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Chapter 1

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CHLOROPHYLL FLUORESCENCE AND PHOTOSYNTHESIS: FLUORESCENCE TRANSIENTS

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Abbreviations

A	Intersystem intermediate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CCCP	Ketomalononitrile 3-chlorophenylhydrazone (carbonyl cyanide 3-chlorophenylhydrazone)

Chl	Chlorophyll
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethyl urea
DCPIP	2,6-Dichlorophenol-indophenol
FCCP	Ketomalononitrile 4-trifluoromethoxyphenylhydrazone (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
PMS	Phenazine methosulfate
PQ	Plastoquinone
PSU	Photosynthetic unit
Q	Quencher of fluorescence, also primary electron acceptor for system II
P680	Energy trap for system II having one of its absorption bands at about 680 nm
P700	Energy trap for system I
X	Primary electron acceptor for system I
Z	Primary electron donor for system II

1. Introduction

1.1 General

When a chlorophyll (Chl) molecule, excited by light, returns from its first singlet excited state to its ground state, there is light emission within a few nanoseconds. This emission is red in color, and is the fluorescence of Chl. Of the plant pigments *in vivo*, Chl a and the algal biliproteins (phycobilins) fluoresce weakly; other pigments of photosynthetic importance do not have any measurable fluorescence.* These, including Chl a and phycobilins dissipate their electronic excitation energy also by other processes, of which the most important is the transfer of energy to other Chl a molecules—ultimately to certain special Chl a molecules (the reaction centers) where the energy is used for photosynthesis. The fluorescence characteristics of a molecule, e.g., the lifetime of the excited state, the quantum yield, the degree of polarization of fluorescence, the excitation and the emission spectra, are governed both by its chemical nature and by interaction with its environment. Thus fluorescence of Chl a *in vivo* has been used as a powerful tool in the analysis of photosynthesis, particularly of the process of excitation energy transfer (Weber, 1960; Duysens, 1964; Butler, 1966a,b; Goedheer, 1966; Govindjee *et al.*, 1967; Hoch and Knox, 1968; Fork and Amesz, 1969).

Both Chl a fluorescence and photosynthesis draw on the excited Chl a population, and thus a change in the photosynthetic rate is reflected as a change in the yield of fluorescence. This chapter† will place emphasis on the dependence of the efficiency of Chl a fluorescence on processes such

* Some intermediates of photosynthesis, e.g., the reduced form of nicotinamide adenine dinucleotide phosphate, NADPH, do fluoresce, but this is not under discussion here.

† No attempt has been made to cite and discuss all the papers in the field. Moreover, the emphasis is on work with intact cells.

as the electron and ion transport, which are components of what is collectively referred to as photosynthesis. The reader's familiarity with the general outline of the present theories of photosynthesis is assumed. When necessary, reference can be made to particular topics in several recent reviews as those by Hind and Olson (1968) (electron transport), Avron and Neumann (1968) (phosphorylation), Rabinowitch and Govindjee (1969) (general mechanisms), Boardman (1970) (two pigment systems), Cheniae (1970) (O_2 evolution), Fork and Amesz (1970) (intermediates of photosynthesis), and Packer *et al.* (1970) (ion movements and structural changes in chloroplasts).

The present chapter will deal specifically with the change in the fluorescence yield of Chl a with the time of illumination (i.e., the fluorescence induction, the fluorescence transient, or the Kautsky effect) and its relation to the photosynthetic reactions. [For a review of the older work on fluorescence transients, see Wassink (1951) and Rabinowitch (1956).] If one takes a dark-adapted suspension of algae, or a leaf, and shines bright light on it, Chl a fluorescence yield changes in a characteristic way (see Section 1.4). These changes have been arbitrarily divided into two broad categories—*fast changes* that are over within a second or two (at moderate-to-high intensities), and *slow changes* that may last for several minutes. The fast fluorescence changes reflect the momentary oxidation–reduction state of the photochemical reaction center, the rates of reactions associated with it, and with the intermediates of the electron transport chain. The slow changes, on the other hand, reflect the physical state of the pigment systems and the associated ionic changes and the rate of photophosphorylation. We believe that the slow changes are also affected by the oxidation–reduction reactions and the fast changes by the physical state of the pigment systems, but the extent of these effects is not yet clear. It is because of these relationships that the study of fluorescence transients has the potential of providing information regarding the mechanism of photosynthesis.

We must recognize some basic properties of photosynthesis before we can embark on a discussion of the above relationships. The two major concepts to be considered are (1) the existence of *photosynthetic units*, i.e., groups of several hundred Chl molecules that somehow cooperate to evolve O_2, and (2) the existence of *two pigment systems* and the operation of *two light reactions* (I and II) in photosynthesis.

1.2 Photosynthetic Unit; Function and Interaction

The ratio of Chl to the assimilated carbon dioxide (or liberated oxygen), under experimental conditions ensuring the optimal utilization of light for photosynthesis, was found by Emerson and Arnold (1932a,b) to be

about 2500:1. This off-balance stoichiometry was taken as evidence for the existence of photosynthetic units (PSU), i.e., groups of pigment molecules acting in concert to collect and utilize photons. A few years later Gaffron and Wohl (1936) arrived at the same conclusion on the basis of results reported by Warburg and Negelein (1925). In Warburg's experiment, each Chl molecule absorbed one photon every 12 minutes. Assuming that each photon will eventually cause a one-electron reduction, it would take a Chl a molecule about 50 minutes to collect four quanta to reduce one carbon dioxide molecule. (To collect eight quanta, it would take 100 minutes.) This unrealistic state of affairs (when O_2 can, in fact, be evolved in less than a second after illumination) prompted Gaffron and Wohl to propose a speeding up of photosynthesis by means of groups of cooperating Chl molecules.

The previous discussion suggests that the size of the PSU per molecule of oxygen evolved is 2500 Chl; its size per electron transferred is $2500/4 \simeq 600$ Chl as four electrons must be transferred from H_2O to CO_2 to evolve one O_2. Since each electron transfer requires two separate light reactions (see Section 1.3), the size of PSU per primary light reaction is $600/2 = 300$ Chl molecules. The above conclusion is based on the assumption that a minimum of eight quanta are required for the evolution of one O_2 molecule (see Emerson and Chalmers, 1955; R. Govindjee *et al.*, 1968). Thus 300 Chl molecules make up one PSU, although smaller units have been identified in higher-plant mutants (Schmidt and Gaffron, 1968, 1969; Wild, 1968, 1969). The discovery that photosynthesis proceeds by means of two sequential photoreactions (Section 1.3) necessitates the grouping of the units, in accordance with the photoreactions they perform, as PSU I and PSU II. The pigment systems contained in them are referred to as pigment systems I and II.

A photosynthetic unit has a light-gathering (or "antenna") part (to be referred also as the bulk pigments) to which all pigment molecules except one belong, and a reaction center which contains the remaining Chl a molecule. The latter is "distinguishable" because of its proximity with the oxidoreduction couple and possibly with an enzyme. The bulk pigments consist of Chl a and Chl b (higher plants and chlorophytes), Chl c (bacillariophytes and phaeophytes), phycobilins (cyanophytes and rhodophytes), and carotenoids (i.e., xanthophylls and carotenes, all plants). These pigments enrich the plants with wider absorption bands, and therefore with a more efficient photon-harvesting apparatus.

Both pigment systems I and II contain the accessory pigments as well as Chl a, but they differ in the relative abundance of these pigments in them (Fig. 1). Pigment system I contains a larger proportion of the long waveforms of Chl a (Chl a 678, Chl a 685–705, the numbers refer to

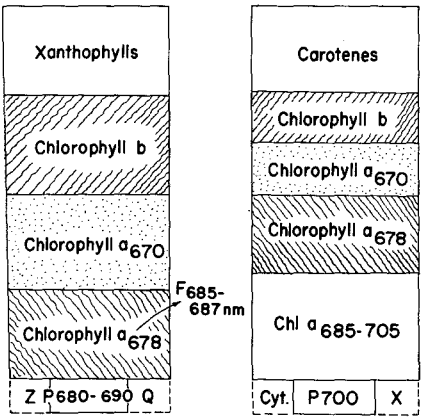


FIG. 1. Composition of the two pigment systems in photosynthesis.

their red absorption maxima), a smaller proportion of the short waveform of Chl a (Chl a 670) and of Chl b (or phycobilins, etc.), most of the carotenes, and all of the reaction center Chl a molecules (P700) having one of their absorption bands at 700 nm (for P700, see Kok, 1956, 1957a,b, 1959, 1961; Kok and Gott, 1960). This system does not have a fluorescence transient, and is weakly fluorescent at room temperature (Briantais, 1966; Brown, 1969). The pigment system II contains a larger proportion of Chl a 670, of Chl b (or phycobilins), most of the xanthophylls, a smaller proportion of the long waveforms of Chl a, and all of the reaction center Chl a molecules P680–690 (for P680–690, see Döring *et al.*, 1967, 1968, 1969). This system, relative to system I, is strongly fluorescent and shows the fluorescence transient (Briantais, 1966). The main fluorescence peak of plants at 685–687 nm originates in Chl a 678 of this system (Cho and Govindjee, 1970b). Most of the data on the time course of fluorescence yield reflect the changes in the system II.

The arrangement of the two pigment systems *in vivo* is open to speculation. There is evidence that they are present in separate membranes that are opposed to each other—system I on the outer side and system II on the inner side (Arntzen *et al.*, 1969; Briantais, 1969). There are two basic models for the arrangement of pigments in these membranes (see Robinson, 1966). There is the "lake" (the statistical or the multicentral) model in which reaction centers are embedded in a lake of pigments. A quantum, not trapped by one reaction center, has a probability of migrating to another reaction center. There is also the "isolated puddles" model in which each unit has it own reaction center. If the reaction center

is closed, the quantum is lost as fluorescence. In this model one lifetime of fluorescence is associated with the puddles of pigments that have open traps and another with those that have closed traps. The lake model for system II predicts a linear dependence of lifetime on the quantum yield of fluorescence, since most of the fluorescence comes from system II. Such a linear relationship is indeed found (Tumerman and Sorokin, 1967; Briantais *et al.*, 1970). Thus it seems that the lake model may be the favored one for system II. However an intermediate situation cannot be excluded.

When the excitation encounters a closed reaction center, it may be transferred to another unit. Evidence for the interunit energy transfer in system II was obtained by Joliot and Joliot (1964). These authors argued that if there was no such transfer, and each unit worked independently (as in the isolated puddles model), the rate of system II reaction (O_2 evolution) would be linearly proportional to the concentration of the open traps. However, if there was interunit transfer, the system II reaction rate would be higher than expected at times when all the traps were not open. This happens because the excitation quanta, not used by the unit they were absorbed in, enhance the probability for photochemistry by a factor of $1/1 - e'p$, where e' is the fraction of the open centers, and p is the probability of the interunit transfer. In such a situation, a nonlinear relationship for system II reaction versus concentration of open traps would be expected. Indeed such a relationship was observed by Joliot and Joliot (1964) who calculated the p to range from 0.45 to 0.55 (see also Delosme, 1967).

For photosystem I, there is evidence for both the "isolated puddles" and "lake" models. Probabilities of interunit transfer of the excitation energy in excess of 0.5 were reported for several algae by Fork and Amesz (1967). In isolated spinach chloroplasts, however, the rate of photoreaction I was found to be a linear function of the fraction of the open reaction center I (P. Joliot *et al.*, 1968) indicating an absence of interunit transfer in this system. It appears that there are variations from plant to plant, and even in the same plant under different conditions.

