Light Emission by Plants and Bacteria

Edited by

GOVINDJEE
Departments of Physiology and Biophysics and Plant Biology
University of Illinois at Urbana–Champaign
Urbana, Illinois

JAN AMESZ
Department of Biophysics
Huygens Laboratory of the State University
Leiden, The Netherlands

DAVID CHARLES FORK
Department of Plant Biology
Carnegie Institution of Washington
Stanford, California

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Foreword

Studies of the absorption and emission of ultraviolet and visible radiation occupy a central position among the methodologies employed in the investigation of living systems at the molecular level. A detailed examination would indeed show that this position is likely to be a permanent one. On the one hand, we can count the elementary quanta in these spectral regions, and are thus close to reaching the physical limits of detection. On the other, quanta of visible and near-ultraviolet light correspond to the highest energies that can be absorbed without producing irreversible changes in the absorbing molecules. The number of organic chromophores that undergo photochemical reactions after absorption of visible or ultraviolet photons is only a very small fraction of those commonly found in organisms, while those that are rapidly and reversibly deactivated after excitation are in the majority, a distinction of importance in the evolutionary selection of the photochemical mechanisms that are at the basis of both vision and photosynthesis. As many of the reversibly deactivated chromophores have detectable fluorescence, we are not limited in our studies of light emission of plants and bacteria to those cases in which photochemistry competes with radiative deactivation. We can also analyze the far more common situations in which dark chemistry of all kinds competes with the emission of light. This favorable circumstance has been recognized and exploited for at least forty years.

In the 1920s and 1930s we began to understand the emission of "cold light" as various forms of luminescence came to be explained by the quantum theory. S. I. Wavilov showed that fluorescein solutions convert almost all of the photons of excited light into fluorescence, and E. Gaviola was able to demonstrate, at about the same time, that the lifetimes of photo-excited states in solution are a few nanoseconds, the length predicted by the classical radiation theory. Also at this time Francis Perrin recognized the importance of the polarization of the radiation as a quantitative measure of the molecular rotational motions in liquids, and Gaviola and P. Pringsheim demonstrated the existence of long-range electronic energy transfer among identical fluorophores in solution.

These observations and theoretical formulations are still the basis of most of today's experiments. Although our views on each of these observations have been considerably refined through the years, nothing of similar fundamental importance has been added since then. What has radically changed is our ability to observe these phenomena in the real world. While the classical observations listed above were limited to some of the strongest emitters, such as fluorescein, and only when studied under exceptionally favorable circumstances, we can now make similar measurements on virtually any system. In the past twenty years, developments in instrumentation to detect and measure optical phenomena have undergone a virtual revolution, exceeded only by the complementary growth in the techniques for the digital manipulation and analysis of the experimental data. Progress in the ability to make observations in the shortest time domains has been particularly impressive. Light emission is now studied by methods that exploit both the impulse response and the harmonic analysis, with the result that the pico- and nanosecond regions can be explored in such reliable detail that, paradoxically, we now know the molecular properties that find expression within these short times much better than those that emerge in micro- or milliseconds.

Absorption, fluorescence, and Raman spectroscopy methods, which involve the controlled interaction of radiation with systems of interest, are being extended daily to new domains of interest in biology, but the analysis of the spontaneous emission of light by plants and bacteria has a special place beside them. It reveals the natural biological activities in a fashion which requires little or no interference with the object under study, and is thus often crucial in the interpretation of experiments which involve the forced behavior of the systems.

This book presents a current view of the emission of light by plants and bacteria discussing its physical basis as well as its biological significance. From such an approach, biophysics is emerging as a distinct science, an accomplishment similar to that of biochemistry a generation ago. It may well be recognized in the future that in this emergence of biophysics the study of light from organisms and their derived components played a major role. Indeed, "in the beginning there was light."

GREGORIO WEBER
Department of Biochemistry
Roger Adams Laboratory
University of Illinois
Urbana, Illinois 61801

Preface

Many plants and bacteria emit light as a result of numerous different reactions. The light emitted by these organisms is of great importance in biology since it provides a nondestructive, noninvasive, extremely sensitive, and versatile tool for measuring and studying various reactions of the living system. This book deals with light emission from plants and bacteria, with special emphasis on light emission from photosynthetic organisms.

When a molecule absorbs a photon, it goes into an excited state. Deexcitation may occur by loss of energy as heat (internal conversion) or as light (prompt fluorescence). *Prompt fluorescence* lasts from picoseconds to hundreds of nanoseconds after the cessation of initial illumination. In some instances, of which photosynthesis is by far the most prominent example, excitation energy is stored in the form of chemical energy (photochemistry). If the excited state is created by chemical reactions, the emitted light is *chemiluminescence* (*bioluminescence* in living systems). *Delayed fluorescence* (also called delayed light emission) is a special type of chemiluminescence in which the substrates for the chemical reaction are produced as a consequence of photochemistry, and may last from nanoseconds to hours after the cessation of initial illumination. If light is emitted during the transition from a triplet state to a ground state, it is called *phosphorescence;* this emission has a lower energy, i.e., it occurs at a longer wavelength than the prompt fluorescence and lasts for a longer period of time (e.g., microseconds) after the cessation of initial illumination. *Thermoluminescence* is delayed fluorescence that is observed on heating of a preilluminated and cooled sample. This book includes discussions of all these types of light emission from biological samples.

During the past fifty years, chlorophyll or bacteriochlorophyll fluorescence has been extensively used to obtain information, among other things, on (1) the composition, organization, and orientation of the pigment systems; (2) the efficiency and the pathway of excitation energy transfer from one pigment to another; and the regulation of excitation energy distribution and redistribution among and within the

photosystems; (3) photosynthetic models and photochemical efficiencies; and (4) the nature of the early electron carriers, the times involved in these early electron-transport steps, and the sites of action of inhibitors. Since almost all photosynthetic reactions from the time light is absorbed until CO_2 is fixed (see Govindjee: "Bioenergetics of Photosynthesis," Academic Press, 1975; and Govindjee: "Photosynthesis," Volumes 1 and 2, Academic Press, 1982, all in the Cell Biology series) could potentially affect fluorescence, it is often a difficult task to interpret the data without extreme caution and understanding of all the parameters involved, especially when making practical applications. However, the time scale of measurement usually defines the reactions being monitored, and in many cases fluorescence monitors a single reaction with great precision and reliability. Examples of the above-mentioned uses can be found in several chapters in this book.

This volume is divided into six parts. Part I provides an introduction (Chapters 1–5), Part II (Chapters 6–12) deals with the relationship of light emission to the various photosynthetic reactions *in vivo,* Part III (Chapter 13) discusses bioluminescence, Part IV (Chapter 14) describes light emission from bacteriorhodopsin and rhodopsin, Part V (Chapters 15–18) deals with the special light emission characteristics and their relationship to specialized pigment systems in various groups of bacteria and plants, and Part VI (Chapter 19) deals with the practical applications of light emission from algae and higher plants.

L. N. M. Duysens (Chapter 1) discusses the historical development in the area of chlorophyll *a* (and bacteriochlorophyll) fluorescence and develops equations relating emission kinetics to the concentration of photosynthetic components. W. A. Arnold (Chapter 2) relates how he discovered delayed fluorescence, thermoluminescence, and the electric field effect on delayed light in plants. J. R. Norris and G. van Brakel (Chapter 3) summarize the energy trapping and the primary photochemistry in photosynthesis of purple bacteria, which is the best understood system to date. J. Lavorel, J. Breton, and M. Lutz (Chapter 4) present the principles of the methods of measurement of light emitted by photosynthetic systems. The authors have also included a discussion of resonance Raman measurements. Part I ends with a chapter by G. R. Seely and J. S. Connolly (Chapter 5) which deals with fluorescence of photosynthetic pigments *in vitro*. This chapter also provides an introduction to the physical processes of light absorption, singlet-state decay processes, excitation energy transfer, fluorescence lifetimes, quantum yields, and polarization of fluorescence, and helps the reader appreciate the potential of light emission measurements both *in vitro* and *in vivo*. N. Murata and Kimiyuki Satoh (Chapter 6) summarize absorption and

fluorescence emission by intact cells, chloroplasts, and chlorophyll–protein complexes. This chapter serves as an introduction to Chapters 16, 17, and 18. I. Moya, P. Sebban, and W. Haehnel (Chapter 7) discuss the results and significance of the lifetime of excited states and quantum yield of chlorophyll *a* (and bacteriochlorophyll) fluorescence *in vivo*. R. van Grondelle and J. Amesz (Chapter 8) present an introduction to excitation energy transfer in photosynthetic systems. This chapter provides the basis for excitation energy-transfer measurements mentioned in Chapters 15, 16, 17, and 18. A. J. Hoff (Chapter 9) provides a thorough discussion of triplets, phosphorescence, and magnetic resonance measurements. H. van Gorkom (Chapter 10) presents a brief but lucid discussion of the fluorescence measurements in the study of photosynthetic electron transfer. This chapter provides information on a topic that is of paramount importance to current research in several laboratories. P. A. Jursinic (Chapter 11) discusses the current concepts and status of delayed fluorescence. Part II ends with a chapter by P. V. Sane and A. W. Rutherford (Chapter 12), who summarize the current concepts and status of thermoluminescence from photosynthetic membranes. J. W. Hastings (Chapter 13) presents the current status of bioluminescence in both bacteria and dinoflagellates. The author also explains the similarities between bioluminescence and delayed fluorescence in this sole chapter of Part III. R. Govindjee and T. Ebrey (Chapter 14) provide a summary of light emission from bacteriorhodopsin and rhodopsin and relate these studies to the photocycle of these pigment proteins in this sole chapter of Part IV. J. Amesz and H. Vasmel (Chapter 15) discuss the absorption and fluorescence properties of photosynthetic bacteria (green as well as purple). D. C. Fork and P. Mohanty (Chapter 16) review the absorption and fluorescence properties of cyanobacteria, red algae, and cryptomonads and discuss the special features and unique properties of these interesting organisms. Govindjee and Kazuhiko Satoh (Chapter 17) discuss the absorption and fluorescence properties of all greenish (chlorophyll *b*–containing) and brownish (chlorophyll *c*–containing) algae. A brief discussion of evolution is also included. Part V ends with the chapter by J.-M. Briantais, C. Vernotte, G. H. Krause, and E. Weiss (Chapter 18), who discuss chlorophyll *a* fluorescence of higher plants, both from leaves as well as from chloroplasts. G. Renger and U. Schreiber (Chapter 19, the sole chapter of Part VI) summarize the practical applications of fluorimetric methods to algae and higher plant research. We hope that many more future applications are to come, and that this book will be used to obtain background knowledge in this growing field of biology.

This volume emphasizes the physiological, biophysical, and biochem-

ical relevance of light emission studies in plants and bacteria. The aim of most of the authors was to include a review of the historical developments of the major concepts, critical analysis of experimental approaches, and an exposition of recent findings. It is hoped that the individual chapters will serve as a reference source that integrates experimental results and theoretical considerations from a large number of research publications as well as from the authors' laboratories. Since both background and up-to-date information are included, this book should serve not only as a reference source for researchers but also as an introductory book for graduate and advanced undergraduate students in general biology, cell biology, microbiology, plant physiology, biochemistry, biophysics, and agriculture. We hope the readers will benefit from this book by absorbing the exciting information contained on light emission from both plants and bacteria.

A major setback in the preparation of this book was the untimely death of Professor Warren L. Butler, who was scheduled to write a chapter on "Photosynthetic Models." We felt that no one could replace Warren, so this chapter was omitted. We have, however, attempted to include the important concepts involved in the various chapters of this work. We are aware of Warren's pioneering research on several topics relevant to this volume: photosynthetic models and excitation energy transfer in normal and developing plants; absorption spectroscopy of highly scattering systems; lifetime and polarization of fluorescence; two-light effect on chlorophyll *a* fluorescence; inhibition and reconstitution of electron transport in photosynthesis; low-temperature reactions; the redox potential and function of primary electron acceptors; quenching of chlorophyll *a* fluorescence; and the role of cytochromes in photosynthesis. In view of this, and of the personal loss we all feel, we dedicate this book to the memory of our friend Warren L. Butler.

GOVINDJEE
JAN AMESZ
DAVID CHARLES FORK

Warren Butler and Photosynthesis: The Early La Jolla Years

Warren Butler in 1979. Photograph by Helga Ninnemann.

