorophyll-protein complexes, implies that the energy is transferred by the "weak coupling" mechanism and the overall energy migration can be visualized as a random hopping motion.

The reason that Porphyridium has a higher p than Chlorella may be due to the fact that the rate of radiationless loss in Porphyridium is greater than in Chlorella, as shown in Chapter III. The extent of energy migration is less in Porphyridium than in Chlorella.

Both explanations for the measured decrease in p are plausible. It is difficult to design an experiment that can prove or disprove the existence of loose chlorophyll-protein complexes. Action spectra or emission spectra of the polarization of fluorescence are inconclusive

because both results can be explained by loose chlorophyll-protein complexes or by energy migration. Trying to isolate only the photosynthetic unit and measuring its polarization of fluorescence would also be inconclusive because the isolation techniques are not good enough to isolate only the pure photosynthetic unit without any loose chlorophyll. Using different concentrations of CTAB (cetyl trimethylammonium bromide) on cabbage chloroplasts, Weber[56] found that the polarization of fluorescence goes up with increasing concentration of CTAB. This result could mean that the increasing concentration of detergent makes an increasing number of loose chlorophyll molecules. This could also mean that the increasing concentration of detergent makes smaller particles so that the extent of energy migration is less. (The latter could be an explanation for the higher degree of polarization in the smaller system I than in the larger system II particles measured by Cederstrand and Govindjee.[63]) If loose chlorophyll-protein complexes are assumed to exist, they constitute only three to five percent of the total chlorophyll molecules in vivo, quantum yield measurements of oxygen are not accurate enough to detect a three to five percent waste of energy. Although one would expect no fluorescence transients from these loose complexes because they do not perform any photochemistry, measurements of fluorescence transients cannot be used to detect these complexes because one third of the total fluorescence does not show any transient. Experimentally, then, it is very difficult to prove or disprove the existence of loose chlorophyll-protein complexes in the cell.

It is highly unlikely, however, that the cell, having its own machinery to control and to direct the synthesis of the photosynthetic unit,

would waste energy to make imperfect units which in turn will compete with regular units for photons. If we can assume that the polarization of fluorescence is not caused by loose chlorophyll-protein complexes, the decrease in the polarization of fluorescence, due to the closing of the reaction centers, is the first experimental evidence which shows that energy transfer between Chl a molecules in vivo is by the Förster mechanism.

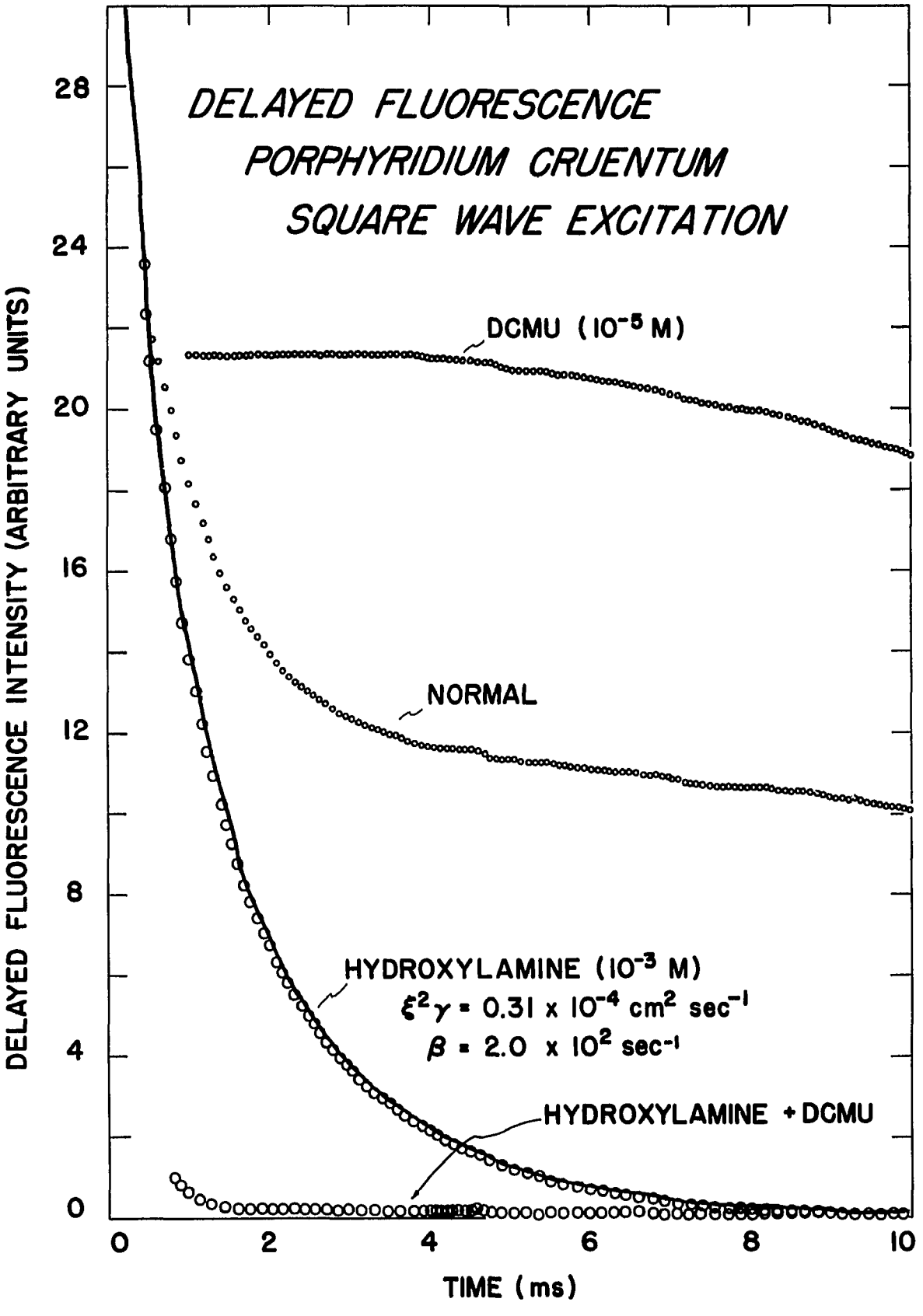
V. DELAYED LIGHT EMISSION AND INDUCED LUMINESCENCE: RESULTS AND DISCUSSION

A. DELAYED LIGHT EMISSION

Delayed light emission from the red alga Porphyridium cruentum was measured. This was an extension of the work done on Chlorella and Anacystis by the author in collaboration with Stacy, Swenberg and Govindjee.[101] The experiment was done to show that in Porphyridium as well as Chlorella and Anacystis the delayed emission can be explained by a triplet fusion model in which the production of the triplets is mediated by the photosystem II reaction center. (For a review of our theory and a general introduction to this problem, see Chapter I, section E.) The reasons for adding DCMU and hydroxylamine to the cell will be discussed later.

Delayed light emission from Porphyridium cruentum excited by a rectangular pulse of 632.8 nm light 4.5 msec in width repeated 25 times per second is shown in Figure 27. Addition of 10^{-3}M hydroxylamine to the normal cell causes the delayed emission to have a fast decay. This decay is not exponential. Addition of 10^{-5}M DCMU to the normal cell causes the fast decay to disappear. In the time range measured, the delayed light emission with DCMU added decays slowly and its intensity is higher than that given off by normal cells or normal cells with hydroxylamine added. The delayed light emission of the normal cell looks like it is intermediate between the two DCMU and hydroxylamine cases. Addition of 10^{-5}M DCMU and 10^{-3}M hydroxylamine to the cell causes a dramatic decline in the intensity of the delayed light emission.

Figure 27. Intensity of square wave excited delayed light emission(labelled fluorescence) versus time for normal Porphyridium cruentum and for Porphyridium with 10^{-5}M DCMU, with 10^{-3}M hydroxylamine and with both 10^{-3}M hydroxylamine and 10^{-5}M DCMU. The open circles are experimental points. The smooth line is the theoretical curve.


DELAYED FLUORESCENCE
PORPHYRIDIUM CRUENTUM
SQUARE WAVE EXCITATION
DCMU (10^{-5} M)
NORMAL
HYDROXYLAMINE (10^{-3} M)
$\xi^2\gamma = 0.31 \times 10^{-4}$ cm^2 sec^{-1}
$\beta = 2.0 \times 10^2$ sec^{-1}
HYDROXYLAMINE + DCMU
DELAYED FLUORESCENCE INTENSITY (ARBITRARY UNITS)
TIME (ms)
0
4
8
12
16
20
24
28
0
2
4
6
8
10

No effect either on the shape of the decay or on the intensity of the emission is detected upon applying a magnetic field as high as 13.2 kilogauss.

The solid curve in Figure 27 is a plot of the theoretical curve given by equation 20 in the first chapter. Equation 20, as derived in the introduction, is the predicted kinetic curve for delayed light emission based on the model that delayed light emission is caused by a bimolecular reaction (i. e., triplet-triplet fusion). The method to fit the curve is as follows. Using the observed intensity of delayed emission at 1 msec, F_o was calculated with equation 20 for an arbitrarily chosen constant $\xi^2\gamma$ (the bimolecular fusion rate constant, γ, multiplied by the constant ξ^2 which is defined in equation 15) and β (the monomolecular decay rate of the triplet). $\xi^2\gamma$ and β were then varied over a range of several orders of magnitude. The F_o, $\xi^2\gamma$ and β values were then used to generate a series of theoretical curves. This was done with the digital computer I. B. M. System 360. The theoretical curve that fit the experimental curve best was chosen.

The constant β has the proper units of reciprocal time. The β used to match the experimental curve is 2×10^2 sec^{-1}. The constant $\xi^2\gamma$ formed from the theoretical curve has no meaning because it has arbitrary units of the computer output. The units of $\xi^2\gamma$ are reciprocal density multiplied by reciprocal time. To fit $\xi^2\gamma$ into its proper units, we use the fact that $(\xi^2\gamma F)^{1/2}$ has the units of reciprocal seconds For the hydroxylamine case, two milliseconds after the incident light was turned off we have:

$$\xi^2\gamma F = (2.7 \times 10^{-8})\,(4.7 \times 10^3)\,(10^6) = 127\ (sec^{-2})$$

These numerical values are from the computer output. F now has to be evaluated into its proper units of $(sec)^{-1}$ $(cm^2)^{-1}$. Two milliseconds after the incident light was turned off, the signal given off by the photomultiplier was measured by the oscilloscope to be about two millivolts. Since the load resistor of the photomultiplier was 150K, the current given off by the photomultiplier was (2mv/150K) 1.3×10^{-2} microamps. The cathode radiant sensitivity of the photomultiplier is 3.2×10^{-2} microamps per microwatt. The gain at the photomultiplier bias voltage of 1300 volts is 1.5×10^4. Hence the light signal is equal to $\left(\frac{1.3 \times 10^{-2} \mu a}{(3.2 \times 10^{-2} \mu a/\mu w)(1.5 \times 10^4)}\right) =$ 0.27×10^{-5} microwatts. The photomultiplier, however, collects only about one third of the radiation given off by the algae. The total fluorescence should equal about 0.81×10^{-5} microwatts. Since the average energy of the emitted photon is 1.7 ev, the total fluorescence is equal to $\left(\frac{0.81 \times 10^{-5} \times 10 \text{ ergs/sec}}{1.6 \times 10^{-12} \text{ ergs/ev} \times 1.7 \text{ ev}}\right) = 0.3 \times 10^7$ photons/sec To find the area which this fluorescence comes from, we assume that the cross-sectional area of one alga cell is 12×10^{-8} cm^2. At an absorbance of 0.5, we estimate that there are 6×10^8 cells/cm^3. Since the volume excited by the laser beam is only 0.1 cm^3, we calculate the total excited area to be $(1.2 \times 10^{-7}$ cm^2/cell) $(6 \times 10^8$ cells/$cm^3)$ $(0.1$ $cm^3) = 7.2$ cm^2. F is then calculated to be $\left(\frac{3 \times 10^7 \text{ photons/sec}}{7.2 \text{ cm}^2}\right) = 4.1 \times 10^6$ photons/sec cm^2. Since $\xi^2\gamma F$ is 127 sec^{-2}, $\xi^2\gamma$ in its proper unit is $\left(\frac{127}{4.1 \times 10^6}\right)\left(\frac{sec\ cm^2}{sec^2}\right) =$ 0.31×10^{-4} cm^2/sec.

Knowing the value of F and $\xi^2\gamma$ one can calculate the triplet state density n_T. From equation 17

$$n_T = \left(\frac{2F(t)}{\gamma\phi}\right)^{1/2} \tag{41}$$

At t = 2 milliseconds F is calculated to be 4.1×10^6 photons/sec cm^2 and γ is 0.31×10^{-4} cm^2/sec if one assumes that $\frac{k}{k + q_1}$ is small so that ξ^2 is approximately one. Assuming ϕ, the fluorescence yield, to be 3×10^{-2}, [130] the triplet density is calculated to be 3×10^6 cm^{-2}. The lamellae area of a Chl a molecule has been calculated to be 2×10^{-14} cm^2. [131] The ratio of chlorophyll molecules which are in the triplet state to the total number of chlorophyll molecules is calculated to be only 6×10^{-8} when the incident light has been turned off for two milliseconds. As mentioned in the introduction, the most sensitive flash photolysis instrument used by Porter and Strauss[78] to detect triplet-triplet absorption is only capable of resolving absorbance changes down to 0.5%. Hence the best experimental techniques are not sensitive enough to observe the low concentration of triplets that could account for the delayed emission observed.

Although the time course of delayed emission can be explained by the triplet-triplet fusion model, it should be pointed out that the present results do not exclude the electron-hole model. In an electron hole picture, γ would become the recombination rate and β the change in reciprocal lifetime.

Only the time course of delayed light emission for cells with the hydroxylamine added can be fitted with the theoretical curve from the triplet-triplet fusion model. However, the kinetic curves of the delayed emission fornormal cells with DCMU and of cells with hydroxylamine

and DCMU together cannot be theoretically fitted by equation 20, no matter what the values of constants $\xi^2\gamma$ and β are. This is reasonable if one notes that equation 20 is derived with the assumption that q_2, the rate of chemical back reaction, is zero. Without this assumption one cannot find a close form solution to the three different equations. With q_2 not equal to zero, we can only discuss the kinetic behavior qualitatively. To explain why cells with DCMU added have a slower decay, we have to understand the function of DCMU in the cell. Assuming that the reaction center II is comprised of an energy trap,[6] an oxidant Z[9] and a reductant Q,[8] then one can represent the reaction center as Z (Chl) Q. DCMU is postulated[8] to inhibit the oxidation of Q by the electron transport chain after it has been reduced by light. As Q is reduced and Z oxidized by light, the trapping center is inoperative to receive any further energy, this is shown by the increase of fluorescence yield when DCMU is added. We will discuss this further in the following section. Hence, with the reaction center as Z^+ (Chl) Q^- there will be triplets produced by the recombination of Z^+ and Q^-, with a slow rate constant q_2. The rate of producing Z^+ (Chl) Q^- obviously is proportional to the rate of photons absorbed. Using a rectangular pulse of light 25 times per second, the rate of producing Z^+ (Chl) Q^- is much faster than its decay to Z (Chl) Q. Hence at steady state the number of Z^+ (Chl) Q^- is much greater than Z (Chl) Q. When the incident light is turned off, the delayed emission is due predominantly to the triplets which are produced by this chemical back reaction.

No exact theoretical fit of equation 20 to the decay of delayed light emission of normal cells is possible. The shape of this decay can

qualitatively be explained as being an intermediate to the case when hydroxylamine is added and the case when DCMU is added. In the case when hydroxylamine is added, the triplet excitons are produced mainly by intersystem crossing at the trap. In the case where DCMU is added, the triplet excitons are produced predominantly by a chemical back reaction. In the normal case, at steady state conditions a certain amount of reaction centers will remain in the Z^+ (Chl) Q^- state for a short time due to the fact that at steady state some of the electron transport intermediates are reduced so they cannot reoxidize Q. Within that time Z^+ (Chl) Q^- could decay by a back reaction to form a triplet state. Hence in the normal case, triplets can be produced both by intersystem crossing and by chemical back reaction.

