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# PRACTICAL FLUORESCENCE

## *Theory, Methods, and Techniques*

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Chapter 13

# CHLOROPHYLL FLUORESCENCE AND PHOTOSYNTHESIS[a]

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## I. INTRODUCTION

The physical and chemical processes that lead to the reduction of carbon

dioxide and the oxidation of water in plants have been the subject of much study in the last decade [1, 2]. In 1957 Emerson [3, 4] discovered that the rate of photosynthesis was greater when red light (absorbed mainly by chlorophyll b) and far-red light (absorbed mainly by chlorophyll a) were used simultaneously than when they were used separately (the Emerson enhancement effect). To explain this phenomenon, it was suggested that photosynthesis requires two light reactions, sensitized by two pigment systems [5]. It is now widely accepted that two light reactions, arranged in series, are involved in this process [6] (Fig. 131). The first light reaction, arbitrarily called reaction II by Duysens and Amesz [7], leads to the reduction of a cytochrome and the oxidation of water to molecular oxygen; the second light reaction (reaction I) leads to the oxidation of the reduced cytochrome and the reduction of $NADP^+$ (nicotinamide adenine dinucleotide phosphate). Along the electron pathway from water to $NADP^+$ a fraction of light energy is utilized to synthesize ATP molecules from ADP and inorganic phosphate. With sufficient NADPH and ATP available, enzymatic reduction of carbon dioxide to the carbohydrate level (Calvin-Benson cycle) becomes possible.

The experiments of Emerson and Arnold [8] had established that a group of about 2400 chlorophyll a molecules somehow cooperate in evolving one oxygen molecule and reducing one carbon dioxide molecule. Since four hydrogen atoms (or electrons) must be transferred from water to carbon dioxide to achieve this process, about 600 chlorophyll molecules must cooperate in the transfer of one electron. In the two-light-reaction model of photosynthesis each electron must be transferred in two steps, so that the group of 600 chlorophyll molecules has to be divided in two parts, probably of 300 molecules each. These groups are usually referred to as photosynthetic units (PSU). Photosynthetic units of the pigment system that sensitize light reaction I ($PSU_I$) and those that sensitize light reaction II ($PSU_{II}$) are spectrally distinguishable.

Each PSU consists of a light-collecting part, comprising the majority of the pigment molecules ("bulk") and a "reaction center," or "trap," that participates directly in the primary photochemical reaction. Light energy absorbed by the bulk pigments is funneled (by a resonance-transfer mechanism) to the "trap," where the primary oxidation-reduction reaction (electron transfer) takes place. The trap in system I has been identified [9] as a chlorophyll a molecule with an absorption peak at 700 nm on the long-wavelength side of the absorption band of the bulk with a peak at 675 nm; it is referred to as "P700." The nature of the trap in system II has not yet been definitely established, but there are indications that it is also a chlorophyll a molecule (P680-P690) [10-12].

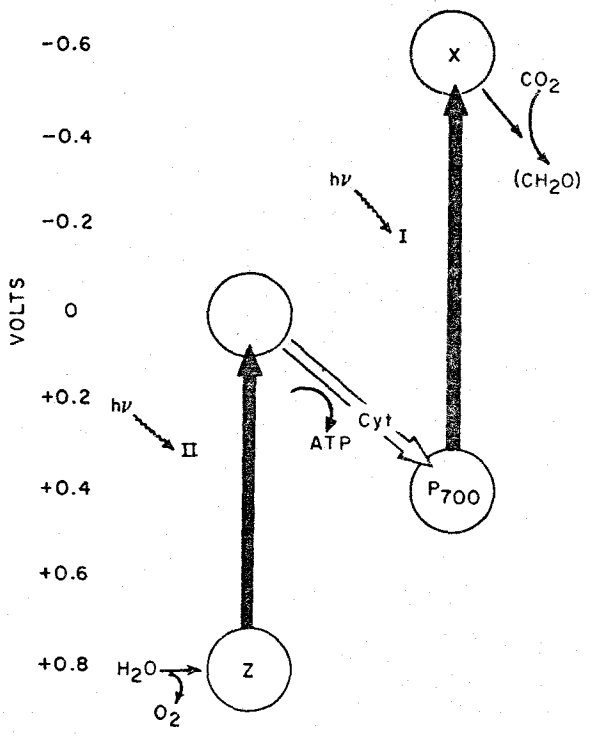



FIG. 131. Hydrogen (or electron) transfer in photosynthesis is now conceived as a two-step process involving two pigment systems. (The scale on the right represents the oxidation-reduction potential $E_o'$ of the intermediates in photosynthesis.) The light energy collected by pigment system II, trapped by pigment 680-690 (P680-690), oxidizes the primary H donor ZH to Z and reduces the unknown primary acceptor Q, the quencher of chlorophyll a fluorescence (also known as C550, for an absorbance change at 550 nm; in the text we use the symbol A for it). Molecular oxygen is evolved when water reacts with Z. The electrons are passed downhill to cytochrome f (Cyt), synthesizing ATP in this process. The light energy collected by pigment system I, trapped by pigment 700 (P700), oxidizes cytochrome f and reduxes another unknown acceptor P430 (or X). The reduced X, in turn, can reduce $NADP^+$ to NADPH via an iron protein, ferredoxin. The NADPH and ATP--the end products of the light reactions of photosynthesis--are then utilized to synthesize carbohydrate $(CH_2O)$ from carbon dioxide. A backreaction of reduced entities (XH, NADPH, etc.) with oxidized Cyt (or any component in the intersystem chain) leads to a cyclic flow of electrons that could be coupled to the formation of more ATP.

We now ask: How are the absorbed light quanta distributed equally between the two pigment systems for the efficient operation of photosynthesis? Two different hypotheses have been suggested [13]. In one of them, the "spillover" hypothesis, the excess light energy absorbed in pigment system II can spill over into system I (but not vice versa), leading to balanced excitation of the two systems in the part of the spectrum where more light is absorbed by system II and to a dropoff of the yield where light is absorbed preferentially in system I. The spillover is energetically possible because system I absorbs light of longer

wavelengths than system II. This hypothesis explains the high quantum yield of photosynthesis at wavelengths below 680 nm and the "red drop" in the action spectrum of photosynthesis beyond 680 nm (observed in green plants and algae).

In the second, "separate-package," hypothesis, excess light quanta absorbed in pigment system II are not transferred to pigment system I. An approximately balanced excitation of both systems is achieved by the presence in both systems of the same pigments in somewhat different proportions. For example, in the green alga Chlorella system II contains relatively more chlorophyll b, and system I relatively more long-wavelength forms of chlorophyll a. According to this hypothesis, the action spectrum of photosynthesis (and also of chlorophyll a fluorescence as system I is weakly fluorescent) must show drops wherever one of the two systems absorbs more than the other. This must cause a "fine structure" in the $\Phi = f(\lambda)$ curve (where $\Phi$ stands for quantum yield). Some such structure has been noted in the action spectrum of photosynthesis [5, 13] and of chlorophyll a fluorescence [14-16]. It has been suggested [17] that the spillover may occur only between system II and the trap (P700) in system I, but not the bulk of the latter system. Energy transfer can occur only if P700 is in the reduced, colored state. This means that energy transfer from system II to system I is possible only when the electrons flow from system II to P700, keeping it in the reduced state; if P700 is oxidized (i.e., bleached), the energy transfer stops. This hypothesis permits to reconcile certain observations favoring the spillover mechanism with other observations favoring the separate-package hypothesis.

A light quantum absorbed in a pigment molecule in the bulk of one of the two pigment systems faces three alternatives. Within the lifetime of its excited state it may be (a) lost by rapid "radiationless relaxation" into thermal energy; (b) reemitted as fluorescence; or (c) transferred to other pigment molecules. The energy quanta that reach a trap have an additional choice--to be converted into chemical energy by an "uphill" electron transfer. The efficiency of "quantum conversion" in the traps seems to be close to 100% when the electron-transfer chain operates at its highest rate (for recent estimates see Mar et al. [18]).

The quanta reemitted as fluorescence are presumed to originate mainly (or exclusively) in the bulk of the photosynthetic unit. When the trap is less than 100% effective in the conversion of light energy that reaches it into chemical energy, some energy could become available for trap fluorescence. However, since the difference in excitation energy between the bulk and the trap at room temperature is not large compared with kT (k - Boltzmann's constant), the photochemically unused quanta have a good chance of returning from the

trap to the bulk to be emitted there. At very low temperatures, however, the energy barrier opposing the diffusion of quanta back into the bulk becomes prohibitive, and emission from the trap can become significant.

Fluorescence, in photosynthetic organisms, represents a waste of light energy; this waste is, however, quite low since the yield of chlorophyll a fluorescence in living cells is only about 3%. In spite of the low yield of fluorescence, and complexity due to overlapping of the absorption and fluorescence spectra of the various pigments, a systematic analysis of the fluorescence intensity and spectrum has yielded important information concerning the composition of the two pigment systems and the primary mechanisms of photosynthesis. The intensity of emission is related to the efficiency of the two postulated primary processes, and its spectrum (and the action spectrum of its excitation) is indicative of the properties of the two postulated pigment systems. Fluorescence provides a nondestructive tool for monitoring the transformations of the pigments in photosynthesis.

Prior to the discovery of the two light reactions in photosynthesis, reviews on the relation of chlorophyll fluorescence to photosynthesis were written by Franck [19], Rabinowitch [20], and Weber [21]. More recently Butler [22], Clayton [17], Robinson [23], and Govindjee and Papageorgiou [24] have reviewed the subject in the context of the two-light-reaction model. In this chapter we discuss mainly the complexity of the chlorophyll a fluorescence spectrum in vivo, with emphasis on experiments made in our laboratory in Urbana.