Warren Butler received a Bachelor of Science degree in physics from Reed College in 1949 and his Ph.D. in biophysics from the University of Chicago in 1955 as the last graduate student of James Franck. He joined the United States Department of Agriculture laboratory in Beltsville,

Maryland in 1956, and was a major participant with Harry Borthwick, Sterling Hendricks, Karl Norris, and Bill Siegelman in the initial isolation and biophysical characterization of phytochrome. The development of spectrophotometric instrumentation capable of measuring very small absorbance changes in extremely turbid and highly scattering samples was absolutely essential to these studies. These studies and all of Warren's subsequent work in photobiology utilized state-of-the-art sophistication in spectrophotometric techniques. The Charles F. Kettering research award given to Warren in 1963 recognized this work as well as that in chloroplast development and the origins of variable yield chlorophyll *a* fluorescence in chloroplasts. These studies and his subsequent research in photosynthetic electron transport and excitation energy transfers were recognized by Warren's elections in 1976 to membership in the National Academy of Sciences, to the American Academy of Arts and Sciences, and to the French Academy of Sciences as a Foreign Associate. He also received a Guggenheim Fellowship in 1977 to work with Paul Mathis at the Saclay laboratory in France.

A sabbatical in 1964–1965 with Britton Chance at the Johnson Foundation (JF) of the University of Pennsylvania served to form a bridge to studies on photosynthetic electron transport, which were a major focus of his subsequent work, and also a transition to an academic position as Professor of Biology at the University of California/San Diego (UCSD) in La Jolla. Warren worked with M. Avron and Chance at the JF on the function of the *b*-type cytochromes in the chloroplast electron chain. Warren thought highly of the perspective to the problem proposed by Avron, the use of uncouplers and ADP in "crossover" experiments. This approach to the respiratory chain had been pioneered by Chance. It was thought that a well-defined crossover site existed between cytochromes *b* and c_1-c in the respiratory chain. Oxidation of cytochrome *b* and reduction of the *c*-type were observed to accompany the addition of uncouplers or cofactors of phosphorylation.

Warren arrived at La Jolla from Pennsylvania in the fall of 1965. I arrived shortly after, and Warren assigned the cytochrome *b* problem to me. We searched for crossover effects in the chloroplast cytochrome system. The initial task was to identify the number and spectral peaks of the *b*-type cytochromes. Using the dual wavelength spectrophotometry technique that Warren had mastered at the JF, we eventually detected light-induced absorbance changes of two cytochromes in the *b*-spectral region, with reduced α-band maxima of the reduced cytochromes at 559–560 nm (cytochrome *b*-559) and 563 nm (cytochrome *b*-563 or b_6). Discussions held with Geoff Hind at a 1966 Brookhaven meeting proved helpful since, at that time, we were seeing mostly the cyt *b*-559, and he

the cyt *b*-563. Our paper on light-induced redox changes of the two *b* cytochromes was published shortly after Keith Boardman and Jan Anderson's on digitonin separation of *b*-559 (photosystem II) and *b*-563 (photosystem I). Warren continued to work on the function of cytochrome *b*-559. His final study on this problem was a valuable contribution concerning the role of high potential *b*-559 in O_2 evolution and the reconstitution of water-splitting activity in liposomes. It was presented at a symposium at the International Photosynthesis Congress in Brussels in the summer of 1983.

My direct collaboration with Warren ended with a study of the redox properties of the variable (chlorophyll *a*) fluorescence yield of photosystem II after Mike Cusanovich in Martin Kamen's lab taught us the titration technique. During our stay at La Jolla we (Bernie Epel, Helga Ninnemann, Dave Hopkins, Lee Pratt, Tak Yamashita, and I) learned from Warren the problems involved in measuring small absorbance changes in turbid samples and the artifacts associated with stray light, actinic light leak, and fluorescence. We also learned "Warren's principle," that the details of the construction and response of our instruments must be known inimately in order to avoid such artifacts. We worked on photosynthetic electron transport and phytochrome in the midst of the construction of the La Jolla campus.

Warren survived "a trial by fire" one day, while lecturing on cell biology to the first UCSD sophomores, which I prayed would never happen to me. The students rigged a tape recorder in the rafters which was tripped in the middle of Warren's lecture to provide a parallel, satirical version of the lecture. Warren was startled for a moment, and then finished the lecture masterfully by playing off the recorder as a second person on stage.

We all enjoyed the hospitality of Warren, Lila, and the children, as well as each other's company, on camping trips to the Anza Borrego desert. Warren was a gregarious host. This spirit carried over to scientific discussions. It added a special joy to scientific discussions with Warren, as well as to his public lectures.

Warren's death has deprived us of this spirit and his unique experimental insights. The photosynthetic research community is reminded of its loss whenever we gather at a meeting to discuss new findings and ideas in photosynthetic energy transduction.

WILLIAM A. CRAMER
Department of Biological Sciences
Purdue University
West Lafayette, Indiana 47907

I

Introduction

1

Introduction to (Bacterio)chlorophyll Emission: A Historical Perspective

LOUIS NICO MARIE DUYSENS
Department of Biophysics
Huygens Laboratory of the State University
2300 RA Leiden, The Netherlands

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ABBREVIATIONS AND SYMBOLS

ATP	Adenosine triphosphate
BChl	Bacteriochlorophyll
BPheo	Bacteriopheophytin
Chl	Chlorophyll
Cyt	Cytochrome
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea; diuron
I	Intermediate
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate

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P	Primary electron donor of reaction center
P680	Primary electron donor of photosystem II
P700	Primary electron donor of photosystem I
PQ	Plastoquinone
PC	Plastocyanin
PSII	Photosystem II; includes reaction center complex and associated pigments
Q or Q_A	First (plasto)quinone electron acceptor
RC	Reaction center
Tris	Tris(hydroxylmethyl)aminomethane

ABSTRACT

This chapter starts with a short description of the historical development of hypotheses about the properties of fluorescing pigments, the photosynthetic unit, and excitation energy transfer between pigments in photosynthesizing organisms. The concepts developed are essential for the interpretation of the emission phenomena.

The light emission from Chlorophyll *a* in photosystem II is strongly quenched by the oxidized states of the first plastoquinone acceptor, Q_A, and of the primary donor, P^+, but less quenched by the reduced states of these electron transfer components (see Fig. 1). The same is true for the corresponding states of the quinone acceptor in purple bacteria. However, in these bacteria the reduced state of the primary donor is a stronger quencher than the oxidized state. Other quenching factors are a high concentration of protons in the lumen of the thylakoid, a pigment distribution designated as state II and caused by prolonged illumination with light of wavelengths mainly absorbed by PSII, the oxidized equivalents of the plastoquinone pool, and carotenoid triplet states. These factors probably largely determine the emission kinetics in intact cells.

Equations are derived with which it is possible to relate the emission kinetics to the varying concentrations of the redox states of substances affecting the emission, and the range of validity of these equations is discussed. By using these equations it is possible to obtain quantitative information about the kinetics of photosynthetic redox components and the other factors mentioned in the preceding paragraph from measurements of the emission kinetics.

I. Introduction

For a long time it has been known that certain pigments, including the main photosynthetic pigment chlorophyll (Chl) *a*, emit upon excitation so-called fluorescence or luminescence light of wavelengths slightly beyond the longest wavelength absorption band. The (bacterio)chlorophyll [(B)Chl] emission occurs from the lowest excited electronic state, and is called fluorescence when it occurs directly after light absorption. Delayed fluorescence, delayed light emission, or luminescence occurs when the electronic excitation energy is converted into some other form of energy (e.g., that of a radical pair) and then is partly reconverted to the electronic excitation energy and emitted. The discovery of this phenomenon is described by Arnold (Chapter 2 in this volume).

With the onset of photosynthesis large changes in Chl emission occur; these were the first phenomena observed that were caused directly by photosynthetic reactions. It took a long time before these phenomena were correctly interpreted and the main factors responsible for the emission changes characterized and identified.

Among substances causing changes in Chl *a* emission yield are electron-transferring components of the photosynthetic reaction center. The efficiency of emission quenching of the oxidized form of such a component is different from that of the reduced form. Fluorescence phenomena provide information not only about the kinetics of redox components, which is often difficult to obtain by other methods, but also about regulatory reactions of photosynthesis.

The relatively large fluorescence changes can be measured in a technically simple manner and with a better signal-to-noise ratio than other properties, e.g., absorption changes. These are some of the reasons why measurement of fluorescence has been one of the methods most applied in the study of photosynthesis. Emission studies are frequently used in investigations of photosynthetic processes in intact cells and in parts of whole plants, which are difficult or impossible to study by absorption difference spectroscopy because of light scattering.

The historical development of hypotheses concerning the properties of fluorescing pigments, the photosynthetic unit, and energy transfer, which are necessary for a quantitative interpretation of the emission measurements, will be described. It seems that the major factors affecting emission have been identified. For details, see other chapters in this volume. Quantitative relations between the fluorescence yield and the concentrations of various quenching photosynthetic intermediates or states will be stressed here, since this subject has not been treated elsewhere in this volume. Emission and excitation spectroscopy, polarization, lifetime of fluorescence, and other microsecond or submicrosecond phenomena will not be discussed here. For these phenomena, see van Grondelle and Amesz (Chapter 8), Moya *et al.* (Chapter 7), and van Gorkom (Chapter 10).

II. Stokes and Successors; Fluorescence Characteristics of Photosynthetic Pigments

The first important study of the fluorescence of pigments, specifically photosynthetic pigments, was made by the great English theoretical and experimental physicist G. G. Stokes (1864), using the sun or a carbon arc as a light source, two small crossed prisms as dispersing elements, and his eye as the detector. His conclusions formed the basis for investiga-

tions of photosynthetic fluorescence, energy transfer, primary reactions, and photochemistry in general. These conclusions were that the shape of the fluorescence spectrum of a pure pigment is independent of the wavelength of the excitation light and that this spectrum occurs at and beyond the longest wavelength band of the absorption spectrum.

Stokes's observations were essentially qualitative, but he remarked that strong fluorescence was excited by wavelengths corresponding to the absorption bands of the pigments. Later investigators used photographic plates, but, probably because of technical difficulties, no useful results were obtained. Quantitative measurements of fluorescence became practically possible, although not routine, after the development of photoelectric cells and low-noise amplifiers, and became technically simple after the introduction of photomultipliers. In contradistinction to the retina, which is a two-dimensional detector, these zero-dimensional photoelectric devices require two monochromators, which each reject more than 95% of the light in principle available for the measurement. The processing of the data by hand was complicated and time-consuming (see, e.g., Duysens, 1952). These experiences prompted me to introduce analog-to-digital conversion and computer processing in photosynthetic research as soon as these techniques became commercially available. Two-dimensional detectors (such as special television cameras having a high dynamic range) and associated information processors have now become commercially available, so that in principle the large light losses mentioned above can be avoided with a corresponding gain in time or signal-to-noise ratio. For methods, see Lavorel *et al.,* Chapter 4, this volume.

With much better precision than was possible with the eye, the measurements with photoelectric devices showed that the shape of the fluorescence spectrum of a pigment was indeed, in general, independent of the exciting wavelength. In addition, it was shown that the number of quanta emitted was proportional to the number of quanta absorbed, independent of the wavelength of the absorbed quanta, and the proportionality factor was defined as the fluorescence quantum yield. With the exception of the carotenoids, all photosynthetic pigments have a fluorescence yield in solution of 20% or more (see Seely and Connolly, Chapter 5, this volume).

Exact quantum mechanical calculations and predictions of absorption and fluorescence properties of pigments are generally beyond the capacity of computers, but approximate calculations based on simplified models provide insight in the properties found experimentally and are heuristically useful (Turro, 1978).

Except for a displacement of the maximum to a longer wavelength, the shape of the fluorescence spectrum of Chl *a in vivo* is similar to that

in vitro. However, the *in vivo* fluorescence yield, which is approximately 3%, is about 10 times lower than that in solution (see, e.g., Latimer *et al.*, 1956). The yield in solution can be lowered by adding certain substances, called (fluorescence) quenchers, e.g., benzoquinone. In general, molecules interact with each other appreciably only if they are adjacent to each other or at distances of a few angstroms. However, if the excited molecule is in "resonance" with another pigment molecule, the excitation can be transferred to this molecule over distances well in excess of 10 Å, as discussed below. Quantitative interpretation of photosynthetic fluorescence phenomena became possible only after it was realized that the excited state causing fluorescence was transferred over several hundred Chl *a* molecules; the fluorescence yield can thus be strongly affected by the quenching or dequenching of only one of these molecules.