Since the action of hydroxylamine in the cell is to replace water as the donor of electrons,[132, 133] which will be discussed in the next section, we question why then the kinetics of delayed light emission is different for the normal and the hydroxylamine case. To understand the reason, let us look at the new oxygen data of Joliot et al.[109] and Kok et al.[110]. They measured the amount of oxygen evolved per flash with a series of short high intensity flashes of light. The result they obtained shows that after a long dark period, the first flash produces very little oxygen and the third flash produces more than twice the amount of oxygen measured at steady state. These findings show that charges are stored and that the cooperation of these stored charges is necessary to produce oxygen. (The present author has evolved two kinetic models of oxygen evolution, they will be discussed along with the models of Joliot and Kok in Appendix I.) The

normal cell after the first flash, then, can have a reaction center with Z partially oxidized and Q oxidized, for example, Z^+ (Chl) Q. We postulate that hydroxylamine inhibits the production of oxygen by destroying this intermediate by feeding an electron to Z^+ so that the reaction center becomes Z (Chl) Q. In cells with hydroxylamine added no Z^+ is stable enough to undergo chemical back reaction with Q^-, and delayed light emission is solely caused by intersystem crossing at the trap.

Adding DCMU and hydroxylamine together should eliminate the production of triplet excitons completely. Since DCMU blocks the re-oxidation of Q^- and hydroxylamine keeps the Z reduced, the reaction center will remain as Z (Chl) Q^-. Hence production of triplets by inter-system crossing is not possible due to the closed trap, and the production of triplets by chemical back reaction is not possible due to the reduced Z. This agrees exactly with experimental results. As shown in Figure 27, in cells with DCMU and hydroxylamine added the delayed emission almost disappears.

To further explain the possibility that delayed light emission in the seconds region may be due to a chemical back reaction, we follow the behavior of Q by means of fluorescence transient measurements. We also perform chemiluminescence and thermoluminescence experiments to see if, thermal and chemical perturbations will increase the back reaction.

B. FLUORESCENCE TRANSIENTS

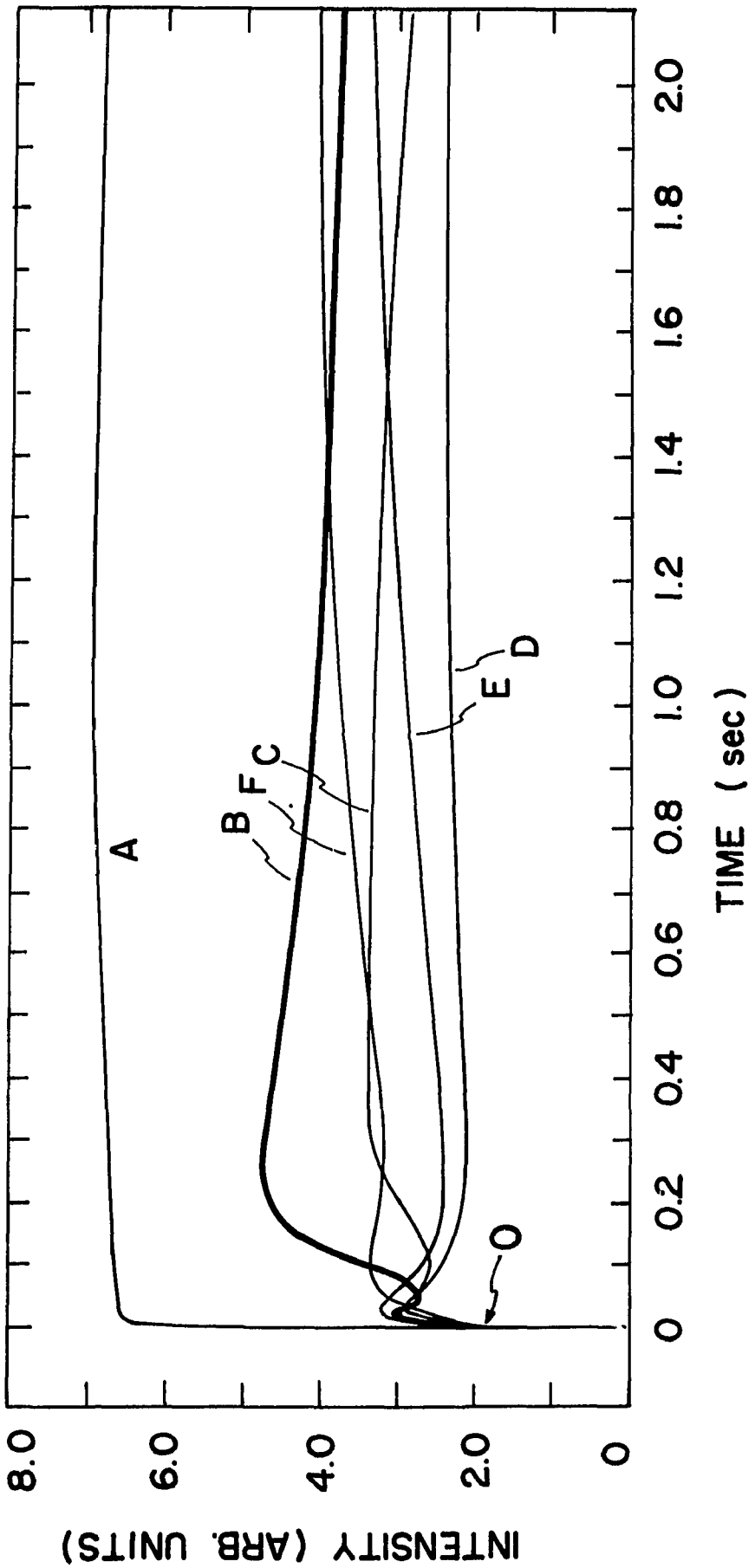
The study of the fast fluorescence transient and its relation to the oxidation and reduction state of Q has been discussed by many workers.[8, 116, 134, 135]

Fluorescence rise is believed to follow the reduction of the quencher Q to QH; the latter being a non-quencher. Hence, Q, one of the substrate believed to be involved in the chemical back reaction, can be monitored by fluorescence transient measurements.

The fluorescence transient of Porphyridium cruentum is shown in Figure 28. The exciting light was green (λ, 540 nm, broad band) with an intensity of 4.4×10^4 ergs/cm^2 sec. The fluorescence was monitored at 685 nm with a 6.6 nm band width. The sample was kept in the dark for five minutes before the fluorescence transient was taken. The normal Porphyridium fluorescence transient (Curve B) is similar to that of Chlorella reported by Munday and Govindjee.[116] As 10^{-3}M hydroxylamine is added, the transient has the O-I-D phase of the normal cell but the P level completely disappears (Curves C, D, E and F). On adding 10^{-5}M DCMU to the cell, the fluorescence yield increases and after a brief transient (0.01 sec), it remains steady with time (Curve A). On adding 10^{-5}M DCMU and hydroxylamine together to the cell, the fluorescence yield is the same as that when DCMU alone is added to the cell. The delayed emission, as was discussed in the previous section, is completely different. On adding DCMU and hydroxylamine to the cell, the delayed emission is not the same as that when only DCMU is added.

Although both DCMU and hydroxylamine inhibit the evolution of oxygen, it is clear from the fluorescence transient and DLE data that they act differently. The similarity in the transient of the normal cell with the cell with hydroxylamine added in the O-I-D phase indicates that the hydroxylamine does not stop the electron flow in the electron transport chain. Measurements

Figure 28. The fluorescence transient of Porphyridium cruentum. Curve A is the transient of Porphyridium with 10^{-5}M DCMU and with both 10^{-5}M DCMU and 10^{-3}M hydroxylamine; Curve B, normal Porphyridium; Curves C-F, Porphyridium with 10^{-5}M, 10^{-4}M, 10^{-3}M and 10^{-2}M hydroxylamine respectively. (λ excitation, 540 nm, broad band; intensity, 4.4×10^{4} ergs/cm^{2} sec.)


INTENSITY (ARB. UNITS)
8.0
6.0
4.0
2.0
0
TIME (sec)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
A
B
F
C
E
D
O

with chloroplasts indeed showed that hydroxylamine acts as an electron donor by replacing water.[132] The cause of the disappearance of the P' level in the transient of cells with hydroxylamine added is unclear. It is widely accepted that DCMU inhibits the evolution of oxygen by blocking the electron transport after Q. Q remains in the reduced QH state and the reaction center can no longer act as a quencher of fluorescence, hence the fluorescence rises. When hydroxylamine is added with DCMU, it does not change the state of QH, and hence the fluorescence remains the same as when only DCMU is added.

The initial transient (O-I) observed in cells with DCMU added represents the rate at which QH is being formed and also shows that after a long dark period all Q is in the oxidized state. The initial transient of cells with DCMU added is shown in Figure 29. Chlorella pyrenoidosa was kept in the dark for five minutes and then illuminated with 540 nm light with an intensity of 4.4×10^4 ergs/cm^2 sec. The fluorescence was measured at 685 nm with a half bandwidth of 6.6 nm. The transient of Chlorella cells with 10^{-5}M DCMU after the sample was kept in the dark for five minutes and for one minute were identical (Figure 29) This result shows that the Q^- produced by the light will all be reoxidized within one minute or less. Since Q^- cannot be reoxidized by the next intermediate in the electron transport chain because it is blocked by DCMU, a possible reoxidation step for Q^- is the combination with Z^+ to produce delayed light. Since the delayed emission has decayed to a very small value after the light was turned off for one minute, it can be inferred that most of the Q^- has been reoxidized to Q by Z^+.

The bottom curve in Figure 30 shows the initial fluorescence transient upon the addition of 10^{-2}M hydroxylamine and 10^{-5}M DCMU to cells which have been kept in the dark for five minutes. The transient is similar to that of cells with only DCMU added. One would expect this result since hydroxylamine replaces water as a donor of electrons to reduce Q and would not influence the rate at which QH is formed. The top curve in Figure 30 is the transient taken after the cells were kept in the dark for one minute following the previous measurement. The fluorescence transient is now not observed. In another experiment it was found that the top curve remains the same even after it was kept in the dark for one hour following a flash of light. This result can be explained by assuming hydroxylamine reduces Z^+ as fast as it is formed, as was discussed in the previous section. No "stable" Z^+ state is possible. Since Q^- needs Z^+ for chemical back reaction, no chemical back reaction is possible and hence no delayed emission is observed. Q will also remain in the Q^- state and hence fluorescence transients are no longer possible. There should be no variation in the fluorescence yield with time as it is observed experimentally in Figure 30.

In a report published during the preparation of this thesis, P. Bennoun[136] investigated the reoxidation of Q in the presence of DCMU and hydroxylamine by following changes in fluorescence yield. His conclusions agree with those presented above.

C. THERMOLUMINESCENCE

Arnold and Sherwood[137] found that chloroplasts that had been illuminated previously will emit light if they are heated at a rate of 14°C per

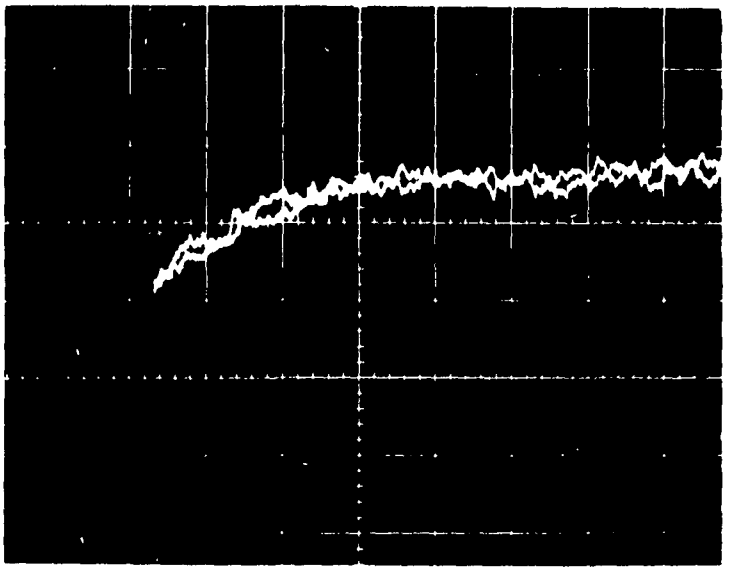


Figure 29. The fluorescence transient of Chlorella pyrenoidosa with 10^{-5}M DCMU. One curve is taken after a five minute dark time. The other curve is taken after a one minute dark time. Horizontal scale is two msec/division. (λ excitation, 540 nm; intensity, 4.4×10^4 ergs/cm^2 sec.)

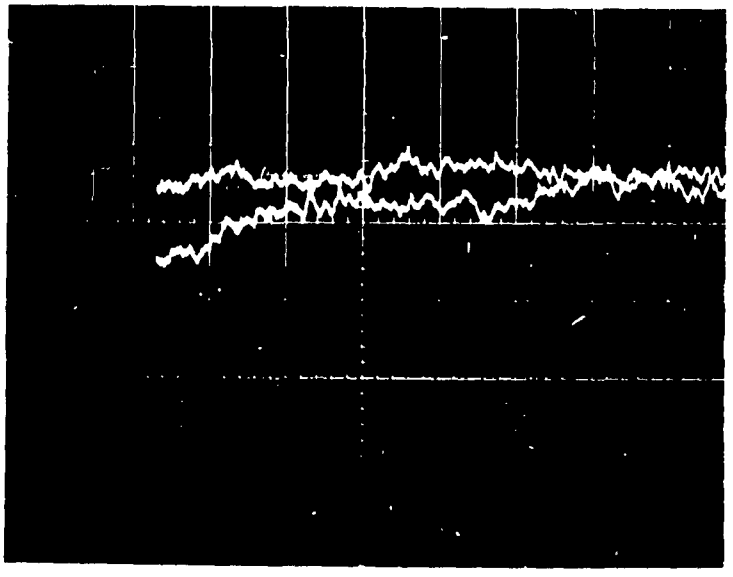


Figure 30. The fluorescence transient of Chlorella pyrenoidosa with 10^{-5}M DCMU and 10^{-2}M hydroxylamine added to the cell after five minutes in the dark. The bottom curve is the initial transient. The top curve is the transient taken one minute after the photograph of the bottom curve was taken. Horizontal scale is two msec/division. (λ excitation, 540 nm; intensity, 4.4×10^4 ergs/cm^2 sec.)

minute. The results were interpreted by Arnold as the recombination of the electrons and holes that were trapped in the photosynthetic unit (see Chapter I, section E, for the discussion of Arnold's model). Alternatively, we may suggest that the increase in temperature enhances the rate of recombination of Q^- with Z^+ to populate the triplet state of the reaction center that leads to light emission. We performed simple temperature jump experiments by injecting boiling water into a sample of algae.

Both Chlorella and chloroplasts isolated from spinach give off light when 1 ml of warm water at 53°C is injected into 1 ml of sample ten seconds after a preilluminating light is shut off. No light is given off unless the sample is preilluminated. Cold water (16°C) replacing the warm water injected after preillumination did not induce the sample to give off any light. Hence the light given off is not due to an osmotic change but a temperature change. Figure 31 shows the light emitted by normal chloroplasts, by chloroplasts with the addition of 10^{-5}M DCMU, by chloroplasts with the addition of 10^{-2}M hydroxylamine and by chloroplasts with both 10^{-2}M hydroxylamine and 10^{-5}M DCMU added. Results with Chlorella are similar except that there is a greater decrease in signal upon the addition of DCMU. In both Chlorella and spinach chloroplasts, upon the addition of hydroxylamine even with DCMU already added, thermoluminescence decreases to a low value.

The above experiment was designed such that the energy storage process is allowed to go normally at room temperature. After the energy is stored, then the temperature is suddenly made to jump. This temperature jump is used to increase the back reaction rate, q_2. It can be seen from

Figure 31 that chloroplasts give off a fairly large signal on raising their temperature suddenly from 16°C to 33°C. DCMU added to the chloroplasts decreases the signal. The reason why this happens is not known. This could be explained by the fact that only Q^- is available for recombination reaction while in normal cells both Q^- and A^- are available for recombination.