Let us now look at the intersystem excitation energy transfer. If the energy absorbed—but not utilized—by system II can be transferred to system I, it is called "spillover" (Myers, 1963). If the two systems are physically separated and the energy transfer from system II to system I is absent, we have the so-called "separate package" situation. In the nineteen sixties, it was a question of "spillover" versus "separate package." P. Joliot *et al.* (1968) found identical action spectra of photoreaction I whether the reaction center II was kept closed or open. If the spillover of energy occurred from system II to system I, one would expect

o see the additional participation of system II pigments in photoreaction I if the reaction center II were kept closed. This would happen because photons absorbed—but not used by photosystem II—would be turned over to and used by photosystem I. However if no spillover of energy occurs, the action spectra of photoreaction I would be unaffected whether the reaction center II were closed or open. Thus the experiments of Joliot *et al.* suggested that "separate package" hypothesis is the correct one. Contrary to this conclusion, Murata (1969b) and Murata *et al.* (1970) have presented evidence in support of the excitation energy spillover (see also Malkin, 1967; Avron and Ben-Hayyim, 1969). Obviously the photosynthetic system is a dynamic one. We expect to find or not find interunit or intersystem transfer depending upon the state of the pigment systems and the photosynthetic units. Duysens (1970) has recently suggested that a movement of membranes, containing the separate pigment systems I and II, away from or toward each other, could easily control the spillover of energy from system II to system I. Similarly the movement of photosynthetic units of the same type with respect to one another could control the interunit energy transfer. If the units are far apart, the "isolated puddles" situation exists; if they are close together, it is equivalent to a "lake." In this picture, one can imagine many intermediate states. We hope that future efforts will be made to define the conditions for each state.

1.3 Two Light Reactions of Photosynthesis

An acceptable model of photosynthesis must make provision for the occurrence of two distinct pigment systems and photoreactions, the presence of a chain of electron transport intermediates (redox couples), and the presence of a phosphorylating mechanism converting adenosine diphosphate (ADP) to adenosine triphosphate (ATP). In addition, it must provide for the possibility of artificial electron transport in which only parts of the photosynthetic electron transport chain are used, and for the kinetics of the Chl a fluorescence yield. Indeed, since fluorescence is a measure of the photochemistry at the reaction centers of photosystem II, fluorescence kinetics may supply a criterion to test the merits of any model of photosynthesis. All models proposed, save one, invoke two (or more!) reaction centers communicating with each other by means of electron transport or a high-energy intermediate, e.g., ATP). The one exceptional model with a single reaction center capable of two distinct photoprocesses (J. Franck and Rosenberg, 1964) is now of historical importance only. It does not provide for the chain of electron transport carriers (experimentally found to exist) and of the two reaction centers P680–690 and P700, now known to exist.

The series model of Hill and Bendall (1960) is the most widely accepted model [cf. with scheme 7.V. of Rabinowitch (1945) and the scheme discussed by Emerson and Rabinowitch (1960)]. However several other models have been proposed in the last ten years. More recently, Govindjee *et al.* (1966), Arnold and Azzi (1968), and Knaff and Arnon (1969b) have proposed alternate models; these will not be discussed here but the references are given so that the reader can consult them to keep an open mind toward future development in this field. Experimental support for the series model was first provided by Duysens *et al.* (1961), Kok and Hoch (1961), and by Witt *et al.* (1961). This model has since been elaborated to show a detailed electron transport path leading from water to nicotinamide adenine dinucleotide phosphate ($NADP^+$; see Hind and Olson, 1968). Given below is a simple version of the series model that does not include the names of most of the intermediates.

The pigment system I sensitizes light reaction I oxidizing P700 and reducing a low potential energy acceptor X that leads to the production of the strong reductant (X^-) and a weak oxidant (A) (Fig. 2):

$$A^-P700X \xrightarrow{h\nu_I} A^-P700^*X \dashrightarrow A^-P700^+X^- \dashrightarrow AP700X^-$$

the label A is applied to a pool of intermediates that includes two cytochromes, a plastoquinone, and plastocyanine. The strong reductant (X^-) ultimately reduces $NADP^+$ to NADPH. The pigment system II sensitizes

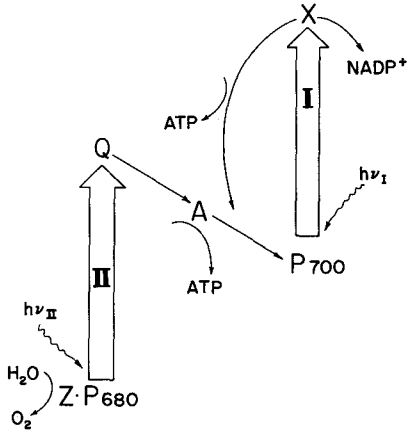


FIG. 2. Two light reactions in photosynthesis (simplified Hill and Bendall Scheme). Z, primary electron donor of light reaction II; P680, energy trap of pigment system II; Q, primary electron acceptor of system II—also a quencher of Chl a fluorescence; A, pool of intersystem intermediate; P700, energy trap of pigment system I; X, primary electron acceptor of system I; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate.

ight reaction II that leads to the production of an oxidant (Z^+) and a weak reductant (Q^-):

$$ZP680Q \xrightarrow{h\nu II} ZP680^*Q \dashrightarrow ZP680^+Q^- \dashrightarrow Z^+P680Q^-$$

(only by analogy to light reaction I). A strong oxidant (Z^{4+}) is formed only after four primary reactions (II), and then it reacts with water to evolve O_2 : $Z^{4+} + 2H_2O \dashrightarrow Z + O_2 + 4H^+$; the weak reductant ($Q^-$) formed in each primary reaction II reacts with a member of the pool (A) to restore Q and A^-: $Q^- + A \dashrightarrow A^- + Q$. During the latter exergonic reactions, molecules of ADP and P_i (inorganic phosphate) are esterified to ATP (*noncyclic phosphorylation*).

The reduced form of pyridine nucleotide NADPH (formed from X^-) then enters the carbon fixation cycle, or it returns its electrons, by one or several pathways, to an intermediate pool carrier. In the latter instance(s), electron transport traces a closed circuit passing through P700. This and the associated phosphorylation are referred to as the cyclic electron transport and the *cyclic phosphorylation.*

The series model offers unbiased explanations for the synergistic effect of the two photoreactions on the oxidoreduction states of electron carriers that are on the oxidizing (Z) or the reducing (X) end of the scheme, and their antagonistic effect on the intersystem pool carriers. It thus accounts for the enhancement of oxygen evolution (Emerson *et al.*, 1957; Emerson and Rabinowitch, 1960; Govindjee and Rabinowitch, 1960; Myers and French, 1960; Fork, 1963; Govindjee, 1963), and the reduction of $NADP^+$ (R. Govindjee *et al.*, 1962, 1964; P. Joliot *et al.*, 968; Avron and Ben-Hayyim, 1969; Sun and Sauer, 1970). It also accounts for the reduction of cytochromes (components of pool A) by photosystem II and their oxidation by photosystem I (Duysens and Amesz, 1962; Cramer and Butler, 1967); for reference to antagonistic effect on other intermediates, see the review of Vernon and Avron (1965).

The above picture of the two light reactions and the two pigment systems is consistent with fluorescence data. Kautsky *et al.* (1960) suggested two light reactions (but not two pigment systems) to explain their data on the time course of fluorescence. It is known that light absorbed by photosystem II causes the fluorescence yield of system II Chl a to increase, whereas light absorbed by photosystem I has the opposite (quenching) effect (Govindjee *et al.*, 1960; Butler, 1962; Duysens and Sweers, 1963; Munday and Govindjee, 1969b; Mohanty *et al.*, 1970). According to Duysens and Sweers (1963) light reaction II reduces a fluorescence quencher (Q), while light reaction I reoxidizes it by the intermediary of the pool A. Further ramifications of this theory and the newer relationships will be discussed later (see Sections 3.6 and 4.4).

1.4 The Fluorescence Induction

Light energy absorbed by the pigment systems has three possible fates: (a) to be used in photosynthesis (k_p), (b) to be radiated as fluorescence (k_f), and (c) to be lost in other processes including energy transfer to weakly fluorescent Chl a and heat (k_h). As a first approximation, the yield of Chl a fluorescence (ϕ_f) is an inverse measure of the efficiency of the photosynthetic process (ϕ_p).

$$\phi_f = k_f/(k_f + k_h + k_p), \qquad \phi_p = k_p/(k_f + k_h + k_p)$$

This is why Chl a fluorescence has often been regarded as the inefficiency index of photosynthesis. Fluorescence simply competes with photosynthesis. This competition is seen from the increase in the fluorescence yield when (a) photosynthesis is poisoned (Kautsky and Zedlitz, 1941; Duysens and Sweers, 1963; U. Franck *et al.*, 1969), or (b) it is light-saturated (Shiau and Franck, 1947; J. Franck, 1949; Brugger, 1957; Krey and Govindjee, 1966; Bonaventura and Myers, 1969), or (c) the temperature is lowered (see U. Franck *et al.*, 1969). Similarly, it is evident from the antiparallel time course traced by the rates of O_2 evolution (or CO_2 uptake) and fluorescence during a large portion of the fast fluorescence transient (Kautsky and Hirsch, 1931; McAlister and Myers, 1940; Delosme *et al.*, 1959; P. Joliot, 1965b; Bannister and Rice, 1968). This competition between O_2 evolution and fluorescence is, however, not general. For example, parallel increase of both fluorescence and O_2 evolution are observed after prolonged illumination (Papageorgiou and Govindjee, 1968a,b; Bannister and Rice, 1968; Bonaventura and Myers, 1969). (Perhaps changes in k_h are responsible for this phenomenon.)

When dark-adapted photosynthetic organisms are subjected to continuous intense illumination, the following changes in the yield of Chl a fluorescence are observed (Fig. 3). At zero illumination time, the Chl a fluorescence yield rises instantly to the initial level O (origin), followed by an increase to an intermediate level I, a dip or a plateau D, and a high peak P. From peak P, there is a decline to a quasisteady state level S. The notation OIPS for the fluorescence transient was used by Lavorel (1959) and P. Joliot and Lavorel (1964). The label D for the first dip was used by Munday and Govindjee (1969a). Preparations of higher plant chloroplasts, in the absence of added oxidants, show the biphasic rise (OIDP) although no clear dip (D) is observed (see Forbush and Kok, 1968). The decay of P to S is extremely slow in such chloroplasts. However in the presence of oxidants or cofactors of the cyclic electron transport, Chl a fluorescence yield in chloroplasts decays

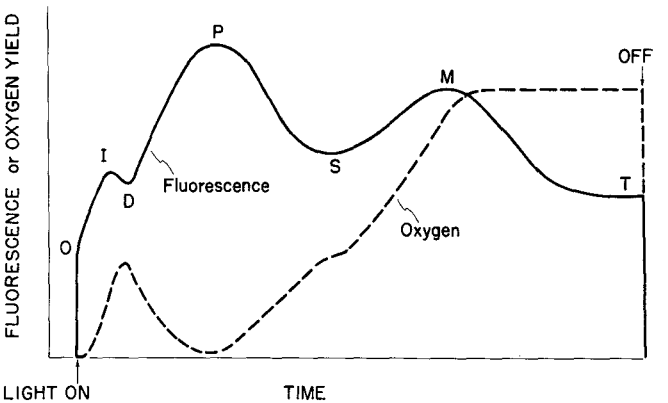


FIG. 3. Changes in the fluorescence yield of Chl a and of the yield of O_2 evolution, as a function of time of illumination (diagram, not drawn to scale).

to a low level (Malkin and Kok, 1966; Murata and Sugahara, 1969).

Both in the chloroplasts and in the algae, the intensity of fluorescence at the level O is referred to as "constant," and the difference in the fluorescence yield (ΔF) between P and O, i.e., P–O, as variable fluorescence. It is generally assumed that it is only the variable fluorescence that reflects changes in photochemistry (cf. Clayton, 1969); the yield of variable fluorescence increases with light intensity saturating at high intensity, but the yield of constant fluorescence is independent of intensity Lavorel, 1963; Munday and Govindjee, 1969a; de Klerk *et al.*, 1969). The nature of fluorescence at O is not yet clear. Its yield remains constant when photochemistry changes; perhaps, it originates from the "bulk" chlorophylls of systems I and II before the energy is trapped at the reaction centers.

In whole cells, the OIDPS transient—the fast change—is over within 2 seconds (at medium intensities), and is known as the *first wave* of fluorescence induction.

Level S is not a real steady-state level, because in whole cells, e.g., of *Chlorella*, *Chlamydomonas*, *Porphyridium*, and *Cyanidium*, it is followed by a rise to a maximum M (or plateau) from where the yield declines to a terminal steady level T. In blue-green algae, e.g., *Anacystis*, *Plectonema*, and *Phormidium*, the decline M to T is extremely slow requiring several minutes (Papageorgiou and Govindjee, 1967). The SMT transient —the slow change—is also known as the *second wave* of fluorescence induction (see Papageorgiou and Govindjee, 1969).