The emission spectrum of chlorophyll a in vivo has a main band with a maximum at 685 $\pm$ 2 nm and a vibrational "satellite" at about 740 nm. These bands originate in a $\pi^* \longrightarrow \pi$ transition to the ground state. Excitation in the blue-violet (Soret) absorption band, leading to the second electronic excited state, is followed by radiationless transition to the lowest excited state, which is so fast that no emission originating in the upper state has ever been reported.

## II. MULTIPLICITY OF CHLOROPHYLL a FORMS IN VIVO

The red absorption band of chlorophyll a in vivo is broad (half-bandwidth $\sim$30 nm) in comparison with the same band of chlorophyll (Chl) a in organic solvents (18-nm half-bandwidth in diethyl ether). This suggests that chlorophyll a in vivo is composed of more than one molecular species or complex. Analyses of the absorption bands of chlorophyll a in suspensions of chloroplasts or whole algal cells encounter several difficulties:

1. Other pigments besides chlorophyll a are present.

2. Light is strongly scattered by the suspension (Rayleigh scattering and selective spectral scattering [25]; this may increase the apparent absorption if

measured in an ordinary spectrophotometer, and the absorption spectra may become distorted.

3. In a suspension light that is less absorbed has a larger effective path length than the more strongly absorbed light ("detour" effect [26]).

4. A mutual shading of the pigment molecules takes place in colored particles (chloroplasts and grana), and some light may pass the medium without hitting any particle at all ("sieve effect" [20, 27]).

5. Fluorescence contributes to the "transmitted" light particularly significantly when absorption is strong.

Two methods have been used to remedy some of these difficulties. In one a diffusing glass (an opal glass) is placed between the sample and the detector, so that almost all forward scattering is collected. In the other an integrating sphere (the Ulbright sphere) is used, so that almost all scattered energy is collected and read as "transmitted" light. Neither of the two methods eliminates the sieve effect or the detour effect.

French [29] constructed a "derivative spectrophotometer" in which the first derivative of the absorbance was plotted automatically as function of wavelength. This procedure accentuates the complex structure of a band, since every inflection in the band envelope appears as a crossing of the absicca or as a peak. The derivative absorption spectra of various algae obtained in this way were analyzed by Brown and French [30] (also Ref. [31]) into several Gaussian components. They were identified as Chl a 672, Chl a 683, and Chl a 694. More recently, a new component, which may be the short-wavelength band of long-wavelength aggregated forms, absorbing at 660 nm has been definitely identified by French and co-workers [32].

Cederstrand et al. [33] measured the absorption spectrum of algae in an integrating regular dodecahedron with a photocell on each of the 12 faces: the red band was interpreted as the sum of two components, Chl a 668 and Chl a 683 (again assuming a Gaussian shape of the components). A third band, due to chlorophyll b, was located at 650 nm. The assignment of a Gaussian shape to the bands of individual chlorophyll a components is not completely arbitrary, since a Gaussian curve matches very closely the red band of chlorophyll a in various solvents.

Das et al. [27] (see also Ref. [28]) have shown that elimination of the so-called sieve effect by sonication of Chlorella does not affect the qualitative results obtained with whole cells, but the peaks of the two major chlorophyll a components are moved (as expected) closer together to 670 and 683 nm. The half-width of both components is about 17 nm, very similar to that of the chlorophyll a band in ether.

The action spectrum of Emerson's enhancement effect [34, 35] (the proportional increase in yield when far-red light (680-720 nm) is given together with the short-wavelength light, compared with the sum of the yields in the separate beams) exhibits a peak or a shoulder at 670 nm, apparently attributable to the Chl a 670 component. (Both peaks, at 670 and at 680 nm, are assigned to chlorophyll a because of their location. The Chl a 670 is probably identical with French's Chl a 672, and Chl a 680 with his Chl a 683.) Cederstrand et al. [33] saw no convincing evidence of the existence of a third component in Chlorella, Anacystis, Porphyridium, and Spinacea (equivalent to French's Chl a 694), because in the long-wavelength tail of the band deviation from Gaussian shape occurs also in solution spectra. However, there is other evidence of its existence. The separate existence of a Chl a 670 component is indicated also by the observation of preferential photochemical bleaching in this spectral area [36].

Fractionation of the two (or three) chlorophyll a forms has been attempted by breaking the cells mechanically and solubilizing the pigment complexes selectively by means of detergents (such as digitonin) or by extraction with solvents of varying polarity. The two types of pigment complex identified in this manner are also Chl a 670 and Chl a 683, the latter being the more labile form [37-39].

## III. FLUORESCENT AND NONFLUORESCENT (OR WEAKLY FLUORESCENT) FORMS OF CHLOROPHYLL a

Duysens [40] and French and Young [41] observed that the light absorbed by the phycobilins of red algae is more efficient in exciting chlorophyll a fluorescence than light absorbed by chlorophyll a itself. This led Duysens [40] to postulate the existence of two forms of chlorophyll a: one fluorescent and the other nonfluorescent (or weakly fluorescent). The fluorescent form must be associated with the phycobilins much more effectively than the "nonfluorescent" one. It is now considered to be part of pigment system II. The "nonfluorescent" form was first assumed to be inactive in photosynthesis, but we now interpret it as belonging to pigment system I, which can perform only light reaction I.

A second indication of the existence of two forms of chlorophyll a with different fluorescence yields was obtained from measurements of the lifetime, $\tau$, of chlorophyll a fluorescence in vivo and in vitro by a direct flash method [42]. The lifetime was found (in Chlorella) to be 1.5 nsec (assuming a simple exponential decay). If one makes the plausible assumption that the "natural" lifetime $\tau_0$ of chlorophyll a in vivo is the same as it is in vitro (15.2 nsec,

calculated by integrating the area under the band envelope), one calculates $\phi = 0.10$ from the relation $\tau = \phi \cdot \tau_0$. However, direct measurements [43] (see also Ref. [44]) of $\phi$ in Chlorella gave significantly lower values, of about 0.03. This discrepancy can be explained if it is postulated that a significant proportion of chlorophyll a in vivo does not contribute significantly to fluorescence. The exact proportion of nonfluorescent (or weakly fluorescent) chlorophyll a could be estimated only if the measurements of both $\tau$ and $\phi$ were done under identical conditions on the same suspension and if $\tau_0$ could be determined directly for chlorophyll in the living cell. (For recent measurements of, and literature on, chlorophyll a fluorescence lifetime in vivo see Refs. [18] and [45] through [47]).

## IV. THE RED DROP IN CHLOROPHYLL FLUORESCENCE YIELD

If the weakly fluorescent or nonfluorescent form of chlorophyll a absorbs more strongly on the long-wavelength side of the red absorption band than the fluorescent form (as we have reason to believe), a decline (red drop) in the quantum yield of fluorescence $\phi_F$ plotted as a function of wavelength $\lambda$ can be expected. Indeed, Duysens [40] has noted such a decline beyond 680 nm in green algae. However, Teale [48], who took great precautions to exclude scattering by using a polarizing filter in the path of fluorescence, a suspending medium of high refractive index (concentrated sucrose, ethylene glycol), and a very dilute suspension of Chlorella, could find no red drip up to 690 nm. We reinvestigated the $\phi_F$ versus $\lambda$ curve for Chlorella sonicates (in which the scattering is very much reduced [49, 50]) and for very dilute suspensions of Chlorella, both by direct measurements and by computing the $\phi_F$ versus $\lambda$ curve according to Stepanov's relation between the absorption and the emission spectrum [15, 50, 51]. Fluorescence was collected from the same surface on which the exciting light fell, thus reducing its reabsorption. As a result of these studies we are convinced that a red drop in the $\phi_F$ versus $\lambda$ curve undoubtedly does exist. It begins at about 680 nm in Chlorella.

The fluorescence yield curve [$\phi_F = F(\lambda)$] in the phycobilin-containing algae is different because almost all the chlorophyll a is present in pigment system I. The system I chlorophyll a, however, is not fluorescent because the long-wavelength-absorbing from (C700, or Chl a 695) drains all the excitation, and the latter is nonfluorescent or weakly fluorescent. Because the absorption by system I chlorophyll a extends to shorter wavelengths in these algae, the red drop begins earlier. Thus in the red alga Porphyridium and in the blue-green alga Anacystis the fluorescence yield begins to decline at about 640 to 660 nm [50, 52].

One is naturally tempted to associate the "long-wavelength" nonfluorescent component of chlorophyll a with Cederstrand's Chl a 680, and the "short-wavelength" fluorescent component with Chl a 670. However, quantitative analysis makes this interpretation difficult to maintain. It rather suggests that the red drop is associated with a third, minor, long-wavelength component (French's Chl a 694?). This means that both main forms, Chl a 670 and Chl a 680, are present in both pigment systems I and II, whereas Chl a 694 may be present in pigment system I only, making the whole bulk of chlorophyll a in this system nonfluorescent (or rather weakly fluorescent).

The red drop in the fluorescence yield should disappear if the nonfluorescent (or weakly fluorescent) form of chlorophyll a could be preferentially destroyed. Under aerobic conditions and at low pH, prolonged sonication of Chlorella cells does lead to preferential bleaching of a long-wavelength form (Chl a 693) of chlorophyll a, as demonstrated by the difference between the absorption spectra of the sonicates prepared by sonication in the absence (pH 7.8) and in the presence (pH 4.5) of air. As expected, the samples deficient in the far-red form of chlorophyll a show a complete absence of the red drop in the action spectrum of chlorophyll fluorescence [49].