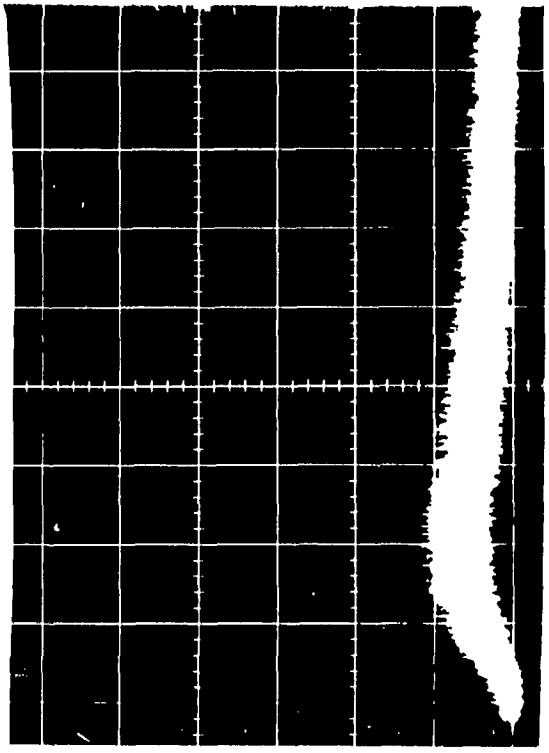
The fact that cells with hydroxylamine added or cells with both hydroxylamine and DCMU added show very little thermoluminescence could again be explained by the fact that hydroxylamine reduces Z^+ of the reaction center so that no stable Z^+ is possible. Q^- therefore has no Z^+ to recombine and hence no emission is observed. This result is consistent with the results obtained from delayed light emission and fluorescence transient measurements discussed in the previous chapter. We will show that it is also consistent with results obtained by chemiluminescence experiments.

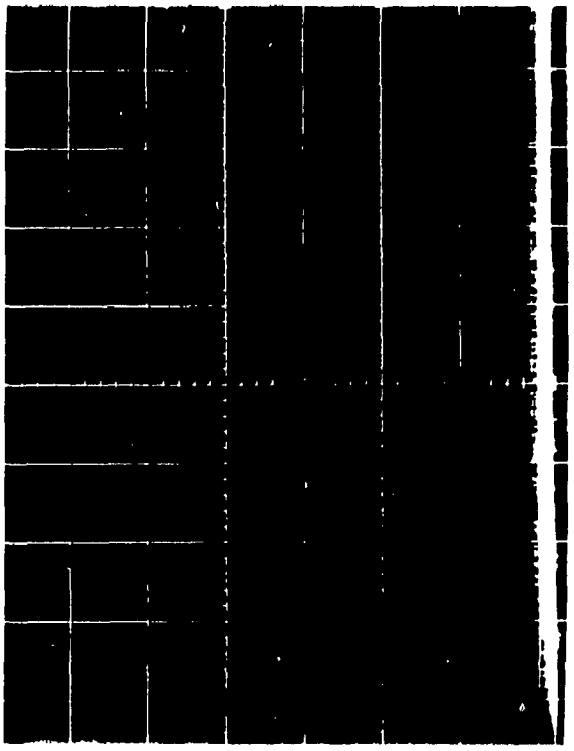
D. CHEMILUMINESCENCE

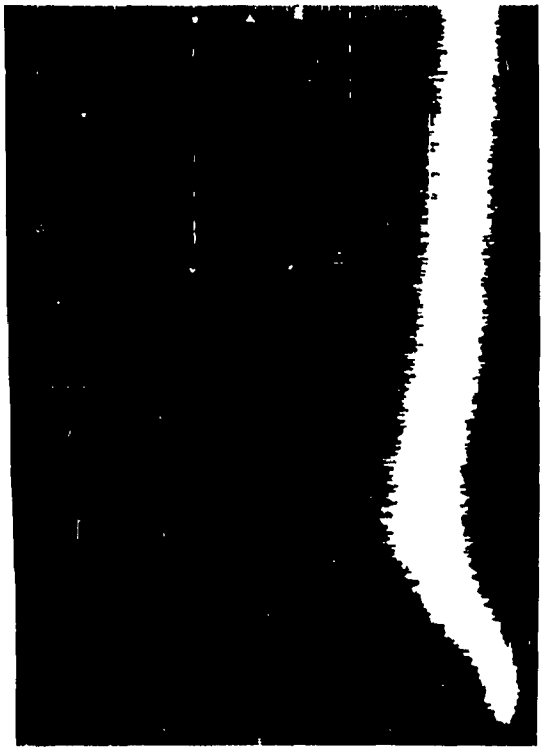
Chemiluminescence was discovered by Mayne and Clayton;[138] they showed that isolated chloroplasts emit light when subjected to an acid-base pH transition. Later Mayne[139] found that the light emission by the chloroplasts requires preillumination. This indicates that the acid-base transition may be serving as a trigger to release stored energy as in thermoluminescence.

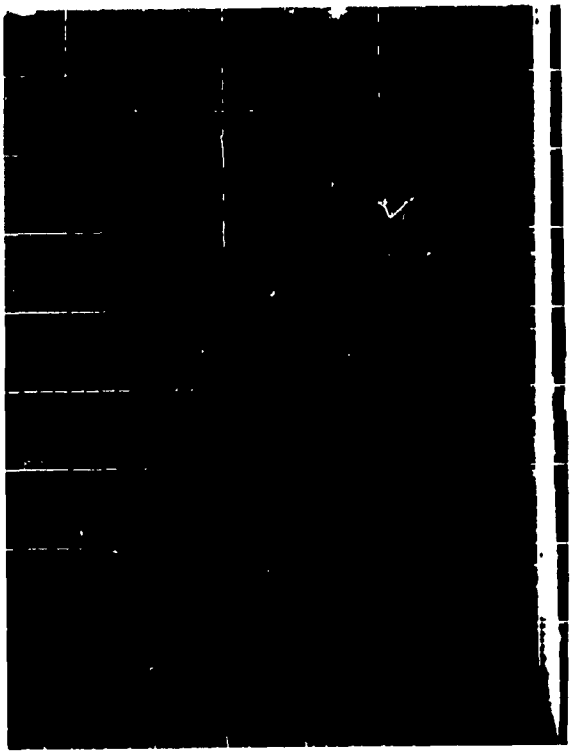
Chemiluminescence data for spinach chloroplasts are shown in Figure 32. (See Chapter II, Section G for method). The results are similar to those

Figure 31. Time course of the luminescence induced by a temperature jump in spinach chloroplasts. The temperature jump is induced by adding water at $53^{\circ}C$ to the suspension at $16^{\circ}C$. A: signal induced in normal chloroplasts; B: in chloroplasts with $10^{-5}M$ DCMU; C: in chloroplasts with $10^{-3}M$ hydroxylamine; D: in chloroplasts with both $10^{-3}M$ hydroxylamine and $10^{-5}M$ DCMU. Preillumination intensity is 3.8×10^{5} ergs/cm^{2} sec.









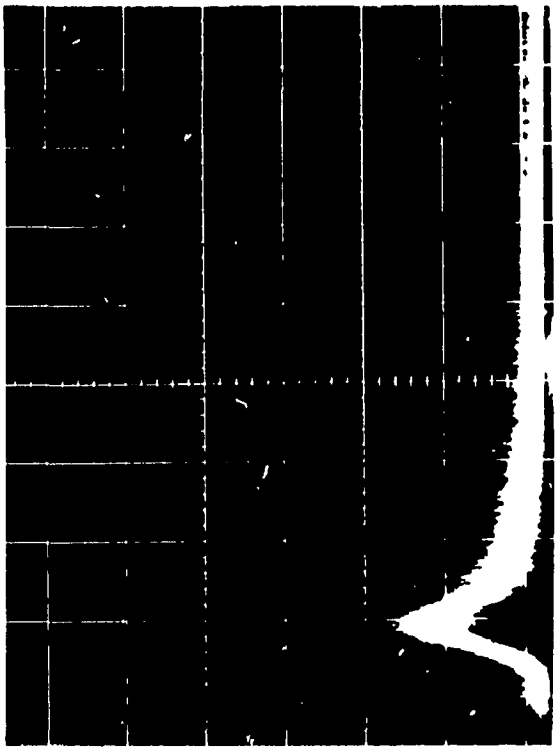
obtained for thermoluminescence. For chloroplasts suspended in buffer solution, chemiluminescence is large. For chloroplasts with 10^{-5}M DCMU added this emission decreases and for chloroplasts with 10^{-3}M hydroxylamine or with hydroxylamine and DCMU, no emission is detected.

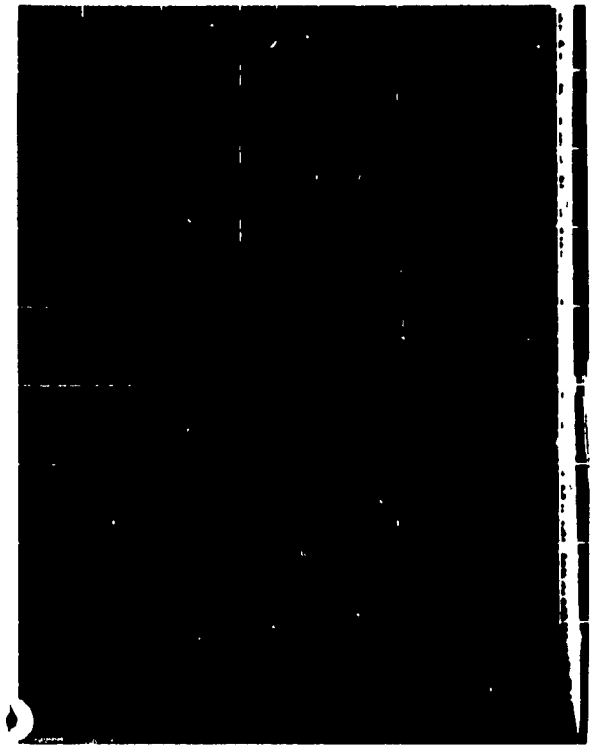
Adding acid and then base to the chloroplast to induce luminescence causes a permanent change in the chloroplast. This is shown in the change of a fluorescence transient which is permanently altered on the addition of acid and base. Addition of hot water to the chloroplast to cause thermoluminescence, however, causes only a temporary change in the fluorescence transient and the transient recovers to that of the normal chloroplast after thirty minutes.

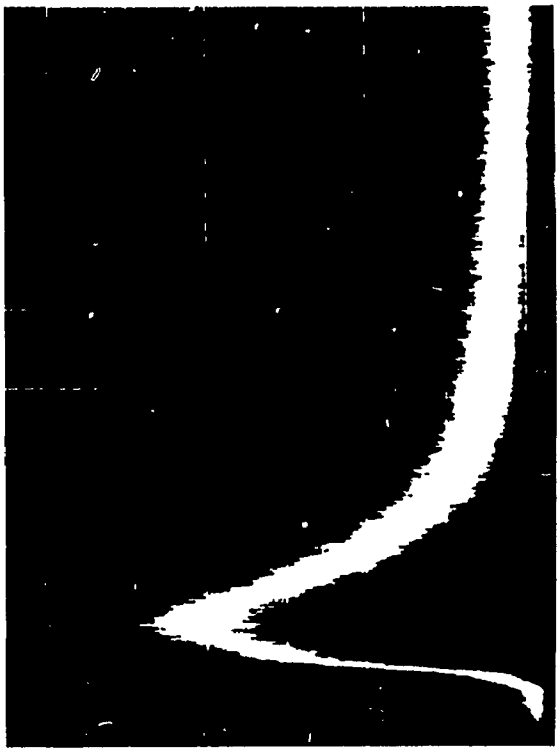
Chemiluminescence has been postulated as a chemical back reaction of Photosystem II triggered by a rapid pH change.[139] Why the emission is higher for normal chloroplasts than the emission in chloroplasts with DCMU added is again open to speculation just as in the case of thermoluminescence It could be due to the fact that in normal cells the second intermediate in the electron transport chain (A^-) can also recombine with Z^+ to cause the emission. The addition of hydroxylamine to normal or DCMU treated cells eliminates light emission drastically. It may be due to the same cause as that in delayed emission and thermoluminescence in that the hydroxylamine reacts with Z^+ so fast that there is very little Z^+ for chemical back reactions.

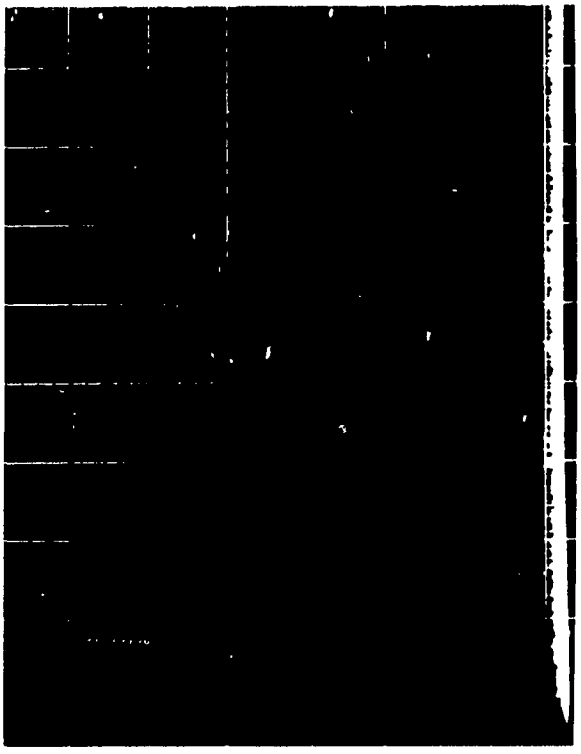
Two chemicals, DCMU and hydroxylamine, play an important role in these studies. Both chemicals, when added to the cell in sufficient concentration, will inhibit oxygen evolution. Fluorescent transient studies

Figure 32. Time course of the luminescence induced by an acid-base transition in spinach chloroplasts. A: signal induced in normal chloroplasts, B: in chloroplasts with 10^{-5}M DCMU, C: in chloroplasts with 10^{-3}M hydroxylamine; D: in chloroplasts with both 10^{-3}M hydroxylamine and 10^{-5}M DCMU. Preilluminating intensity is 3 8 x 10^{5} ergs/cm^{2} sec.









(Figure 28) show, however, that they act differently. DCMU has been shown to block Q^- from being reduced by the next component in the electron transport chain. Hydroxylamine has been shown to replace water as the electron donor to Z^+, which explains why cells with hydroxylamine added have about the same initial transients as the normal cells. From delayed light experiments it was believed that DCMU blocks intersystem crossing at the reaction center because of the accumulation of Q^- which blocks the trapping capability of the reaction center. The large delayed emission observed is believed to be due to the recombination of Q^- and Z^+ at the reaction center to populate the triplet state, which can wander out into the bulk pigment and annihilate with another triplet to form an excited singlet state. Hydroxylamine, however, is believed to block the chemical back reaction which produces the triplet s tate. The reason is that although Z^+ states can exist for a long time in the dark when water is used as the electron donor, hydroxylamine reacts quickly with the Z^+ so that Z^+ cannot exist as a stable state. In cells with hydroxylamine added, intersystem crossing at the reaction center is believed to be the main way to populate the triplet state. The measured decay curve of the delayed emission of cells with hydroxylamine agrees exactly with the theoretical curve calculated with the assumption of intersystem crossing with no chemical back reaction. DCMU, then, blocks intersystem crossing and hydroxylamine blocks chemical back reaction. If both chemicals were added to the same cell, one should expect no delayed light emission as both paths to produce the triplets are blocked. This is indeed observed experimentally in delayed emission measurements.

Since thermoluminescence and chemiluminescence phenomena are explained as luminescence given off by chemical back reaction triggered by heat or pH change, and since hydroxylamine is postulated to eliminate back reactions, one would expect no observable thermoluminescence and chemiluminescence when hydroxylamine is added. This is exactly what is found experimentally (Figures 31 and 32). On the addition of DCMU with hydroxylamine also no luminescence is observed. In cells with DCMU and hydroxylamine, the chemical back reaction does not exist, Q^- is stable since DCMU blocks the reoxidation of Q^- by electron transport intermediates, and hydroxylamine eliminates Z^+ needed for the back reaction. Q^- is found to be stable from fluorescence induction experiments (Figures 29 and 30).

Studies of delayed light emission, thermoluminescence, chemiluminescence and fluorescence transients in cells with DCMU and hydroxylamine indicate the occurrence of the recombination of Q^- with Z^+. From energetic considerations, the energy from the recombination reaction is probably not high enough to populate the excited singlet state of Chl a, but high enough to populate the excited triplet state of Chl a in the reaction center. The triplet then can migrate into the bulk pigment system and collide with another triplet to produce an excited singlet state. The resultant light emitted is the observed delayed emission or induced luminescence. That triplets can also be produced by intersystem crossing mediated by the reaction center is based mainly on the analysis of the time dependence of the luminescence decay.