Leaves of higher plants show a pronounced PSMT phase at elevated

carbon dioxide tensions (McAlister and Myers, 1940; J. Franck *et al.* 1941). Under normal conditions one sees a slow decline from P to T with a shoulder for M. (For variations in fluorescence transients in different organisms, see U. Franck *et al.*, 1969.)

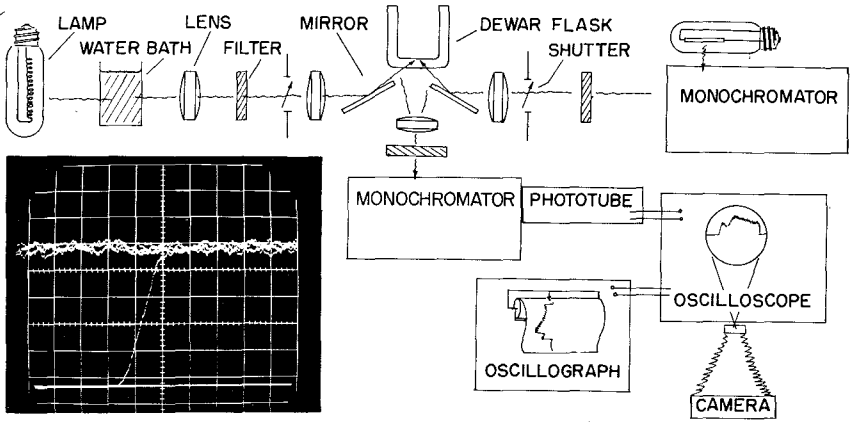
2. Techniques

The general techniques for measuring fluorescence have been discussed at length by Ellis (1966) and by Udenfriend (1962, 1969). In what follows, we will discuss briefly the instruments used for the measurement of the time course of Chl fluorescence *in vivo*.

2.1 Stationary Method

The instruments in this category utilize samples which remain stationary in the sample holder. Changes in chlorophyll fluorescence yield with time are measured directly. In the simplest instrument, the sample is illuminated with a continuous (unmodulated) bright light of fixed intensity. This type of instrument has been used in all the older work on fluorescence induction [for recent use and modifications, see Delosme (1967) and Munday and Govindjee (1969a)]. Figure 4(a) shows the details of an instrument for the direct measurement of fluorescence induction in algae and in chloroplasts. At the intensity used in some experiments ($\sim 10^4$ ergs cm^{-2} sec^{-1}), transient changes of the fluorescence yield of Chl a are observed in a few milliseconds from the instant of illumination. Accordingly, photographic compur shutters are quite adequate for the sharp transition from darkness to light. These, with the narrowin of the iris diaphragm, provide opening times of about 1–2 msec. Much shorter opening times (a few microseconds) are needed if the intensity of excitation is increased to study the purely photochemical aspects of OI transition. This is achieved by shooting a metallic shutter out of the light path (Delosme, 1967). The fluorescence signal is either displayed and photographed on the screen of an oscilloscope or it can be recorded by a fast oscillographic recorder.

In a quite different instrument, one can assay the fluorescence yield with a weak modulated beam, the fluorescence yield changes are affected by other noninterrupted, i.e., continuous, bright actinic beams that cause specific photochemical change (e.g., the reduction of photosynthetic electron transport intermediates) (Duysens and Sweers, 1963). This instrument responds only to changes in the yield by the weak modulated beam because a tunable amplifier passes only the modulated signal. With this instrument, changes in the fluorescence yield can be amplified and measured precisely.



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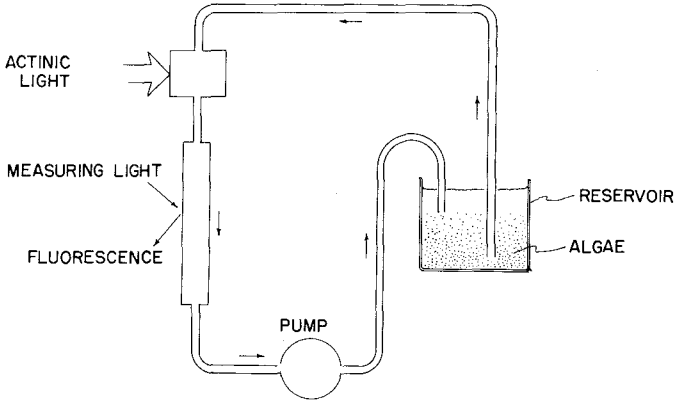

b

FIG. 4. (a) Diagram of a fluorometer for measuring fluorescence transients in algae and chloroplasts. It includes Bausch & Lomb monochromators, an EMI 9558B phototube, a Tektronix 502A oscilloscope, a Midwestern Instruments 801B oscillograph, and compur shutters from Burke and James; the vertically slanting curve in the insert shows the phototube signal during a shutter opening (horizontal scale: 1 msec/division). (After Munday and Govindjee, 1969c.) (b) Block diagram of the flow apparatus (redrawn from Lavorel, 1965).

2.2 Flow Method

Instead of having the fluorescing sample stationary, as in the methods described in the previous section, it can be made to flow at a regulated rate through a transparent capillary tube (Lavorel, 1962; 1965; Vreden-

berg and Duysens, 1965). The light exposure time of the sample, in this case, becomes a function of the flow rate and of the area illuminated. The fluorescence time course can be obtained by first flowing the sample, and then suddenly stopping the flow. Using different flow rates one can measure the yield of the different phases of the transient in a quasi-steady state.

Figure 4(b) shows a portion of the flow apparatus. The sample is drawn into the capillary tube by a syringe and a valve pump, and then returned to a reservoir. In the measuring compartment a strong measuring light assays the fluorescence yield at some point of the transient. When the flow is suddenly stopped, the changes in yield with time are recorded. Changes in fluorescence yield can also be made by an actinic illumination upstream of the flow of algae. The instrument of Vredenberg and Duysens (1965) is very similar to that of Lavorel (1965) except that their measuring beam is weak and is modulated; the changes in the yield are caused by continuous actinic beams.

To measure the emission spectra of variable and constant fluorescence, Lavorel's instrument is used as follows. The sample is subjected to repeated flow–rest cycles; the signal oscillates between a minimum (O) corresponding to the flow part of the cycle, and a maximum (P) corresponding to the rest part. These measurements are repeated at different wavelengths of observation to get fluorescence spectra. By adjusting the flow rate, fluorescence spectra can be obtained at any stage of the fluorescence induction. These spectra provide information regarding the pigment systems involved at various stages of the transient.

3. Fast Fluorescence Yield Changes and Electron Transport

3.1 Reactions at the Reaction Center II

The quantum yield of Chl a fluorescence depends on the rate of the primary photochemical reaction at the reaction center II. A knowledge of the mechanism of this reaction is, therefore, essential for the understanding of the relationship between Chl fluorescence and electron transport.

As noted in Section 1.3, the reaction center of system II is visualized as ZP680Q, where Z and Q are the unknown primary electron donor and acceptor of system II, and P680 is the energy trap of that system. Earlier suggestions of the existence of an energy trap in system II came from low-temperature fluorescence spectra of algae, chloroplasts and subchloroplast fragments (system II). An emission band in the region of 693–698 nm (F698) at 77°K, is preferentially excited by pigment system II, and originates in a Chl a species present in very small quanti-

ies; it has been postulated that this Chl a species fluorescing at 698 nm, and having an absorption band in 680–685 nm region, is the energy trap of system II (Bergeron, 1963; Brody and Brody, 1963; Govindjee, 1963, 1965; Broyde and Brody, 1966; Boardman *et al.*, 1966; Murata, 1968; Donze and Duysens, 1969; Cho and Govindjee, 1970a,b,c). However direct evidence for the existence of an active Chl a in system II (Chl a_{II} or P680–690) was first presented by Döring *et al.* (1967, 1968; 1969; also see Govindjee *et al.*, 1970; Floyd *et al.*, 1971) in spinach, Swiss chard and maize chloroplasts. Using a repetitive flash technique, Döring *et al.* discovered a light-induced absorbance change in pigment system II that decays, at room temperature, with a half-time of 0.2 msec (in contrast to 20 msec for P700). It is absent in system I particles, has peaks at 435, 640, and 682 nm, is abolished when DCMU (10^{-6} *M*) is added to the system, has the same dependence on intensity as O_2 evolution, and is present in a concentration of one per several hundred to a thousand Chl molecules. Floyd *et al.* (1971) have shown that the 680 nm absorbance change occurs also at 77°K, and at that temperature it recovers biphasically with half times of 30 μsec and 4.5 msec (in contrast to P700 that recovers monophasically with a half-time of 30 μsec at 77°K). On the basis of the above observations, this absorbance change appears to be related to the reactions of system II. Döring and coworkers have shown that any condition that leads to the destruction of P680 always stops photosynthesis, but the reverse is not true. For example, if chloroplasts are heated to 50°C for 5 minutes, or washed with high concentrations of tris, or treated (and washed) with "wet" heptane, P680 change remains but electron transport ceases. We consider it likely that P680 is indeed the energy trap of system II. However it is still not clear how this trap operates. Döring *et al.* (1969) believe that it acts as a sensitizer without directly engaging in an oxidation–reduction reaction. But why, then, should it undergo absorbance change? Floyd *et al.* (1971) have expressed the possibility that P680 is oxidized in light and, under normal photosynthesis, recovers its electron indirectly from water. This could be represented as follows.

$$ZP680Q \xrightarrow{h\nu_{II}} ZP680^{*}Q \dashrightarrow ZP680^{+}Q^{-} \dashrightarrow Z^{+}P680Q^{-}$$

We consider this mechanism likely because (1) an excited molecule has a tendency to eject an electron rather than accept one; (2) the level of Chl fluorescence is determined by the abundance of reduced Q; hence oxidized Q quenches the Chl a excitation (Duysens and Sweers, 1963); this would suggest that $ZP680^{*}Q \dashrightarrow Z^{+}P680^{-}Q$ is not possible because fluorescence rises within a few microseconds of illumination (Sybesma and Duysens, 1965; Delosme, 1967), and (3) oxidized Z can accumulate without a change in the fluorescence yield (P. Joliot, 1968).

The nature of the primary electron acceptor (Q) is not clear. There is the possibility that it is a special minor fraction of quinones. Amesz and Fork (1967) showed that 70 μM of certain oxidized quinones can quench 50% of the variable fluorescence rise. They have further demonstrated that this quenching was not due to the stimulation of the electron transport because it occurred even in the presence of the powerful inhibitor of electron transport 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). Kohl and Wood (1969) have shown that a light-induced electron-spin resonance (esr) signal of system II is absent in chloroplasts from which quinones have been extracted with heptane, and this signal is restored if certain plastoquinones are added back. Kohl and Wood suggested that this esr signal may be due to the plastochromanoxyl as well as plastosemiquinone free radicals formed from plastoquinone. If esr signal II indeed arises from the primary electron acceptor of system II, then it follows that Q may also be a type of plastoquinone (PQ). Stiehl and Witt (1969) suggested that two electron chains are arranged in parallel such that a pair of P680 are in contact with a pair of PQ–PQ. In this model, plastoquinone is reduced by P680 to PQ^-–PQ^-; this is followed by a dismutation to PQ^{2-} and PQ; and lastly two single PQ^- molecules are formed in the plastoquinone pool by a redismutation reaction. Stiehl and Witt (1969) attributed to the postulated semiquinone PQ^-–PQ^- the absorbance change they discovered at 320 nm. R. Govindjee *et al.* (1970) found that extraction of quinones with "wet" heptane leads to an increase in the constant fluorescence (level O) and a decrease in the variable fluorescence; this is explained by assuming that heptane also extracts a part of "Q" that may be a quinone-type compound. Thus there is only vague evidence that Q is a type of plastoquinone. In practice, Q is only recognized by its ability to quench fluorescence (Duysens and Sweers, 1963). When it is reduced to QH, fluorescence yield rises. Therefore one can measure the light reaction II by the initial rise in fluorescence with time (OI phase of fluorescence transient). However further reduction of Q eventually becomes limited by the size and the state of the pool A because Q^- reacts with A to restore Q and A^-.