## V. ANALYSIS OF THE EMISSION SPECTRA AT ROOM TEMPERATURE

As mentioned in Section I, chlorophyll a in vivo fluoresces with a peak at 685 $\pm$ 2 nm and a vibrational band at about 740 nm. The main red emission band has been investigated intensively in recent years for evidence of its complexity. Due to experimental difficulties, such as the low quantum efficiency of chlorophyll fluorescence in living tissues, the overlapping of the bands, and the polyphasic structure of the material, special techniques must be applied to obtain meaningful data.

### A. Matrix Analysis

The intensity of fluorescence emitted at a certain wavelength by a mixture of several fluorescent forms with different positions of absorption bands will depend on the wavelength of excitation. If intensity data for a sufficient number of excitation-emission wavelength pairs are available, one can determine the number of individual fluorescing species. Weber [53] developed for this purpose a matrix method of analysis. The intensity of emission at different wavelengths is determined for fluorescence excited at different wavelengths, and matrices of increasing order are formed. If the 2 x 2 matrices do not disappear, there must be at least two fluorescence emitters; if the 3 x 3 matrices are significant, at least three, and so on. Weber's method was applied by Brody and Brody [54]

to Euglena, and by Govindjee and Yang [55] to spinach chloroplasts. The analyses showed that at room temperature there are (at least) two different fluorescent chlorophyll a species.

More recently Williams et al. [14] confirmed this also for Chlorella; they suggested, from consideration of the sign and magniture of different matrices, that only two fluorescent chlorophyll a species seem to be present, and that the one responsible for strongest emission has an emission peak at about 687 nm, whereas the less or weakly fluorescent component emits at about 700 nm. A band at 700 nm has recently been observed by Govindjee and Briantais [56] in the ratio spectrum of "high" to "low" fluorescence yields (see their paper for details). The two 700-nm bands may not be identical.

### B. Polarized and Nonpolarized Fluorescence

The existence of a chlorophyll a component absorbing on the long-wavelength side of the main absorption band was established by Olson et al. [57] and by Lavorel [58]. A dichroism ratio of more than 4 was observed when the analyzer was set parallel and perpendicular to the lamellar plane of the chloroplast at 695 nm; at the shorter wavelengths no dichroism was discernible. The emission spectrum of "polarized" fluorescence showed a maximum at 716 to 720 nm, that of "depolarized" fluorescence, at about 685 nm. The intensity of the former was about $\sim$ 5 to 10% of that of the latter.

Since the red absorption band of chlorophyll a is due to a $\pi \rightarrow \pi^*$ transition, with the oscillator located on the porphyrin plane (and bisecting rings II and IV), the above experiments suggest that at least a part of the chlorophyll a is either lying flat or is tilted at a small angle with respect to the lamellar plane. This orientation can be imposed by the protein-lipid matrix in which the chlorophyll a molecules are embedded. Possibly the weakly fluorescent long-wavelength form of chlorophyll a is simply the oriented fraction. It thus seems that a minor form of chlorophyll a in system I is arranged in a relatively ordered array. Is it the form responsible for the weak, long-wavelength polarized-fluorescence band beyond 700 nm and for the red drop in the overall chlorophyll fluorescence yield in the far-red region? The answer is not yet clear.

Boardman and Anderson [59] found evidence of partial separation of the two postulated pigment systems by differential centrifugation of digitonin-solubilized chloroplast material. Anderson et al. [60] provided further evidence of this separation: the lighter particles, enriched in P700 (i. e., in pigment system I) effectively performed the reduction of $NADP^+$ with reduced 2,6-dichlorophenol indophenol (DCPIPH, a dye with a normal oxidation-reduction potential of about 0. 2eV) as an electron donor, whereas the heavier particles, richer in chlorophyll b (i. e., in pigment system II), effectively reduced DCPIP

with water as electron donor. The degree of polarization of chlorophyll fluorescence was 2.7% in the heavier, and 5.4% in the lighter, fractions [60, 61]. These results are consistent with the conclusion that a fraction of chlorophyll a in pigment system I is ordered and emits more strongly polarized fluorescence. However, such conclusions should be made with caution because the size of the particles may also affect the polarization values.

The excitation spectra [58] of the polarized fluorescence in Chlorella have peaks at about 700, 540, and 400 nm, whereas those of depolarized fluorescence show a red drop beyond 680 nm, again indicating that the oriented chlorophyll a is a part of pigment system I. Our observations on the red alga Porphyridium confirm these findings [62]. The action spectrum of the species fluorescing at 685 nm always follows closely the absorption spectrum of pigment system II, whereas the action spectrum of polarized fluorescence at 720 nm follows the absorption spectrum of system I. Vredenberg [63] also suggested that chlorophyll $a_I$ fluoresces at 720 nm, but he found no correlation between the kinetics of absorbance changes attributable to P700 and the 720-nm fluorescence. (However, if P700 sensitizes the reaction in its triplet state, no correlation between chlorophyll fluorescence and photochemical sensitization can be expected.)

## VI. FLUORESCENCE AT LOW TEMPERATURES

The sharpening of the emission bands at low temperatures is a useful means of reducing the difficulties arising from the overlapping of the bands at room temperature (see Section V). In addition, the quantum efficiency of chlorophyll fluorescence is considerably increased at low temperatures (both photochemical and internal quenching is slowed down). The low-temperature spectra are of particular interest for the identification of the photochemical reaction centers (the traps) since the "trap depth" ($\Delta\lambda \cong 10$-15 nm) increases from 1-2 kT at room temperature, to 5-8 kT at 77°K (liquid-nitrogen temperature), and to 100-150 kT at 4°K (liquid-helium temperature).

S. S. Brody [64] was the first to observe a new emission band, located at 720 nm (F720), in Chlorella cooled to 77°K. Several investigators [5, 54, 55, 65-70] confirmed its existence and observed an additional band at 696 to 698 nm. An extension of this work to liquid-helium temperature (4°K) by Cho et al. [71] and Cho and Govindjee [66, 72] clearly showed the existence of three fluorescence bands, at 689 (F687), 698 (F696), and 725 (F720) nm, respectively. These bands appear at different locations in different organisms. We shall, however, refer to them as F687, F696, and F720.

The overall shape of the red emission band in green cells exhibits a strong

temperature dependence caused by the different behavior of the three components. Govindjee and Yang [55] studied this behavior in spinach chloroplasts, Krey and Govindjee [65] in Porphyridium, and Cho and Govindjee [73] in Chlorella, in the range 77 to 293°K. The F696 band appears only in the temperature range between 77 and 140°K. On warming from 77 to 140°K, both F696 and F720 decrease in intensity (although at different rates), whereas F687 remains constant. At the lower temperatures (40 to 4°K) Cho et al. [71] found that the F687 band increased, whereas the F696 band intensity rose only slightly. These changes can be interpreted as reflecting a reduction in the rate of energy transfer from the bulk of chlorophyll a (emission peak at 685 nm) to the trap in system II (emission peak at 698 nm) at these low temperatures.

Furthermore it was found [55, 65, 66, 72] that excitation in chlorophyll b (in green plants) and in phycobilins (in red or blue-green algae) leads to a lower F720 but higher F687 and F696 bands; whereas excitation in chlorophyll a leads to the opposite result. This suggests that F687 and F696 belong mainly to pigment system II, and F720 belongs mainly to pigment system I, in agreement with the conclusions reached in a preceding section.

These conclusions are further confirmed by experiments on particles prepared by solubilizing spinach chloroplasts with detergents (digitonin) and differential centrifugation, with chloroplasts extracted with acetone and methanol of different concentrations, and with Chlorella cells sonicated in air or argon. Particles--prepared by the digitonin method--that perform light reaction I, according to Boardman and Anderson [59], were found to be richer in F720-emitting material when cooled down to 77°K, whereas particles that perform light reaction II were enriched in F696-emitting material [61, 74]. Shimony et al. [75] obtained fractions enriched in F720-emitting material (Chl a in system I) and fractions poorer in F720-emitting material (Chl a in system II) from the blue-green alga Anacystis. Thomas and Van der Wal [76] reported that chloroplasts extracted with methanol of different concentrations had somewhat different absorption and fluorescence characteristics.

Cederstrand et al. [39] studied the absorption and fluorescence characteristics of (a) chloroplast residues after extraction with aqueous methanol and acetone of different strength, and (b) the corresponding extracts. They found, particularly at low temperatures, an apparent enrichment either of system II or of system I in the residues from extraction with different solvents. For example, chloroplasts extracted with 50% methanol were enriched in F696-emitting material (system II). Very dilute aqueous acetone or methanol apparently extracted whole pigment complexes present in the chloroplasts. Under aerobic conditions and at acid pH, sonication of Chlorella cells [49] leads to preferential bleaching of the weakly fluorescent (or nonfluorescent) chloro-

phyll a (Chl a 693); these samples show no F720 band at 77°K, assumed to be due to the long-wavelength form of chlorophyll a (pigment system I).

The excitation spectra of the F720, F696, and F687 fluorescence bands in Chlorella and Anacystis at very low temperatures (4-77°K) provided the following information [66, 72, 73]:

1. There are two excitation peaks, at about 670 and 680 nm, respectively, which must be due to the two forms of chlorophyll a identified in the absorption spectra [27, 33]; the excitation spectra of both F720 and F696 show these peaks, confirming the earlier suggestion that both pigment systems I and II contain both components. Using narrow slits, it was possible [66] to extend the measurement of the excitation spectrum of F687 to the 680-nm region. It appears that Chl a 680 is responsible for F687. If Chl a 680 is preferentially extracted, one can show that Chl a 670 is responsible for a band at 680 nm (F680) that appears under these conditions. The exact location of the 670- and 680-nm peaks in the action spectra may be slightly different for F720 and F696, suggesting some differences in the environment of these chlorophyll a components in the two systems; this, however, needs further study.