III. Fluorescence Kinetics of Oxygen-Evolving Organisms: The Kautsky Effect, a Gordian Knot

When dark-adapted photosynthetic cells are illuminated with light of constant intensity, the concentration of photosynthetic intermediates and the fluorescence yield change during a few minutes until a steady state is reached. The period of change is called the *induction period.* With light of an intensity about sufficient for saturation of photosynthesis, the Chl *a* fluorescence yield increases within less than a second by a factor of two or three to a maximum, and then, after one or a few oscillations, decreases in a time of the order of a minute to a steady state somewhat higher than the original dark yield. This phenomenon, discovered by Müller (1874), has been called the Kautsky effect, after the investigator who first made the extensive quantitative studies (see, e.g., Kautsky and Hirsch, 1931). After the exciting light is turned off, the fluorescence yield decreases within a second to the original value in the dark. In the period from 1930 to 1950 this was the only measurable phenomenon that could provide direct information about the mechanism of photosynthesis. Numerous studies were carried out in which the fluorescence yield was measured as a function of light intensity, added substances, or other parameters known to or thought to affect the rate of photosynthesis or the yield of Chl *a* fluorescence. Reviews of these investigations, giving data on fluorescence as a function of time and intensity, have been presented by J. Franck (1951), Rabinowitch (1951, 1956), and Wassink (1951).

Although much effort was devoted to the interpretation of the data, few if any of the hypotheses proposed found acceptance outside the "school" of origin. Kautsky, observing that the fluorescence yield tended

to be much higher after a period of anaerobiosis, proposed a molecular oxygen complex as a photosynthetic intermediate (see Rabinowitch, 1956, p. 1428). It is rather remarkable that in a recent publication of ours (van Best and Duysens, 1975), in which results were obtained which could partly explain the oxygen effects observed by Kautsky, his hypothesis was not even mentioned, having been forgotten or rather bypassed. Anaerobicity was used simply as a method for studying the interaction between the electron acceptor Q_A of photosystem II (PSII) and the intermediates between PSI and Q_A (see Fig. 1). For background information on this interaction, see Clayton (1980, pp. 51–58) and Govindjee and Govindjee (1975). Van Best and Duysens noted that in the presence of oxygen a slow dark oxidation of the plastoquinol pool occurred. In its absence a slow reduction by internal hydrogen donors predominated. Since reduced Q_A, which causes a high fluorescence yield (see below), is rapidly oxidized by the oxidized plastoquinone pool, the fluorescence yield increases more rapidly on illumination after a period of anaerobicity than in the presence of air.

J. Franck (1951) used as a starting point for his interpretation the photochemical properties of Chl observed in solution. He assumed that in photosynthesis Chl *a* fluorescence was not directly influenced by the photochemical reaction, because he believed that Chl *a* reacted from a lower state, presumably the triplet state, as had been found for certain reactions in solution. The observed increases in fluorescence yield were explained by the formation of hypothetical substances, "narcotics," which were formed in side reactions and which increased the yield by attachment to the Mg of the Chl molecule. Now it is clear that the binding of Chl in specific ways to special proteins provides it with properties different from those in solution.

In an early paper, Kautsky and U. Franck (1943) reported a decrease of the high fluorescence on illumination, under anaerobic conditions, of the green alga *Ulva lactuca*. They proposed that this quenching is caused by another light reaction, different from the light reaction causing an increase of the low fluorescence after a period of darkness under aerobic conditions. As later experiments indicated (see Duysens and Sweers, 1963), this decrease probably was caused by a second photosynthetic reaction, but Wassink (1951) suggested the then plausible possibility that the rapid quenching observed by Kautsky and Franck was caused by a side reaction.

The interpretations of the fluorescence phenomena by Kautsky (Kautsky and U. Franck, 1943), J. Franck (1951), and Wassink (1951) were completely different. Perhaps the main reason for this disagreement was that the kinetics observed in different species and under dif-

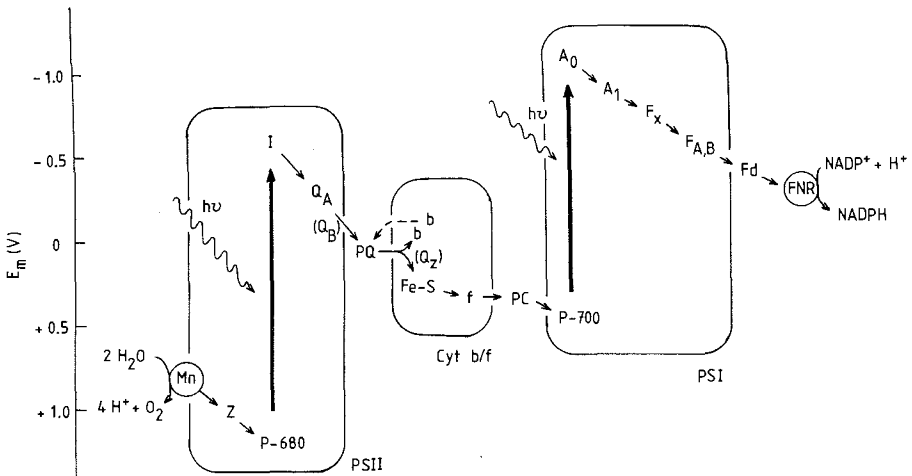

FIG. 1. Scheme of electron transfer in oxygen-evolving organisms. The boxes represent protein complexes embedded in a closed membrane, the thylakoid. The letters indicate prosthetic groups participating in electron transfer, their redox potential being indicated on the vertical axis at the left. PQ is the plastoquinone pool, whose molecules can freely move in the thylakoid membrane between photosystem II complexes (PSII) and cytochrome *b/f* complexes. There are about seven PQ molecules per PSII. The Q's (Q_A and Q_B) are plastoquinone molecules bound to PSII. In two-electron transfer and proton addition reactions driven by two excitations of PSII, Q_B (two-electron acceptor) is reduced via Q_A (one-electron acceptor) to plastoquinol, which exchanges with a PQ molecule of the pool. DCMU inhibits Q_B reduction. The plastoquinol molecule formed reduces, via the Cyt *b/f* complex and a plastocyanin pool (PC), the primary photooxidized donor P700 of PSI. The Cyt *b/f* complex contains two cytochrome *b*-563 molecules and one cytochrome *f* molecule, indicated by *b* and *f*, an iron–sulfur (Fe–S) protein, and a plastoquinone molecule (labeled Q_z. Excitation of PSI leads to the reduction of ferredoxin (Fd), which, via the enzyme FNR (ferredoxin-$NADP^+$ reductase), reduces $NADP^+$ or returns the electron to the Cyt *b/f* complex, resulting in a cyclic light-driven reaction involving PSI. The electron acceptors of PSI, preceding F_d, are iron–sulfur centers labeled F_x and F_A (F_B), A_1 (which may be a plastoquinone), and A_0 (which may be a Chl *a* molecule). Ferredoxin occurs in the aqueous space at the outside of the thylakoid, PC in the inside space. The photooxidized primary donor of PSII, P680, oxidizes water via Z (a plastoquinol molecule) and a manganese (Mn)-containing enzyme, which results in an oxygen molecule and four protons, set free at the inside of the thylakoid. The primary electron acceptor of PSII is designated by I (identified as a pheophytin molecule). The protons are taken up at the outside of the thylakoid in the plastoquinol-forming reaction. Cytochrome *b*-559 (not shown) is associated with PSII. The energy accumulated in the proton gradient across the membrane is used for phosphorylation, when the protons leave the thylakoid via the ATP synthase. Also electron transport through the Cyt *b/f* complex results in phosphorylation via proton transport. Finally, NADPH and ATP are used for reducing CO_2 to carbohydrate. (Drawing from Dekker, 1985.)

ferent conditions were simultaneously affected by more factors than suspected. We now know that about 10 intermediates and states quench the Chl *a* fluorescence, and that the kinetics of these intermediates is influenced by an interplay of various photosynthetic reactions, including the regulatory ones.

Nevertheless, under certain conditions striking regularities were observed. Wassink (1951) stated: "In many cases a decrease of the yield of photosynthesis is accompanied by an increase in the yield of fluorescence." Such an antiparallel relationship between the yields of fluorescence and photosynthesis was observed by McAlister and Myers (1940), who found that upon illumination the rate of carbon dioxide fixation increased while the fluorescence intensity decreased, with similar but mirror-symmetrical kinetics. The authors remarked (p. 23): "The inverse relationship is one which would be expected if it were assumed that the intensity of fluorescence is always a constant fraction of that part of the energy absorbed by Chl which is not taken up by photochemical mechanisms. Although we have no factual basis for such an assumption, we shall adopt this point of view since it will simplify the discussion without greatly limiting its generality." However, a parallel relationship would occur if the fluorescence yield decreased when the photosynthetic yield was diminished by an inhibitor which acts by quenching the Chl fluorescence. As discussed in Sections II and IV, this consciously heuristic idea of complementarity proved to be fruitful because it suggested an inverse linear relationship, which was indeed later observed under certain conditions. This relationship can be derived (see below) from certain models for energy transfer in the pigment system. Thus, heuristic reasonings, even if based on only partly correct assumptions, are often useful in suggesting new experiments or new interpretations of data.

For a suspension of the green alga *Chlorella,* with light intensity as a variable parameter, Wassink *et al.* (1938) observed an approximately constant fluorescence yield, although for the highest intensities the yield of photosynthesis had dropped to less than half of that at low intensity. However, for the purple bacterium *Chromatium,* Wassink *et al.* (1942) observed an inverse relationship between the rate of uptake of hydrogen donor and the BChl fluorescence yield. Wassink and co-workers did not check whether the relationship between fluorescence yield and photosynthetic rate was linear, but these experiments suggested the use of purple bacteria in later fluorescence studies (see Section VI). In the following the term *complementary relationship* will be reserved for the *inverse linear* relationship.

IV. The Complementary Relationship between the Quantum Yields of Fluorescence and of the Photochemical Reaction

Evidence for a complementary relationship with a precision of a few percent between the Chl *a* fluorescence and the rate of oxygen evolution was first obtained by Delosme *et al.* (1959) for the green alga *Chlorella* in the first 5 s of induction at constant intensity.

$$V = c - af \tag{1}$$

In this equation V is the rate of oxygen evolution, f is the fluorescence yield, and c and a are positive constants. By integrating this equation as a function of time, the equation for the so-called complementary area is obtained (also see Lavorel *et al.*, Chapter 4, this volume). For constant light intensity I, the fluorescence yield is maximal ($f = f_{max}$) for $V = 0$. Equation (1) can then be written as: $a(f_{max} - f) = V$. The time integral between t_0 and t_1 of the left-hand side of this equation represents the complementary area, which, in the experimental time curve of the fluorescence, is the area between the horizontal line $f = f_{max}$ and the curve $f = f(t)$ in the time interval t_0 to t_1. The time integral of the right-hand side may be considered proportional to the photochemical production in this time interval.

In general, the complementary relation will not be valid if the pigment system is heterogeneous. For example, if this system consists of two independent systems 1 and 2, which have complementary equations with different a's and f's, the total photochemical production is

$$V_1 + V_2 = c_1 + c_2 - (a_1 f_1 + a_2 f_2)$$

Since the expression in parentheses cannot be written as $a'(f_1 + f_2)$, the preceding equation is different from the complementary relation.

V. Transfer of Excitation Energy and Its Effect on Fluorescence Quenching

As will be discussed below, photosynthetic fluorescence phenomena can be interpreted only on the basis of a model for energy transfer.

In 1932 Emerson and Arnold observed that in short saturating light flashes, spaced at least 10 ms apart, the yield per flash amounted to a maximum of one carbon dioxide or oxygen molecule evolved per 2400 Chl molecules. This phenomenon was explained by the assumption that

one carbon dioxide-reducing enzyme was present per 2400 Chl molecules, and that each enzyme molecule required at least 10 ms for this reduction. This enzyme molecule was called a unit, but Gaffron and Wohl (1936a,b) called the 2400 Chl molecules plus the enzyme molecule the (photosynthetic) unit and suggested that "the energy absorbed at an arbitrary place in the unit fluctuates very rapidly through this unit until it is trapped by the reducing enzyme." Wohl (1937), although not excluding other explanations of Emerson and Arnold's experiments, suggested that energy transfer may take place via the 2400 Chl molecules in the form of excitation energy, but at that time no physical theory was available for describing such a transfer quantitatively.