Recent experiments by Erixon and Butler (1971) show that there is one-to-one correspondence between the redox changes of another compound C-550 (Knaff and Arnon, 1969a) and of Q. Thus, there is the likelihood that Q is identical to C-550.

The chemical nature of Z is also unknown. It seems that oxidized Z decomposes water to oxygen in a process that shows a requirement for Mn ions. One may speculate therefore that Z contains Mn (cf. Cheniae, 1970). Although we know little of its chemical composition, we do know a great deal about the reactions of Z from the recent work of P. Joliot (1968), P. Joliot *et al.* (1969), Kok *et al.* (1970), and Forbush *et al.*

(1971) on O_2 evolution in flashing light (also see Kok and Cheniae, 1966). Let us briefly review the situation.

The reduction of Q is a one-electron process (Kautsky *et al.*, 1960; Delosme, 1967), but to evolve one O_2 molecule (Z response) four electrons must be transferred from H_2O to oxidized Z. P. Joliot *et al.* (1969) illuminated dark adapted photosynthetic specimens by a sequence of strong flashes and monitored the oxygen yield per flash under conditions that each photosynthetic unit received one quantum per flash and there was enough time for Q^- to return to Q between the flashes. The O_2 signal was largest at the third flash, other maxima were at the seventh and the eleventh flashes. There was a periodicity of four, but the oscillations damped off. Forbush *et al.* (1971) recently found that the detailed pattern of this result in chloroplasts is very different if the dark adaptation period is preceded by 1, 2, or 3 light flashes. They suggest, to explain these results, that in chloroplasts long lasting species of Z exist; in darkness there is 80% of Z^+ and 20% Z. Kok's model for O_2 evolution could be formulated as follows (leaving aside the P680 and Q)

$$Z \xrightarrow{h\nu} Z^+ \xrightarrow{h\nu} Z^{2+} \xrightarrow{h\nu} Z^{3+} \xrightarrow{h\nu} Z^{4+} \dashrightarrow Z,$$

$$2H_2O \rightarrow O_2 + 4H^+$$

where solid arrows indicate reactions in single flashes and dotted arrow indicates a dark reaction. To explain the small amount of O_2 evolution in the second flash, Kok and associates assume a small probability of double hit, i.e., during one flash two quanta hit a photosynthetic unit, so that the following reaction is possible:

$$Z^+ \xrightarrow{2h\nu} Z^{3+} \xrightarrow{h\nu} Z^{4+} \dashrightarrow Z.$$

$$2H_2O \rightarrow O_2 + 4H^+$$

To explain the high yield in the third flash, the following reactions are suggested:

$$Z \xrightarrow{2h\nu} Z^{2+} \xrightarrow{h\nu} Z^{3+} \xrightarrow{h\nu} Z^{4+} \dashrightarrow Z.$$

$$2H_2O \rightarrow O_2 + 4H^+$$

$$Z^+ \xrightarrow{h\nu} Z^{2+} \xrightarrow{h\nu} Z^{3+} \xrightarrow{h\nu} Z^{4+} \dashrightarrow Z$$

$$2H_2O \rightarrow O_2 + 4H^+$$

Kok's detailed model also incorporates the idea of some "misses," i.e., the unit receiving the quantum does not do anything. With these assumptions, all the kinetics of O_2 evolution are explained. However we wish to emphasize that there are alternate ways of explaining the kinetic data on O_2 evolution; Mar (1971) has evolved two new detailed models that also qualitatively explain all the results.

It is important to note that if Z is kept oxidized Q cannot be reduced by light, and the fluorescence rise with time cannot be expected. However if Q is present in its oxidized state, it should be possible to reduce it by first reducing Z by an external electron donor. Yamashita and Butler (1969) showed that washing chloroplasts with high concentration of tris (0.8 M; pH 8) stops the electron transport from H_2O to Z, and keeps the fluorescence at a low level. However if hydroquinone (or reduced phenylenediamine) is added, a light-induced fluorescence rise occurs. Q can also be reduced directly by dithionite ($Na_2S_2O_4$).

3.2 The OI Phase

When dark adapted cells are illuminated with light of moderate-to-low intensities, the rate of O_2 evolution is initially zero, and the fluorescence yield is low (all the traps are open as all molecules of Q are in the quenching form). Then, the fluorescence yield increases from O to I, but the rate of O_2 evolution remains zero for a while, accelerating slowly to a maximum. During this phase, fluorescence yield increases simultaneously (Delosme *et al.*, 1959; P. Joliot, 1965b, 1968; Bannister and Rice, 1968). At these intensities of illumination, the OI phase measured by several investigators (cf. Munday and Govindjee, 1969a) does not reflect a purely photochemical reaction as it includes the dark reaction of Q^- and A.

The lag in O_2 evolution—mentioned above—proportionally decreases as intensity of light is increased suggesting the reactions' photochemical nature. This lag, most clearly observed in very weak continuous light (P. Joliot, 1968), exists because two or four oxidizing equivalents must accumulate on Z in one photosynthetic unit before a molecule of O_2 will evolve (see Section 3.1). At time zero, the observed fluorescence is due to the slight inefficiency of the trapping process. During the lag period in O_2 evolution, fluorescence yield rises because of the reduction of Q to Q^-. With the intensity of light used, we deal with two processes—one that closes the traps because of the conversion of Q to Q^-, and the other that reopens the trap in a dark reaction with A: $Q^- + A \dashrightarrow Q + A^-$. We imagine that the rate of this reaction is slow, i.e., the rate of closing of traps exceeds the rate of their reopening. Consequently fluorescence rises during this phase.

The oxygen begins to evolve as soon as some units have accumulated four oxidizing equivalents (if we assume Kok's model for O_2 evolution). The reopening of traps and O_2 evolution occurs because, as mentioned above, the weak photoreductant Q^- is restored to Q by a pool of A. However even during this phase, the closing of the trap exceeds their reopening, and fluorescence rises in parallel with O_2 (Fig. 5).

The purely photochemical O to I rise is observed only with very strong light (Delosme, 1967); almost all Q are reduced before Q^- interacts with A. If this is true, then no trap will be able to accumulate more than one oxidizing equivalent on Z, and no oxygen should evolve during this time (microsecond range), although fluorescence will rise to maximum. To our knowledge, parallel O_2 measurements at such high intensities are not available. Such measurements are not easy to obtain as we have to worry about the response time of the O_2 electrode. However in the presence of DCMU which is assumed to block the reaction of Q^- and A, the pure photochemical OI phase can be measured without any O_2 evolution.

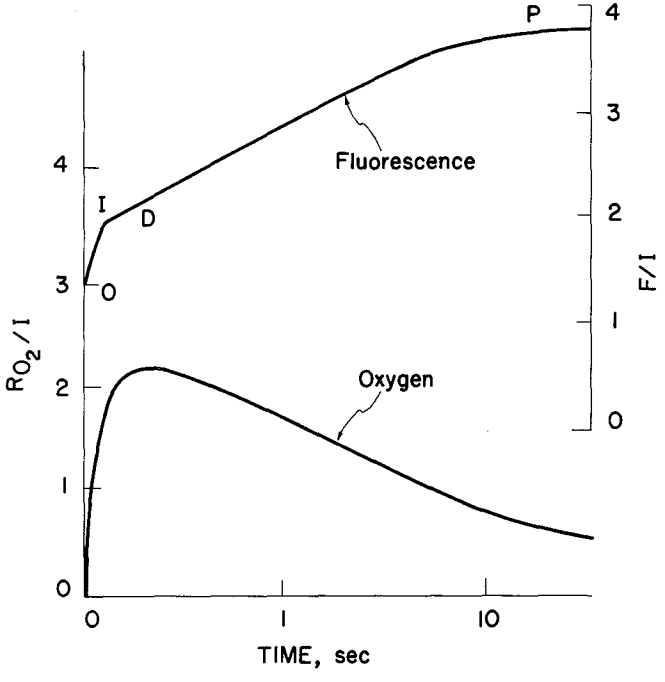

FIG. 5. Time course of the yield of fluorescence and of the rate of O_2 evolution as a function of time of illumination in the green alga *Chlorella*. Temperature, 5°C (redrawn from P. Joliot, 1968).

After the OI phase, a plateau or a decline ID is observed. Obviously a plateau will be observed if the fluorescence yield remains constant meaning thereby that the rates of formation and utilization of Q^- are equal. At such time, a more balanced overall reaction becomes possible, and a peak in O_2 evolution is observed.

The decline ID is observed more clearly in anaerobic cells although it is present in aerobic cells too (Kautsky and Franck, 1943; Munday and Govindjee, 1969a; U. Franck *et al.*, 1969) (Fig. 6). In anaerobic suspensions of *Chlorella,* Kautsky *et al.* (1960) found that if they replaced continuous illumination with periodic light flashes, the ID decline was delayed even when the total number of absorbed quanta remained the same. Thus the ID decline requires light. Munday and

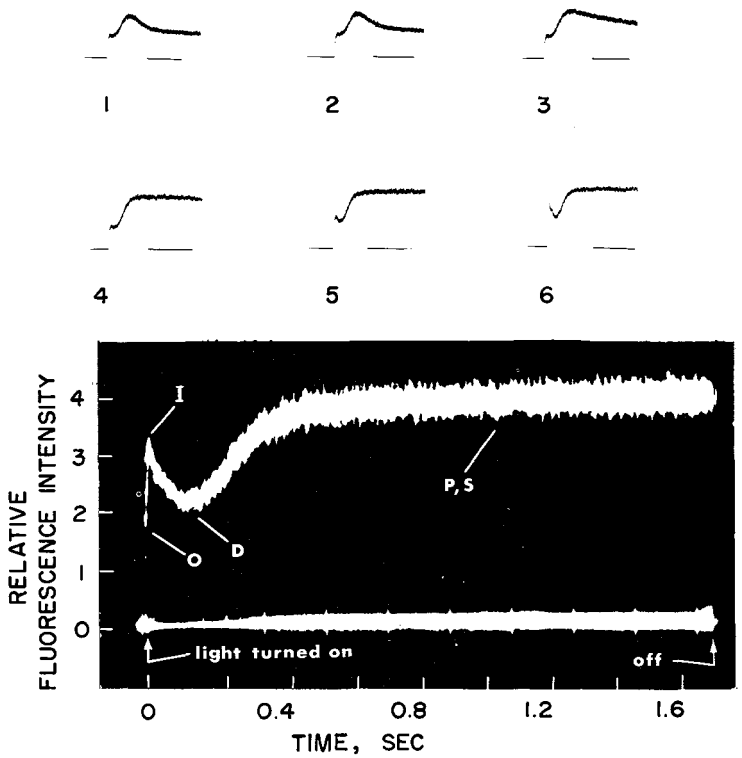

FIG. 6. Changes of the fluorescence transient in *Chlorella* during oxygen removal. The small photographs were taken at 4-minute intervals as 2.0% CO_2 in air was replaced by 2.6% CO_2 in argon. Replacement began a few seconds after photograph 1. The effect was complete by photograph 6, as subsequent transients were identical to that of number 6. The large photograph, from a different anerobic experiment, shows the distinction between O, I, D, and P (after Munday and Govindjee, 1969a).

Govindjee (1969b) found that for the same number of absorbed quanta, the 705 nm (system I) background light gave only a slightly greater ($\sim$ 15%) effect in accelerating the ID decay than the 650 nm (system I + II) light. A reoxidation of Q^- by system I could account for the dip. However this interpretation needs to be modified because 650-nm light should have given a lower effect than what was observed. It appears that both systems I and II are almost equally effective in causing the dip.