In conclusion, in this chapter indirect evidence was given for two other processes which occur at the reaction center besides the oxidation-

reduction reaction, fluorescence and radiationless loss; these processes are intersystem crossing and chemical back reaction.

VI. SUMMARY

The primary events in photosystem II, following the absorption of a photon by the bulk pigment system, are the migration of the excitation energy to a reaction center and the loss of the excitation energy by fluorescence and by non-radiative decay. The primary processes that occur at the reaction center, after it has trapped the excitation energy, are the utilization of the excitation energy for an oxidation-reduction reaction and the loss of the trapped excitation energy by fluorescence or non-radiative decay. Another possible process that occurs in the reaction center is the recombination reaction of the primary oxidant and primary reductant To study the primary processes in the bulk pigment system, we have calculated the rates and efficiencies of the primary processes of the excitation energy in the bulk pigment system by using data from the measurement of fluorescence lifetime (Chapter III). We have studied the mechanism of energy transfer by means of polarization of fluorescence measurements (Chapter IV). To study the primary processes in the reaction center, we use information obtained from delayed light mission, fluorescence transients, chemiluminescence and thermoluminescence measurements (Chapter V).

The lifetime of the excited singlet state of Chl a in vivo was measured both by the flash decay method and the phase shift method using a mode-locked He-Ne laser operating at a frequency of 102.207 Mhz. The flash decay method cannot resolve the question of whether the decay of the excited singlet state has one or two lifetimes due to the poor time response of the

photomultiplier. The relationship between fluorescence yield and lifetime, as measured by the phase shift method, was predicted for one lifetime decay and two lifetime decays. Comparison of the predicted results with existing experimental data shows that the decay of Chl a in vivo has only one major lifetime. This result implies that the photosynthetic units are all connected and that energy from one unit can migrate to another.

Precise fluorescence lifetime of algae with DCMU added was measured by the phase shift method. The measured lifetime was used to calculate the rates and the efficiencies of the major processes that follow after a photon is absorbed by a chlorophyll molecule: fluorescence, radiationless loss, and trapping of the energy in the reaction center. The results show that the rate of radiationless loss for the green alga Chlorella is 5.09×10^8 sec^{-1}, 12.86×10^8 sec^{-1} for the blue-green alga Anacystis, and 9.09×10^8 sec^{-1} for the red alga Porphyridium. The rate of fluorescence for all three algae is calculated to be 6.57×10^7 sec^{-1}. The rate of trapping for Chlorella is calculated to be 1.19×10^9 sec^{-1}, 1.98×19^9 sec^{-1} for Anacystis, and 1.97×10^9 sec^{-1} for Porphyridium. The quantum efficiency for trapping of excitation energy in Chlorella is then calculated to be 66%, in Anacystis, 59% and in Porphyridium, 68%. Measurements of the fluorescence lifetime at $77^{\circ}K$ show that the rate of radiationless loss of Chl a with a fluorescence band at 685 nm is independent of temperature up to $77^{\circ}K$.

Precise measurements of the polarization of fluorescence of algae showed a decrease in polarization when DCMU is added; this chemical "closes" the reaction center II. Assuming that the cause of the small polarization of

fluorescence measured is not due to loose chlorophyll-protein complexes within the chloroplast, this result is interpreted to show that the energy transfer between Chl a *in vivo* is by the Förster "weak coupling" mechanism.

To obtain information concerning the primary events in the reaction center, the delayed light emission in Porphyridium cruentum was measured in the millisecond region after excitation with flashes of light from a He-Ne laser. It was analyzed in terms of a triplet exciton model as proposed by Stacy, Mar, Swenberg and Govindjee[101] based on experiments with Chlorella. This model -- a valid alternative to the electron-hole recombination theory -- can be described as follows. Following light absorption by a bulk pigment molecule, the energy can be emitted as normal fluorescence or be lost in non-radiative decay or be transferred to the reaction center. At the reaction center, the excitation energy can be used for an oxidation-reduction reaction, can decay by fluorescence or internal conversion or lastly it can populate a triplet state by intersystem crossing. The triplet excitons can then either decay, probably by a radiationless transition, or undergo fusion and produce an excited singlet in the bulk. The light emitted from the excited singlet is observed as the delayed light emission. This process occurs in the short time region after the incident light is turned off. At the longer times (0.1 sec) we assume that the triplets can also be produced at the reaction center by chemical back reaction of the primary reduced (Q^-) and oxidized (Z^+) products of the photoreaction. This model then predicts another primary event at the reaction center after the excitation energy is absorbed, namely intersystem crossing.

The delayed light emission curves measured with Porphyridium with 10^{-3}M hydroxylamine were shown to fit exactly the theoretical curve based on the model with the contribution of the chemical back reaction ignored. Hydroxylamine is assumed to stop the chemical back reaction. The results of the delayed light emission of normal Porphyridium and Porphyridium with 10^{-5}M DCMU and with both DCMU and hydroxylamine are qualitatively explained by the triplet fusion model when the contribution of the chemical back reaction is not neglected. Thermoluminescence and chemiluminescence measurements were used to further show that the chemical back reaction is due to the primary reduced (Q^-) and oxidized (Z^+) products of the photoreaction. Addition of hydroxylamine to the cell which is assumed to stop all back reaction completely eliminates both the thermoluminensce and chemiluminescence. Fluorescence transient measurements also show that on the addition of both hydroxylamine and DCMU to the cell, the photoreduced Q^- cannot be reoxidized and remains stable. These results indicate that the primary products in the reaction center are involved in the chemical back reaction, another reaction that can occur at the reaction center.

The details of the chemical reaction, at reaction center II, leading to oxygen evolution are also discussed (Appendix I). Two new alternate models for oxygen evolution are proposed.

APPENDIX

TWO NEW MODELS FOR OXYGEN EVOLUTION IN PHOTOSYNTHESIS

A. INTRODUCTION

From the quantum yield measurements of photosynthesis,[140] it is generally agreed that it takes eight photons to produce a molecule of oxygen from water and to reduce a molecule of CO_2 in green plants. The overall process requires the transfer of four electrons, in two steps, from H_2O to CO_2.[1] The oxygen evolution step requires the transfer of four electrons from water to an intermediate (A), and this requires four photons.[141] How this is done remains a mystery. (For a recent review, see Cheniae.[142]) There is experimental evidence that indicates that photons act successively on the same photochemical center with dark intermediate steps to produce one molecule of oxygen. This rules out the possibility that the primary oxidant produced at one reaction center can migrate and react with another oxidant produced at another reaction center. Allen and Franck[143] showed that after a long dark period no oxygen was given off if algae were illuminated by a single short bright flash of light. Oxygen, however, was given off on subsequent flashes of light following the single flash. These observations were confirmed by Whittingham and Brown.[144] From measurements of the time course of oxygen evolution at low intensities, Joliot[145] found that after a long dark period there was a lag in oxygen evolution. If a preilluminating flash was given, oxygen evolution started immediately on illumination. Joliot interpreted these and other results (not cited here) to mean that in

order to obtain oxygen production two quanta must be absorbed successively in the same photochemical center producing an oxygen atom; oxygen atoms from neighboring units can combine to give an oxygen molecule. Rosenberg[146] has also measured the time course of oxygen evolution at low light intensities and from these studies has suggested that four photoacts on the same reaction center lead to the evolution of one oxygen molecule. From measurements of the rate of oxygen evolution as a function of light intensity at low levels, Kok et al.[110] found that the difference in the area bounded by the time course curve taken right after continuous preillumination (no deactivation) and that taken after ten minutes in dark was independent of intensity at low light levels. With the assumption of constant quantum efficiency, this result implies that photons are needed to fill a finite pool of intermediates before oxygen can be evolved.

More recently, Joliot et al.[109] and Forbush et al.[147] have re-investigated the evolution of oxygen by a series of short saturating flashes of light. Joliot et al.[109] found that the amount of oxygen given off per flash of light showed a damped four cycle oscillation in relation to the numbers of flashes given. On the basis of these experiments, they proposed a new scheme for the mechanism of oxygen evolution in photosynthesis. The main features of this scheme are 1) the reaction center II includes two electron donors (Z) and one electron acceptor (Q), 2) transfer of two electrons from the same donor leads to the formation of one oxygen atom and 3) the reaction center acts as a switch that connects alternately each donor to the acceptor; this is what we call a "flip-flop" mechanism. This switch

works with an efficiency of 85% and is induced by each photoact. Kok et al.[110] made similar experiments and suggested that oxygen evolution is a four quanta process that occurs in a sequence. To fit the damped four cycle oscillations, Forbush et al.[147] proposed that after a long dark period all reaction centers do not deactivate to the ground state and that the damping is caused by failure rate of the trapping centers in the photochemical conversions ("misses," α). Damping is also due to reaction centers which can receive two photons and perform two photochemical acts within the duration of the light flash ("double hits," β). In this communication, we propose two new alternate models (Figure 33c and 33d) that will fit the published data of both Kok et al.[110, 147] and Joliot et al.[109]

B. THEORETICAL TREATMENT

Any model for oxygen evolution must satisfy the following conditions. It must explain the fact that the oxygen evolved per flash, when the photosynthetic units are illuminated by a series of short ($\sim$10μs) saturating flashes of light spaced 300 msec apart, oscillates with a period of four and damps out after four to six periods.[109, 147] It must also explain the difference in oxygen yield sequences by a series of light flashes after various pretreatments with light as observed by Kok et al.[110] and Forbush et al.[147] After 25 flashes were used to attain steady state of oxygen evolution, the chloroplasts were left in the dark for five minutes and then were given a single flash or a sequence of two or three flashes or no flashes at all. After this light pretreatment, the chloroplasts were left in the

dark for 30 minutes. The oxygen yield per flash produced by a sequence of light flashes was then measured and found to be different with different pretreatments. Any model of oxygen evolution must also be able to predict the time course of the rate of oxygen evolution observed in continuous weak light following darkness or different numbers of flashes.[109, 147]

Two general alternative models are possible. The first is that the evolution of an oxygen molecule is due to a two step mechanism in which the reaction center successively accumulates two positive charges to produce an atom of oxygen. Two oxygen atoms quickly combine to produce a molecule of oxygen. The second is that the evolution of an oxygen molecule is due to a four step mechanism in which the reaction centers must successively accumulate four positive charges before a molecule of oxygen is evolved.

1. Two Step Mechanism

Joliot et al.'s model[109] for oxygen evolution is a two step mechanism. This model is drawn schematically in Figure 33A. Detailed calculations of this model show that after the first flash the sum of the amount of oxygen given off by two consecutive flashes is always equal to twice the amount of oxygen given off at steady state, i.e., the sum of the oxygen given off by the second and third flashes is equal to the sum of the oxygen given off by the fourth and fifth flashes. This prediction does not agree with either their own[109] or Forbush et al.'s[147] experimental results. As shown in Figures 34A and 35A the sum of the oxygen given off by the second and third flashes is greater than the sum of the fourth and fifth flashes. Furthermore, in

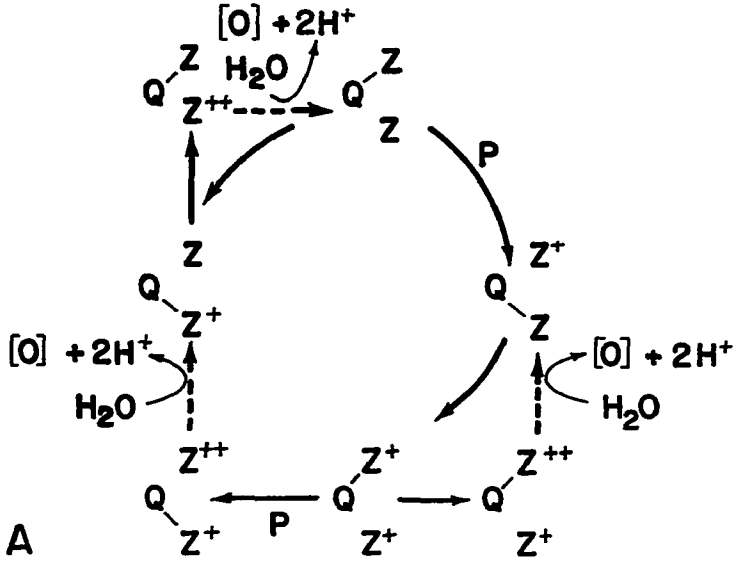
Figure 33. Schematic diagram of four different models of oxygen evolution. Q is the primary electron acceptor; Z is the primary electron donor, solid and broken arrows indicate light and dark reactions respectively.

(A) Model A for oxygen evolution redrawn from Joliot et al.[109] ρ is a probability constant.

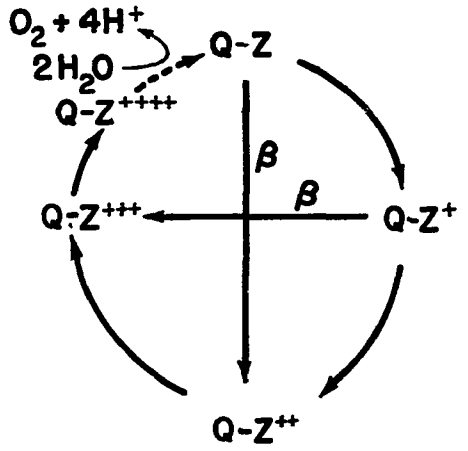
(B) Model B for oxygen evolution redrawn from Kok et al.[110] β is the probability of "double hits." Kok's S_0 has been replaced by Q-Z and S_1 by $Q\text{-}Z^+$ etc.

(C) Model C. q is a probability constant

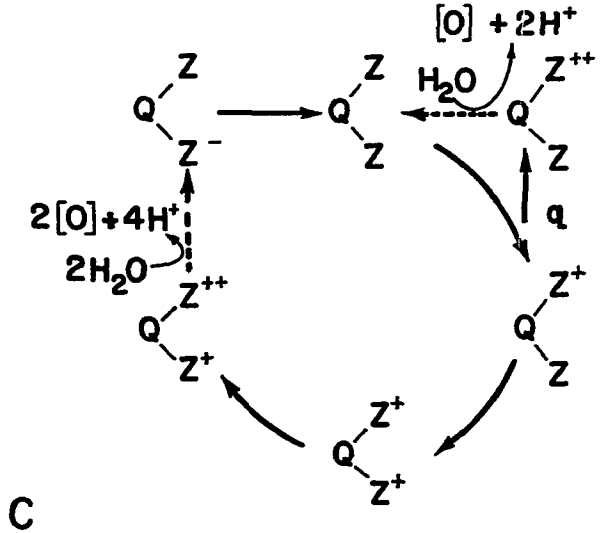
(D) Model D. A, B, ρ are probability constants.