In 1948 Förster published a quantum mechanical theory which allowed the calculation of the number of transfers between pigment molecules from experimentally determined quantities. These quantities are the distance between the molecules, the *overlap integral* between the fluorescence spectrum of the energy-transferring molecule and the absorption spectrum of the energy-receiving molecule, and the fluorescence yield of the former molecule. Förster, assuming incorrectly an *in vivo* fluorescence yield of 30%, estimated that the excitation could be transferred over 10,000 Chl *a* molecules. Franck and Livingston (1949), using too small a yield of 0.1%, estimated that the number of transfers was about 10, which was considered to support an earlier proposal by Franck that transfer to the enzyme mentioned occurred in the form of an intermediate generated by each excited Chl molecule. The 0.1% percent yield was taken from a publication in which several incorrect assumptions were made. Using a corrected value of about 1%, the number of transfers of excitation was estimated to be about 800 in the state f_{max} (Duysens, 1952). Gaffron and Wohl's unit hypothesis was revived in the following form, which proved to be very fruitful for suggesting and interpreting fluorescence and other experiments (see the following sections). In plants, excitation energy is transferred via Chl molecules to a pigment, P, which absorbs in the region of the Chl fluorescence spectrum, so that efficient energy transfer from Chl to P is possible according to the Förster mechanism. In photosynthetic bacteria, bacteriochlorophyll (BChl) replaces Chl and the size of the unit is smaller. The primary reaction of photosynthesis is the transfer of an electron from excited P to an electron acceptor (see Norris and van Brakel, Chapter 3, this volume). The excitation of the unit has a lifetime of about 1 ns, which requires the acceptor to be present in a complex with P; this complex was called the reaction center (complex). Since the reaction is assumed to be a 1-quantum reaction and not a 10-quantum one (the quantum requirement for oxygen evolution; see Emerson, 1958), the unit size is 2400/10,

i.e., 240 Chl molecules per reaction center, allowing a high efficiency of trapping.

VI. Quenching of Emission in Purple Bacteria

Application of the hypotheses formulated in Section V to purple bacteria resulted in the following predictions (Duysens, 1952). Light energy absorbed by an arbitrary BChl molecule, later called an antenna molecule, is transferred via other antenna molecules to a reaction center molecule, P. On the basis of the Förster equation, efficient transfer was calculated to occur for the estimated distances between the BChl molecules if the ratio P/BChl was not much less than 1%; in addition, P should have an appreciable extinction coefficient in the region of the fluorescence spectrum of BChl, i.e., around 900 nm, which would ensure good resonance or overlap between the fluorescence and the absorption spectrum. Because at that time I knew only that the hypothetical reaction center was a pigment, I called it P, an abbreviation now generally used for the primary donor in all photosynthetic reaction centers.

It had been observed by Wassink *et al.* (1942) that the fluorescence yield of a suspension of purple bacteria was higher for low concentrations of hydrogen donor than for higher concentrations. Since at low concentrations of hydrogen donor, P could be expected to accumulate in the oxidized state P^+, P^+ was assumed to quench the excitation less than P. This would be the case if P^+ had a lower extinction coefficient than P. In other words, in the region around 900 nm, a bleaching of not much less than 1/100 of the extinction of the long-wavelength BChl band should occur on illumination because of the oxidation of P, especially in the absence of a hydrogen donor. This bleaching should be correlated with the increase in fluorescence yield.

All of the predictions above have been verified experimentally (see Duysens, 1952; Duysens *et al.,* 1956; Vredenberg and Duysens, 1963). Indeed, Vredenburg and Duysens found, for a purple bacterium, that a decrease of the absorption band around 890 nm, caused by P oxidation, was correlated with an increase in fluorescence yield; the inverse of the fluorescence yield was a linearly decreasing function of the fraction of reaction centers in the state P^+.

A simple model is given below for which exact equations have been derived, which have a form similar to that of largely heuristic equations often used in the literature. These equations will be applied to the pigment system of purple bacteria, but may be generalized to be applicable to other systems.

After light absorption and transfer by antenna pigment molecules, excited P, P*, is formed. From P* an electron is transferred in less than 1 ns via an intermediate acceptor bacteriopheophytin (BPheo), I, to a ubiquinone acceptor, Q, which is reduced to the semiquinone form, Q^- (see Parson and Ke, 1982, for a discussion of the primary photochemistry of photosynthesis). In light of moderate intensity the reaction centers occur predominantly in three states, PQ, PQ^-, and P^+Q, designated by the numbers or subscripts $k = 1, 2,$ and 3 (Kingma *et al.*, 1983). The concentration of the state P^+Q^- is low because of relatively rapid recombination or transfer of the charges. If the reaction centers are in state 3 or 2, the emission yields are about three or two times higher than in state 1. Let n_k be the fraction of reaction centers in state k ($k = 1$–3) and n_0 the number of BChl molecules per reaction center; then the inverse of the emission yield f, f^{-1}, is found experimentally to be a linear function of the n_k's, or

$$f = n_0 P_{e0} / \Sigma\, n_k P_{lk} \tag{2}$$

where Σ is the sum over the k's and the P's are constants; P_{e0} is the probability that the excitation of an antenna BChl molecule is converted into emission, and P_{lk} is the probability that the excitation of a BChl molecule of the antenna ($k = 0$) or a reaction center in the states $k = 1$–3 is photochemically converted or lost (except by transfer). The fractions n_2 and n_3 were measured by absorption spectroscopy, using the characteristic difference spectra of $Q^- - Q$ and $P^+ - P$.

Equation (2) can be derived in an exact and simple way if it is assumed that the energy transfer frequency is so high that the excitation will visit a representative part of the molecules of the matrix (Duysens, 1979). This implies that the energy will pass through reaction center pigments several times in the photoactive, or "open," state.

If we assume that the n_0 antenna molecules and the reaction center in its various states have equal emission probabilities, the total probability of emission for one round of transfer through a unit is given by the numerator of Eq. (2). A more precise expression for the numerator would be $\Sigma\, n_k P_{ek}$, where the P_{ek}'s are the emission probabilities of the states of the reaction centers. Usually, the numerator of Eq. (2) is correct well within 1%, since n_0 is large compared to 1 and the transfer rate is high.

The denominator of Eq. (2) represents the sum of the losses and thus the probability of total loss or conversion in one round of transfer. The right-hand side of the equation, which is the ratio of emission and deexcitation during one round of transfer through the unit, is by definition the emission yield, f. Analogously, it follows that the quantum yield

for the conversion of the photoactive state PIQ (state 1) of the reaction center, p, is given by

$$p = n_1 P_{11}/\Sigma \tag{3}$$

Σ being the same sum as that in the denominator of Eq. (2).

The emission probability P_{e0} in Eq. (2) can also be written as $k_{e0}/(k_{10} + k_h)$, where the various k's are the rate constants for emission, loss (emission and internal conversion), and hopping (transfer) frequencies for an excited antenna molecule. If a practically irreversible charge separation $P^*IQ \rightarrow P^+IQ^-$ occurs with rate constant k_{11}, then $P_{11} = k_{11}/(k_{11} + k_h)$. If these expressions are substituted on the right-hand sides of Eqs. (2) and (3) and the numerator and denominator are multiplied by $k_h + k_{10}$, Eq. (2) becomes

$$f = n_0 k_{e0}/\Sigma\, n_k k_{1k} \tag{2'}$$

In an analogous way an alternative equation (3′) can be obtained. In state PIQ^- in purple bacteria the charge separation is reversible, and then k_{12} is only a formal rate constant, which is a function of forward and backward rate constants. The equations can be simplified by noting that k_h is much larger than $n_0 k_{10}$ and all other k's.

More quenchers can be taken into account by adding further terms $n_k P_{1k}$ to the denominators of Eqs. (2) and (3). Examples of this are given in the following sections.

In the literature, pigment systems in which an excitation, absorbed anywhere, can in principle reach any reaction center have been called matrix or lake systems or connected photosynthetic unit systems (for a discussion of energy transfer, see Pearlstein, 1982). In such systems, different equations generally obtain for different patterns of energy transfer (see Duysens, 1979). The equations derived above are valid for models for which the conditions formulated under Eq. (2) obtain. We will call such a model a matrix model with rapid transfer, or simply a matrix model [see also paragraphs following Eq. (6)].

At least one system has been observed (see next section) which is not a matrix system described by Eqs. (2) and (3). This system consists of so-called separate units, between which no energy transfer occurs. If it is assumed that a unit consists of a reaction center, in either a strongly quenching photoactive state 1 or a weakly quenching nonphotoactive state 2, then the fluorescence yield is given by

$$f = n_1 f_1 + n_2 f_2 \tag{4}$$

where the f_k's are the fluorescence yields in states 1 and 2.

A model intermediate between the matrix and separate unit models is that of weakly connected units. Before being transferred from one unit to another, the excitation is assumed to visit the antenna molecules and the reaction center of the unit. If the excitation also visits a representative part of the units, the fluorescence yield is given by

$$f = \Sigma\, n_k M_{ek} / \Sigma\, n_k M_{1k} \qquad (5)$$

where the n's are the fractions of units with reaction centers in the states k, and the M's the probabilities for emission (e) and for trapping or loss (l) of excitation in these units.

If the photoactive state is state 1, the rate of photochemical trapping is

$$p = n_1 M_{11} / \Sigma\, n_k M_{1k} \qquad (6)$$

As follows from the derivation, Eqs. (1)–(6) are exact for identical separate units and for the matrix and connected unit models, if the excitation transfer rate is sufficiently high compared with the loss and trapping rates. More precise conditions have been given above. If these conditions are not fulfilled, these equations are correct only to a first approximation. If the simplifying conditions mentioned are relaxed, exact derivations become difficult and the equations become so complicated that even computer calculations may be impractical.

After this chapter was essentially completed, den Hollander and Duysens (1985) generalized Eq. (2) for a matrix model in which the rate constant for energy transfer was *not* assumed to be much larger than the rate constant for trapping. By exact random walk calculations it was shown that for a unit size larger than 50, to a great approximation the generalized Eq. (2) is obtained by replacing in Eq. (2) the constants P_{1k} ($k = 1, 2$) by

$$P'_{1k} = 1/[1.52 + (1 - P_{1k})/P_{1k}]$$

If, on the other hand, the rate constant of energy transfer is large compared to that of trapping, then $P_{1k} \ll 1$, P'_{1k} approximately equals P_{1k}, and the original Eq. (2) is obtained again.

Although the equations for the matrix and the other models are derived in different ways, the complementarity relation, Eq. (1), will be shown to be valid for both the matrix and the separate or connected unit models. Complementarity implies that only two states, say 1 and 2, are to be taken into account. After eliminating n_2 by substituting $n_2 = 1 - n_1$ and setting the other n's equal to 0, Eq. (5) can be written as

$$f = A + B/(C + n_1 D) \qquad (7)$$

where A, B, C, and D are constants. This expression is obtained by dividing the linear function in n_1 in the numerator of Eq. (5) by the corresponding linear function in the denominator. From Eq. (6) an expression with a similar right-hand side is obtained with different values for A and B but the same values for C and D. After elimination of the denominator $C + n_1D$ from the rewritten Eqs. (5) and (7), a linear relationship between f and V is obtained, which has been called the complementarity relation, Eq. (1). The same procedure can be applied to Eqs. (2) and (3), and an even simpler elimination is possible with the equations for the separate unit model. This shows that the complementarity relation is valid for all three models considered. This probably is not strictly correct for the exact equations derivable by means of random walk theory. As shown in the last paragraph of Section IV, the complementarity relation completely fails for a heterogeneous pigment system, e.g., one consisting of two independent photosystems with different properties. Such systems may be analyzed in terms of two or more homogeneous systems.

VII. Quenching of Photosystem II Fluorescence by Electron Acceptors; Yield

A. Fluorescence Quenching by Q_A

As mentioned in Section III, Kautsky and U. Franck (1943) had evidence that light reactions may cause not only a rapid increase but also a rapid decrease in the fluorescence of oxygen-evolving organisms. These experiments were extended by Kautsky *et al.* (1960), who discussed the idea of two light reactions, but not two photosystems, in photosynthesis. Govindjee *et al.* (1960) found in steady-state experiments that the sum of the Chl *a* fluorescence intensities excited by two beams of different wavelengths was larger than the total fluorescence excited by the two beams applied simultaneously; this indicated that the beams had qualitatively different effects and was consistent with the idea of two photosystems and two photoreactions (see also Butler, 1962). However, these effects were not usefully related to changes in components participating in the electron transfer chain, which was driven, as concluded from absorption difference spectroscopy (Duysens and Amesz, 1962; cf. Kok and Hoch, 1961), by two photochemical reactions in series.