2. The ratio of fluorescence intensity excited at 440 nm (Chl a absorption) to that excited at 480 nm (Chl b absorption) is greater for F720 than it is for F687 and F696. This confirms that F720 is excited more effectively by absorption in chlorophyll a (preferentially in pigment system I) and F687 and F696 by absorption in chlorophyll b (preferentially in pigment system II). Near identity of the excitation spectra for F687 and F696 confirms our earlier suggestion [62] that F696 belongs to the same system as F687 (system II). Williams et al. [14] concluded from their analyses that the "F700" band, observed in Chlorella fluorescence in vivo at room temperature, belongs to system I; this shows that it is not identical with the low-temperature F696 band. We now consider the possibility that there are two emission bands in this spectral region--the system I component is present both at room temperature and at 77°K, and the system II component appears at low temperatures (see earlier discussions [77, 78]) and under conditions when photosynthesis is saturated or abolished.

3. The highly efficient energy transfer from chlorophyll b to chlorophyll a appears to be independent of temperature. This is suggested by the constancy of the ratio of chlorophyll fluorescence excited at 440 nm (absorption in Chl a) to that excited at 480 nm (absorption in Chl b) when the temperature was varied from 4 to 77°K. However, the efficiency of energy transfer from phycobilins to chlorophyll a was dependent on temperature--just as for the transfer from Chl a 680 to the trap of system II, noted above.

## VII. LIFETIMES OF THE EXCITED STATES

The decay period of chlorophyll a fluorescence in vivo has been estimated from flash fluorometry [42, 79] and phase fluorometry [80, 81] to be in the range of 1 nsec; it was assumed that the decay of chlorophyll a fluorescence in vivo follows a simple first-order exponential function. Under conditions of interrupted photochemical deexcitation, such as low-temperature [18, 81] and 3-(3, 4-dichlorophenyl)-1, 1-dimethyl urea (DMCU) poisoning [18, 47, 82], the lifetimes were approximately doubled, corresponding to increased quantum yield of fluourescence under these conditions.

Murty and Rabinowitch [83] employed shorter flashes and a faster detecting system than those employed by S. S. Brody and Rabinowitch [42] to trace the decay curve of chlorophyll a fluorescence in various algae. The plot of the logarithm of the fluorescence intensity against time showed [83] not a single straight line but a curve with two linear portions. From the slopes of these curves two time constants were calculated: one corresponding to a decay period $\tau_1$ of 1 to 2 nsec, and the other to a decay period $\tau_2$ of 4 to 5 nsec. This experiment suggested the existence of two components of chlorophyll a fluorescence, decaying at different rates. The authors suggested, as one of the alternatives, that the faster decaying component originates in pigment units with a higher chlorophyll a concentration, in which the excitation is rapidly transferred to the reaction site, lowering the quantum yield of fluorescence. (A similar conclusion has been reached by Pearlstein [84] on theoretical grounds.) This needs further investigation, as Singhal and Rabinowitch [85] (see also Refs. [45] and [46]) could not repeat Murty's earlier observation; it is possible that an "artifact" in the exciting lamp was responsible for the $\tau_2$ observed earlier.

According to Robinson [23], the more rapid photochemical deexcitation of the chlorophyll a singlets in comparison with the slower rates of intersystem crossing renders the participation of the chlorophyll a triplets in the primary reactions unlikely. Assuming that at 77°K the singlets can either fluoresce (with a measured lifetime of 3.1 nsec) or cross over to the triplet state, an intersystem-crossing rate of 0.26 $nsec^{-1}$ can be calculated on the basis of the $\tau_0$ value of 15.2 nsec. This rate represents an upper limit since, at room temperature, other competing deexcitation processes will reduce it further. The rate of chemical deexcitation in vivo at room temperature, however, calculated on the basis of the 0.03 quantum yield of fluorescence, is much higher ($\sim$6 $nsec^{-1}$) than the upper limit of the rate of intersystem crossing, so that the latter cannot compete favorably with the former. The low fluorescence yields indicate a fast chemical turnover, with the participation of the singlet

states of the reaction centers. This concept is by no means universally accepted. For example, Franck and Rosenberg [86] suggested that reaction I proceeds through the triplet state. There is also the possibility that triplets are not involved in the main path of photosynthesis, but they are produced in a side pathway--either directly at the reaction center by intersystem crossing or by a backreaction of photoreaction II. An explanation of this nature has been recently formulated and analyzed by Stacy et al. [87] and Lavorel [88]. They explain the delayed fluorescence in algae on the basis of a triplet-triplet fusion theory.

## VIII. VARIATION IN FLUORESCENCE YIELD WITH LIGHT INTENSITY

There are several pathways for the dissipation of energy by a population of excited chlorophyll a molecules: radiative deexcitation (fluorescence), photochemical quenching, nonradiative deexcitation (internal conversion into heat energy), and transfer to a fluorescent or nonfluorescent species. The following equation can be written for the yield $\phi_F$ of chlorophyll a fluorescence:

$$\phi_F = \frac{k_F}{k_F + k_R + k_C [A]}, \tag{207}$$

where $k_F$ is the rate of radiative deexcitation, $k_C$ is the rate of a bimolecular quenching process, [A] is the concentration of the quenching partner, and $k_R$ is the sum of nonradiative and nonphotochemical deexcitation rates. Since the availability of the photochemical quencher [A] is limited by a sequence of dark enzymatic reactions in photosynthesis, it is expected that at saturating light intensities the quantum yield of fluorescence will reach an upper limit corresponding to [A] = 0, whereas at low intensities a lower limit will be established corresponding to a constant value [A] = $A_0$.

In pure dilute chlorophyll a solutions fluorescence intensity F is proportional, within wide limits, to the incident intensity I; that is, the quantum yield $\phi_F$ of fluorescence is independent of light intensity. Chlorophyll a fluorescence in vivo shows, however, a dependence of $\phi_F$ on I, as expected from Eq. (207). This was clearly shown by, among others, Franck [19], Wassink (see Ref. [20]), and by Brugger [89], who plotted F as a function of I. They found that the slope of this curve, at "high" intensities (i.e., in light strong enough to "saturate" photosynthesis), is twice that at low intensities (where the quantum yield of photosynthesis, $\phi_p$, is maximal and constant). Latimer et al [43], who measured the absolute quantum yields of fluorescence in Chlorella, confirmed these findings, showing that the quantum yield of chlorophyll a

fluorescence increased from about 0.025 and approached 0.05 with increasing intensity of the exciting light. (The quantum yield of photosynthesis ($O_2$ evolution) is known to be about 0.12 at low intensities [15, 90] and declines steadily at high intensities).

Krey and Govindjee [65] measured chlorophyll a fluorescence F as a function of exciting light intensity I in the red alga Porphyridium cruentum. They could confirm the nonlinearity of the F = f(I) curve only in the case when excitation took place in the pigment phycoerythrin (i. e., in system II); no dependence of $\phi_F$ on I could be observed when excitation took place in chlorophyll a itself (i. e., in system I). This difference can be understood if the reaction centers of pigment system I operate in the triplet state, whereas those in pigment system II operate in the singlet state, because only in the second case is there a competition between sensitization and fluorescence.

## IX. CHANGES IN FLUORESCENCE YIELD WITH TIME AND THE TWO-LIGHT EFFECT: THE ACTIVATION REACTION

The yield of chlorophyll fluorescence in vivo undergoes complex but reproducible changes with time (the Kautsky effect); not only the intensity but also the spectral composition of the fluorescence is altered. (For literature and other details see a recent review by Govindjee and Papageorgiou [24]).

When dark-adapted Chlorella cells are exposed to strong light, the fluorescence yield rises instantaneously to an initial level (0) that is independent of photochemical processes. It then rises to a level that remains constant (or decreases slightly) for a brief period (ID), in the millisecond range, and then rises again to a peak (P) (reached after 0.25 to 1 sec). Within about 1 to 2 sec it decreases to an almost steady level (S); another peak is observed after about 30 to 50 sec (M), after which the fluorescence declines to its final steady level (T). The exact shape of the F = f(t) curve, and the time of occurrence of the various characteristic points, varies with the conditions of the experiment [91-95].

To explain the induction curve of chlorophyll fluorescence, Kautsky et al. [96] suggested that its yield depends on the presence of the oxidized form of an oxidation-reduction intermediate (A) that quenches the fluorescence. This quencher is reduced by the first light reaction

$$A + e \xrightarrow{h\nu} A^- \tag{208}$$

and reoxidized by a dark reaction

$$B^+ + A^- \longrightarrow B + A; \tag{209}$$

a second light reaction

$$B + X \xrightarrow{h\nu} B^+ + X^- \qquad (210)$$

completes the sequence.

According to Kautsky, the fluorescence plateau reached in the millisecond range is due to the removal of the quencher (A) by a first-order photochemical process. However, the rate of the regeneration of the dark reaction becomes significant when the concentration of $B^+$ is increased by the second light reaction; this causes the first plateau in the induction curve. The renewed rise to the peak reached after about 0.25 sec follows as $B^+$ is used up. Kautsky al. did not explain the decay of this peak.