What could this be due to? It appears that both pigment systems are involved in the ID decline as follows. A^- can be reoxidized in two ways: by system I, and by a reaction with O_2 molecules involving system II. Reoxidation of the pool A by O_2 (a Mehler type reaction; Mehler and Brown, 1952) has been suggested by Murata *et al.* (1966a) and Malkin (1968) as an explanation of the lower fluorescence yield at weak excitation intensities. This flow of electrons to O_2 is also suggested by the oxidation of photosynthetic carriers (Q^-, A^-) in darkness. (See also de Kouchkovsky and Joliot, 1967, for sites of action by O_2.) Kok *et al.* (1966) suggested that a part of A pool is sensitive to O_2. That molecular O_2 may quench fluorescence by a reaction with A^- can also be inferred from the higher fluorescence yields in anaerobic than in aerobic cells poisoned with DCMU. The intensification of fluorescence yield on the addition of sodium dithionite to DCMU poisoned *Chlorella*, observed by Homann (1968a,b), may also be due to the elimination of this O_2 effect. This, of course, implies that in an aerobic condition some Q are never converted to Q^-.

In apparent contradiction to the oxidation of A^- by O_2 as being the cause of the fluorescence dip (D) is the appearance of a prominent dip obtained with anaerobic algae. It must be borne in mind, however, that the O_2 evolution spike ("gush") is higher in anaerobic than in aerobic samples (Vidaver and Chandler, 1969). Moreover, the decline from the peak is more rapid in anaerobic than in aerobic cells as if O_2 produced is used up more quickly in the former case. This, along with the effective system I reaction, could explain the greater dip in anaerobic samples.

3.3 The DPS Phase

After the completion of the OID phase, the rates of oxygen evolution and fluorescence yield proceed in an antiparallel fashion (Delosme *et al.*, 1959; P. Joliot, 1968; Bannister and Rice, 1968) (Fig. 5). The rise in the fluorescence yield (DP) is associated in time with a decline in the rate of oxygen evolution, while the decline in fluorescence yield (PS) is simultaneous with an increase in O_2 evolution. The indication, therefore, is that the competition between photochemistry and fluores-

cence is an important determinant of the Chl a fluorescence yield at this stage. The kinetic pattern suggests that the sum of the quantum efficiencies of primary photochemistry (ϕ_p) and fluorescence (ϕ_f) may be constant. (The rise and fall of O_2 during the entire OP phase represents the well-known O_2 gush.) The notion of complementarity, however, is of only qualitative significance since the momentary magnitude of the rate of internal conversion cannot be assessed.

In general, the rise DP is attributed to the accumulation of reduced Q and A as a result of system II reaction (Kautsky *et al.*, 1960; Duysens and Sweers, 1963). The decline in O_2 evolution during this phase is due to the depletion of the oxidized pool A. When the oxidized pool A is almost empty, the O_2 evolution is at its minimum; this point in time coincides with the time at which P occurs. The O_2 gush is over.

This simple picture of DP rise may have to be slightly modified because Munday and Govindjee (1969c) found that if methyl viologen (1,1-dimethyl-4,4′ dipyridilium chloride), which accepts electrons only from X^- the primary reductant produced by PSI (Kok *et al.*, 1965), is added to *Chlorella*, the DP rise is completely eliminated. Munday suggested that since system I has already begun to act near D (see Section 3.2), the cause of the accumulation of A^- and Q^- lies beyond A in the electron chain. It is the accumulation of X^-. This is where the real "traffic jam" occurs evidently because the Calvin cycle is too slow to start functioning by the time of the peak P. This accumulation of X^- leads to the accumulation of A^-, and thus, of Q^-. When methyl viologen is provided, electrons are quickly drawn from X^-. Thus there is no accumulation of Q^-, and fluorescence rise DP is abolished. The simple explanation given in the previous paragraph is not complete, however, because it does not account for the fact that A^- could be reoxidized to A by system I light, making impossible the complete reduction of A and Q at the time of P. For this reason we believe that Munday's explanation may be closer to reality. His view is also in agreement with the fact that the "O_2 gush" is clearly seen in whole cells of algae, after a dark period where CO_2 reduction is delayed. This is also confirmed with chloroplasts in the absence of added oxidants; here an O_2 gush and an OIDP rise are observed without any CO_2 fixation.

Additional mechanisms must be invoked to explain a recent experiment by Duysens (1970) who found that a brief (microseconds) bright flash of system I light can instantly quench fluorescence yield at P. This experiment is difficult to explain by any of the above theories of DP rise that involve the accumulation of A^-. System I light can only slowly reoxidize A^- to A and thus Q^- to Q causing quenching of fluorescence—this would be too slow to explain Duysens' experiment. Hence one has

to postulate a separate mechanism for a more direct quenching of system II fluorescence by bright system I light flashes. Obviously further work is needed to fully understand the DP transient.

The decay of the Chl a fluorescence yield along PS is the least understood part of the fluorescence transient. The fact that this decay is associated with a simultaneous rise in the rate of O_2 evolution points to the reoxidation of A^- as a possible cause of this fluorescence decline. Perhaps the Calvin cycle begins to operate then, and the "traffic jam" at X^- is removed allowing a balanced system I and system II reaction.

Whenever the net electron transport is at its maximum and is not limited by the oxidant in the system, there is no P. This is the situation with methyl viologen treated *Chlorella* cells, and with ferricyanide treated chloroplasts. When there is no P there is no P to S decline. However, in chloroplasts without added oxidants, there is DP rise, but no PS decline. This is so because all the intermediates (Q, A, X) are reduced during the DP rise, but there is no way to reoxidize them.

The above explanation of P to S, i.e., due to reoxidation of Q^- and A^-, has difficulties. If light is turned off at the quasi-steady state S, where we imagine that most A and Q are in their oxidized states, and then turned on again, the OP rise cannot be observed as if something else has happened. Duysens and Sweers (1963) proposed that a quencher Q′ (not capable of reduction by light) is formed. Recently, the concept of such a quencher has been abandoned in favor of another theory in which changes on and in the chloroplast membranes are suggested to occur (see Section 4.4.1). We believe that a comprehensive theory for P to S decline will probably include an interplay of such physical changes and of chemical changes in terms of the oxidation–reduction states of the intermediates Q, A and X; such a theory remains to be formulated.

3.4 Preillumination Effects

Preillumination of the photosynthetic tissue can alter the pattern of Chl a fluorescence kinetics in a manner that depends both on the light-induced shifts in the oxidation–reduction states of the intermediates, and on the slow physical changes (see Section 4.4.1). The picture becomes more complex when we consider that the pool A can interact with both the presystem II oxidants and the postsystem I reductants. Govindjee *et al.* (1966) and Munday and Govindjee (1969b) found that preillumination (or continuous background illumination) of *Chlorella* with weak system I light depresses the levels I and P, DP rise is delayed, PS is slowed down, and the level of S is raised. The decrease in I and P was ascribed to a shift of the A^-/A equilibrium to a more oxidized

position, an argument supported by the greater effectiveness of far-red (system I) light in causing this effect. Vredenberg (1969) reported similar results with a red alga *Porphyra;* his interpretations were similar to those given above. These fluorescence transient changes are consistent with the data on O_2 evolution. For example, system I preillumination increases the O_2 evolution in system II light (French, 1963; Govindjee and Govindjee, 1965).

The increase in the "S" level, mentioned above, is possibly due to long term effects (see Section 4.4). These effects are also evident in the increased S level observed at higher intensities of illumination (Lavorel, 1959). Treatment with stronger light eliminates the minimum S, the fluorescence decay proceeds monotonously from P to the terminal level T (Bannister and Rice, 1968).

3.5 More about the Pool "A"

The pool A is not homogeneous. At least two kinetically distinct entities are thought to exist (A_1 and A_2). When Forbush and Kok (1968) plotted the amount of DCPIP reduced per flash of light as a function of the duration of the flash, they observed a biphasic rise. If an instantaneous equilibration of DCPIP with pool A is assumed, the amount of reduced DCPIP would be proportional to the amount of A reduced by the flash. The observed biphasic rise was interpreted as evidence for two subpools of A—a fast-reacting A_1 and a slower A_2. Malkin (1966) and Forbush and Kok (1968) obtained a good bit of their experimental data on fluorescence transients with their theoretical curves based on the assumption that two subpools of A exist.

How fast is the reaction between Q and A? P. Joliot (1965a) estimated the half-time constant ($t_{1/2}$) to be 3 msec at 5°C and 1 msec at 20°C. Forbush and Kok (1968) determined $t_{1/2}$ to be 0.6 msec from the measurements of the decay of fluorescence yield in isolated chloroplasts subjected to a saturating flash. It must be kept in mind that this half-time corresponds to a bimolecular rate; $t_{1/2} = 1/k_1\ [A]_{total}$, where $[A]_{total}$ represents all A in the oxidized form after darkness.

The half-time obtained from the kinetics of DCPIP reduction (as discussed above) is 4 msec for the fast-reacting component A_1. Since this half-time equals $1/k_1[Q]$ total, the ratio of [Q] to $[A_1]$ was estimated (Forbush and Kok, 1968) as $[A_1]/[Q] = 4/0.6 = 7$. As $[A_1] = \frac{1}{3}\ [A]_{total}$, they obtained [Q]/[A] to be 1/20. Similar values were earlier obtained by Malkin and Kok (1966) and P. Joliot (1965a) from measurements of area over the fluorescence rise curve (the OP transient) with and without DCMU, and from the measurements of the "O_2 gush" respectively.

Another approach, that has been used recently by Kok *et al.*, (1969), was to measure the relative number of electrons flowing through P700 as induced by either a short or long flash. The estimate of $[A]_{total}/[Q]$ by this method was 10. This ratio is somewhat smaller than that observed when reactions through system II alone were used for calculations. They explain this difference by assuming that photosystem I reacts only or mainly with one of the two subpools.

3.6 Discussion: Theories on Chl Fluorescence Kinetics

Earlier theories on the induction of Chl a fluorescence invoked processes which quenched the Chl a excitation and which were supposed to compete with photosynthesis. J. Franck and his co-workers (1941, 1945; Shiau and Franck, 1947) ascribed the fast fluorescence rise (O–P) to a chemical inactivation (narcotization) of Chl a. The narcotic substances were oxidized metabolites, whose later depletion reactivated Chl a and depressed fluorescence yield (P–S). To account for the same decay, Kautsky and Hormuth (1937) suggested quenching of Chl a excitation by oxygen. A fluorescence quencher, consumed by a photochemical reaction and regenerated by a subsequent thermal reaction was suggested by Ornstein *et al.* (1938) to account for the fast fluorescence transient (OPS).

The currently prevailing hypothesis for the fast change, discussed in Sections 3.2–3.4, is similar to the above suggestion of Ornstein *et al.* Kautsky *et al.* (1960) explained the fluorescence transient (OIPS) in terms of the two light reactions. In addition Duysens and Sweers (1963) explained the fluorescence transient within the framework of a two-pigment system–two light reactions hypothesis. The quantum yield of the system II Chl a fluorescence increases when the primary electron acceptor (Q) and the intermediate pool (A) are reduced by system II light. The yield decreases when they become oxidized (mainly) by system I light. In principle then, the magnitude of the variable Chl a fluorescence, i.e., the fluorescence level above the level 0, reflects the momentary proportion of reduced (closed) photosystem II reaction centers. Several kinetic treatments have been devised. Starting from a number of assumptions, several investigators have attempted to theoretically reproduce the course of the fast fluorescence transient (Malkin, 1966; Murata *et al.*, 1966b; Delosme, 1967; Munday, 1968; Munday and Govindjee, 1969a; Clement-Metral and Lavorel, 1969). The physical identity of each kinetic variable, that appears in the final expressions of these treatments, is not known. A further shortcoming of such treatments is the restricted applicability of the derived kinetic expressions to only the rise portion of the fast transient (OIP); some theories, in fact, do not even allow D. Because

of their present limited utility we will not discuss these kinetic theories here.

Finally, there are suggestions that there may be more than one quencher of fluorescence. Delosme (1967) proposed a second quencher and had called it R. Recently R. Govindjee *et al.* (1970) have suggested two quenchers Q_1 and Q_2 to explain their data on the fluorescence transient of heptane-extracted chloroplasts. Cramer and Butler (1969) obtained two midpoint potentials when they titrated Q. So in view of present evidence, the possible existence of two quenchers needs to be explored. Speculations have even been made that one of these quenchers may not be a real chemical, but simply a state of the matrix surrounding active Chl a_{II}.