Both pigment systems driving these reactions contain Chl *a*, but the Chl *a* of PSII, which is closely associated with oxygen evolution, has an appreciably higher fluorescence yield than that of PSI. Photosystem I has a stronger absorption than PSII at the red side of the long-wave-

length absorption band (i.e., beyond 690 nm). Photosystem II usually has stronger absorption than PSI in the bands of the accessory pigments, such as Chl *b* in higher plants and green algae and the phycobilins in red algae. Light of a wavelength mainly absorbed by PSI or II is called light I or II. Light I of moderate intensity oxidizes within less than a second the electron transfer components between the two photochemical reactions; light II brings about their reduction. One of these components is cytochrome (Cyt) *f*. DCMU (diuron), which is a powerful inhibitor specific for oxygen-evolving organisms, was found to block the reduction of cytochrome by light II, but not the oxidation by light I (see Fig. 1).

It was argued (Duysens and Sweers, 1963) that if the redox state of an electron transfer component between the two photochemical reactions directly affected the fluorescence yield, this yield should (because of the limited quantity of redox equivalents present) rapidly (within 1 s) increase and decrease when switching from light II to light I and vice versa. This phenomenon was indeed observed in all species of different groups studied. We note here that these experiments were performed with an apparatus employing a weak modulated "measuring" beam, by means of which the fluorescence yield changes were monitored independently of the stronger emissions caused by the actinic lights I and II (see Lavorel *et al.*, Chapter 4, this volume).

In the presence of DCMU, even at a low intensity, a rapid and major increase of the fluorescence yield occurred in the red alga *Porphyridium cruentum,* with only one quantum absorbed per ~150 Chl *a* molecules, a number of the same order as that of Chl molecules in a photosynthetic unit. For the green alga *Chlorella,* Kautsky *et al.* (1960) found, in the presence of another inhibitor, phenylurethane, a value of about 400 Chl *a* molecules.

On the basis of the experiments discussed in the two preceding paragraphs and other evidence, Duysens and Sweers (1963) hypothesized that the fluorescence yield (of PSII) was low when the acceptor of photoreaction II, called Q, was in the oxidized state, and high when it was in the reduced state, Q^-. They also concluded that DCMU inhibited electron transfer between Q and Cyt *f* (see Fig. 1). Q was later called Q_A.

B. Structure of the Pigment System of PSII

In the preceding section we discussed the pigment system in purple bacteria as a matrix system. Joliot and Joliot (1964), who first derived heuristically a special equation of the form of Eq. (6), concluded from the kinetics of the fluorescence and the rate of oxygen evolution that, in chloroplasts, PSII consisted of weakly connected units. Later it was

found that in spinach chloroplasts two different systems II were present. The major system was a matrix system, the so-called PSIIα system; the other, the PSIIβ system, consisted of separate units between which no energy transfer occurred (Melis and Homann, 1976; Melis and Duysens, 1979). For chloroplasts treated with Tris, van Gorkom *et al.* (1974) found that the equation for the matrix model obtained. Possibly, the fluorescence of the (small) separate unit system was quenched as a consequence of the Tris treatment. The fractions of reduced Q, in the two investigations mentioned last, were determined by absorption difference spectroscopy.

In *Chlorella,* the inverse of the fluorescence yield [Eq. (2)], $1/f$, was plotted as a function of the concentration, n_4, of the externally added quencher dinitrobenzene, in the states Q ($n = 1$) and Q^- ($n_2 = 1$). The effect of this quencher can be taken into account in the matrix model by adding a term $n_4 P_{14}$ to the denominator of Eqs. (2) and (3). In accordance with the matrix model, two parallel lines were obtained (Sonneveld *et al.,* 1980a). This result showed further that only a negligible part of the fluorescence was due to PSI.

These experiments indicate that in PSII a matrix system, called α, is present, and in addition, at least in higher plants, there is a smaller separate unit system, called β (see also van Gorkom, Chapter 10, this volume).

C. Fluorescence Quenching by Plastoquinone Pool

Between Q and Cyt *f*, an electron transfer pool of 5–10 plastoquinone molecules is present. Vernotte *et al.* (1979) found that the Chl fluorescence in state Q^- was 10–20% higher when this pool was reduced than when it was oxidized. However, if the chloroplasts were treated with detergents, plastoquinone belonging to the pool showed a much higher quenching than in untreated samples (van Gorkom *et al.,* 1974).

VIII. The State I to State II Shift

As stated above, upon excitation with light of moderate intensity, such as that readily obtained from a small slide projector with band filters, the intermediates of the photosynthetic electron transport chain may be expected to attain their quasi-steady-state concentrations rapidly (within 1 s). This follows from the fact that at such an intensity, more than 10 quanta/s are absorbed per reaction center, while the concentration of electron equivalents of intermediates is of the order of 10 per reaction center.

In the experiments discussed in Section VII, light II and light I were switched on and off within a few seconds, and rapid and relatively large changes in fluorescence yield occurred, caused by redox changes in Q_A. If light II was left on longer than a few seconds, however, the rapid initial increase in fluorescence was followed by a slow more or less oscillatory decrease, lasting for tenths of a second. Duysens and Sweers (1963) concluded that this decrease, because of its slowness, could not be caused exclusively by direct quenching by electron chain components, but must be caused by a slow side reaction. The state of low fluorescence, produced in light II within about 10 s, survived after darkening for tens of seconds. Later it was shown (see, e.g., Duysens and Talens, 1969) that this state was removed more rapidly by illuminating with light I than by darkening. It was also shown that the generation of the quenching state was promoted not by the presence of Q_A^-, but by the presence of a reduced intermediate between Q and reaction center I, and that the removal occurred when this intermediate was in the oxidized state. For reasons discussed below, I later called these states *(pigment) states* I and II (or 1 and 2) (see discussion by Duysens, 1972); Bonaventura and Myers (1969) had called these states *light states* I and II. With all Q_A in the reduced state, the fluorescence yield in pigment state I is roughly 30% higher than in state II.

Bonaventura and Myers (1969) concluded, from a comparison of fluorescence yield and oxygen production in the two states, that reaction center II received more light energy (had a higher cross section) in state I than in state II. The opposite was the case for reaction center I. Thus the pigment system adapts itself to the prevailing spectral distribution of the actinic illumination. At nonsaturating light intensities the photosynthetic efficiency is improved and the fluorescence intensity and thus possibly photodamage is reduced. It had been suggested earlier (Duysens and Sweers, 1963) that in state II part of acceptor Q was present in a nonphotoactive quenching form Q′. This hypothesis for explaining the low fluorescence yield in state II proved incorrect, since in state II the oxygen yield in short saturating light flashes, which is a measure of the amount of photoactive Q_A, was the same as in state I (Duysens, 1972); also consistent with the hypothesis of Bonaventura and Myers was the observation that the oxygen yield in nonsaturating flashes was 15% lower in state II than in state I. Independently, Murata (1969b) had found that at 80°K, in the red alga *P. cruentum,* the fluorescence band around 730 nm, which is associated with PSI, was somewhat higher in state II than in state I. The opposite was the case for the fluorescence bands at 685 and 695 nm, which are mainly due to PSII (see a review by Lavorel and Etienne, 1977, and chapters by Briantais *et al.,* Fork and Mohanty, and Govindjee and Satoh, this volume).

It was suggested (Duysens, 1972) that the transition from state I to state II consisted of "a change in the thylakoid membrane by which the pigments of the two reaction centers [i.e., the pigments associated with PSII and PSI] are moved closer towards each other," which quenches the fluorescence of the pigments, thus diminishing the fluorescence yield of PSII.

F. Bennett (see Bennett *et al.,* 1980) discovered that the shift from state II to state I was brought about by the phosphorylation of the so-called light-harvesting protein complex, which contains part of the antenna Chl molecules of PSII (see the review on membrane components by Kaplan and Arntzen, 1982). The negative charges of the phosphate groups may cause the phosphorylated Chl-bearing proteins to move from PSII to PSI. The phosphorylating enzyme is active only in the presence of an at least partly reduced plastoquinone pool, which identifies the reduced intermediate postulated earlier (see above) between photoreactions I and II. For further details, see Briantais *et al.* (Chapter 18, this volume).

At longer periods of not too weak illumination a superimposed decrease in fluorescence yield occurred, which had a cause quite different from that of the shift from state II to state I. This effect will be described in the next section.

IX. Fluorescence Quenching on Protonation of the Thylakoid Interior; Cation Effects

Several groups observed that the fluorescence of PSII was markedly (of the order of 50%) quenched on "energization" of the thylakoid membrane (see reviews by Papageorgiou, 1975, and Lavorel and Etienne, 1977). This quenching could be clearly demonstrated by illumination in the presence of DCMU and phenazine methosulfate. Wraight and Crofts (1970) observed that the quenching was reversed by eliminating the pH gradient rather than the membrane potential, and they concluded that the pH gradient was mainly responsible for the "high energy quenching." Recent experiments by Krause *et al.* (1982) support this conclusion (see Briantais *et al.,* Chapter 18, this volume). There is some evidence (Papageorgiou, 1975) that the Chl quenching may be an indirect effect of the pH on the protein to which the Chl is bound.

Another effect causing pigment shifts in isolated chloroplasts was discovered by Homann (1969) and Murata (1969a). When metal cations, specifically magnesium ions, were removed from chloroplasts by washing, the fluorescence yield in the state Q_A^- was appreciably lowered. The high yield could be restored by adding magnesium at a concentration of

a few millimoles per liter. There have been many publications on this subject, especially by Barber and co-workers (see, e.g., Barber, 1976), who demonstrated that in chloroplasts the phenomena could be explained in terms of electrostatic membrane effects, and that they depended not on the kind but on the charge of the cation. *In vivo,* rather high magnesium concentrations occur, which change on illumination, but they may not become low enough to decrease the fluorescence yield.

In many earlier publications the state I–II shift was attributed to the magnesium effect. If this were true, in intact cells during illumination with light II a higher concentration of magnesium would be expected outside the thylakoids, and thus a higher fluorescence yield would be expected in state Q_A^- than after a dark period. The opposite was observed. In intact cells presumably the phosphorylation of the proteins of the light-harvesting complex mainly determines the shift between pigment states I and II, although it cannot be excluded that magnesium effects also play a role *in vivo.*

X. Interactions between Various Quenching and Other Processes Affecting the Fluorescence Yield; Methods for Analysis

The quenching effects due to oxidation of the quinone acceptor Q_A, acidification of the intrathylakoid space (proton quenching), and the state I–II shift may be expected to occur simultaneously during photosynthesis, their relative contributions being determined by the state of the biological system and external conditions.

For a number of quenchers in purple bacteria (see Section VI) and in PSII (see Section VII), Eq. (2), based on a matrix model, has been found to describe the experimental data with good precision. In these experiments, the (relative) concentrations of intermediates were determined independently by means of absorption difference spectroscopy or another technique. Using Eq. (2), it is possible to determine the concentrations of a number of quenchers from measurements of fluorescence yields alone, e.g., under steady-state illumination or even when the metabolic state is changing, as during the induction period (Kautsky effect).

The fraction of reaction centers, q, with the acceptor in the state Q_A can be determined as follows. Let the rate of quenching by a "metabolic" quenching process, such as intrathylakoid acidification, be proportional to x. Then the fluorescence yield will be

$$f(q) = 1/(a + bq + x) \tag{8}$$

The values given for a, b, and x may be interpreted as follows (see Section II.). The value of a is proportional to the sum of the rates of losses in the antenna molecules and losses in the reaction centers with all acceptors Q_A in the reduced state; the value of b is proportional to the rate of trapping in the reaction centers with all centers in the state Q_A; and the value of x is proportional to the sum of the rates of all additional losses. The proportionality constants are the same in these cases.

Equation (8) was obtained from Eq. (2) by elimination of the fraction of reaction centers with acceptors in the reduced state, which equals $1 - q$.

After a period of darkness, $x = 0$ and $q = 1$. Shortly after a strong light pulse, $q = 0$. For the determination of a, b, x, and q, the following four measurements of the fluorescence yield are sufficient:

1. The maximum fluorescence yield after a strong light pulse:

$$f(0) = 1/a \tag{8a}$$

2. The fluorescence yield after a dark period:

$$f(1) = 1/(a + b) \tag{8b}$$

3. The fluorescence yield in the metabolic state with quenching x, after a strong short flash, when $q = 0$:

$$f(0, x) = 1/(a + x) \tag{8c}$$

4. The fluorescence yield before this strong flash:

$$f(q, x) = 1/(a + bq + x) \tag{8d}$$

From these equations and the corresponding four fluorescence yield measurements, a, b, x, and q can be successively determined.