Lavorel [97] suggested that the fluorescence yield is a sum of two contributions. One, invariant with time, is independent of any photochemical events; the other, a time-dependent one, is due to a photoactive form of chlorophyll a. With an experimental setup in which an algal suspension flowed at controlled speed through a capillary, a segment of which was illuminated, he succeeded in recording the spectra of the two components of fluorescence. The variable fluorescence contained relatively more of the 685-nm than the 717-nm fluorescence species. This was confirmed by Rosenberg et al. [98] and by Munday [99].

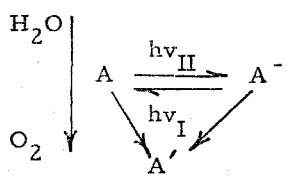
Two light reactions were postulated in Kautsky's scheme, but he reported no attempts to separate them by exciting with light of different wavelengths. The first observation that the yield of fluorescence can be modified by adding far-red light (absorbed in pigment system I) to short-wavelength light (absorbed in system II) was made by Govindjee et al. [100] in experiments analogous to those by which the photosynthetic enhancement effect was demonstrated. They found that fluorescence excited with red or blue light (absorbed in pigment system II) was lowered (quenched) when far-red light (which by itself produced no fluorescence) was added. Butler [101, 102] used strong red (system II) and far-red (system I) actinic light to establish a steady-state concentration of the postulated oxidation-reduction intermediate and followed with a weak exciting beam to observe the fluorescence yield. (The actinic light was eliminated during the fluorescence measurement.) Butler confirmed the antagonistic effects of the two lights on chlorophyll a fluorescence, in whole leaves. The quenching of fluorescence by far-red light was maximal at 705 nm [103]. This maximum depends, however, on the intensity of the actinic light; it is shifted to longer wavelengths at higher light intensities. This band may be due to the same long-wavelength chlorophyll a form that is responsible for the 720-nm emission band at 77°K.

Another method employed to establish a steady photochemical state before

measuring fluorescence is to excite fluorescence with a weak modulated beam, superimposed on a strong, constant background light [104]. The two-light effect on fluorescence was confirmed by this method. Duysens and Sweers [104] proposed that in reaction II--sensitized by light absorbed in pigment system II-- the reductant (indirectly, water) reduces an intermediate, which they called Q, we shall continue to use Kautsky's notation, A, for the sake of uniformity in this presentation. The oxidation-reduction potential $E_o'$ of the primary electron acceptor, pigment system II, has been estimated to be +180 mV, over a pH range from 6 to 9 [105]. However, Butler et al. [106] reported two $E_o'$ values for Q: -35 and -270 mV; they considered only the first value to be of significance. Recently Knaff and Arnon [107], and Eriyon and Butler [108] have described an absorbance change at 550 nm due to compound 550, or C550, which has been suggested by the latter authors to be identical with Q.

If A is associated with the trap, and the reaction can occur in the excited-singlet state of the latter, it must cause a quenching of chlorophyll a fluorescence in pigment system II. The steady-state fluorescence yield of pigment system II is governed by the proportion of oxidized "traps," that is, of traps associated with A (as contrasted to those associated with $A^-$) since only the oxidized form can be utilized for the photochemical reaction. At very high intensities of light absorbed in system II, there is a preponderance of $A^-$ (since light reaction I is unable to reoxidize $A^-$ at a high enough rate). The increase in fluorescence at the higher light intensities is due to this shift in system II. This has been confirmed by observations on the excitation spectra of fluorescence of the green alga Scenedesmus [109] and on the emission spectra of the red alga Porphyridium [65, 110]. On excitation, pigment system I depresses the fluorescence yield because $A^-$ is oxidized to A.

Duysens and Sweers [104] postulated an additional dark backreaction via another intermediate ($Q'$) to account for the inability to observe an increase in fluorescence yield if enough dark period was not provided between light exposures. (We use A' to remain consistent with the terminology used here.) Their scheme is very similar to that of Kautsky et al. [96]:




(211)

The rise in fluorescence yield is attributed to the removal of A by light reaction II. On poisoning with DCMU, this rise can be observed in its "pure" form,

without subsequent decline. This indicates that DCMU inhibits the reactions that regenerate A. The brief ID plateau (in the millisecond range) is due to (as in Kautsky's scheme) the indirect oxidation of $A^-$ by system I, involving one or more additional oxidation-reduction intermediates. Finally, the decay from the peak (at about 0.25 sec) is due to both system I action and the dark back-reaction via the so-called A'. The latter also accounts for the higher steady-state fluorescence value at the end of the decay period as compared with the first plateau in the millisecond range. Both $A^-$ and A' are photochemically inactive forms; one can assume that the higher yield at the steady state is due to the fact that part of the quencher A exists in the nonphotochemical form A'. Recent experiments (see review [24]) suggest that A' may be nothing else but a physical state of the system in which there is a high spillover of energy from pigment system II to the weakly fluorescent pigment system I.

Since A is the primary electron acceptor, the rate of oxygen evolution will depend on its concentration, and an inverse linear relationship should exist between it and the fluorescence yield. This prediction has been substantiated by the experiments of Delosme et al. [111].

Kautsky et al. [96] estimated the relative concentration of the fluorescence quencher as [A]:[Chl] ≃ 1:400, whereas the estimate of Duysens and Sweers [104] gave [A]:[Chl $a_2$] ≃ 1:150. A somewhat higher ratio was found by Malkin and Kok [112]. These figures indicate that the quencher is present in amounts approximately equal to the number of reaction centers. In all likelihood Kautsky's A (or Duysens' Q) is not a usual plastoquinone, because the latter is known to be present in much larger quantities. Plastoquinone is an intermediate that participates in the electron-transfer chain between the two photosynthetic systems, operating at a site close to system II. (However, there are suggestions [113] that Q may be a type of quinone, and there may be two quenchers, $Q_1$ and $Q_2$, instead of one.)

After a dark period, simultaneous recordings of the fluorescence kinetics and oxygen-evolution rate [111, 114] showed that the inverse relationship is not valid over the entire fluorescence-rise curve. During the first phase (referred to as "activation phase" in the earlier literature) fluorescence and oxygen-evolution rate increase in parallel. (In weak light there is a lag in $O_2$ evolution, but not in the fluorescence rise; also, in the presence of DCMU, when there is no $O_2$ evolution, the fluorescence yield rises.) During the second ("complementary") phase these processes are antiparallel. (McAllister and Myers [115] had first observed this phenomenon with the techniques available in 1940.) The duration of the "activation phase" is inversely proportional to the intensity of the exciting light. In the complementary phase these is a linear

relationship between the fluorescence yield (F/I) and the rate of oxygen evolution (V/I) of the form

$$\frac{F}{I} = a\frac{V}{I} + b. \tag{212}$$

This relationship between the yields is independent of light intensity since the constants a (which is negative) and b are independent of it. To explain his kinetic results Joliot [114] postulated (a) that the initial fluorescence variations originate in pigment system II and (b) that a photochemical quencher designed by him as E at that time (we shall continue to use A for it) is activated during this period. Schematically the reaction sequence given by Joliot can be rewritten as follows:

$$A_iH \underset{k_1}{\overset{k_2I}{\rightleftharpoons}} AH \underset{BH}{\overset{B}{\rightleftharpoons}} A \underset{}{\overset{k_2I}{\rightleftharpoons}} A^* \begin{cases} \nearrow O_2 \\ \searrow H_2O \end{cases} \tag{213}$$

In this scheme B is the form of another intermediate and is produced by light reaction I. Both $A_iH$ and A can quench the fluorescence, but AH does not. In the very beginning of the excitation period the photochemical quencher is assumed to exist in the form $A_iH$. During the activation phase it is transformed to AH, with parallel increase in the fluorescence intensity (due to the removal of $A_iH$), and in the rate of oxygen evolution (due to the production of $A^*$). At the end of the activation phase the form $A_iH$ is consumed, and only the forms A (a quencher) and AH (a nonquencher) are present.

In the "complementary" phase the transformation of AH to A and the subsequent photochemical reaction results in a decrease in fluorescence and increase in oxygen evolution. These initial explanations have now been replaced by more intricate theories in which four oxidizing equivalents accumulate on the donor side (Z) before oxygen evolution occurs, and the "activation" is included in the "main" pathway as the formation of less than four equivalents [116-118].

It has been shown [92, 119] that preillumination with far-red light (pigment system I), even if followed by a brief dark period (0.5 sec), affects the induction of fluorescence by largely eliminating the 0.25-sec peak (P). These experiments indicate the presence of a "pool" of intermediate B formed by far-red light, as suggested earlier by French [120] on the basis of flashing-light experiments on oxygen evolution and as suggested recently by Kok et al. [121] and Witt et al. [122]. Govindjee et al. [119] also found that, after far-red preillumination, the steady-state fluorescence at about 2 to 3 sec is slightly higher than the brief plateau in the millisecond range. Joliot's reaction

sequence can explain these results. Prolonged far-red light treatment converts BH to B, so that when pigment system II begins to operate, the nonquencher AH is rapidly transformed to the quencher A, by the dark bimolecular reaction, thus causing a delay in the rise and a reduction in the height of the 0.25-sec peak.

Experiments on oxygen evolution in flashing light support the idea of an activation reaction in photosynthesis. Allen and Franck [123] reported that no oxygen is produced in algae by a single short light flash (~1 msec). However, when a longer flash (~25 msec) or two brief flashes with a spacing of a few seconds were given, or when weak preillumination was provided before the flash, oxygen was evolved. Whittingham and co-workers [124, 125] confirmed and extended these observations. Joliot [126], who developed a very sensitive oxygen electrode, provided more precise data on this phenomenon, which can also be ascribed to the need for an activation reaction (for recent theories see Refs. [116] through [118]).