4. Slow Fluorescence Yield Changes: The SMT Phase

4.1 General

The slow change in algae consists of a rise of the Chl a fluorescence yield from the level S to a broad maximum M and a subsequent slower decay to the terminal level T (Fig. 7). The decay MT is faster in the green and the red algae than in the blue-greens. (Adequate dark periods or preilluminations with far-red light are needed to repeat these transients.) During the SMT phase, the fluorescence yield of phycobilins (in

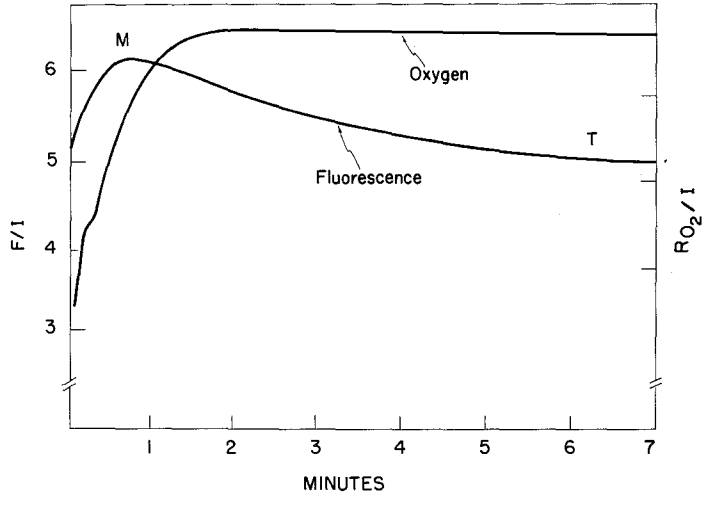

FIG. 7. Time course of the Chl a fluorescence yield and of the rate of oxygen evolution in *Chlorella*. Excitation λ, 480 nm; incident intensity, 3×10^3 ergs cm^{-2} sec^{-1}; observation λ, 685 nm (after Papageorgiou and Govindjee, 1968b).

red and blue-green algae) does not change (cf. French and Young, 1952; Govindjee *et al.*, 1966). Figure 8 shows the fluorescence spectra of the blue-green alga *Anacystis nidulans* at S and M levels. The difference spectrum (M–S) shows only an increase in the Chl a fluorescence yield, but no decrease in the yield of phycocyanin fluorescence. This suggests that the increase in the yield of Chl a fluorescence is not due to an increase in energy transfer from phycobilins to Chl a. However, these data do not exclude the hypothesis that this increased Chl a yield is due to a decreased energy transfer (spillover) from Chl a of system II to Chl a of weakly fluorescent system I.

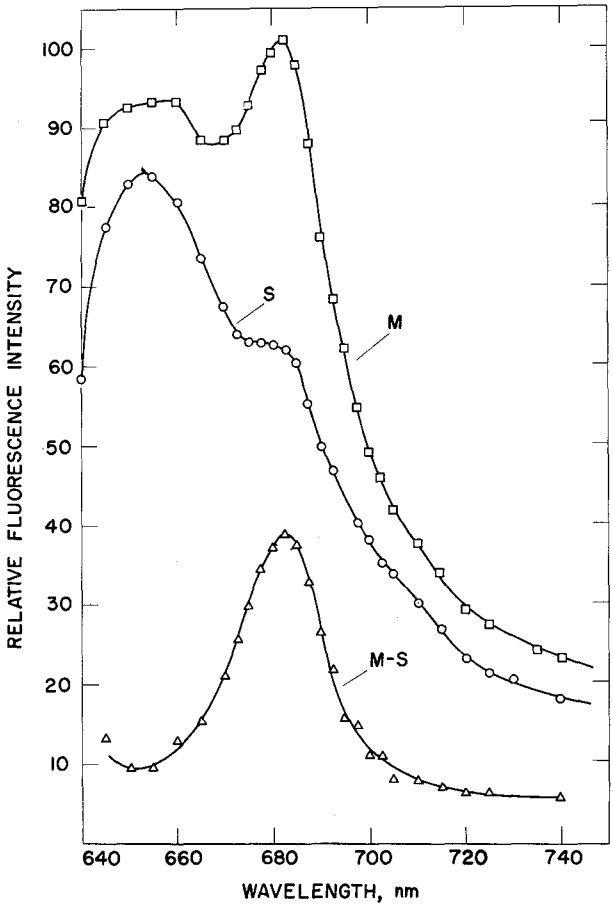

FIG. 8. Emission spectra of normal *Anacystis nidulans* at 3 seconds (level S) and 10 minutes (level M) of light exposure; M-S, the difference spectrum; excitation λ, 590 nm; observation λ, 685 nm (after Papageorgiou and Govindjee, 1968a).

Only the light absorbed by photosynthetic pigments can cause the slow fluorescence change—with green algae it is seen using light absorbed by Chl a or Chl b, while with the blue-green algae the slow change is seen using light absorbed mainly by phycocyanin. This emphasizes the photochemical character of the process and its possible relation to photosynthesis. A relationship with system I-sensitized cyclic electron transport is also inferred because DCMU-poisoned blue-green algae display a slow fluorescence change with light absorbed either by Chl a (system I) or phycocyanin (system II) (Govindjee *et al.*, 1966; Papageorgiou and Govindjee, 1967; Duysens and Talens, 1969).

As in any photochemical process, the SMT change depends upon the intensity of exciting light. At very light intensities, SMT change is absent. On increasing the intensity of excitation, the rates and amplitudes of the induction waves increase and saturate. The light intensity that saturates the yield at M is different than the intensities that saturate S and T (Papageorgiou, 1968). For the dependence of O, P, and S, in the fast transient, on light intensity, see Lavorel (1963), Govindjee *et al.* (1966), and Munday and Govindjee (1969a).

4.2 Electron Transport and the Slow Fluorescence Change

The complementarity between the yield of Chl a fluorescence and the rate of O_2 evolution (the rate of noncyclic electron transport), that characterizes the DPS phase of the fast transient, is absent in the SMT phase. During the SM portion, the rate of O_2 evolution rises together with the yield of Chl a fluorescence, and attains a constant level while the fluorescence decays along MT (Fig. 7). Whatever may be the relationship of the slow fluorescence to photosynthesis, it is not a competitive one. Therefore, the fraction of reduced photosystem II reaction centers is of secondary significance in determining the yield of the variable Chl a fluorescence at this stage.

Photosynthetic electron transport is, however, in some way a contributing factor for the slow change. Moreover cyclic electron flow is implicated in the SMT phase more directly than noncyclic. Although, DCMU-poisoned *Chlorella* does not exhibit the SMT change when exposed to light of moderate intensity ($\sim$10 kergs cm^{-2} sec^{-1}), higher intensities of light ($\sim$50 kergs cm^{-2} sec^{-1}) cause this slow fluorescence change (Bannister and Rice, 1968). The evidence for a cyclic flow of electrons *in vivo* is rather indirect (see Teichler-Zallen and Hoch, 1967; Rurainski *et al.*, 1970), but it is generally believed that in DCMU-poisoned cells only cyclic electron flow operates. It can be inferred that intense light supports cyclic electron transport of sufficient magnitude to somehow cause the fluorescence change. The contribution of the cyclic

electron transport also explains the slow fluorescence change of DCMU-poisoned *Anacystis nidulans* (Govindjee *et al.*, 1966) and of *Schizothrix calcicola* (Duysens and Talens, 1969).

Bannister and Rice (1968) were able to demonstrate the relationship of the cyclic electron flow to the slow fluorescence change from the following. They found that a mutant of *Chlamylomonas* having its non-cyclic—but not the cyclic—electron flow impaired was still capable of the slow fluorescence change. On the other hand, mutants missing both types of electron transport did not show the slow fluorescence change. A relationship with the cyclic electron flow (a system I reaction) does not, however, imply that we are now looking at system I fluorescence. It simply means that the system II fluorescence is somehow influenced by system I reactions.

4.3 Phosphorylation and the Slow Fluorescence Change

Strehler (1953) proposed a relationship between photophosphorylation and the fluorescence induction phenomena. He demonstrated a correlation between the time course of Chl a fluorescence yield and of the ATP content in *Chlorella*. Recently further evidence for the involvement of photophosphorylation has been acquired by the use of uncouplers of phosphorylation (Papageorgiou, 1968; Papageorgiou and Govindjee, 1968a,b). These uncouplers prevent the synthesis of ATP while permitting electron transport. In fact the electron transport is accelerated in chloroplasts performing Hill reactions since in the presence of uncouplers a rate limiting step is bypassed (Good *et al.*, 1966). In whole cells, however, the action of uncouplers is not so simple. A decrease in the concentration of photoproduced ATP could lead to a decrease in the rate of reactions of the Calvin cycle, and this indirectly decreases electron transport.

The powerful uncoupler of photophosphorylation FCCP (*p*-trifluoromethoxyphenyl hydrazone of ketomalonyl nitrile) at concentrations as low as 0.4 μM reduces the amplitude of the slow fluorescence change in *Schizothrix calcicola* by a factor of three (Duysens and Talens, 1969). At higher concentrations (1–10 μM), when it only partially inhibits the electron transport in whole cells, FCCP abolishes the MT phase in *Chlorella* and the rise SM in *Anacystis* (Papageorgiou, 1968; Papageorgiou and Govindjee, 1968a,b) (Fig. 9). FCCP not only abolishes the slow fluorescence induction of DCMU-poisoned *Chlamydomonas*, but also lowers the Chl a fluorescence yield, an observation for which no adequate explanation exists (Bannister, 1967; Bannister and Rice, 1968).

Murata and Sugahara (1969; also see Govindjee *et al.*, 1967) found that the fluorescence yield of DCMU-treated chloroplasts is slowly

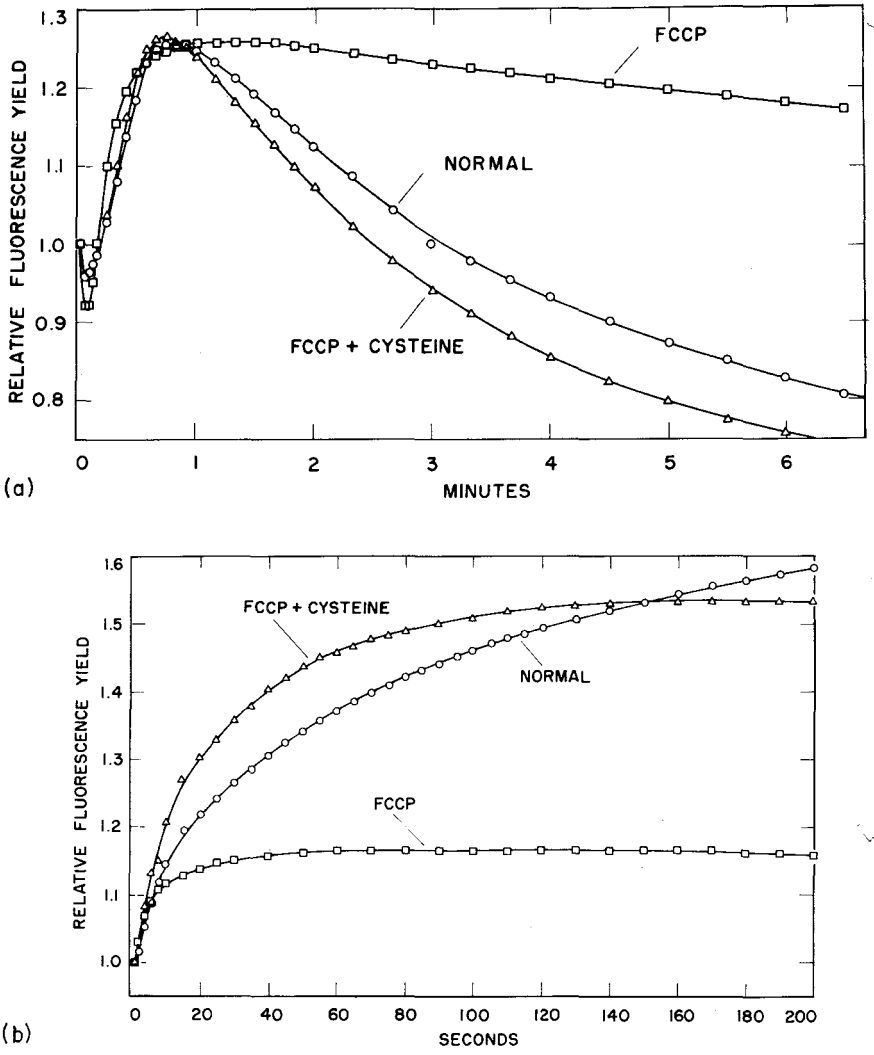

FIG. 9. (a) Time course of the relative fluorescence yield in *Chlorella pyrenoidosa* normalized at S. Control; with $3 \times 10^{-5} M$ FCCP; with $3 \times 10^{-5} M$ FCCP and $10^{-3} M$ cysteine; excitation λ, 480 nm; observation λ, 685 nm (after Papageorgiou and Govindjee, 1968b). (b) Time course of the relative fluorescence yield in *Anacystis nidulans* normalized at S. Control; with $3 \times 10^{-5} M$ FCCP; with $3 \times 10^{-5} M$ FCCP and $10^{-3} M$ cysteine; excitation λ, 590 nm; observation λ, 685 nm (after Papageorgiou and Govindjee, 1968a).

quenched by the addition of PMS. This depression of the fluorescence yield by PMS was reversed if an uncoupler of phosphorylation CCCP (*p*-chlorophenyl hydrazone of ketomalonyl nitrile) was added to the sample containing DCMU and PMS. The effect of CCCP, described above, seems opposite to that found by Bannister (1967). It is difficult to compare the two experiments because of the different systems and effective concentrations of uncouplers used. However this experiment supports the idea that uncouplers of phosphorylation influence the slow fluorescence changes.