If the measured yields of the left-hand sides of Eqs. (8a)–(8d) are, e.g., 1.0, 0.25, 0.44, and 0.30, respectively, these equations give the following values for a, b, x, and q: 1, 3, 1.27, and 0.42. For the same values of a and b but $x = 0$ and $f(q) = 0.86$, it follows that $q = 0.054$. Although in both cases the ratio between the fluorescence quenching by q and the maximum fluorescence is 0.14, the fraction of q in the first case is much higher because of the nonlinearity of Eq. (8). Therefore the use of this ratio as a measure of q should be avoided.

Bradbury and Baker (1984) measured reproducibly fluorescence kinetics after a dark period (Kautsky effect). At a certain time, t, after the start of the constant-intensity illumination, a much stronger intensity was applied and the rapid fluorescence yield increase, ascribed to the reduction of Q_A, was determined. This experiment was repeated for

various times t. The fluorescence values used in the preceding section were estimated from kinetic curves (Bradbury and Baker, 1984) with the exception of that of $f(1, 0)$, which was assumed because it could not be read from the figures.

A better method for reducing Q_A is to use a saturating short flash, e.g., a xenon flash, followed after about 50 μs by a short weak flash for measuring the fluorescence yield (Sonneveld *et al.*, 1980b). The disadvantage of the method in which relatively weak prolonged illumination is used is that not all Q_A may be reduced, but nevertheless more than one electron may be transported per reaction center, which may cause additional fluorescence yield changes.

At the relatively high intensities used by Bradbury and Baker, most of the x quenching may well have been proton quenching. Using chloroplasts, Horton (1983) showed that the kinetics of the fluorescence quenching under various conditions was qualitatively correlated with the quenching of the fluorescence of added aminoacridine, which is a measure of intrathylakoid proton accumulation. This proton accumulation was furthered by a high rate of electron transport and a high ATP/(ADP · phosphate) ratio. This is understandable, since protons are generated by electron transport and used up by phosphorylation.

In most experiments at a low light intensity, the x quenching in light II was probably caused by a pigment state I–II shift, since it was reversed more rapidly in light I than in darkness (Duysens and Talens, 1969).

Methods for determining the components of x *in vivo* during photosynthesis have not yet been tried, but the experiments and equations discussed in this section may suggest a number of approaches.

XI. Quenching of Photosystem II Fluorescence by the Oxidized Primary Electron Donor and a Carotenoid Triplet State

Butler *et al.* (1973) observed that the rise time of the fluorescence yield at liquid nitrogen temperature with continuous illumination was about the same as that of Cyt *b*-559 oxidation and longer than that of Q_A reduction indicated by "C550" absorption kinetics. These phenomena were explained by the assumptions that the PSII reaction center in the state P^+Q^-, just as in the state PQ, is a fluorescence quencher and that P^+ oxidizes Cyt *b*-559. In more direct experiments, fluorescence quenching by P^+ was indeed found under (unphysiological) conditions which strongly delayed P^+ reduction (see van Gorkom, Chapter 10, this volume).

In a paper submitted later than the 1973 paper, Butler (1972) suggested that the microsecond fluorescence rise at room temperature, occurring after a nanosecond light flash, was also caused by a microsecond P^+ reduction. However, this rise is largely caused by the disappearance of another quencher, a carotenoid triplet (see below). Correcting for this triplet quenching, Sonneveld *et al.* (1980b) obtained evidence that under physiological conditions P^+ is also a fluorescence quencher. It was concluded from the recovery kinetics of the Chl *a* fluorescence following excitation by a short flash, given in the state PQ at room temperature, that the primary electron donor oxidized in the first flash was reduced in about 30 ns, and that after a few flashes it was reduced in several hundred ns. These conclusions are similar to, although not identical with, recent conclusions based on absorption difference spectroscopy of P at 820 nm (Brettel *et al.*, 1984).

Another quencher can be clearly observed in a high-intensity xenon flash of about 15 μs by which Q_A is reduced in about 5 μs. After an initial rise of the fluorescence yield, a dramatic decrease in yield occurs. During this flash the concentration of the oxidized primary donor remains low because of its rapid reduction. The fluorescence decrease is caused by a carotenoid triplet formed by transfer from a Chl *a* triplet, which is formed from excited singlet Chl *a* with the same rate constants as in solution (den Haan, 1977; also see a review by Lavorel and Etienne, 1977). The carotenoid triplet decays in a few μs. For a discussion on triplets, see Hoff (Chapter 9, this volume).

Because of the rapidity of the back reactions of P^+ and the carotenoid triplet, the concentrations of these quenchers are negligible at intensities of the order of magnitude of sunlight, and these quenchers thus *do not* affect fluorescence phenomena at normal intensities.

XII. Concluding Remarks

For the study of PSII, measurement of the kinetics of chlorophyll fluorescence has been the most popular and, up to now, perhaps the most fruitful technique. The reason for this popularity is the relative ease of precise measurements: at the onset of illumination changes in fluorescence yield of a factor of two or more may occur. The existence of most of the known components and reactions of PSII has been revealed or foreshadowed by studies of the kinetics of chlorophyll emission. A mixed blessing is the fascinating and sometimes frustrating variety of components affecting these kinetics. Verification and characterization of components and reactions have often been possible by more direct

methods such as absorption difference spectroscopy. Verification has not yet been possible for a number of hypothetical intermediates and reactions, e.g., those responsible for the luminescence components decaying in 0.1 and 1 μs observed after excitation of PSII in state Q_A^- by a 30-ns laser flash (Sonneveld *et al.,* 1980a). For a discussion of luminescence, see Jursinic (Chapter 11, this volume).

We expect that further study of the fluorescence and luminescence kinetics in the μs and ns time ranges, correlated with studies of absorption kinetics, will help to elucidate important new details of the mechanism of the primary and associated reactions of PSII and other photosynthetic systems.

In the longer time ranges numerous interesting fluorescence studies have been carried out. In the future such studies should become increasingly useful for following electron transfer, phosphorylation, and Calvin cycle and regulatory reactions in chloroplasts (see this volume, Chapter 18, Briantais *et al.*), intact cells (Amesz and Vasmel, Chapter 15; Fork and Mohanty, Chapter 16; Govindjee and Satoh, Chapter 17), whole leaves, and even plants under natural conditions (Renger and Schreiber, Chapter 19).

Finally, the reader is referred to Chapter 12 by Sane and Rutherford for a discussion of thermoluminescence. For non-Chl- or BChl-containing systems, consult Hastings (Chapter 13) (bioluminescence) and R. Govindjee and Ebrey (Chapter 14) (fluorescence of a unique class of bacteria, called halobacteria).

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Delayed Light, Glow Curves, and the Effects of Electric Fields

WILLIAM ARCHIBALD ARNOLD
Biology Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37830

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ABSTRACT

This chapter presents a short history of the discovery of delayed light, glow curves, and the effects of electric fields in photosynthetic systems.

I. Delayed Light

In 1950 I was in the Biology Division of Oak Ridge National Laboratory working on the problem of photosynthesis in green plants; the experiments involved the green alga *Chlorella* and isolated chloroplasts from higher plants. In June, Dr. Bernard Strehler came to the Laboratory. Strehler had a brand-new Ph.D., a tremendous amount of energy, and lots of ideas about almost everything. One day he appeared at the laboratory door and said, "Arnold, how would you like to make one of the fundamental discoveries in plant physiology?" My answer was "OK, if it won't take too long."

Strehler then explained that he had been thinking about photosynthesis and that it seemed to him that ATP (adenosine triphosphate) must be required and that it would be silly for the plants to use respiration as the source. So the chloroplasts must make ATP.

Strehler had done his thesis on bioluminescence (see Chapter 13 by Hastings, this volume) under the direction of Dr. William McElroy at the Johns Hopkins University. This work had led to the invention of a simple method of measuring ATP, and the device consisted of a small light-tight box with two compartments. A test tube could be put into the first compartment through a small light-tight door; then a shutter could be opened so that the photomultiplier in the second compartment could

Light Emission by Plants and Bacteria

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"see" the test tube. The test tube contained a "concoction" made from dried firefly tails that would emit light upon addition of ATP.

We mixed a suspension of chloroplasts with the firefly suspension, illuminated the mixture for a minute or so, and put it into the box. The photomultiplier gave a signal for some seconds. We thought at first that we had made Strehler's "fundamental discovery in plant physiology." But when we ran the controls, firefly suspension alone, we found that the light was coming from the chloroplasts. Simple experiments with colored-glass filters showed that the light emitted by the chloroplasts was red and was probably coming from chlorophyll.

Light emission from green plants *seconds* after illumination was totally unexpected. We knew from the absorption spectrum of chlorophyll that the natural lifetime was very short (10^{-8} s). The quantum yield of fluorescence in the living plant was only a few percent, so the lifetime must be 50–100 times smaller still.

Dr. Hans Gaffron (to whom Volume II of *Photosynthesis* was dedicated; see Govindjee, 1982) had told me about an experiment in which living green leaves had been glued to the top of a phonograph turntable. Light from a high-intensity incandescent lamp was filtered through several centimeters of copper sulfate solution and concentrated by a lens to a small spot on the surface of the leaves. By looking at the spot through a red filter (which did not pass the blue exciting light) the fluorescence of the chlorophyll could be seen. When the turntable was made to spin at a high rate the small red spot was not drawn out into a line. We now know that the delayed light is something like 100 times lower in intensity than is the fluorescence. Had the delayed light been a little brighter it would have been seen in the turntable experiment.

We then went to the University of Illinois at Urbana to talk with Dr. Robert Emerson and Dr. Eugene Rabinowitch about the meaning of the experiment.

By April 1951 we had enough information about this new light emission for Strehler to make an announcement at the Federation Meetings in Cleveland (see Strehler and Arnold, 1951a).

In 1954 Arnon, Allen, and Whatley (see Arnon *et al.*, 1954; Frenkel, 1954) showed that illuminated chloroplasts did indeed make ATP. Some years later Strehler repeated our original experiment with a filter on the photomultiplier so that it could not "see" the red light from chlorophyll but could see the firefly light. The experiment did show the ATP.

I will list some of the main findings about the delayed light. (1) The action spectrum for the production of delayed light is the same as that for photosystem II (PSII) (see Strehler and Arnold, 1951b). (2) The emission spectrum of delayed light is the same as that for the fluores-

cence (see Arnold and Davidson, 1954; Azzi, 1966). (3) There are two kinds of delayed light; the recombination light has a lifetime of 2–3 ms. That does not depend on the temperature. I will discuss this under Glow Curves. (4) The main fraction of the delayed light from 10^{-5} s to 3 h seems to be due to the formation of excited chlorophyll by the reversal of early steps in photosynthesis. (5) During the decay the intensity is roughly proportional to the reciprocal of the time in the dark (see Arnold, 1957; Strehler, 1957; Arnold and Davidson, 1963). (6) A plot of the logarithm of the intensity versus the logarithm of the time in the dark seems to show small waves, suggesting that the decay is made up of a number of different components. These waves were first seen by Dr. Walter Bertsch, who held a postdoctoral research position in our laboratory.

Results on delayed light emission, after the initial discovery, have been summarized by, among others, Lavorel (1975), Malkin (1977a,b), Govindjee and Jursinic (1979), and P. Jursinic (Chapter 11, this volume).

II. Glow Curves

I first learned about thermoluminescence in a geology course in college. An energy storage in crystals had been known since 1602 from the work of the alchemist Vincenzo Cascariolo. A large number of inorganic crystals, after irradiation with ultraviolet light or X-rays, were known to emit light on being heated. A number of people at Oak Ridge National Laboratory were making glow curves on small glass rods to estimate gamma rays, and I had been given a copy of the famous paper by Randall and Wilkins (1945). So to try to make glow curves on samples of green plants was obvious (see Arnold and Sherwood, 1957).

Since the delayed light was seen at room temperature the glow curve must start very much lower, e.g., at the temperature of dry ice or liquid nitrogen.

The curve made by heating the sample from the low temperature to 100°C consists of a half-dozen or so peaks. The idea that the delayed light consists of a number of components was made more convincing by this experiment than by the logarithmic plot of the decay curve (see Arnold and Azzi, 1971).

The theory given by Randall and Wilkins could be used to calculate activation energies for the various peaks, which seemed to be reasonable for the storage of energy in photosynthesis.

However, these low-temperature experiments had two surprises for us. Tollin and Calvin (1957) found that delayed light with a lifetime of 2

to 3 ms could be seen at temperatures below −100°C after a short bright flash (also see Tollin *et al.,* 1958; Arnold, 1977). After repeating the experiment a few times no more delayed light was emitted unless the sample was warmed to room temperature and refrozen in the dark. I have called this the *recombination light* since it seems to be made by the recombination of a free electron and a free hole. How this component is to be used in our picture of photosynthesis is not yet clear.