The common feature of the three reaction schemes (Kautsky, Duysens, and Joliot) proposed to account for the cause of induction in chlorophyll fluorescence in vivo is a photochemical substrate that can be directly reduced by pigment system II and indirectly reoxidized by pigment system I, both processes proceeding with a quantum efficiency of unity. This picture permits an explanation of the antagonistic effect of lights absorbed in system II and system I, respectively, and of complementarity between fluorescence intensity and the rate of oxygen evolution. However, so far no chemical identification of the postulated primary photochemical reactants has been possible, and additional complications due to changes in the efficiency of energy spillover for system II to system I spoil this simple relationship.

## X. VARIATIONS IN FLUORESCENCE: CHANGES IN INTENSITY AND SPECTRA

Light-induced variations in the quantum yield of chlorophyll a fluorescence have been employed in the study of the complexity of chlorophyll a in vivo (see Sections VIII and IX).

Butler and Bishop [109] compared the excitation spectra of an intact leaf after red and far-red preillumination and observed that it was the pigment system II fluorescence that increased at high light intensities, whereas a band at 705 nm was virtually independent of the preillumination of the sample. Difference emission spectra of Porphyridium, constructed by Krey and Govindjee [110] by subtracting the emission spectrum obtained in low light (below the saturation of photosynthesis) from the spectrum obtained in high light, exhibit a band at 693 nm in addition to the main 687-nm band (pigment system II). Recently we have obtained [56] similar results with Chlorella. The increase

in 687-nm fluorescence confirms Butler and Bishop's conclusions. The 693-to 700-nm band was, however, new. From its spectral location, the expected Stokes' shift, and the consideration that, when photosynthesis is saturated, some emission from the traps may become possible, the 693- to 700-nm band has been tentatively assigned to the trap in system II.

An alternative means of modifying the quentum yield of fluorescence, in living cells, is the interruption of the photosynthetic electron flow by selective poisons, such as certain derivatives of phenylurea (e. g. , DCMU) and 9,10-phenanthroline. Poisoning with DCMU results in the diminution of oxygen evolution (although the possibility of its complete abolition is questionable) and an increase in the fluorescence yield by a factor of more than 2. Under conditions of interrupted photochemical quantum conversion one may expect that previously nonfluorescent species, such as energy traps, may also emit some fluorescence, although most of the excess fluorescence must originate in the bulk, as pointed out in Section I. Indeed, "difference spectra" obtained by comparison of DCMU-poisoned with nonpoisoned Porphyridium or Chlorella cells [56, 65] reveal the existence of a large 685-nm band (bulk) and an additional band at 692 to 700 nm, which for the above-stated reasons may also originate in the trap of system II.

The occurrence of an effective energy transfer from accessory pigments (e. g. , phycocyanin, Chl b) to chlorophyll a is well known [40, 41, 79, 127-129]. M. Brody and Emerson [130] and Brody and Brody [131] reported changes in the rate of oxygen evolution and the quantum yield of fluorescence in Porphyridium induced by prolonged preillumination--changes they ascribed to changes in the efficiency of energy transfer from phycoerythrin to chlorophyll a. Such a modification could be achieved by growing Porphyridium in light of different wavelengths. Ghosh and Govindjee [132] obtained similar results with the blue-green alga Anacystis; they suggested that decreased efficiency of energy transfer from phycocyanin to "fluorescent" chlorophyll a in pigment system II may mean a more effective transfer to the weakly fluorescent form of the same pigment in system I.

Papageorgiou and Govindjee [77, 93] studied the effect of prolonged illumination on normal and DCMU-poisoned Anacystis; they found that not only the quantum efficiency of fluorescence changes in a specific way but also the spectral distribution of fluorescence is altered, the effect being largest at about 695 nm. In order to interpret their kinetic data the authors assumed that light-induced configurational changes may occur and affect both the rate of energy transfer (from phycobilins to Chl a) and the quantum yield of chlorophyll a fluorescence (for more recent ideas see recent reviews [24, 133, 134].

The effects of prolonged illumination on the quantum efficiency of

chlorophyll fluorescence were also studied in Chlorella [94]. Under constant exciting-light conditions the fluorescence yield, after the initial induction effects are over in about 1 sec, rises for 30 to 40 sec (S → M phase); this rise is followed by a slower decline to a lower steady level (T) than that observed after 2 to 3 sec. The rates of change, as well as the final established level of the yield, depend on the light intensity, the pH of the medium [135], and the integrity of the electron-transport chain and phosphorylation. In whole cells poisoning by DCMU or 9,10-phenanthroline abolishes the transients and inhibits electron transport in photosynthesis. When phosphorylation is uncoupled from electron transport by ammonium chloride and p-trifluoromethoxyphenylhydrazone of ketomalonyldinitrile (FCCP), the fluorescence transients are also affected. The compound FCCP, which is known to uncouple phosphorylation and increase electron transport in the Hill reaction, may affect the whole cells differently. In whole cells, where carbon dioxide is the oxidant and ATP is needed in the Calvin cycle, uncoupling of phosphorylation would reduce the rate of the Calvin cycle, thus reducing, rather than accelerating, electron transport. Increased fluorescence may then be expected. We found that in whole Chlorella cells FCCP, in a concentration of $5 \times 10^{-5}$ M, completely eliminates the (M → T) decay after the 30- to 40-sec fluorescence peak, and the fluorescence remains at a constant level. The dependence of the long-time light-induced fluorescence changes on the same parameters (pH, uncouplers of phosphorylation, etc.) that affect the light-induced configurational (volume and scattering) changes in chloroplasts may indicate a close relationship between fluorescence changes and energy-preserving processes like phosphorylation. Recently Mohanty et al. [95] found that glutaraldehyde, which abolishes configurational changes, also abolishes slow fluorescence changes. Closer analysis of this subject cannot be undertaken without further data on short-time fluorescence induction and observations on isolated chloroplasts.

## XI. FLUORESCENCE INTENSITY AND PARTIAL REACTIONS

Prior to the discovery of the two light reactions, Lumry et al. [136] studied the relationship of chlorophyll fluorescence and the Hill reaction, which is the reduction of an added oxidant (other than $CO_2$) and the simultaneous oxidation of water to molecular oxygen. Quenching of chlorophyll a fluorescence was observed when Hill oxidants were added to chloroplasts. Kok [69] observed quenching of chlorophyll a fluorescence in chloroplasts when $NADP^+$ and ferredoxin were added; this quenching was abolished by the addition of DCMU. Further addition of 2,6-dichlorophenol indophenol plus ascorbate did not cause renewed quenching. These experiments show a clear relationship between chlorophyll a fluorescence yield and the efficiency of electron transport

involving pigment system II (or both systems I and II, but not system I alone).

Govindjee and Yang [137] observed a strong quenching of chlorophyll $a_2$ fluorescence (687 nm) when phenazine methosulfate (PMS) was added to DCMU-treated spinach chloroplasts. (Excitation was at 610 nm to avoid absorption by PMS.) It is known that PMS accelerates "cyclic" phosphorylation (i. e., phosphorylation coupled with reversal of light reaction I), which is sensitized by system I. One could suggest that the yield of chlorophyll a fluorescence is also affected by the "physical state" of the chloroplast membrane, which may change during "phosphorylation." These results can also be explained if we suggest that under these conditions spillover of excess energy takes place from the strongly fluorescent pigment system II to the weakly fluorescent pigment system I, resulting in the quenching of fluorescence. However, a direct reaction of PMS with pigment system II cannot be excluded.

## XII. CONCLUDING REMARKS

Absorption and fluorescence spectrophotometry has been used successfully in the study of photosynthetic pigments in vivo. Chlorophyll a is the most important pigment since it is present in all photosynthesizing plants and algae, and a special "complex" of chlorophyll a--designated as P700--participates in the primary oxidation-reduction reaction of photosynthesis. Although only one form of chlorophyll a can be identified in organic solvent extracts, the evidence presented in this chapter suggests that several modifications of it exist in vivo; they may be different holochromic forms or different aggregates of chlorophyll a. Figure 132 shows the probable distribution of the different forms of chlorophyll a and b in the two pigment systems of photosynthesis in higher plants and green algae. In the red and blue-green algae chlorophyll b is replaced by the phycobilins and most of the chlorophyll a is in pigment system I.

Some of the chlorophyll a forms are designated as Chl a 670, Chl a 680, Chl a 695, and P700. These forms (see Table 87) have been identified mainly from the absorption spectra, the difference absorption spectra, the action spectra of the light reactions, and the action spectra of chlorophyll a fluorescence. It is generally believed that the two pigment systems (I and II) contain both chlorophyll b and a (Chl a 670 and Chl a 680), but in different proportions. (In phycobilin-containing algae most of the Chl a 670 and Chl a 680 belong to pigment system I.) However, Chl a 695 may be present exclusively in pigment system I. A form of chlorophyll a designated as Chl a 660 may simply be the short-wavelength band of this aggregate form of Chl a 695, as suggested by S. S. Brody.