[O] +2H+
H2O
P
[O] +2H+
H2O
[O] +2H+
H2O
P

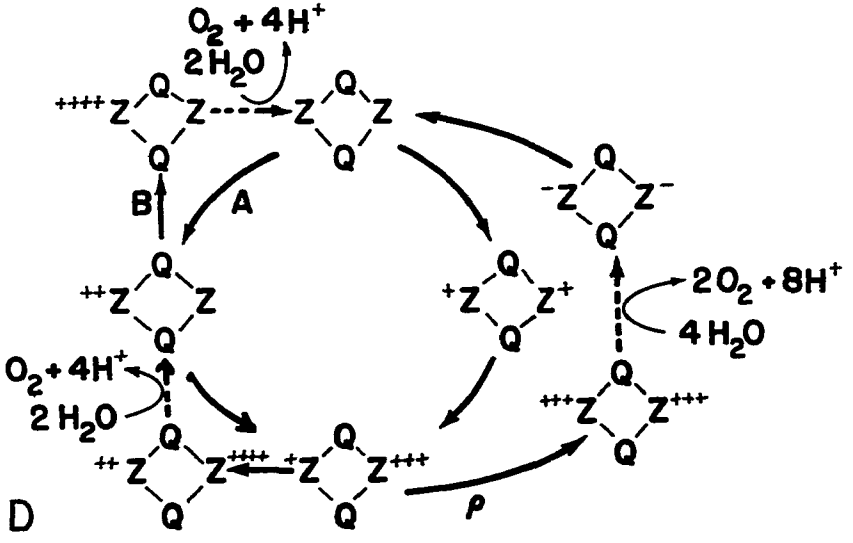
A


O2 + 4H+
2H2O
β
β

B


[O] +2H+
H2O
2[O]+4H+
2H2O
q

C


O2 + 4H+
2H2O
B
A
2O2 + 8H+
4H2O
O2+4H+
2H2O
ρ

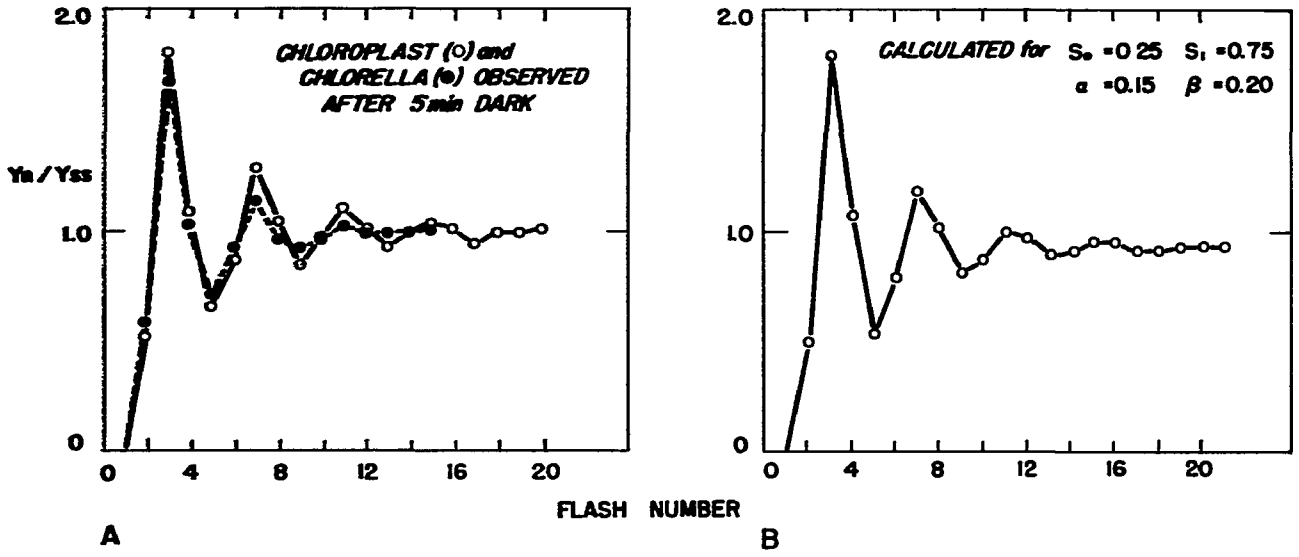
D

Figure 34. (A) The amount of oxygen evolved by illumination with a series of light flashes of saturating intensity after the cells were kept five minutes in the dark. The dark time between each flash was 300 msec. Y_n is the oxygen emitted by the n^{th} flash of light. Y_{ss} is the oxygen emitted after steady state conditions have been reached. The solid line is for isolated spinach chloroplasts. The dotted line is for the green alga Chlorella. (Experimental data of Joliot et al.[109])

(B) Predictions based on Kok's model.[110] S_o = concentration of Q-Z species, S_1 = concentration of $Q\text{-}Z^+$ species, α = "misses", β = "double hits."

(C) Predictions based on model C. γ = inefficiency index, q = a probability factor.

(D) Predictions based on model D. A, B, ρ = probability constants, γ = inefficiency index of the trap.


CHLOROPLAST (○) and
CHLORELLA (●) OBSERVED
AFTER 5min DARK
CALCULATED for S₀ =0.25 S₁ =0.75
α =0.15 β =0.20
Yn / Yss
2.0
1.0
0
0
4
8
12
16
20
FLASH NUMBER
A
B

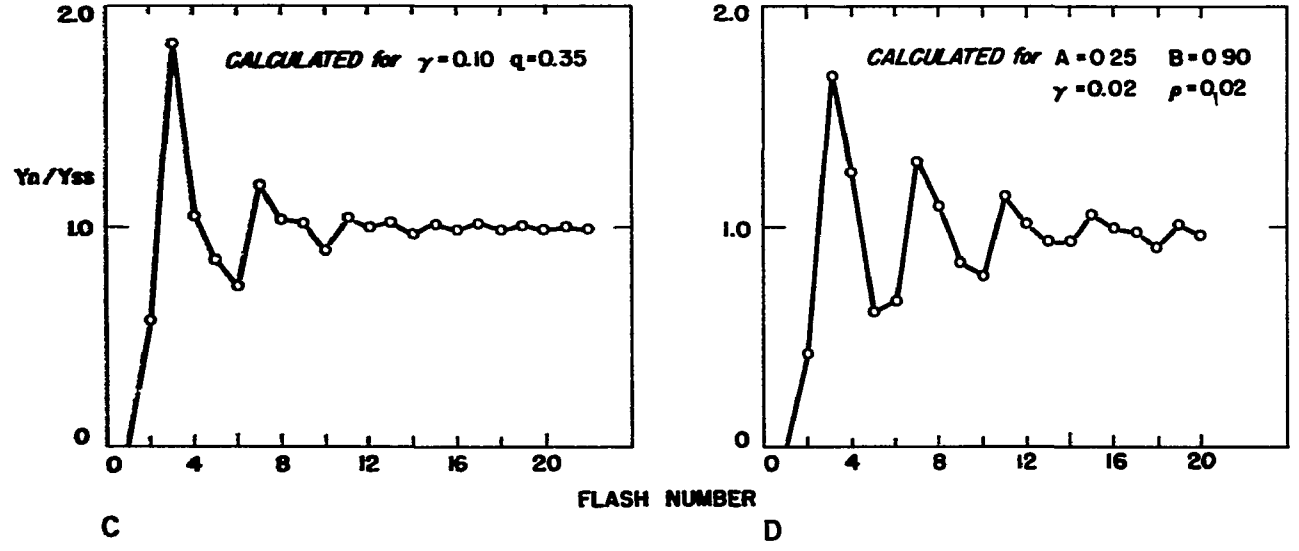
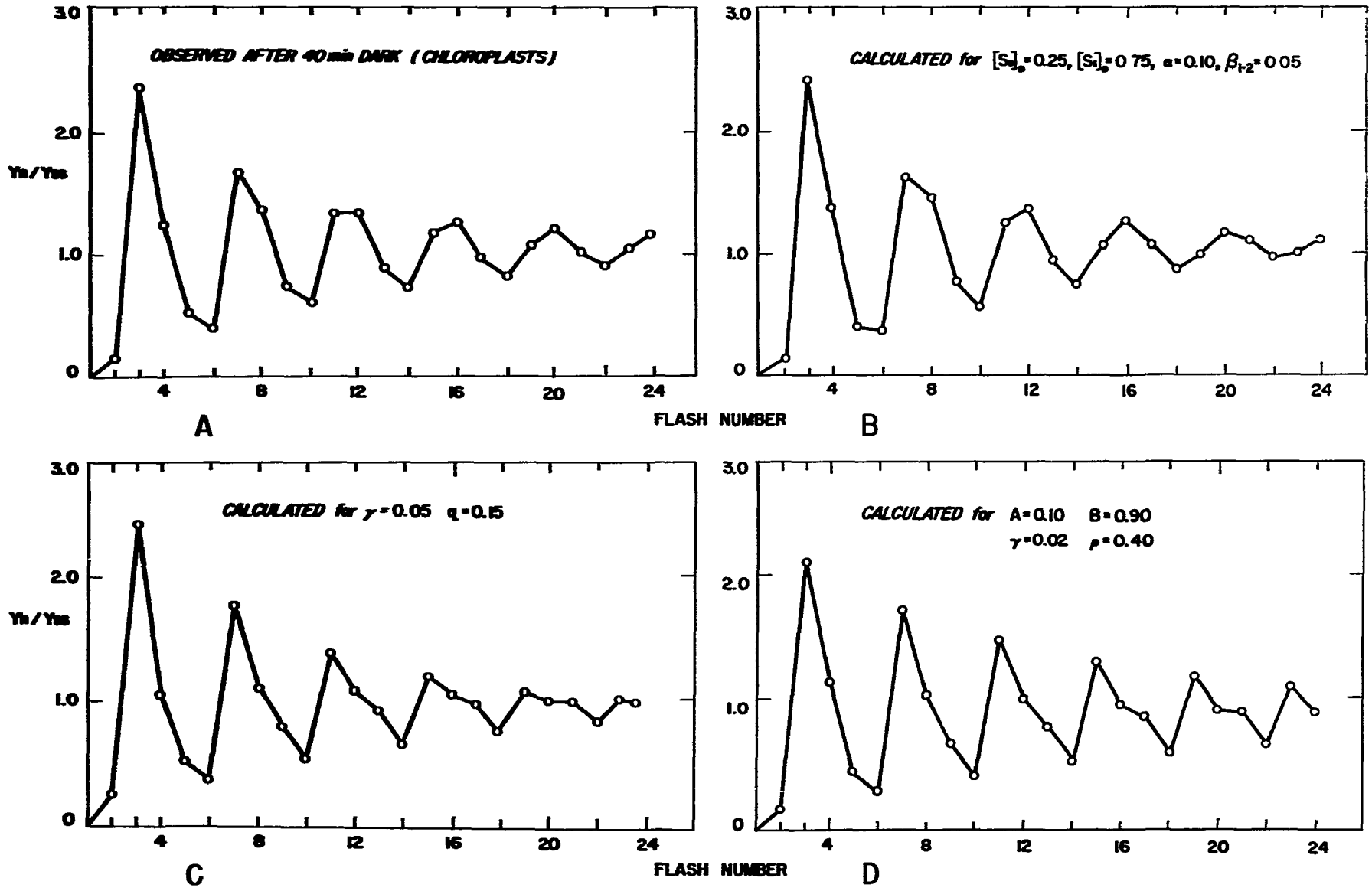

CALCULATED for γ =0.10 q=0.35
CALCULATED for A =0.25 B =0.90
γ =0.02 ρ =0.02
Yn / Yss
2.0
1.0
0
0
4
8
12
16
20
FLASH NUMBER
C
D

Figure 35. (A) The amount of oxygen evolved by illumination with a series of light flashes of saturating intensity after a long period of darkness following continuous illumination. The dark time between flashes was one sec. Isolated spinach chloroplasts were used.
(Experimental data of Forbush et al.[147])

(B) Predictions based on Kok's model.[110, 147] The symbols in this and following figures C and D have the same meaning as in Figure 33.

(C) Predictions based on model C.

(D) Predictions based on model D


OBSERVED AFTER 40 min DARK (CHLOROPLASTS)
CALCULATED for [S2]0=0.25, [S1]0=0.75, α=0.10, β1-2=0.05
CALCULATED for γ=0.05 q=0.15
CALCULATED for A=0.10 B=0.90
γ=0.02 ρ=0.40
Yn/Yss
FLASH NUMBER
A
B
C
D
0
1.0
2.0
3.0
4
8
12
16
20
24

Kok et al.[110] and Forbush et al.'s[147] experimental results the sum of the oxygen from the second and third flashes is larger than twice the amount of oxygen given off at steady state. Joliot et al.'s model[109] also assumes that after a long period of darkness all the trapping centers deactivate to one ground state. This cannot explain why the oxygen given off per flash should be different if three flashes of light were given before the long period of darkness and if no light were given. Joliot et al.'s model using a two step mechanism, then, cannot explain all the experimental results. This, of course, does not rule out the possibility that oxygen is evolved after the accumulation of two charges only.

We have evolved a two quanta step model (Model C) that will fit the experimental results The model is shown in Figure 33C. We assumed as in Joliot et al.'s model that there are two donors (Z) to one acceptor (Q). After a long period in the dark, we assumed that there are two stable states (Q^{Z}_{Z}) and (Q^{Z}_{Z-}). On absorbing the first flash of light, one Z is oxidized and the Q is reduced, i.e., (Q^{Z}_{Z}) becomes ($^{-}Q^{Z}_{Z+}$) and (Q^{Z}_{Z-}) becomes ($^{-}Q^{Z}_{Z}$). After a short period of dark time (10^{-4} sec.), Q^{-} becomes re-oxidized to Q (via "A") and ($^{-}Q^{Z}_{Z+}$) becomes (Q^{Z}_{Z+}). On the second flash of light we assume that (Q^{Z}_{Z+}) has a probability of q to change into ($^{-}Q^{Z}_{Z++}$) and a probability of 1 - q to change into ($^{-}Q^{Z^{+}}_{Z+}$). In the subsequent dark period ($^{-}Q^{Z}_{Z++}$) will transform back to (Q^{Z}_{Z}) by a reaction with water giving off an atom of oxygen. (Two oxygen atoms from neighboring units would combine to give one oxygen molecule.) Hence, in this model, the probability q can be determined by the amount of oxygen given off after

the second flash. On absorbing the third flash of light, $(Q^{Z^+}_{Z^+})$ will become $(^{-}Q^{Z^{++}}_{Z^+})$. We assume then that this species with three charges cooperates in such a way that at each Z two oxygen atoms are evolved within the same unit; thus, a molecule of oxygen is produced.* Three of the four resultant electrons from water are used to reduce the oxidized Z, the fourth electron from water is assumed to be trapped by one of the Z's. This trapped electron is assumed to be very stable and can only be deactivated by a quantum of light. Hence $(Q^{Z^{++}}_{Z^+})$ becomes a very stable $(Q^{Z}_{Z^-})$. On the fourth flash of light as in the first flash $(Q^{Z}_{Z^-})$ attains the initial state (Q^{Z}_{Z}). On the fifth flash of light, this cycle of reactions is repeated. To match the experimental results with this model, we make the last assumption. We assume that the photochemical reaction centers are not 100% efficient but operate with an efficiency of $(1 - \gamma)$. This assumption is very similar to the assumption of "misses" used by Kok and co-workers[110, 147] in their model. We do not believe that the reason why a photochemical reaction will not occur at a unit is due to a photon "missing" a particular unit. Since there is much evidence to support the fact that the units are connected to one another, the reason why a photochemical reaction will not occur is more probably due to the inefficiency of the trapping center itself (see Chapter III).

*We note that it is difficult to visualize this step as a two charge step. What we wish to emphasize is that if there are two plus charges on a reaction center "Z", oxygen atoms can be formed; this does not preclude the evolution of a whole molecule of oxygen if the proper state of Z occurs within the same reaction center. However, we can look at it as evolution of two oxygen atoms at the two Z's and an oxygen molecule being formed just as in the case when two atoms are formed in neighboring reaction centers.

From the model in Figure 33C, one can derive the following recursion relations.

$$[X_1]_{n+1} = (1 - Y)[X_4]_n + Y[X_1]_n + q[X_2]_n \quad (42a)$$

$$[X_2]_{n+1} = (1 - Y)[X_1]_n + Y[X_2]_n \quad (42b)$$

$$[X_3]_{n+1} = (1 - Y - q)[X_2]_n + Y[X_3]_n \quad (42c)$$

$$[X_4]_{n+1} = (1 - Y)[X_3]_n + Y[X_4]_n \quad (42d)$$

where $[X_1]$ is the relative concentration of (Q_Z^Z)

$[X_2]$ is the relative concentration of (Q_Z^{Z+})

$[X_3]$ is the relative concentration of (Q_{Z+}^{Z+})

$[X_4]$ is the relative concentration of (Q_{Z-}^{Z})

n is the number of flashes used.