The second surprise was that Tatake *et al.* (1981) found that some peaks in the glow curve did not follow the Randall and Wilkins theory. This has now been explained (see DeVault *et al.,* 1983).

Reviews on the glow curves have been written by Inoue and Shibata (1982) and by Sane and Rutherford (Chapter 12, this volume), and they should be consulted for further details.

III. Effects of Electric Fields

The stimulation of delayed light by an electric field was found by the same method Strehler and I had used to find the delayed light. One plans an experiment to find something and then finds something else.

Jim Azzi and I tried a number of experiments to separate the components that make up the decay curve of delayed light. It occurred to me that an electric current passed through a chloroplast suspension might, by electrolysis, remove some of the components so that the ones left would stand out.

When we performed the experiment (Arnold and Azzi, 1977) we found that the voltage made the intensity of the delayed light *higher,* not lower as I had expected. By varying the concentration of the ions in the chloroplast suspension we found that the stimulation depended on the voltage and not on the current.

Chloroplasts in distilled water, where "blebs" form, makes the stimulation much larger. With the voltage *on,* the delayed light can be 10–100 times the intensity with no voltage.

I will list some of the main findings (Arnold and Azzi, 1977). (1) For the delayed light seen from 20 ms to 5 min after illumination, a given voltage makes the intensity a certain number of times larger. (2) The voltage does not affect the decay curve except to make the intensity higher. (3) The stimulation is very fast; 60-cycle ac shows 120 peaks per second. The peak in the delayed light signal is not more than 0.2 to 0.3 ms behind the peak in voltage. (4) Voltage gives very little if any stimulation of the delayed light at 2 ms (the recombination light). (5) Voltage *on* at the time of illumination has an effect on the delayed light, but this has not really been examined.

Most of the experiments that I have discussed above were made with green plant material, algae, leaf plugs, or isolated chloroplasts. A few experiments used blue-green algae, before they were called cyanobacteria. And a few used the purple bacteria. These experiments showed that the bacterial photosynthesis system also emits the delayed light (see Arnold and Thompson, 1956).

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3

Energy Trapping in Photosynthesis of Purple Bacteria

JAMES RUFUS NORRIS, Jr.
GERARD van BRAKEL
Chemistry Division
Argonne National Laboratory
Argonne, Illinois 60439

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ABBREVIATIONS AND SYMBOLS

ADMR	Absorption detected magnetic resonance
ATP	Adenosine triphosphate
BChl	Bacteriochlorophyll
BPheo	Bacteriopheophytin
Chl	Chlorophyll
ENDOR	Electron nuclear double resonance
EPR	Electron paramagnetic resonance
ESE	Electron spin echo
FDMR	Fluorescence detected magnetic resonance
hfc	Hyperfine coupling constant
$H-H_A$, $H-H_B$	BPheo where $H-H_A$ is the primary electron acceptor
$M-M_g$	"Special pair" donor BChl
Mg_A, Mg_B	Bridging intermediate acceptor BChl
NMR	Nuclear magnetic resonance
ODMR	Optically detected magnetic resonance
P870	Donor BChl *a* absorbing at 870 nm

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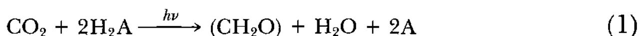
P960	Donor BChl *b* absorbing at 960 nm
P^F	Primary radical pair state of bacterial photosynthesis
$^1P^F$, $^3P^F$	Singlet and triplet P^F
P^R, $^3P^R$	Triplet states of the primary donor, P870 or P960
PS	Photosystem
Q_A, Q_B	Secondary and tertiary electron acceptors (quinones)
RC	Reaction center
RYDMR	Reaction yield detected magnetic resonance
$T\|0\rangle$, $T\|+\rangle$, $T\|-\rangle$	Triplet sublevels

ABSTRACT

Energy trapping is the fundamental process of photosynthesis. The crucial aspects of this complicated sequence of events involve chemical reactions, a series of rapid and efficient electron transfer steps that ultimately result in the harvesting of electromagnetic radiation in the form of biochemical energy. Emphasis is placed on the multistep nature of photosynthesis that leads to "permanent" capture of light energy. Prevention of back reactions and the achievement of high quantum yields are discussed in terms of the structure of the reaction center components. These components include electron donors and acceptors that function in the chemical trapping process. The possible functions of the protein in relation to the donor and acceptors are also discussed. The role of magnetic resonance in understanding the chemical trapping process is reviewed. Although the emphasis in this chapter is on photosynthesis of purple bacteria, the basic concepts are applicable to photosynthesis of all other organisms.

I. Introduction

Photosynthesis is the process by which green plants trap the energy of sunlight while assimilating CO_2 from the air. This complicated process produces the food for all animals, including man, and consequently is at the basis of all life. The overall process was summarized by van Niel (1931) as



$$CO_2 + 2H_2A \xrightarrow{h\nu} (CH_2O) + H_2O + 2A \quad (1)$$

In green plants 2A is equivalent to oxygen. However, A can be numerous other substances, including sulfur in sulfur bacteria, or many organic substances stemming from degradation of biological material. (CH_2O) represents the final product of carbon fixation used to store energy. Photosynthesis thus can be defined as the transformation of visible and near-visible light energy into chemical free energy for synthesis of biological material (see Govindjee, 1982, for further details). Within this fundamental definition of photosynthesis we will discuss some of the structural and mechanistic features of bacterial photosynthesis that result in the harvesting of electromagnetic radiation. For

earlier descriptions, see Okamura *et al.* (1982) and Parson and Ke (1982).

II. Energy Trapping in the Photosynthetic Process

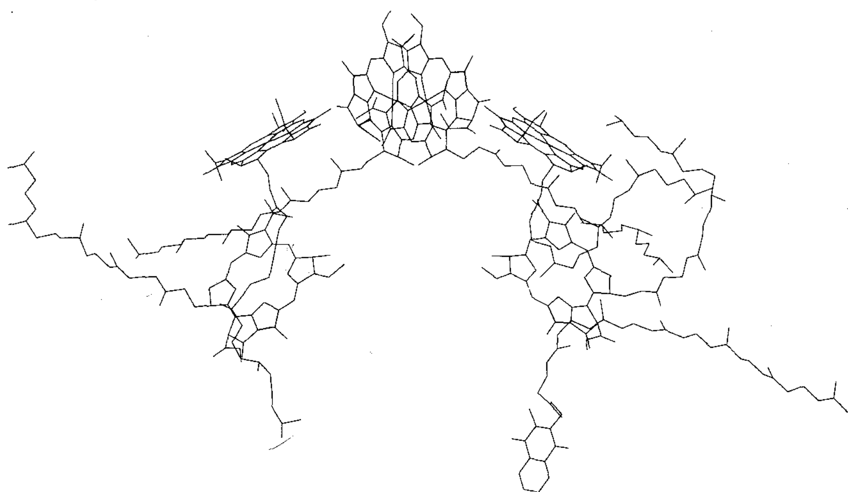
A. Fundamentals of Energy Trapping

The initial step of photosynthesis requires the absorption of photons by a pigment system. This "antenna" efficiently captures light by pseudolocalizing the energy as excited singlet states on a relatively small number of pigment molecules (Knox, 1977; Pearlstein, 1982, 1984). The captured energy rapidly migrates to a special protein complex known as a reaction center (RC) in which these pseudolocalized singlet states initiate electron transfer chemistry. Pairs of separated radicals are quickly formed by these electron transfer reactions (Fajer *et al.*, 1975; Kaufmann *et al.*, 1975; Parson *et al.*, 1975; Rockley *et al.*, 1975; Thurnauer *et al.*, 1975; Bowman *et al.*, 1981; Shuvalov and Parson, 1981; Norris *et al.*, 1982). Electron flow occurs across a membrane and chemically traps the energy. To complement the electron displacement, protons migrate across the membrane in the opposite direction, providing a proton gradient for ATP synthesis.

B. Primary Photochemistry in Bacterial Systems

The first photoinduced charge separation occurs in approximately 4 ps (Kaufmann *et al.*, 1975; Rockley *et al.*, 1975; Shuvalov and Parson, 1981) and results in the $^{1}P^{F}$ radical pair, an excited state composed primarily of bacteriochlorophyll (BChl) cation (M–Mg) and bacteriopheophytin (BPheo) anion ($H{-}H_A$) embedded in the RC protein complex (Fig. 1) (Fajer *et al.*, 1975; Parson *et al.*, 1975; Thurnauer *et al.*, 1975; Bowman *et al.*, 1981; Shuvalov and Parson, 1981; Norris *et al.*, 1982). In the next step after the radical pair formation the electron on the primary acceptor, BPheo ($H{-}H_A$) is quickly transferred to an additional acceptor molecule, a quinone (Q_A), in ~150 ps (Kaufmann *et al.*, 1975, Rockley *et al.*, 1975). The initial radical pair P^F lives much longer (~15 ns), as illustrated in Fig. 2, if the secondary acceptor Q_A is removed or reduced (Dutton *et al.*, 1972, 1973; Fajer *et al.*, 1975; Parson *et al.*, 1975; Thurnauer *et al.*, 1975; Bowman *et al.*, 1981; Shuvalov and Parson, 1981; Norris *et al.*, 1982).

The combination of rapid (~4 ps) charge separation and long lifetime (~15 ns) for the state P^F seems to require an additional, intermediate



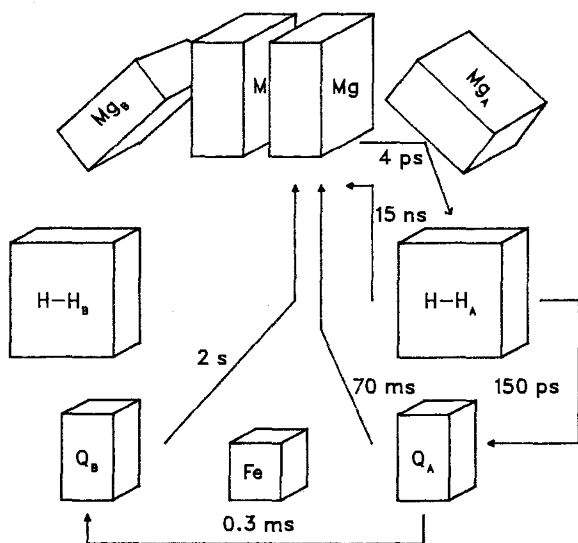


FIG. 1. (Top) Structure of the reaction center of the purple nonsulfur bacterium *Rhodopseudomonas* (*Rps.*) *viridis* as determined by X-ray analysis of the crystalline preparation. Only the components of the electron transport chain(s) are shown. (Redrawn from Deisenhofer *et al.*, 1984.) (Bottom) A simplified representation of the donor–acceptor complex based on the X-ray data (Deisenhofer *et al.*, 1984; Michel, 1982) and on spectroscopic data for *Rps. sphaeroides*. The rectangles define the aromatic ring systems of bacteriochlorophyll (M–Mg and Mg), bacteriopheophytin (H–H), the quinones (Q), which are ubiquinone and menaquinone, and Fe^{2+}. M–Mg is the primary electron donor, a dimer of bacteriochlorophyll *a* (*Rps. sphaeroides*) or *b* (*Rps. viridis*). Subscripts A and B label the two potential electron transfer pathways, of which only pathway A appears active. The arrows show the various electron transfer reactions with their half-times. Note that Q_B is absent in the crystal.

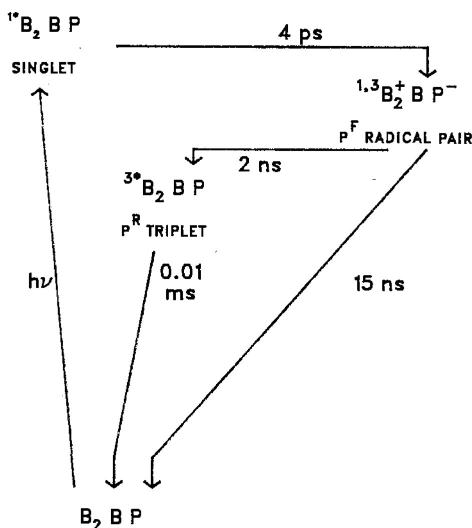


FIG. 2. Reactions in reaction centers of *Rps. sphaeroides* in which electron transfer to the secondary electron acceptor Q_A is blocked. B, Bacteriochlorophyll *a*; P, bacteriopheophytin *a*; 1, singlet; 3, triplet; * denotes an excited state.

state that may precede stable radical pair formation (Haberkorn *et al.*, 1979; Shuvalov and Parson, 1981; Schenck *et al.*, 1982). An additional BChl acceptor that mediates electron transfer from the primary donor to the primary acceptor $H{-}H_A$ is invoked, although not proved, an intermediate that leads to rapid charge separation but slow back reaction. In Fig. 1 this bridging intermediate is denoted by Mg_A. The geometric arrangement of BChl and BPheo is crucial to the pseudolocalization of energy in the RC as well as to the "permanent" trapping of this energy as charge separation.