The inhibition of the slow fluorescence change by uncouplers of phosphorylation is particularly pronounced at low intensities of light. At an incident intensity of 3.1 kerg cm^{-2} sec^{-1} ($\lambda = 480$ nm) half-maximal inhibition in *Chlorella* is obtained at 4.5 μM of FCCP. This inhibition of the slow fluorescence change is reversed on adding cysteine hydrochloride (Papageorgiou, 1968; Papageorgiou and Govindjee, 1968a, 1969). These observations parallel those of increased uncoupling activity of FCCP at low light (Avron and Shavit, 1963) and the reversal of its activity by aminothiols (Heytler, 1963).

Another powerful uncoupler, atabrin, proved to be as effective as FCCP in suppressing the slow fluorescence change in algae and in isolated chloroplasts.

Park *et al.* (1966) have shown that phosphorylation and certain electron transport reactions that lead to CO_2 fixation do not occur if intact algae cells are fixed with glutaraldehyde. Such cells, however, are capable of DCPIP reduction and certain systems I reactions. A recent experiment by Papageorgiou and Mohanty (1969) on glutaraldehyde fixed *Porphyridium* cells show the complete absence of slow fluorescence change. This experiment confirms that electron transfer, involving system II only, is not enough per se to cause slow fluorescence changes (see Section 4.2). In addition, we can argue that since there was no phosphorylation in the fixed cells, there was no slow fluorescence change strengthening our view that phosphorylation and associated processes are somehow related to slow fluorescence changes in whole cells.

4.4 Discussion

4.4.1 General Theories

Wassink and Katz (1939) had recognized that several features of the slow fluorescence change were different from those of its fast counterpart. The slow change had proved to be insensitive to the oxygen content of the gas phase of the sample (Wassink and Katz, 1939; Kautsky and Eberlein, 1939). Carbon dioxide, on the other hand, appeared to play

some role since the "single-wave" kinetics in wheat and in *Hydrangea* were converted to a "double-wave" form at elevated carbon dioxide concentrations (McAlister and Myers, 1940; J. Franck *et al.*, 1941).

Any mechanism describing the events during the slow fluorescence change must take into account (1) the requirement of cyclic electron transport and (2) the apparent independence from the noncyclic electron transport. All hypotheses are essentially similar in the sense that they invoke a slow photoprocess which modifies the photosynthetic and emissive capacity of photosystem II units. This process is coupled to and is controlled by the cyclic electron transport and associated photophosphorylation.

According to Bannister and Rice (1968), the slow fluorescence rise SM is caused by some kind of "activation" of "inactive" photosynthetic units that leads to an increase in O_2 evolution as well as in fluorescence yield; this hypothesis gives no explanation for the slow fluorescence decay MT of the green algae.

Recently Murata (1969a,b) has provided evidence for changes in the spillover of energy from the strongly fluorescent system II to the weakly fluorescent system I—an increase in the fluorescence yield is due to a decrease in this transfer, and a decrease in the yield is due to an increase in the transfer. Thus the PS and MT decline would be interpreted as a consequence of an increase in this transfer and the SM rise to a decrease in this transfer. The experimental results supporting the existence of such a change in the spillover of energy are as follows. Murata (1969a) compared the 77°K fluorescence spectra of algae that were preilluminated with system II light with those that were kept in the dark. He found the fluorescence efficiency of Chl a of system II, which fluoresces mainly at 685 nm and 695 nm, to be lower and the fluorescence efficiency of Chl a of system I, which fluoresce mainly at 720 nm, to be higher in the preilluminated than in the nonpreilluminated sample. This suggests that preillumination with system II light causes an increase in the efficiency of energy transfer from system II to system I. A decrease in this transfer is caused by treating chloroplasts with Mg^{2+} (and other divalent ions). This is shown from the observed increase in the fluorescence yield of system II Chl a concomitant with a decrease in the fluorescence yield of system I Chl a (Murata, 1969b; Murata *et al.*, 1970; Mohanty, 1969). The decrease in transfer from system II to I is further confirmed by the observed increase in the rate of system II reaction (DCPIP reduction) and a decrease in the rate of system I reaction ($NADP^+$ reduction with added $DCPIPH_2$, in the presence of DCMU).

The hypothesis of Murata (1969a) is consistent with that of

Bonaventura and Myers (1969) who proposed a variable distribution of the absorbed photons in the two photosystems. Bonaventura and Myers (1969) defined two states of the chloroplast: (1) *light state 1* in which quanta absorbed by system II remain mostly in system II and are not transferred effectively to system I; this state is created by illumination with system I light, or by prolonged darkness; and it has a high fluorescence yield, (2) *light state 2* in which quanta absorbed by system II are transferred to system I; this state is created by prolonged illumination with system II light; and it has a low fluorescence yield. To quantitate this variable distribution of quanta in the light states, Bonaventura and Myers defined a fraction α/α_{max}, where α is the fraction of photons delivered to system II, and α_{max} is the maximum value that α can attain. For state 2, this fraction is 0.9, and for state 1, it is 1.0. The difference is only 10%. Duyens (1970) has given a visual picture to these states (Fig. 10). He explains the transitions from one state to another as an energy dependent movement of pigment molecules of system II away from or closer to system I. When they are away from each other, state 1 exists, fluorescence yield is high, α is 1.0, and energy transfer is minimal. When they are close to each other, state 2 exists, the fluorescence yield is low, α is 0.9 and consequently energy transfer from system II to system I is maximal. Thus in addition to the control of fluorescence yield by the concentration of Q, we have to concern ourselves with the changes in the movement of chloroplast membranes.

The above hypotheses have been used to explain the changes in fluorescence yield in algae. A rapid quenching followed by an increase of the fluorescence yield of system II was observed upon addition of system I light in *Porphyridium* (Murata, 1969a). When the system I light was turned off, the yield increased further and then decayed slowly to the original level [confirmed by Bonaventura and Myers (1969) and Mohanty *et al.* (1970) in *Chlorella*]. Murata explained the increase in fluorescence yield after prolonged illumination of system I light by a decrease in energy transfer from system II to system I, that is, by the conversion of state 2 to state 1.

When Bonaventura and Myers (1969) replaced system II with sys-

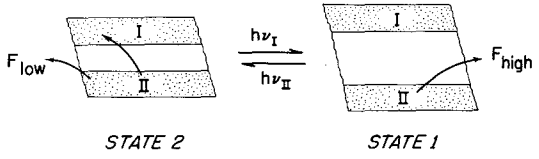

FIG. 10. A hypothetical picture of the two "states" based on the ideas of Duysens (1970), Bonaventura and Myers (1969), and Murata (1969b) (see text).

tem I light, a slow increase in the fluorescence yield—measured by a weak system II light—accompanied a parallel increase in the rate of O_2 evolution in *Chlorella*. This reminds us of the SM rise in *Chlorella* where too, both fluorescence and O_2 rise in parallel (Papageorgiou, 1968; Bannister and Rice, 1968; Papageorgiou and Govindjee, 1968a,b). This fluorescence rise is also explained as due to conversion of state 2 to state 1.

On the other hand, replacement of system I with system II light caused a slow increase in the fluorescence yield although the rate of O_2 evolution did not change significantly (Bonaventura and Myers, 1969). This kinetic pattern is almost identical to the MT phase in *Chlorella* when O_2 rate remains constant while fluorescence yield declines (Papageorgiou, 1968; Bannister and Rice, 1968; Papageorgiou and Govindjee, 1968b). This fluorescence decline is explained as due to the conversion of state 1 to state 2.

Now the PS decline in the fluorescence yield can be explained as resulting from conversion of highly fluorescent state 1 to weakly fluorescent state 2. In this picture, the Q′ of Duysens and Sweers (1963) is equivalent to state 2. It must be converted to state 1 by a long dark period or far-red illumination before the OIDP rise can be observed again. This explains the inability to repeat OIDS without such a treatment.

There are some difficulties however, if PSMT changes are indeed entirely due to changes in the "states" of chloroplasts, i.e., P and M occur when chloroplasts are in state 1 and S and T when they are in state 2, then why do we obtain different types of changes in the rate of O_2 evolution during P to S (O_2 declines) as compared to the M–T phase (O_2 remains constant)—when both result from the conversion of state 1 to state 2. Also, why does the rate of O_2 evolution increase when state 2 is transformed into state 1 during the SM phase? Obviously other factors are involved and the slow fluorescence changes are not exclusively caused by the proposed changes in the states of the chloroplast membranes.

Addition of system I light at different points on the SMT transient showed different effects depending upon whether it was added on the SM or the MT phase, even when points having identical fluorescence yields were chosen, representing intermediate but identical "states" of the chloroplast (Fig. 11). On the SM rise part little, but on the MT decline part significant quenching was observed (Mohanty *et al.*, 1970). This observation suggests that identical "states" are not identical in their photochemical reactions. We believe that the SMT fluorescence transient

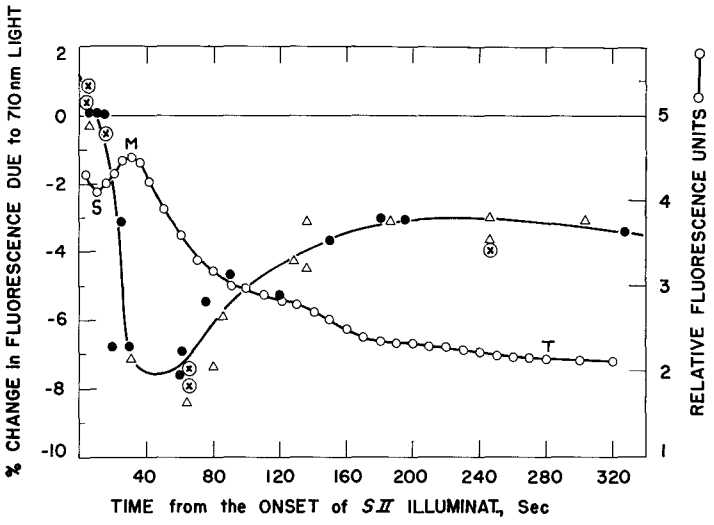

FIG. 11. Percent changes in the fluorescence yield of Chl a due to the addition of 710 nm light at various times during the fluorescence transient (exciting light, blue). Experiments with three different cultures (thick line with open triangles, solid circles, and crossed circles) are shown: the scale to the left. For comparison, the fluorescence transient for the cultures used in these experiments is represented by the curve with a thin line and small open circles: the scale to the right (after Mohanty *et al.*, 1970).

reflects both changes in oxidation–reduction intermediates as well as changes in "states" of the membrane although the exact pattern is not clear. (For the role of changes in ionic gradient, see Section 4.4.2.)