The amount of oxygen evolved by the n + 1th flash can be calculated from the equation·

$$\frac{1}{2}[O_2]_{n+1} = q[X_2]_n + 2(1 - Y)[X_3]_n \quad (43)$$

To calculate the amount of oxygen evolved per flash by a series of flashes, we must first calculate the initial concentrations of the two stable states X_1 and X_4 after a long period of darkness and the amount of oxygen

evolved after a large number of flashes so that we can use that amount as the normalization constant. At steady state, when the amount of oxygen evolved per flash becomes constant, the concentration of X_1, X_2, X_3, X_4 also remains constant. Hence by simple algebraic manipulation of the recursion relations, one can show that at steady state:

$$[X_1] = \frac{1 - Y + q}{4 - 4Y + 2q} \tag{44a}$$

$$[X_2] = \frac{1 - Y + q}{4 - 4Y + 2q} \tag{44b}$$

$$[X_3] = \frac{1 - Y}{4 - 4Y + 2q} \tag{44c}$$

$$[X_4] = \frac{1 - Y}{4 - 4Y + 2q} \tag{44d}$$

where $$[X_1] + [X_2] + [X_3] + [X_4] = 1$$

The amount of oxygen evolved at steady state is then:

$$\frac{1}{2}[O_2]_{ss} = \frac{1 - Y + q}{q_4 - 4Y + 2q} + 2(1 - Y)\frac{1 - Y}{4 - 4Y + 2q} \tag{45}$$

To compare with experimental results, $[O_2]_{ss}$ will be set to unity, and for all $[O_2]_n$ calculated, $[O_2]_n$ must be divided by $[O_2]_{ss}$.

After reaching steady state of oxygen yield and turning off the flashing light, we assume that X_2 and X_3 deactivate back to X_1. Hence after a long dark period, the relative concentration of the X's are:

$$[X_1]_o = 1 - \frac{1 - Y}{4 - 4Y + 2q}$$

$$[X_2]_o = 0 \tag{46}$$

$$[X_3]_o = 0$$

$$[X_4]_o = \frac{1 - \gamma}{4 - 4\gamma + 2q}$$

After substituting these values into the recursion relation, one can calculate the relative concentrations of X_1, X_2, X_3 and X_4 following each succeeding flash. Using these calculated X_1, X_2, X_3, X_4 values, one can calculate the amount of oxygen evolved per flash by a series of flashes.

If after reaching steady state of oxygen yield, the flashing light was turned off for five minutes, then one flash was given and the chloroplasts were allowed to sit in the dark for 30 minutes, the initial concentrations of $[X_1]$ and $[X_4]$, the two assumed stable states would be changed. Just before the single flash was given, the concentration of X_1 and X_4 is equal to $[X_1]_o$ and $[X_4]_o$. On applying the single flash, most of the $[X_4]_o$ will change into $[X_1]_o$ and will not decay back to $[X_4]_o$ in the dark because $[X_1]_o$ is stable. The states that $[X_1]_o$ will change into following a single flash will deactivate back to $[X_1]_o$. Hence one can easily show that following a single flash:

$$[X_1]_o = 1 - \frac{\gamma(1-\gamma)}{4 - 4\gamma + 2q}$$

$$[X_2]_o = 0$$

$$[X_3]_o = 0 \qquad (47)$$

$$[X_4]_o = \frac{\gamma(1-\gamma)}{4 - 4\gamma + 2q}$$

Using the same argument, with two flashes given, the initial conditions would be:

$$
\begin{aligned}
[X_1]_o &= 1 - \frac{Y^2 (1 - Y)}{4 - 4Y + 2q} \\
[X_2]_o &= 0 \\
[X_3]_o &= 0 \\
[X_4]_o &= \frac{Y^2 (1 - Y)}{4 - 4Y + 2a}
\end{aligned}
\tag{48}
$$

If three flashes were given, the initial conditions would be:

$$
\begin{aligned}
[X_1]_o &= 1 - (1-Y)^2 (1 - Y - q) \; 1 - \left[\frac{1 - Y}{4 - 4Y + 2q}\right] \\
[X_2]_o &= 0 \\
[X_3]_o &= 0 \\
[X_4]_o &= (1 - Y)^2 (1 - Y - q) \; 1 - \left[\frac{1 - Y}{4 - 4Y + 2q}\right]
\end{aligned}
\tag{49}
$$

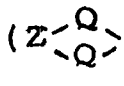
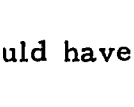
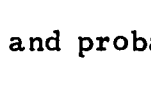
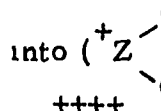
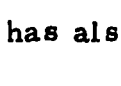
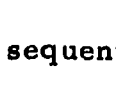
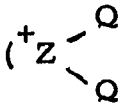
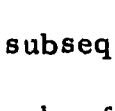
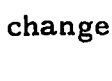
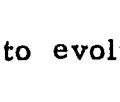
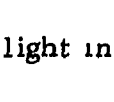
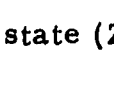
Using these initial conditions to calculate the amount of oxygen evolved per flash, one can easily see that the oxygen yield per flash would be different for each initial condition.

2. Four Step Mechanism

In the four step mechanism, four charges must be accumulated before a molecule of oxygen can be evolved. In the linear four step mechanism of Kok and co-workers there exist four intermediate states S_0, S_1, S_2 and S_3 (Figure 33B). Schematically, we have interpreted their S_0 to be $(Q\text{-}Z)$, S_1 to be $(Q\text{-}Z^+)$, S_2 to be $(Q\text{-}Z^{++})$ and S_3 to be $(Q\text{-}Z^{+++})$. On

absorbing a quantum of light, S_0 goes to S_1, S_1 to S_2, S_2 to S_3, and S_3 on absorbing a photon undergoes a dark reaction with water molecules which transforms S_3 back to S_0 and an oxygen molecule is evolved. Clearly, if after a long period of darkness the only existing state is S_1, then the oxygen produced by sequential flashes of light will oscillate with a period of four and will not damp out. As noted in the introduction, the perturbations to this cyclic reaction are assumed to be due to the possibility that not every S state will move to the next on applying a flash of light ("misses") and also that some intermediate states can have double excitations ("double hits"). Kok et al.[110] and Forbush et al.[147] assume that within the time of a flash of light used by their experiments only S_0 and S_1 can use two photons to change into S_2 and S_3 respectively. To explain why oxygen produced on the third flash should be greater than oxygen produced on the fourth flash, they made the further assumption that the intermediate states S_2 and S_3 relax back to the S_1 state in the dark and that S_1 is an "infinitely" stable state. This assumption also explains the differences in the flash yield sequences observed after various pretreatments with light.

We have evolved an alternate four step model for oxygen evolution (model D) in which two reaction centers are needed to evolve a molecule of oxygen instead of one. (There is indirect evidence that two reaction centers may act together.[148]) It also assumes that within the time of the flash, there is no "double excitation" within one reaction center as in Kok's model. This model is shown in Figure 33D. We assume that after a long period in the dark, most of the "double" reaction center is in the state


. (This should have been written as  but we use the simpler form.) On absorbing one quantum of light in each reaction center of the twin (i.e., a total of two quanta), this state has a probability A to become  and probability 1 - A to become  . On absorbing the next two quanta of light after a period of darkness (300 msec), we assume that both states can change into  . State  has also the probability B to change into  which on the subsequent dark reaction changes back to the initial state  after giving off a molecule of oxygen from a reaction with two molecules of water. State  , on absorbing two more quanta, can undergo two alternate light reactions. One reaction changes it to  which on the subsequent dark reaction changes into after giving off a molecule of oxygen from a reaction with water molecules. The other reaction changes  with a probability of ρ into which is then postulated to undergo a dark reaction with four molecules of water to evolve two molecules of oxygen and a stable state  . This state will not deactivate in the dark. On absorbing another two quanta of light in the double reaction center,  changes back to the initial state  . As in Kok's model, we will assume that the photochemical reaction centers are not 100% efficient but operate with an efficiency of $(1 - Y)$. From Figure 33D, the following recursion relations can be derived:

$$[X_1]_{n+1} = B[X_4]_n + (1 - \gamma)[X_5]_n + \gamma[X_1]_n$$

$$[X_2]_{n+1} = (1 - \gamma - A)[X_1]_n + \gamma[X_2]_n$$

$$[X_3]_{n+1} = (1 - \gamma)[X_2]_n + (1 - \gamma - B)[X_4]_n + \gamma[X_3]_n \qquad (50)$$

$$[X_4]_{n+1} = A[X_1]_n + (1 - \gamma - \rho)[X_3]_n + \gamma[X_4]_n$$

$$[X_5]_{n+1} = \rho[X_3]_n + \gamma[X_5]_n$$

where $[X_1]_n$ is the relative concentration of the twin (or double) reaction center ($Z \begin{smallmatrix} Q \\ Q \end{smallmatrix} Z$) following the n^{th} flash

$[X_2]_n$ is the relative concentration of ($^{+}Z \begin{smallmatrix} Q \\ Q \end{smallmatrix} Z^{+}$) following the n^{th} flash

$[X_3]_n$ is the relative concentration of ($^{+}Z \begin{smallmatrix} Q \\ Q \end{smallmatrix} Z^{++}$) following the n^{th} flash

$[X_4]_n$ is the relative concentration of ($^{+}Z \begin{smallmatrix} Q \\ Q \end{smallmatrix} Z^{+++}$) following the n^{th} flash

$[X_5]_n$ is the relative concentration of ($^{-}Z \begin{smallmatrix} Q \\ Q \end{smallmatrix} Z^{-}$) following the n^{th} flash

The amount of oxygen evolved by the $n + 1^{th}$ flash can be calculated from the equation:

$$[O_2]_{n+1} = B[X_4]_n + (1 - \gamma + \rho)[X_3]_n \qquad (51)$$

As in model C, to calculate the amount of oxygen evolved per flash when it becomes constant after a large number of flashes, one can use the recursion relations of equation 50 and the fact that $[X_i]_{n+1} = [X_i]_n$

where i = 1, 2, 3, 4. The calculated concentration of X_4 and X_3 at steady state can then be substituted into equation 51 to obtain the oxygen evolved at steady state.

Again, in a similar calculation as in model C, the initial concentrations of X_i can be calculated after a long dark period following steady state oxygen evolution per flash. Similarly as in model C, we can calculate the initial concentration of $[X_i]$ after a long dark time following one, two or three flashes of light which were given after five minutes of a dark period that, in turn, followed steady state conditions. From these initial concentrations and substituting into the recursion relations, one can obtain the amount of oxygen evolved per flash by a series of flashes following different light pretreatment.

C. COMPARISON WITH EXPERIMENTAL RESULTS

1. Comparison with Joliot's Experimental Data

The experimental results of Joliot et al.[109] are shown in Figure 34A. The predicted results of the three models (B, C and D) fit fairly well with the experimental data. (Model A was rejected on the grounds discussed in section B1.) Using Kok's model, with the probability of "misses" (α) assumed to be 0.15 and the probability of "double hits" (β) assumed to be 0.20, we obtain a good fit to the experimental results. This is shown in Figure 34B. Using model C to match Joliot's data, γ, the probability of loss in the reaction center, is set equal to 0.10 and q, the probability that $(Q^{Z^+}_{Z})$ will go to $(Q^{Z^{++}}_{Z})$, is set equal to 0.35. The predicted

values are shown in Figure 34C. With model D, the values of the parameter used to match Joliot's data are A = 0.25, B = 0.90, γ = 0.02 and ρ = 0.20. The predicted oxygen flash yield is shown in Figure 34D. In all three cases, the calculated results agree with the experimental results in that they show a damped oscillation with a period of four, no oxygen output in the first flash and maximum oxygen output in the third flash. The match with experimental results of flash numbers greater than five, however, is not too good for Models C and D. The reason for this is assumed to be due to "errors" with each flash which cause slight variations in the probabilities of γ and q in model C and in A, B, γ and ρ in Model D.

2. Comparison with Kok's Experimental Data

The experimental data of Forbush et al.[147] are shown in Figure 35A. Again, the predicted values from the three models (B, C and D) fit fairly well with the experimental results. Figure 35B shows Forbush et al's calculation with their model. They assumed that α is 0.10 and β is 0.05. Figure 35C shows the predicted values as calculated with model C. The parameters assumed are γ as equal to 0.05 and q as equal to 0.15. The predicted values calculated with model D are plotted in Figure 35D. In this case A is assumed to be 0.10, B, 0.90, γ, 0.02, and ρ, 0.40. Again, the match with experimental results of flash numbers greater than five is not very good with model C or D. That the probability used in Model C or D changes slightly with each flash is assumed, this explains the discrepancy.

3. Comparison with Flash Yield Sequences After Various Light Treatments

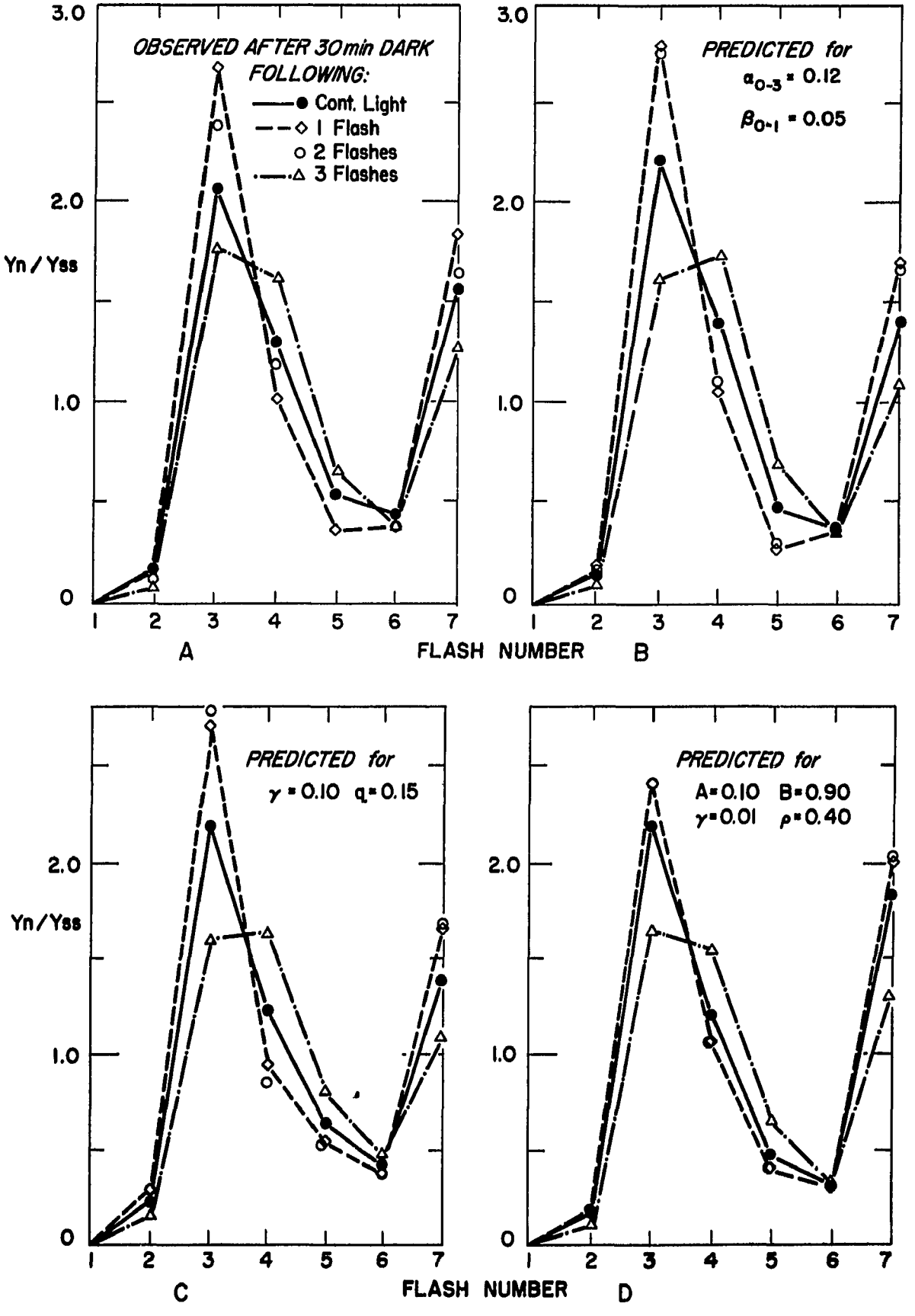
The experimental results of Kok et al.[110] and Forbush et al.[147]

Figure 36. (A) Flash yield sequences observed after various light pretreatments. 25 flashes were used to attain steady state, followed by five minutes of darkness. Then either none, one, two or three flashes of light were given in a sequence spaced one sec apart. This was followed by 30 minutes of darkness before a series of flashes were given. (Experimental data of Forbush et al.[147])

(B) Predictions based on Kok's model.[110, 147] The symbols in this and following figures C and D have the same meaning as in Figure 33.

(C) Predictions based on model C.

(D) Predictions based on model D.