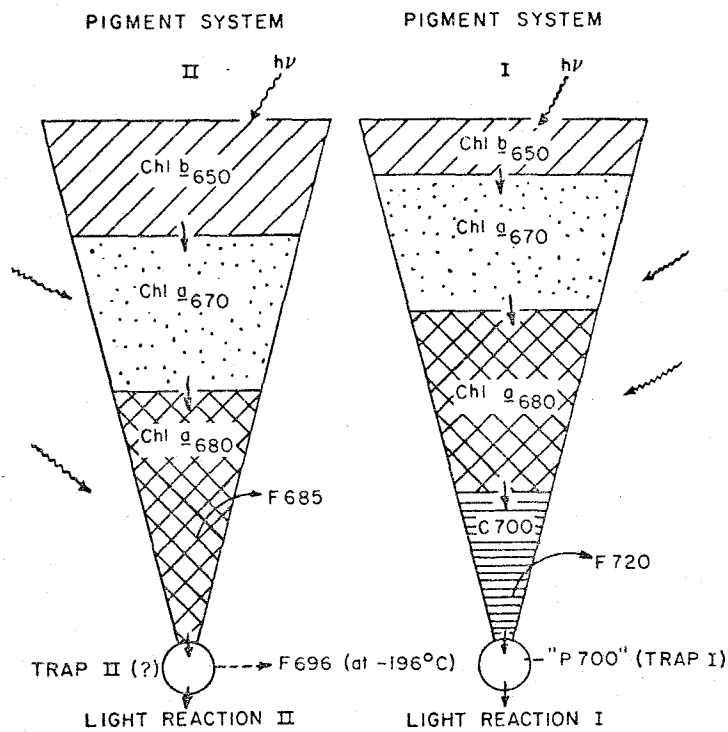



FIG. 132. A working hypothesis for the distribution of the chlorophylls in the two pigment systems (I and II) in higher plants and green algae. The two systems seem to contain both chlorophyll a (Chl a) and chlorophyll b (Chl b 650), but in different proportions. (In red and blue-green algae the phycobilins replace Chl b.) It is suggested that the long-wavelength form of chlorophyll a (C700, Chl a 695) is present only in pigment system I. Chl a 660 may be a sub-band of this aggregate form of chlorophyll a. The two "bulk" chlorophylls (Chl a 670 and Chl a 680) are almost equally distributed in the two systems, pigment system II containing more Chl a 670. (In red and blue-green algae a larger proportion of Chl a is in pigment system I.) The energy trap (trap I) of system I is P700; trap II has not been definitely identified, but it may be identified with the recently discovered P680-P690. It has also been suggested that the new emission band that appears at 696 nm (F696) when plants are cooled to 77°K originates in trap II. At room temperature most (80%) of the main fluorescence band at 685 to 687 nm (and its 740-nm satellite) originates from system II, whereas a band at 700 nm originates mainly in system I; however, a band at 700 nm also appears in system II when photosynthesis is saturated or abolished.

TABLE 87

In vivo Chlorophyll a Absorption Bands

| Band maximum (nm) | Method of observation | Refs. |
|---|---|---|
| 668-672 and 678-683 | Derivative absorption spectrophotometry | 28-31 |
| | Direct analysis of absorption spectra | 27, 28, 33 |
| | Action spectra of Emerson enhancement | 34, 35 |
| | Fractionation and separation of pigment complexes | 37-39, 59, 74 |
| | Excitation spectra of chlorophyll a fluorescence at low temperatures | 66-70, 72 |
| 694-705[a] (+ Chl a 660?) | Derivative absorption spectrophotometry | 28-31 |
| | Chlorophyll a dichroism | 57 |
| | Action spectra of the quenching of fluorescence by far-red light | 102 |
| | Action spectra of chlorophyll a fluorescence at 77°K | 22, 55, 66 |
| | Action spectra of polarized fluorescence | 58 |
| P700 | Difference absorption spectrophotometry | 9 |

[a]This band is composed of more than one species.

In higher plants and algae fluorescence measurements (see Table 88) at room temperature have revealed the presence of at least two emission bands at 687 nm (with a satellite band at 740 nm) and at about 700 nm. The former may be mainly present in pigment system II, and the latter in system I. However, a band at 695 to 700 nm also appears in system II when photosynthesis is saturated or abolished. At low temperatures (4 to 140°K), however, three bands are clearly observed in all the organisms so far studied; these bands are at 687, 696, and 720 nm. The exact locations of these bands differ with different organisms. These 687- and 696-nm bands originate mainly in the pigment system II. Portions of the 687- and 696-nm bands may also come from system I. The 720-nm band, however, originates mainly in system I.

TABLE 88

In vivo Chlorophyll a Fluorescence Bands

| Band maximum (nm) | Method of observation | Refs. |
|---|---|---|
| 683-687 | Emission spectra at room temperature | Well known |
| | Emission spectra at low temperature | 5, 54, 55, 64-72 |
| | Induced variations in the quantum yield of fluorescence | 56, 65, 77, 95, 97, 98, 104 |
| 693-696[a] | Emission spectra at low temperatures | 5, 54, 55, 64-72 |
| | Induced variations in the quantum yield of fluorescence | 56, 65, 77, 110, 119 |
| 716-720[a] | Emission spectra at room temperature (by matrix analysis) | 14, 54, 55 |
| | Emission spectra at low temperatures | 5, 54, 55, 64-72 |
| | Emission spectra of polarized fluorescence | 57, 58[b] |

[a]These bands are composed of more than one species.

[b]There is some doubt as regards this assignment.

## REFERENCES


1. L. P. Vernon and M. Avron, Ann. Rev. Biochem., 34, 269 (1965); G. Hind and J. M. Olson, Ann. Rev. Plant Physiol., 19, 249 (1968).
2. G. W. Robinson, Ann. Rev. Phys. Chem., 15, 311 (1964).
3. R. Emerson, R. Chalmers, and C. Cederstrand, Proc. Natl. Acad. Sci. U.S., 43, 133 (1957).
4. R. Emerson and E. Rabinowitch, Plant Physiol., 35, 477 (1960).
5. Govindjee, Natl. Acad. Sci. -Natl. Res. Council, 1145, 318 (1963).
6. E. Rabinowitch and Govindjee, Scientific American, 213, 74 (1965); Photosynthesis, Wiley, New York, 1969.
7. L. N. M. Duysens and J. Amesz, Biochim. Biophys. Acta, 64, 243 (1962).

8. R. Emerson and W. Arnold, J. Gen. Physiol., 15, 391 (1932).

9. B. Kok, Biochim. Biophys. Acta, 21, 399 (1956).

10. G. Doring, H. H. Stiehl, and H. T. Witt, Z. Naturforschg., 22b, 639 (1967).

11. G. Doring, J. L. Bailey, W. Kreutz, and H. T. Witt, Naturwissenschaften, 55, 220 (1968).

12. Govindjee, G. Doring, and R. Govindjee, Biochim. Biophys. Acta, 205, 303 (1970).

13. J. Myers, Natl. Acad. Sci.-Natl. Res. Council, 1145, 301 (1963).

14. P. Williams, N. R. Murty, and E. Rabinowitch, Photochem. Photobiol., 9, 455 (1969).

15. R. Govindjee, E. Rabinowitch, and Govindjee, Biochim. Biophys. Acta, 162, 539 (1968).

16. M. Bazzaz, Ph. D. thesis, University of Illinois, Urbana, 1972.

17. R. K. Clayton, in The Chlorophylls, Physical, Chemical, and Biological Properties (L. P. Vernon and G. R. Seely, eds.), Academic Press, New York, 1966, p. 610.

18. T. Mar, Govindjee, G. S. Singhal, and H. Merkelo, Biophys. J., in press (1972).

19. J. Franck, in Photosynthesis in Plants (J. Franck and W. E. Loomis, eds.), Iowa State College Press, Ames, Iowa, 1949, p. 293.

20. E. I. Rabinowitch, Photosynthesis and Related Processes, Vols. I and II (parts 1 and 2), Interscience, New York, 1945, 1951, 1956, Chapters 23, 24, and 37c.

21. G. Weber, in Comparative Biochemistry of Photoreactive Systems (M. B. Allen, ed.), Academic Press, New York, 1966, p. 49.

22. W. L. Butler, in Current Topics in Bioenergetics (D. R. Sanadi, ed.), Vol. I, Academic Press, New York, 1966, p. 49.

23. G. W. Robinson, Brookhaven Symp. Biol., 19, 16 (1966).

24. Govindjee and G. Papageorgiou, in Photophysiology (A. C. Giese, ed.), Vol. 6, Academic Press, New York, 1971, p. 1.

25. P. Latimer, Ph. D. thesis, University of Illinois, Urbana, 1956.

26. W. L. Butler, Optical Soc. Amer., 52, 292 (1962).

27. M. Das, E. Rabinowitch, L. Szalay, and G. Papageorgiou, J. Phys. Chem., 71, 3543 (1967).

28. E. Rabinowitch, L. Szalay, M. Das, N. R. Murty, and Govindjee, Brookhaven Symp. Biol., 19, 1 (1966).

29. C. S. French, in Photobiology, Proceedings of the 19th Annual Biology Colloquium, Oregon State College, 1958, p. 52.

30. J. Brown and C. S. French, Plant Physiol., 34, 305 (1959).

31. J. Brown, in Methods in Enzymology, Vol. 23, Part A (A. San Pietro, ed.), Academic Press, New York, 1971, p. 477.

32. C. S. French, J. S. Brown, and M. C. Lawrence, Carnegie Inst. Wash. Year Book, 70, 487 (1971).

33. C. N. Cederstrand, E. Rabinowitch, and Govindjee, Biochim. Biophys. Acta, 126, 1 (1966).

34. Govindjee and E. Rabinowitch, Science, 132, 355 (1960); Biophys. J., 1, 73 (1960).

35. C. S. French, J. Myers, and G. C. McCloud, in Comparative Biochemistry of Photoreactive Systems (M. B. Allen, ed.), Academic Press, New York, 1960, p. 361.