Papageorgiou and Govindjee (1968a,b) had earlier suggested that slow fluorescence changes are due to light-induced changes in the structure of the photosynthetic apparatus. A clue to this was the requirement of phosphorylating electron transport for the slow changes to occur. Phosphorylation is known to be accompanied by changes in the conformation of the thylakoids to a degree that depends upon the rate of electron transport and its phosphorylating capacity (Packer *et al.*, 1970). Whenever phosphorylation is absent, slow changes are absent. (The absence of slow changes in chloroplasts capable of "swelling" and "shrinkage" does not disprove the proposed relationship because the conformational changes may or may not be always related to the observed configurational changes, and vice versa.) It is proposed that the changes in the fluorescence yield need not be only due to changes in energy transfer from system II to system I. The structural changes

could lead to movement of Chl a molecule—within the pigment system II—away from each other leading to a decreased concentration quenching and thus an increase in the fluorescence yield. In this situation, the rate of internal conversion would decrease without affecting the trapping efficiency. On the other hand, a decrease in the fluorescence yield could be due to a movement of Chl a in system II toward each other causing an increased concentration quenching, i.e., an increase in the rate of internal conversion and a decreased fluorescence yield without affecting the trapping efficiency. This would explain the M to T decline without any change in the rate of O_2 evolution.

Whether one believes in the movement of system II and system I, or of Chl a within the system II, one expects light induced conformational changes of the membrane system to cause it. Suggestions for conformational changes have accumulated from several sources. Brody *et al.* (1966) observed spectral changes in fluorescence spectra of *Euglena* chloroplasts treated with concentrated salts [NaCl, $MnCl_2$, $(NH_4)_2SO_4$] and urea; they ascribed these changes to the conformational changes of the lipoprotein matrix. Murata (1969b) and Homann (1969) have demonstrated an increase of the steady-state fluorescence yield of Chl a in DCMU-poisoned chloroplasts by the addition of 3–5 m*M* Mg^{2+}. Other cations also have some effect, but the effect is largest with Mg^{2+} (Murata *et al.*, 1970). Mg^{2+} also causes the largest volume changes in chloroplasts suspended in a low salt medium (Izawa and Good, 1966), and conformational changes are known to accompany volume changes. That chloroplast conformation regulates electron flow *in vivo* has received experimental support from the work of Heber (1969): at high light intensity, excessive photoshrinkage of chloroplasts occurring in a variety of leaves does suppress the rate of electron flow.

Light-induced changes in the chloroplast structure have been investigated by following light scattering changes and by electron microscopy (Murakami and Packer, 1970). Two types of structural changes have been distinguished—decrease in the membrane thickness (conformational change), which is thought to be brought about by proton uptake and a decrease in the spacing between the membranes that causes a flattening of the entire chloroplast (configurational change). (This flattening effect has been recently correlated with changes in the ATP level by Nobel *et al.*, 1969.) This only suggests that light-induced conformational changes occur in chloroplasts, but they cannot yet be related to the theories proposed above concerning fluorescence changes. Finer resolution in observing systems I and II containing membranes, and the ability to observe these changes as a function of time are needed before we can really attribute fluorescence changes to structural changes.

4.4.2 Ionic Control of Excitation Trapping

The smallest chloroplast substructure, capable of complete photosynthesis, is the thylakoid. The membranous thylakoid envelope (the lamella) serves two purposes. First, it supplies the matrix on which the photosynthetic pigments and the enzymes are organized. Second, the lamella functions as a selective osmotic barrier separating the thylakoid interior from the stroma. The permeability of this barrier is variable and it appears to be subject to regulation by the photosynthetic processes. These processes include electron (or H-atom) transport within the membrane, and H^+ transport from the outside to the inside of the thylakoid. In an elegant theory, Mitchell (1966) has proposed that electron transport alternating with H-atom transport leads to a net movement of H^+ from the outside to the inside of the thylakoid. This creates a chemical potential gradient on the membrane. A collapse of this gradient is responsible for the ATP production. The alternating electron and H-atom transfer is visualized as follows. In the thylakoid membrane, the O_2 evolving and the P700 oxidation sites are on the inner side of the membrane, whereas the plastoquinone and $NADP^+$ reduction sites are on the outer side of the membrane. In light reaction II, electrons are removed from Z to reduce Q. Water reduces oxidized Z by donating electrons, H^+ are deposited on the inside of the thylakoid membrane and O_2 is evolved. Plastoquinone is reduced by Q^- and a H^+ is picked up from the outside to complete this reaction as this reduction requires a H-atom. The next step is the reduction, by plastoquinone, of P700 which accepts electrons only, so a H^+ is again deposited on the inside of the membrane. As a result of light reaction I, P700 is oxidized, and X is reduced. The X^- reduces $NADP^+$ on the outer side of the membrane requiring H^+ that is picked up from the outside of the thylakoid. Thus, accompanied by electron (and H-atom) transfer from H_2O to $NADP^+$, there is a net accumulation of H^+ on the inside of the thylakoid. This not only causes a pH gradient (ΔpH) and an osmotic component, but an electrical field component ($\Delta\psi$) as well. A new detailed model of how all this comes about has recently been proposed by Kreutz (1970).

Witt and associates (Rumberg, 1964; Emrich *et al.*, 1969) have indirectly measured $\Delta\psi$ by following absorption changes that arise in certain pigments because of the Stark effect (or the electrochromic shift). A field strength of 10^5 V/cm has been calculated to exist (Schliephake *et al.*, 1968; Wolff *et al.*, 1969); this is sufficiently strong to cause changes in the pigments embedded in the thylakoid. Junge and Witt (1968) found that it takes one molecule of the antibiotic gramicidin D to obliterate the ionic and osmotic response of one thylakoid. Such a

"punctured" thylakoid exhibits only the very fast electrochromic absorption changes as the $\Delta\psi$ is dissipated rapidly due to the indiscriminate permeability of the membrane.

Further support for the Mitchell theory was also given by Uribe and Jagendorf (1967). Chloroplasts were first incubated in the dark at an acidic pH (4) and then rapidly transferred to an alkaline pH (8). This resulted in a net synthesis of ATP. This experiment provided a direct test of the hypothesis that the pH gradient is the driving force for the synthesis of ATP in chloroplasts. (It was, however, not clearly shown that a pH-induced electron flow was absent during the acid–base transition.) Formation of an ionic gradient has been shown to cause volume changes (swelling and shrinkage of chloroplasts) and changes in internal structure (see Itoh *et al.*, 1963; Packer, 1963; Izawa, 1965; Hind and Jagendorf, 1965; Dilley, 1966; Nobel, 1969). These changes are the consequence of the H^+ transport, and are thus consistent with the Mitchell theory. However reservations should be made before Mitchell theory is accepted in its present form (Slater, 1967).

We now consider the hypothesis that the rates of excitation trapping and of electron transport may be under the control of the ionic gradients. Rumberg and Siggel (1969) have demonstrated the control of the rate of electron flow through P700 by the extent of acidification of the chloroplast interior. In their experiment, the rate of P700 reduction (measured as the decay rate of the light-induced absorbance change at $\lambda \sim 700$ nm) was monitored at the end of an illumination period the duration of which determined the steepness of the pH gradient. The results indicated that a long illumination (i.e., a steeper H^+ gradient) caused a slower rate of P700 reduction. On the other hand, in the case of thylakoids that had been rendered leaky by the addition of gramicidin D, the rate of P700 reduction was found to be independent of the length of the preillumination period. These results show that a large H^+ ion gradient causes a reduction in the rate of electron transport.

Let us elaborate this idea of a control mechanism. Perhaps, the trapping of the quanta at the reaction center and the electron and ion transports are coupled and interdependent through a feedback control. Rapid trapping of the excitation causes an equally rapid electron transport and buildup of H^+ gradient. The latter is assumed to exert an inhibitory effect on the rate of the electron flow, the suppression of which results in an increase in the fraction of closed reaction centers causing, in turn, a reduction in the trapping rate. (This is supported by the experiments of Rumberg and Siggel described above.) However the collapse of the ionic gradient by the formation of the high energy inter-

mediate, and subsequent synthesis of ATP from it, will speed up the electron transfer.

We are now in a position to provide a description of the slow fluorescence and oxygen yield change on the basis of the above hypotheses. At P (Fig. 3), the ionic gradient will be assumed maximal, reaction centers closed, and the electron transport low (state 1; Section 4.4.1). During the P to S phase, the membrane potential collapses by the formation of the high-energy intermediate (cf. state 1 to state 2 transition). Thus, the inhibitory pressure of the H^+ gradient is removed. This causes an increase in the rate of electron transport as observed (O_2 evolution rate increases). We believe that the decline in the fluorescence yield from P to S is a result of two factors—(1) increase in the rate of electron transport, and (2) changes in the conformation of the membranes as the membrane potential collapses (Section 4.4.1).

The increase in the O_2 evolution during SM phase is due to the onset of CO_2 reduction that utilizes the pool of ATP present in cells. The H^+ transport, and thus the membrane potential again increases during this phase. This increase in potential causes the change in the conformation

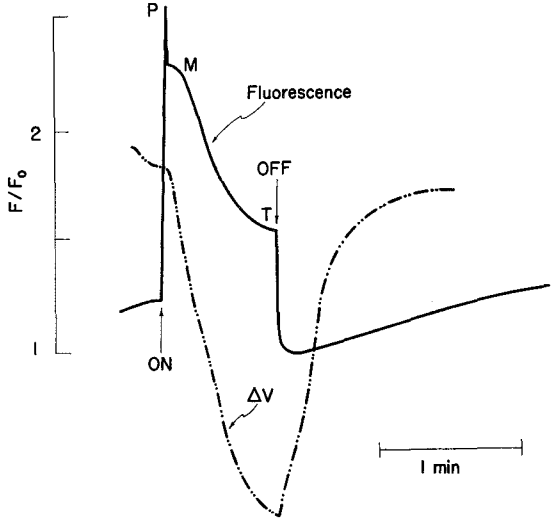

FIG. 12. Kinetics of light-induced changes in Chl a fluorescence yield and in potential (ΔV) across plasmalemma and tonoplast in *Nitella translucens*. Intensity of fluorescence exciting light was of the order of 10^{-11} nEinsteins cm^{-2} sec^{-1}; intensity of actinic light was approx. 3 nEinsteins cm^{-2} sec^{-1}; a downward movement of the potential recording means an increase in potential (less negative) (after Vredenberg, 1971).

of the membrane opposite to that during PS decay and the fluorescence yield rises. The inhibitory effect of the membrane potential becomes apparent again at M just as it does at P.

The fluorescence decline during MT is due to the same reasons as those causing PS except for one major difference. Although the potential gradient collapses and high-energy intermediate builds up just as in P to S, the rate of O_2 evolution remains invariable due to an enzymatic saturation in the CO_2 reduction cycle that does not permit a further rise in the rate of electron transport.

In recent years, evidence has accumulated for the existence of changes in the membrane potentials during the slow fluorescence transient. Vredenberg (1971) has made simultaneous measurements of membrane potentials across plasmalemma and tonoplast and the slow fluorescence transient PT. He found approximately parallel changes in the alga *Nitella translucens*—when the fluorescence yield declined, the membrane potential increased (less positive) (Fig. 12). If we assume that these changes reflect changes in the thylakoid membranes, Vredenberg's experiment may be a demonstration that in intact cells changes in light energy conversion—that lead to fluorescence yield changes—occur by a mechanism controlled by transport processes across the membranes.

5. Summary

Analyses of the fast fluorescence transient (discussed in Section 3) have yielded information regarding the excitation energy transfer, the working of the photosynthetic units, the primary photochemistry of pigment system II, the pool of intersystem intermediates, and the interaction of systems I and II.

The slow fluorescence induction, which remained a complex and enigmatic phenomena for many years, has begun to provide information concerning the changes in the physical status of the pigments *in vivo*. The notion that the dissipation of the Chl a excitation *in vivo* is subject to ionic and osmotic control, as well as to control by electron transport, has gained experimental support in recent years. In the future, we believe the Chl a fluorescence kinetics may be used for the elucidation of the processes that control the transport of excitation quanta, electrons and ions; Chl a is nature's "intrinsic" probe.

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