OBSERVED AFTER 30 min DARK
FOLLOWING:
Cont. Light
1 Flash
2 Flashes
3 Flashes
PREDICTED for
α_{0-3} = 0.12
β_{0-1} = 0.05
PREDICTED for
γ = 0.10 q = 0.15
PREDICTED for
A=0.10 B=0.90
γ=0.01 ρ=0.40
Yn / Yss
Yn/Yss
FLASH NUMBER
A
B
C
D

are shown in Figure 36A. As noted in section B, the chloroplasts were illuminated with a series of flashes until the oxygen yield came to a steady state. They were left in the dark for five minutes. Then the chloroplasts were either left in the dark or were given one or two or three flashes. They were then left in the dark for thirty minutes before being illuminated with a series of light flashes. The predicted results calculated by Forbush et al.[147] using their model are shown in Figure 36B. The parameter α is 0.12 and β is 0.05 in this case. The predicted results calculated from model C are shown in Figure 36C. The parameters used are γ as equal to 0.10 and q as equal to 0.15. Using model D, the predicted results are plotted in Figure 36D with A equal to 0.10, B equal to 0.90, γ equal to 0.02 and ρ equal to 0.40. All the thoeretical curves match qualitatively the experimental curves. The main discrepancy occurs in the oxygen yield of the third flash after a two flash pretreatment. In all the theoretical cases the oxygen yields per flash for one or two flash pretreatments are very similar to each other. (In the experimental case, the oxygen yield of the third flash after two flash pretreatment is lower than that of one flash pretreatment.)

4. Comparison of the Kinetics of Oxygen Production with Low Light Intensities after Different Numbers of Activating Flashes

Joliot et al.[109] and Forbush et al.[147] have calculated the kinetics of oxygen production with low light intensities after different numbers of activating flashes from the recursion relations based on their models. We will show that the kinetics of oxygen production with low light intensities after different numbers of activating flashes can be theoretically calculated from the experimental flash yield data. This means that if one can match

the flash yield data from a particular model, one can also match the kinetics of oxygen production at low light intensities.

The kinetics of the oxygen production with low light intensities after different numbers of activating flashes can be calculated from the oxygen yield per saturating flash of light in a series of light flahses. Let:

N_1 = the number of units that have received no photon from the weak continuous light (to be abbreviated as WCL)

N_2 = the number of units that have received one photon from WCL

N_3 = the number of units that have received one photon from WCL, and in which Q^- has been reoxidized to Q.

N_4 = the number of units that have received the second photon from WCL

N_5 = the number of units that have received the second photon from WCL, and in which Q^- has been reoxidized to Q.

Then,

$$N_1 \xrightarrow{kI} N_2 \xrightarrow{k_1} N_3 \xrightarrow{kI} N_4 \xrightarrow{k_1} N_5 \xrightarrow{kI} N_6 \longrightarrow \cdots$$

where,

I is the intensity of light

k is the rate in which N_1 is converted into N_2 and N_3 to N_4, etc. at unit intensity of light

k_1 is the dark rate in which Q^- goes to Q (via A).

Hence,

$$\begin{aligned}
\frac{dN_1}{dt} &= -kIN_1 \\
\frac{dN_2}{dt} &= kIN_1 - k_1N_2 \\
\frac{dN_3}{dt} &= k_1N_2 - kIN_3 \\
&\vdots \\
\frac{dN_i}{dt} &= kIN_{i-1} - k_1N_i
\end{aligned} \tag{52}$$

In general, the solution for the above set of differential equations can be found. The solution for N_{2i+1}, assuming $k_1 \gg kI$ is:

$$N_{2i+1}(t) = N_1^{o} \frac{(k_1)^1(kI)^i}{(k_1 - kI)^i} \frac{t^1}{i!} e^{-kIt} \tag{53}$$

where $i = 0, 1, 2, 3, \ldots$

N_1^{o} = total number of units

In weak continuous light, without any preilluminating flash, the amount of oxygen evolved at a given time depends upon the amount of oxygen N_1^{th} units will evolve and the number of N_i^{th} units. The amount of oxygen evolved by the N_i^{th} unit is exactly the amount of oxygen that will be evolved after the i^{th} flash in a series of flashes (Y_i). Since the reaction between the stored charges and water is a dark reaction, we will designate k_2 as the rate of this reaction. Hence the kinetics of oxygen production at low light intensities should follow

$$[O_2](t) = k_2 \sum_{i=0}^{\infty} \left(\frac{Y_{2i+1}}{Y_{ss}} \right) N_{2i+1} \qquad (54)$$

where $i = 0, 1, 2, 3, 4, \ldots$

and $[O_2](t)$ is the amount of oxygen evolved normalized at steady state

After n^{th} preillumination flash, the amount of oxygen that the N_1^{th} number of units will evolve will be equal to $Y(i+n)$. Hence

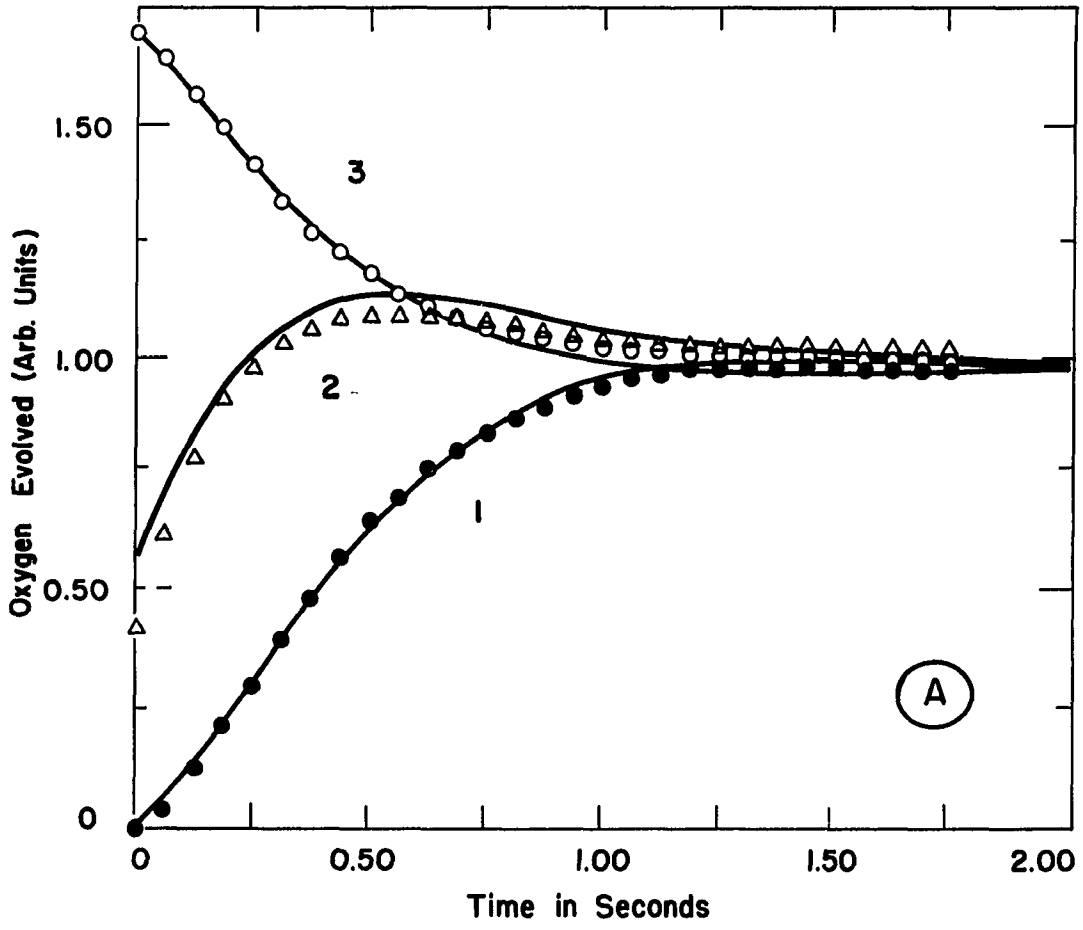
$$[O_2]_n(t) = k_2 \sum_{i=0}^{\infty} \left(\frac{Y_{2i+1+n}}{Y_{ss}} \right) N_{2i+1} \qquad (55)$$

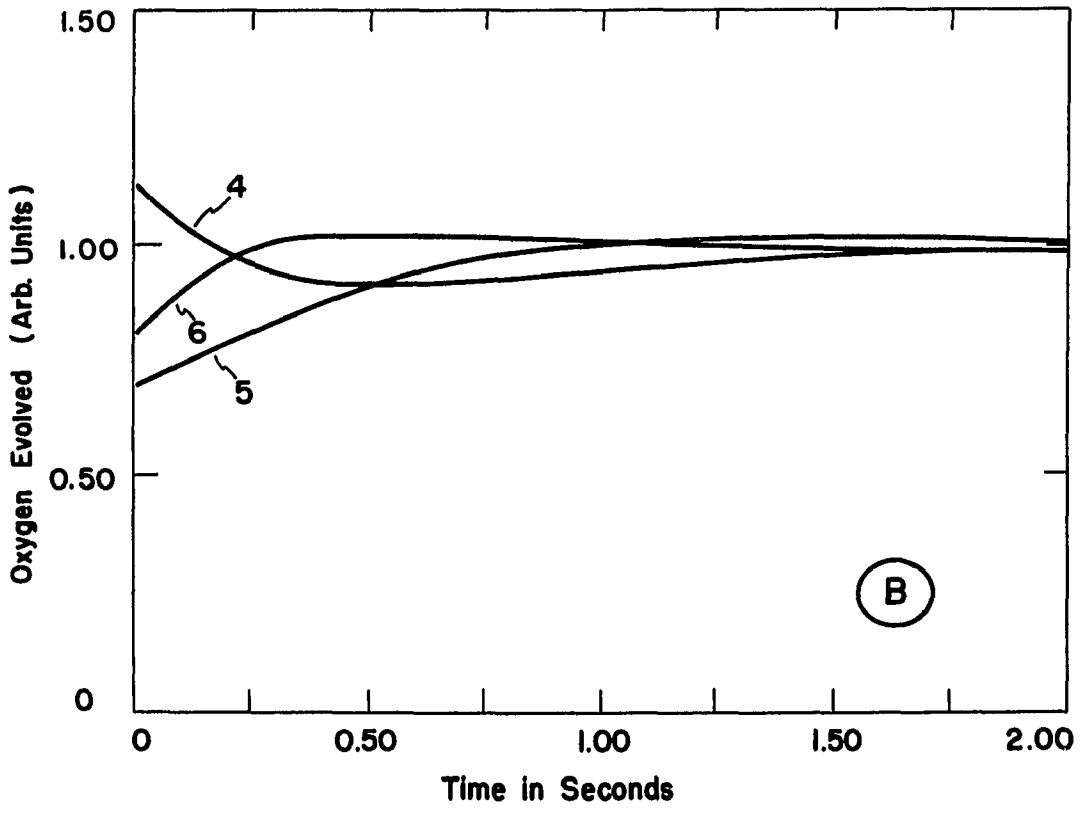
These equations were numerically evaluated. The results are shown in Figure 37. The value of k_1 used was 10^4 which was the experimental value obtained by Kok et al.[149] The value of kI used was 2.5 chosen for the best fit to the experimental data. Since k_2 and N_1^o were arbitrary constants that would not change the shape of the induction curve, they were set at 1. Figure 37A compares the theoretical curves (solid lines) obtained from equations 53 and 55 and the experimental flash yield data of Joliot's with the time course curves obtained also by Joliot et al.[109] after one, two, and three flashes respectively. Our calculated curves match very well with Joliot's experimental data. Figure 37B shows the predicted curves for oxygen evolution after the fourth, fifth and sixth flashes respectively, these curves are very similar to the experimental curves obtained by Kok et al.[110]

These results show that the kinetics of the oxygen production with low light intensities after different numbers of activating flashes can be

Figure 37. (A) The kinetics of oxygen evolution of Chlorella illuminated by weak modulated light (λ = 685 nm, intensity = 700 ergs/cm^2 sec, modulation frequency = 25 Hz) The solid dots, experimental values obtained by preilluminating the algae for 30 seconds, after which they were allowed to stay in the dark for 70 seconds before the weak modulating light was turned on; the triangles, experimental values obtained as before but with one flash given 20 msec before the weak modulating light was turned on; the open circles, experimental values obtained as before but with two short flashes. (Experimental data of Joliot et al.[109]) The solid lines (1 - 3) are the thoeretical curves calculated from equations 53 and 55 in the text using the experimental oxygen flash yield data of Joliot et al.[109]

(B) Theoretical curves predicted from experimental oxygen flash yield data. Line 4 is the predicted curve after three flashes of preillumination, line 5, after four flashes, line 6, after five flashes.


1.50
1.00
0.50
0
Oxygen Evolved (Arb. Units)
3
2
1
A
0
0.50
1.00
1.50
2.00
Time in Seconds


1.50
1.00
0.50
0
Oxygen Evolved (Arb. Units)
4
6
5
B
0
0.50
1.00
1.50
2.00
Time in Seconds

calculated from the oxygen flash yield data. Hence, any model for oxygen evolution that will explain the oxygen flash yield data will explain the kinetics of oxygen production with low light intensities. An exception is the model of Kok et al.[110]. In their model, they postulated that there are "double hits" which occur when the cells are illuminated by flashes of light. At low light intensities, "double hits" should not occur. They have taken this into account and have calculated, based on their model, the kinetics of oxygen production at low intensities.[147] Their theoretical kinetic curves match with their experimental kinetic curves.

D. DISCUSSION

Models C and D are proposed as new explanations for the mechanism of oxygen evolution to the model (B) proposed by Kok et al.[110] and Forbush et al.[147] Our analyses (see Figures 34, 35, 36, 37) of the three models (B, C and D) indicate that all are valid alternatives and that none of them can be declared as the correct one yet. However, it is accepted (see ref. 147) that the model of Joliot et al.[109] (model A) may not be considered, in its present form, because it cannot explain the data of Kok and co-workers.

In Kok's model, the accumulation of four positive charges in one trapping center leads to the evolution of oxygen. Analysis of model C shows that it is possible to explain the existing experimental data on oxygen evolution by proposing that oxygen can be evolved from the accumulation of two positive charges in one reaction center. Analysis of model D shows that a four-charge hypothesis that uses two reaction centers acting together also explains the existing experimental data.

One important difference between the model by Kok and co-workers and the model C or D is the basis for oxygen evolution after the second flash. Within the time of the illuminating pulse of light ($\sim 10\mu s$), some photosynthetic units are capable of being hit twice by photons and are able to do two photochemical reactions (Kok). (It is difficult to imagine how Q^- returns to Q within this time to do the second photoreaction because Kok et al.[149] have shown that the half time of the recovery of Q is about 0.5msec.) Also, it follows that if the time duration of the pulse of light is kept very short, no oxygen should evolve after the second flash. Weiss and Sauer[150] showed that no oxygen was evolved after the second flash when 20 and 40 nanosecond laser flashes and 28 microsecond Xenon light flashes were used. The minimum dark time between flashes that they used, however, was 15 seconds, so that they could not obtain oscillations of the oxygen yield produced by a sequence of flashes. This may be due to the fact that precursors built up by a flash of light deactivate before the next flash of light is given. Hence part of the reason why oxygen was not observed after the second flash may be due to deactivation of the precursors built up by the first flash of light. However, if an experiment can be done with flash time of 20 nanoseconds spaced approximately 300 msec apart, the amount of oxygen evolved after the second flash in a series of flashes following a long dark period should decide whether the two proposed models or Kok's model is the correct one. In Kok's model there should be no oxygen evolved; in models C or D oxygen evolution should be observed.

In conclusion, other models besides the one proposed by Kok and co-workers can also explain existing experimental results. An experiment is

proposed that can decide between our models (C and D) and the model B of Kok, Forbush and McGloin.

E. SUMMARY

Two new alternate models for oxygen evolution are proposed. The first one assumes that oxygen can be evolved from an accumulation of two charges, this modified two-charge hypothesis explains the existing experimental data unlike Joliot and co-workers' two-charge "flip-flop" model. The second one assumes that accumulation of four charges are needed for oxygen evolution, this model differs from the four charge model of Kok and co-workers because it does not require a "double hit" on the same reaction center but in it each oxygen evolving site has two bound reaction centers II. The alternate four-charge hypothesis also explains the existing experimental data. We conclude that Kok et al.'s model is not unique, and the two models presented here should be considered as valid alternate models for oxygen evolution in green plants.