36. L. M. Vorobyova and A. A. Krasnovsky, Biokhimiya, 21, 126 (1956).

37. M. B. Allen, J. C. Murchio, S. W. Jeffrey, and S. A. Bendix, in Studies on Microalgae and Photosynthetic Bacteria, Japanese Society of Plant Physiologists, University of Tokyo Press, Tokyo, 1963, p. 407.

38. J. Brown, C. Bril, and W. Urbach, Plant Physiol., 40, 1086 (1965).

39. C. N. Cederstrand, E. Rabinowitch, and Govindjee, Biochim. Biophys. Acta, 120, 247 (1966).

40. L. N. M. Duysens, Ph. D. thesis, University of Utrecht, The Netherlands, 1952.

41. C. S. French and V. M. K. Young, J. Gen. Physiol., 35, 873 (1952).

42. S. S. Brody and E. Rabinowitch, in Proceedings of the First National Biophysics Conference, Yale University Press, 1959, p. 110.

43. P. Latimer, T. T. Bannister, and E. Rabinowitch, in Research in Photosynthesis (H. Gaffron et al., eds.), Interscience, New York, 1957, p. 107.

44. L. Szalay, M. Török, and Govindjee, Acta Biochim. Biophys. Acad. Sci. Hung., 2, 425 (1967).

45. W. J. Nicholson and J. Fortoul, Biochim. Biophys. Acta, 143, 577 (1967).

46. A. Muller, R. Lumry, and M. S. Walker, Photochem. Photobiol., 9, 113 (1969).

47. H. Merkelo, S. Hartman, T. May, G. S. Singhal, and Govindjee, Science, 164, 301 (1969).

48. F. W. J. Teale, Report to U. S. Army Research and Development in Europe (DA-91-508-EUC-281), 1959.

49. M. Das and Govindjee, Biochim. Biophys. Acta, 143, 570 (1967).

50. M. Das, E. Rabinowitch, and L. Szalay, Biophys. J., 8, 1131 (1968).

51. L. Szalay, E. Rabinowitch, N. R. Murty, and Govindjee, Biophys. J., 7, 137 (1967).

52. G. Papageorgiou and Govindjee, Biochim. Biophys. Acta, 131, 173 (1967).

53. G. Weber, Nature, 190, 27 (1961).

54. S. S. Brody and M. Brody, Natl. Acad. Sci. -Natl. Res. Council, 1145, 455 (1963).

55. Govindjee and L. Yang, J. Gen. Physiol., 49, 763 (1966).

56. Govindjee and J. -M. Briantais, Fed. Europ. Biochem. Soc. Letters, 19, 287 (1972).

57. R. A. Olson, W. H. Jennings, and W. L. Butler, Biochim. Biophys. Acta, 88, 318, 331 (1964).

58. J. Lavorel, Biochim. Biophys. Acta, 88, 20 (1964).

59. N. K. Boardman and J. Anderson, Nature, 203, 166 (1964).

60. J. M. Anderson, D. C. Fork, and J. Amesz, Biochem. Biophys. Res. Commun., 23, 874 (1966).

61. C. N. Cederstrand and Govindjee, Biochim. Biophys. Acta, 120, 177 (1966).

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

63. W. Vredenberg, Ph. D. thesis, University of Leiden, 1965.

64. S. S. Brody, Science, 128, 838 (1958).

65. A. Krey and Govindjee, Biochim. Biophys. Acta, 120, 1 (1966).

66. F. Cho and Govindjee, paper presented at the annual meeting of the Biophysical Society held at Houston, Texas, February 1967; Biophys. J., 9, A119 (1969); Biochim. Biophys. Acta, 216, 139 (1970).

67. F. F. Litvin, A. A. Krasnovsky, and G. T. Rikhireva, Dokl. Akad. Nauk SSSR, 135, 1528 (1960).

68. J. Bergeron, Natl. Acad. Sci. -Natl. Res. Council, 1145, 527 (1963).

69. B. Kok, Natl. Acad. Sci. -Natl. Res. Council, 1145, 45 (1963).

70. J. H. C. Goedheer, Biochim. Biophys. Acta, 88, 304 (1964); ibid., 102, 73 (1965).

71. F. Cho, J. Spencer, and Govindjee, Biochim. Biophys. Acta, 126, 174 (1966).

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

73. F. Cho and Govindjee, Biochim. Biophys. Acta, 205, 371 (1970).

74. N. K. Boardman, Ann. Rev. Plant Physiol., 21, 115 (1970).

75. C. Shimony, J. Spencer, and Govindjee, Photosynthetica, 1, 113 (1967).

76. J. B. Thomas and U. P. Van der Wal, Biochim. Biophys. Acta, 79, 490 (1964).

77. G. Papageorgiou and Govindjee, Biophys. J., 7, 375 (1967).

78. P. Mohanty, R. Zilinskas-Braun, Govindjee, and J. P. Thornber, Plant Cell Physiol., 132 (in press, 1972).

79. G. Tomita and E. Rabinowitch, Biophys. J., 2, 483 (1962).

80. O. D. Dmietrievsky, V. L. Ermolaev, and A. N. Terenin, Dokl. Akad. Nauk SSSR, 114, 751 (1957).

81. W. L. Butler and K. Norris, Biochim. Biophys. Acta, 66, 72 (1963).

82. A. Müller and R. Lumry, Proc. Natl. Acad. Sci. U.S., 54, 1479 (1965).

83. N. R. Murty and E. Rabinowitch, Biophys. J., 5, 655 (1965).

84. R. M. Pearlstein, Proc. Natl. Acad. Sci. U.S., 52, 824 (1964).

85. G. S. Singhal and E. Rabinowitch, Biophys. J., 9, 586 (1969).

86. J. Franck and J. Rosenberg, J. Theoret. Biol., 7, 276 (1964).

87. W. T. Stacy, T. Mar, C. E. Swenberg, and Govindjee, Photochem. Photobiol., 14, 197 (1971).

88. J. Lavorel, Progr. Photosynth. Res., 2, 883 (1969).

89. J. E. Brugger, in Research in Photosynthesis (H. Gaffron et al. eds.), Interscience, New York, 1957, p. 113.

90. R. Emerson, Ann. Rev. Plant Physiol., 9, 1 (1958).

91. J. C. Munday and Govindjee, Biophys. J., 9, 1 (1969).

92. J. C. Munday and Govindjee, Biophys. J., 9, 22 (1969).

93. G. Papageorgiou and Govindjee, Biophys. J., 8, 1299 (1968).

94. G. Papageorgiou and Govindjee, Biophys. J., 8, 1316 (1968).

95. P. Mohanty, G. Papageorgiou, and Govindjee, Photochem. Photobiol., 14, 667 (1971).

96. H. Kautsky, W. Appel, and H. Amann, Biochem. Z., 332, 277 (1960).

97. J. Lavorel, in La Photosynthese, Coll. Intern. C.N.R.S., 119, 159 (1963); Biochim. Biophys. Acta, 60, 510 (1962).

98. J. L. Rosenberg, T. Bigat, and S. Dejaegere, Biochim. Biophys. Acta, 79, 9 (1964); J. L. Rosenberg and T. Bigat, Natl. Acad. Sci. -Natl. Res. Council, 1145, 122 (1963).

99. J. C. Munday, Ph. D. thesis, University of Illinois, Urbana, 1968.

100. Govindjee, S. Ichimura, C. Cederstrand, and E. Rabinowitch. Arch. Biochem. Biophys., 89, 322 (1960).

101. W. L. Butler, Plant Physiol. Suppl., 36, IV (1961).

102. W. L. Butler, Biochim. Biophys. Acta, 64, 309 (1962).

103. W. L. Butler, Biochim. Biophys. Acta, 66, 275 (1963).

104. L. N. M. Duysens and H. E. Sweers, in Studies on Microalgae and Photosynthetic Bacteria, Japanese Society of Plant Physiologists, University of Tokyo Press, Tokyo, 1963, p. 353.

105. B. Kok and O. Owens, cited by B. Kok and G. M. Cheniae, in Current Topics in Bioenergetics (D. R. Sanadi, ed.), Vol. 1, Academic Press, New York, 1966, p. 1.

106. W. Butler, W. A. Cramer, and T. Yamoshita, Biophys. J., 9, A-28 (1969).

107. D. Knaff and D. Arnon, Proc. Natl. Acad. Sci. U. S., 63, 963 (1969).

108. K. Eriyon and W. Butler, Biochim. Biophys. Acta, in press, 1971.

109. W. L. Butler and N. I. Bishop, Natl. Acad. Sci. -Natl. Res. Council, 1145, 91 (1963).

110. A. Krey and Govindjee, Proc. Natl. Acad. Sci. U. S., 52, 1568 (1964).

111. R. Delosme, P. Joliot, and J. Lavorel, Compt. Rend., 249, 1409 (1959).

112. S. Malkin and B. Kok, Biochim. Biophys. Acta, 126, 413 (1966).

113. R. Govindjee, Govindjee, J. Lavorel, and J. -M. Briantais, Biochim. Biophys. Acta, 205, 361 (1970).

114. P. Joliot, Biochim. Biophys. Acta, 102, 116 (1965).

115. E. D. McAllister and J. Myers, Smith Inst. Misc. Coll., 99, 1 (1940).

116. G. Forbush, B. Kok, and M. P. McGloin, Photochem. Photobiol., 14, 307 (1971).

117. P. Joliot, A. Joliot, A. Bouges, and G. Barbieri, Photochem. Photobiol., 14, 287 (1971).

118. T. Mar and Govindjee, J. Theoret. Biol., in press, 1972.

119. Govindjee, J. C. Munday, and G. Papageorgiou, Brookhaven Symp. Biol., 19, 434 (1966).