REFERENCES

1. E. Rabinowitch and Govindjee: Photosynthesis, John Wiley and Sons, New York, 1969.

2. R. K. Clayton: Molecular Physics in Photosynthesis, Blaisdell, New York, 1965.

3. G. W. Robinson Ann. Rev. Phys. Chem., 15, 311, 1964.

4. L. N. M. Duysens: Progress in Biophysics, 14, 1, 1964.

5. L. N. M. Duysens: Ph.D. Thesis, The State University, Utrecht, The Netherlands, 1952.

6. G. Döring, G. Renger, J. Vater and H T. Witt: Z. Naturforschg. 24b, 1139, 1969.

7. Govindjee, G. Döring and R. Govindjee: Biochim. Biophys. Acta, 205, 303, 1970.

8. L. N. M. Duysens and H. E. Sweers: Studies on Microalgae and Photosynthetic Bacteria (edited by Japanese Society of Plant Physiologists, p. 353), U. of Tokyo Press, 1963.

9. P. Joliot: Photochem. Photobiol., 8, 451, 1968.

10. R. Emerson and W. J Arnold: J. Gen. Physiol., 16, 191, 1932.

11. R. Emerson and C. M. Lewis. Am. J. Botany, 26, 808, 1939.

12. J. B. Thomas, O. H. Blaauw and L. N. M. Duysens: Biochim. Biophys. Acta, 10, 230, 1953.

13. N. K. Boardman: Ann. Rev. of Plant Physiol., 21, 115, 1970.

14. R. Emerson, R. V. Chalmers and C. Cederstrand: Proc. Natl. Acad. Sci., U.S., 43, 133, 1957.

15. R. Emerson and E. Rabinowitch: Plant Physiol. 35, 477, 1960.

16. Govindjee and E. Rabinowitch: Biophys. J., 1, 73, 1960.

17. Govindjee, S. Ichimura, C Cederstrand and E. Rabinowitch: Arch. Biochem. Biophys. 89, 322, 1960.

18. W. L. Butler: Biochim. Biophys. Acta, 64, 309, 1962.

19. B. Kok and G. Hoch: In Light and Life (edited by W. D. McElroy and B. Glass, p. 397), Johns Hopkins Press, Baltimore, Maryland, 1961.

20. L. N. M. Duysens, J. Amesz and B. M. Kamp: Nature, Lond., 190, 510, 1961.

21. L. N. M. Duysens and J. Amesz: Biochim. Biophys. Acta, 64, 243, 1962.

22. R. Hill and F. Bendall: Nature, 186, 136, 1960.

23. G. Hind and J M. Olson: Ann. Rev. Plant Physiol., 19, 249, 1968.

24. M. Avron and J. Neumann: Ann. Rev. Plant Physiol., 19, 137, 1968.

25. B. Kok and E. A. Datko: Plant Physiol., 40, 1171, 1965.

26. A. San Pietro: In Biological Oxidations (edited by T. P. Singer, p. 515), John Wiley and Sons Inc., New York, 1968.

27. J. A. Bassham: In Plant Biochemistry (edited by J. Bonner and J. E. Varner, p. 875), Academic Press, New York, 1965.

28. M. D. Hatch and C. R. Slack: Ann. Rev. Plant Physiol., 21, 141, 1970.

29. Govindjee, J C. Munday and G. Papageorgiou: Brookhaven Symp. in Biology, 19, 434, 1966.

30. D. B. Knaff and D. I. Arnon: Proc Natl. Acad. Sci., U.S., 64, 715, 1969.

31. S. S. Brody and E. Rabinowitch: Science, 125, 555, 1957.

32. G. Tomita and E. Rabinowitch: Biophy J., 2, 483, 1962.

33. N. R. Murty and E. Rabinowitch: Biophy. J., 5, 655, 1965.

34. W. J. Nicholson and J. I. Fortoul: Biochim. Biophys. Acta, 143, 577, 1967.

35. G. S. Singhal and E. Rabinowitch: Biophy. J. 9, 586, 1969.

36. O. Dimitrievsky, V. Ermolaev and A. Terenin: Dokl. Akad. Nauk. S. S. S. R., 114, 75, 1957.

37. W. L. Butler and K. H. Norris: Biochim. Biophys. Acta, 66, 72, 1963.

38. A. Müller, R. Lumry and H. Kokubun: Rev. Sci. Instr. 36, 1214, 1965.

39. A. Müller and R. Lumry: Proc. Natl. Acad. Sci., 54, 1479, 1965.

40. H. Merkelo, S. Hartman, T. Mar, G. Singhal and Govindjee: Science, 164, 301, 1969.

41. A. Müller, R. Lumry and M. [illegible]alker: Photochem. Photobiol. 9, 113, 1969.

42. S. S. Brody: Ph.D. thesis, University of Illinois, Urbana, 1956.

43. P. Joliot, R. Dolosme and A. Joliot: In Currents in Photosynthesis (edited by J. B. Thomas and J. C. Goedheer, p. 359), Rotterdam, Ad Donker, 1966.

44. J. Lavorel: Biochim. Biophys. Acta, 60, 510, 1962.

45. R. K. Clayton: J. Thoret. Biol., 14, 173, 1967.

46. J. Lavorel. J. Chem. Phys. 47, 2235, 1967.

47. R. M. Pearlstein· Proc. Natl. Acad. Sci., U.S., 52, 824, 1964.

48. C. S. French and V. M. K. Young· J. Gen. Physiol., 35, 873, 1952.

49. A. Krey and Govindjee: Proc. Natl. Acad. Sci., U.S., 52, 1568, 1964.

50. L. A. Tumerman and E. M. Sorokin: Molekulyarnaya Biologiya, 1, 628, 1967.

51. J. M. Briantais, Govindjee and H. Merkelo: Presented at the International Conference on the Photosynthetic Unit at Gatlinburg, Tenn., 1970.

52. O. Simpson: Proc. Roy. Soc. (London), 238, 402, 1957.

53. F. Perrin: Ann. Physique, 12, 169, 1929.

54. J. H. C. Goedheer: Ph.D. Thesis, The State University, Utrecht, 1957.

55. W. Arnold and E. S. Meek: Arch. Biochem. Biophys., 60, 82, 1956.

56. G. Weber: Trans. Faraday Soc., 50, 554, 1954.

57. G. Weber: Report to U.S. Army Research and Development in Europe (DA-91-508-EUC-281), 1958.

58. F. W. J. Teale: Biochim. Biophys. Acta., 42, 69, 1960.

59. J. Lavorel· Biochim. Biophys. Acta, 88, 20, 1964.

60. J. H. C. Goedheer· In Biochemistry of Chloroplasts (edited by T. W. Goodwin, p. 75), Academic Press, New York, 1966.

61. R. A Olson: Natl. Acad. Sci. - Natl. Res. Council Publ. 1145, 545, 1963.

62. Govindjee: In Currents in Photosynthesis (edited by J. B. Thomas and J. H. C. Goedheer, p. 93), Rotterdam, Ad Donker, 1965.

63. C. Cederstrand and Govindjee: Biochim. Biophys. Acta, 120, 177, 1966.

64. Z. Bay and R. M. Pearlstein· Proc. Natl. Acad. Sci., U.S., 50, 1071, 1963.

65. T. Förster: Ann. Physik, 2, 55, 1948.

66. R. A. Olson, W. L. Butler and W H. Jennings: Biochim. Biophys. Acta, 54, 615, 1961.

67. R. A. Olson, W L. Butler and W H. Jennings: Biochim. Biophys. Acta, 58, 144, 1962.

68. R. A. Olson, W. H. Jennings and W. L. Butler: Biochim. Biophys. Acta, 88, 318, 1964.

69. R. A Olson, W. H. Jennings and W. L. Butler: Biochim. Biophys. Acta, 88, 331, 1964.

70. W. L. Butler, R. A. Olson, and W. H. Jennings: Biochim. Biophys. Acta, 88, 651, 1964.

71. F. Cho and Govindjee. Biochim. Biophys. Acta, 216, 139, 1970.

72. F. Cho and Govindjee: Biochim. Biophys. Acta, 216, 151, 1970.

73. W. Arnold and R. K. Clayton: Proc. Natl. Acad. Sci., U.S., 46, 769, 1960.

74. E. Rabinowitch: Faraday Soc. Discussions, 27, 161, 1959.

75. R. A. Floyd, B. Chance and D. DeVault: Biochim. Biophys. Acta, in the press.

76. W. Arnold and H. K. Maclay: Brookhaven Symp. in Biology, 11, 1, 1958.

77. K. J. McCree: Biochim. Biophys. Acta, 102, 90, 1965.

78. G. Porter and G. Strauss: Proc. Roy. Soc., A295, 1, 1966.

79. E. Rabinowitch. J. Phys. Chem., 61, 870, 1957.

80. A. S. Davydov: Theory of Molecular Excitons, McGraw Hill, New York, 1962.

81. T. Förster: In Comparative Effects of Radiation (edited by M. Burton, J. S. Kirby-Smith and J. L. Magee, p. 300) John Wiley, New York, 1960.

82. T. Förster· In Modern Quantum Chemistry (edited by O. Sinanoglu, p. 93), Academic Press, New York, 1965.

83. G. Hoch and R. S. Knox: In Photophysiology (edited by A. Giese, p. 225 of Vol. 3), Academic Press, New·York, 1968.

84. G. Robinson: Brookhaven Symp. in Biology, 19, 16, 1967.

85. B. L. Strehler and W. Arnold: J. Gen. Physiol., 34, 809, 1951.

86. W. Arnold and J. B. Davidson· J. Gen. Physiol., 37, 677, 1954

87. W. Arnold and J. Thompson· J. Gen. Physiol. 39, 311, 1956.

88. J. Lavorel· Prog. Photosynth. Res., 2, 883, 1969.

89. W. Bertsch, J. R. Azzi and J. B. Davidson Biochim. Biophys. Acta, 143, 129, 1967.

90. L. W. Jones: Proc Natl. Acad. Sci., U.S., 58, 75, 1967.

91. G. Tollins, E. Fujimori and M. Calvin: Proc. Natl. Acad. Sci., U.S. 44, 1035, 1958.

92. W. E. Arthur and B. L. Strehler: Arch. Biochem. Biophys., 70, 507, 1957.

93. G. Tollins and M. Calvin: Proc. Natl. Acad. Sci., U.S., 43, 87, 1957.

94. P. B. Sweetser, C. W. Todd and R. T. Hersh: Biochim. Biophys. Acta, 51, 509, 1961.

95. W. F. Bertsch, J. B. Davidson and J. R. Azzi: Natl. Acad. Sci. - Natl. Res. Council Publ. 1145, 70, 1963.

96. W. F. Bertsch and J. R. Azzi: Biochim. Biophys. Acta, 94, 15, 1965.

97. C. A. Parker and T. A. Joyce: Nature, 210, 701, 1966.

98. R. H. Ruby· Photochem. Photobiol. 8, 299, 1968.

99. W. F. Bertsch, J. West and R. Hill: Biochim. Biophys. Acta, 172, 525, 1969.

100. W. Arnold and J. R. Azzi: Proc. Natl. Acad. Sci., U.S., 61, 29, 1968.

101. W. Stacy, T. Mar, C. E. Swenberg and Govindjee: Photochem. Photobiol., in the press.

102. R. Govindjee, E. Rabinowitch and Govindjee: Biochim. Biophys. Acta, 162, 539, 1968.

103. W. F. Bertsch· Prog. Photosynth. Res., 2, 1, 1969.

104. B. Kok, H. J. Rurainski, and O. Owens· Biochim. Biophys. Acta, 109, 347, 1965.

105. G. Robinson: Proc. Natl. Acad. Sci., U.S., 49, 21, 1963.

106. R. C. Johnson, R. E. Merrifield, P. Avakian and R. B. Flippen: Phys. Rev. Letters, 19, 285, 1967.

107. R. E. Merrifield J. Chem. Phys. 48, 4318, 1968.

108. W. Stacy: Ph.D. Thesis, University of Illinois, Urbana, 1970.

109. P. Joliot, G. Barbieri and R. Chabaud. Photochem. Photobiol. 10, 309, 1969.

110. B. Kok, B. Forbush and M. McGloin: Photochem. Photobiol. 11, 457, 1970.

111. M. Brody· Ph.D. Thesis, University of Illinois, Urbana, 1958.

112. J. A. Bergeron: Natl. Acad. Sci. - Natl. Res. Council Publ., 1145 527, 1963.

113. Govindjee and E. Rabinowitch: Biophys. J. 1, 74, 1960.

114. C. Shimony, J. Spencer and Govindjee: Photosynthetica, 1, 113, 1967.

115. J. C. Munday and Govindjee: Biophys. J. 8, 1, 1969.

116. L. E. Hargrove, R. L. Fork and M. A. Pollack: Appl. Phys. Lett., 5, 4, 1964.

117. F. Cho and Govindjee: Biochim. Biophys. Acta, 205, 371, 1970.

118. E. P. Clancy: J. Opt. Soc. Am. 42, 357, 1952.

119. G. Weber: J. Opt. Soc. Am. 46, 962, 1956.

120. D. H. McMahon: Rev. Sci. Instru., 42, 727, 1969.

121. F. DeMartini and K. Wacks: Rev. Sci. Instru., 38, 866, 1967.

122. B. Mayne and R. K. Clayton· Proc. Natl. Acad. Sci., U.S., 55, 494, 1966.

123. E. A. Bailey and G. K. Rollefson: J. Chem. Phys., 21, 1315, 1953.

124. G. Papageorgiou and Govindjee: Biophys. J. 8, 1299, 1968.

125. G. Weber: In Comparative Biochemistry of Photoreactive Systems (edited by M. B. Allen, p. 395) Academic Press, New York, 1960.

126. Y. G. Shiau and J. Franck: Arch. Biochem. 14, 253, 1947.

127. W. P. Williams, N. R. Murty and E. Rabinowitch: Photochem. Photobiol., 9, 455, 1969.

128. P. K. Mohanty: Personal Communication, 1969.

129. F. Cho: Ph.D. Thesis, University of Illinois, Urbana, 1969.

130. P. Latimer, T. T. Bannister and E. Rabinowitch: Science, 124, 585, 1956.

131. J. B. Thomas, K. Minnaert and P. F. Elbers: Acta Bot. Netherlands, 5, 315, 1956.

132. S. Valinkova: Compt. Rend. Acad. Bulg. Sci., 17, 283, 1964.

133. S. Izawa, R. L. Heath and G. Hind: Biochim. Biophys. Acta, 180, 388, 1969.

134. J. Lavorel: Plant Physiol. 34, 204, 1959.

135. R. Delosme: Biochim. Biophys. Acta, 143, 108, 1967.

136. P. Bennoun: Biochim. Biophys. Acta, 216, 357, 1970.

137. W. Arnold and H. Sherwood: Proc. Natl. Acad. Sci., U.S., 43, 104, 1957.

138. B. C. Mayne and R. K. Clayton: Proc. Natl. Acad. Sci., U.S., 55 494, 1966.

139. B. C. Mayne: Photochem. Photobiol. 8, 107, 1968.

140. R. Emerson: Ann. Rev. Plant Physiol. 9, 1, 1958.

141. M. Delerieu: Thesis, 3rd Cycle, University of Paris, 1968.

142. G. Cheniae: Ann. Rev. Plant Physiol. 21, 467, 1970.

143. F. L. Allen and J. Franck: Arch. Biochem. Biophys. 58, 124, 1955.

144. C. P. Whittingham and A. H. Brown· J. Exptl. Botany, 9, 311, 1958.

145. P. Joliot Biochim. Biophys. Acta, 102, 116, 1965.

146. J. Rosenberg: Biophys. Soc. Abstr. 9, A29, 1969.

147. B. Forbush, B. Kok, and M. McGloin: Photochem. Photobiol., in the press.

148. H. H. Stiel and H. T. Witt: Z. Naturforschg. 24b, 1958, 1969.

149. B. Kok, S. Malkin, O. Owens and B. Forbush: Brookhaven Symp. in Biology, 19, 446, 1966.

150. C. Weiss and K. Sauer: Photochem. Photobiol., 11, 495, 1970.

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