The extent of charge separation associated with P^F is inadequate to ensure permanent chemical trapping so that additional electron transfer is required to obtain a stable condition. Secondary and tertiary chemical reactions increase the effective charge separation and thereby ultimately overcome back reactions. Secondary and tertiary charge separation involve quinone molecules (Q_A and Q_B) mediated by an Fe^{2+} ion (Fig. 1).

C. *Increasing the Lifetime of P^F*

Q_A can be prereduced or [in *Rhodopseudomonas* (*Rps.*) *sphaeroides*] chemically removed from the RC protein, thus preventing the additional charge separation. When Q_A cannot accept an electron, the initial

photoinduced charge separation (P^F) is eventually reversed. As a consequence, the electron on the primary acceptor $H-H_A$ returns to the primary donor M–Mg (Fig. 2) in a relatively slow reverse reaction requiring 10–20 ns. These blocked reaction centers provide a model system for the study of the primary chemical trapping mechanism. The longer-lived P^F state is often required in the study of photosynthetic charge separation by direct time domain electron magnetic techniques (see Section IV).

Reversal of charge separation state P^F as depicted in Fig. 2 involves two distinct routes, one with singlet electron spin (all electrons paired) and the other with triplet electron multiplicity (two unpaired electrons) (Thurnauer *et al.,* 1975). Obviously, charge separation and subsequent annihilation is rather complicated, even in this simplified model system, designed to focus on the early aspects of chemical trapping. Singlet annihilation of P^F directly results in the donor ground state, whereas the triplet back-reaction pathway of P^F produces the lowest excited triplet state of the donor, P^R. For a discussion of triplet states in photosynthesis, see Hoff (Chapter 9, this volume). Ultimately, P^R is restored to the ground state of the primary donor in about 10–100 μs. The scheme of Fig. 2 is insufficient to explain all observations. Back reactions involving $^3P^R$ and $^3P^F$ as well as additional states, perhaps states involving the bridging intermediate, are probably required (Shuvalov and Parson, 1981; S. G. Boxer, personal communication).

III. Components of Chemical Trapping

A. *Optical Aspects of the Primary Electron Donor*

One of the most unusual properties of the M–Mg primary donor complex is associated with the optical absorption properties of the reaction center. Since the optical maximum of monomeric BChl *a in vitro* is near 760 nm, the wavelength of P870 has been red-shifted from 760 nm to 860–870 nm in BChl *a*-containing bacteria. In organisms containing BChl *b* the primary donor absorbs near 960 nm, and is designated P960. The *in vivo* shifts result primarily from a combination of two mechanisms. (1) The primary donor consists of two BChl molecules folded over each other, and the resulting interaction between the two halves accounts for part of the shift. The molecular structure determined by X-ray diffraction of single crystals of purified reaction centers of *Rps. viridis* (Deisenhofer *et al.,* 1984) is consistent with a special pair of BChls inter-

acting via Mg–carbonyl bonds and macrocycle ring overlap. (2) The other component of the red shift mechanism invokes the environment, probably the proteins. Complementary to this overall view, model systems composed of BChl dimers have red-shifted optical properties quite similar to those of the primary donor (Scherz and Parson, 1984a,b).

Recent attempts to amplify these explanations for the redshift of the primary donor include the measurements of Davis *et al.* (1981) and the calculations of Eccles and Honig (1983) and Pancoska *et al.* (1983) on the effect of charges in the neighborhood of porphyrins. These charges may originate in amino acid residue of the surrounding proteins. Also, the possibility that the red shift is produced by a chemical alteration of a BChl molecule has been considered (Pearlstein *et al.,* 1982; Hanson, 1984; Hanson *et al.,* 1984).

High-resolution ENDOR studies can be interpreted in terms of strong interactions between the two monomers of the dimer in the form of a "supermolecule" (Lendzian *et al.,* 1981; Lubitz *et al.,* 1981, 1984a; Möbius *et al.,* 1982). As a result, the exciton bands of the special pair dimer may be difficult to calculate accurately. Strong interactions have been invoked in the red shift explanation of Scherz and Parson (1984a,b) mentioned above.

B. *Electron Acceptors*

1. BRIDGING INTERMEDIATE VERSUS PRIMARY ACCEPTOR

BPheo, labeled $H-H_A$ in Fig. 1, is the first unambiguous acceptor site of the electron from the primary donor (M–Mg in Fig. 1). However, an additional BChl, Mg_A of Fig. 1, may be required to transfer the electron from M–Mg to the acceptor $H-H_A$ as shown in Fig. 1. Whether the mechanism is "superexchange" involving Mg_A energy levels above M–Mg and $H-H_A$ or involves a true intermediate site of lower energy has not been established. In any case, removal of this intervening bridge without altering the location of donor or acceptor may result in slow or negligible electron transfer. Of considerable importance in this respect are the results of the X-ray diffraction studies, which support the existence of a "bridging" BChl molecule (Deisenhofer *et al.,* 1984). Finally, model compounds that mimic photosynthetic charge separation work via a bridging moiety (Moore *et al.,* 1984; Wasielewski and Niemczyk, 1984; Wasielewski *et al.,* 1985). However, Borisov *et al.* (1983) have given theoretical and experimental evidence against electron localization on BChl.

2. SECONDARY ACCEPTOR COMPLEX Q_A

The quinone Q_A of Fig. 1 is part of the secondary acceptor system necessary to ensure permanent energy trapping. *Rps. sphaeroides* and *Rhodospirillum (R.) rubrum* contain ubiquinone-10, *Chromatium (C.) vinosum* menaquinone (Feher and Okamura, 1978), and *Rps. viridis* Q_A is menaquinone-9 (Pucheu *et al.,* 1976; Gast *et al.,* 1985). Under normal photosynthetic circumstances, reduction of Q_A is very rapid (~100 to ~200 ps) and proceeds with near-unity quantum yield (Kaufmann *et al.,* 1975). The lifetime of reduced Q_A is about 35 μs in chromatophores (isolated membranes) (Bowyer *et al.,* 1979).

X-Ray and EPR data show (Deisenhofer *et al.,* 1984; Norris and Gast, 1985) that the RC contains two potential pathways for charge separation, labeled A and B in Fig. 1. The X-ray data clearly show that a single primary donor is shared by two quinones, Q_A and Q_B, and that at first sight bacterial photosynthesis could operate with two parallel pathways. This "dual-pathway model" would involve both quinones as equivalent electron acceptors. Such a model does not appear consistent with the bulk of the experimental evidence. The choice of a single pathway, the A pathway of Fig. 1, is most likely dictated primarily by the structure of the protein and not by the general symmetry of the donor–acceptor molecules. More details on the properties of the quinones can be found in Bowyer *et al.* (1979) and Wraight (1979).

3. TERTIARY ACCEPTOR COMPLEX Q_B

The various quinones in purple photosynthetic bacteria have been reviewed by Parson (1978). In *Rps. sphaeroides* both Q_A and Q_B are ubiquinone-10. In *C. vinosum* Q_B is also an ubiquinone, even though Q_A is a menaquinone (Halsey and Parson, 1974). In *Rps. viridis* the situation appears to be the same as in *C. vinosum* (Pfennig, 1978), while in *R. rubrum* both the secondary, Q_A, and tertiary, Q_B, acceptors are ubiquinones, despite the presence of varying amounts of rhodoquinone in the cell (Bodmer *et al.,* 1981).

Q_B functions as a "two-electron gate" (Wraight, 1979). Several schemes can be drawn for the subsequent reactions (see, e.g., Cramer and Crofts, 1982), but they all have in common the formation a semiquinone on the arrival of the first electron and a hydroquinone after the arrival of the second electron. Two protons must be abstracted from the surrounding medium and the reactions have different transfer rates and pH dependences.

In intact systems the reactions after the secondary acceptor are accompanied by the transfer of protons, which take over the role of the elec-

trons as energy transduction agents. A large amount of quinone, present at 25 molecules per RC and known as the quinone pool, serves this purpose (Parson, 1978).

4. Fe COMPLEX Q_A–Fe–Q_B

An Fe^{2+} ion is present in intact systems and interacts with Q_A and Q_B. The function of Fe^{2+} is still unclear, although many suggestions have been made (Dutton *et al.*, 1978; Dismukes *et al.*, 1984). The magnetic interaction between Fe^{2+} and the reduced state of Q_A appears to be slightly more than with Q_B, which may indicate that the iron is a little closer to Q_A. The distance between Q_A and the iron is estimated to be 7.0 ± 0.8 Å from EPR data (Dismukes *et al.*, 1984). The iron can be removed or replaced by other metals without interfering with the electron transfer from BPheo to Q_A (Feher *et al.*, 1972, 1974; Loach and Hall, 1972).

IV. Studies of Chemical Trapping by Magnetic Resonance Techniques

A. *Introduction to Magnetic Resonance in Photosynthesis*

Each reaction step of photosynthesis—primary to tertiary—involves species containing separated electrons that potentially have unpaired electron spins, which can be studied by EPR and related techniques. The time scale of magnetic resonance is rather slow, and consequently the experiment often necessitates blocking the normal flow of electrons in order to increase the yield and lifetime of a particular radical to permit resonance detection. A typical scheme of altered chemistry is depicted in Fig. 2.

The high-resolution technique that combines EPR and NRM is called ENDOR (electron nuclear double resonance). This technique and its application have been reviewed by Norris *et al.* (1978, 1979) and Hoff (1979, 1982b). ESE (electron spin echo), a pulsed form of EPR, has been reviewed by Thurnauer (1979) and Norris *et al.* (1980). Many magnetic resonance studies of photosynthesis are performed on cations and anion radicals of the donors and acceptors. However, the normal process of photosynthesis also produces radical pairs and triplet states that are suitable for study by EPR as well as by optically detected magnetic resonance (ODMR). One may consult the reviews of Clarke (1982) for a thorough examination of ODMR and of Schaafsma (1982) and Hoff

(1982a) for FDMR (fluorescence detected magnetic resonance). ADMR (absorption detected magnetic resonance; den Blanken and Hoff, 1982, 1983; den Blanken *et al.*, 1983; see also Hoff, Chapter 9, this volume) and RYDMR (reaction yield detected magnetic resonance), which has permitted the direct study of the primary radical pair state of bacterial photosynthesis (Bowman *et al.*, 1981; Norris *et al.*, 1982; Wasielewski *et al.*, 1983a,b, 1984), are recent modifications of ODMR. For recent aspects of radical pairs and related problems in photosynthesis see Boxer *et al.* (1983).

B. ENDOR of the Primary Donor

ENDOR has been used extensively to probe the dimeric nature of the cation of the "special pair." The group of Möbius has pioneered the application of high-resolution liquid solution ENDOR. The technique has been applied to RCs as well as to *in vitro* (B)Chl radical anions and cations. The high-resolution ENDOR results are still interpreted in the same basic terms as the older low-resolution data, i.e., in terms of a special pair of BChl as the primary donor (Lendzian *et al.*, 1981; Lubitz *et al.*, 1981, 1984; Möbius *et al.*, 1982). Feher and co-workers, using ^{15}N-enriched RCs, also measured liquid solution ENDOR of monomer BChl and RCs of *Rps. sphaeroides* (Lubitz *et al.*, 1984b). The measured ^{15}N hyperfine coupling constants (hfc's) obtained by this method appear to be more accurate than those measured by ESE (Bowman, 1984).

O'Malley and Babcock (1984a) have published low-temperature ENDOR results on spinach chloroplasts. Their work is notable because their interpretation involves a monomer structure of the primary donor in plants. However, they have also reached the same conclusion for bacteria (O'Malley and Babcock, 1984b). Their monomer model relies on molecular orbital calculations published by Petke *et al.* (1980) and invokes interpretations similar to the proposals of Davis *et al.* (1979) for the primary electron donor of photosystem (PS) II. Davis *et al.* proposed that the highest occupied molecular orbital of the radical cation of the primary donor of PSII is an environmentally mixed orbital. This monomer model predicts negative signs for the ^{15}N hfc's (Lubitz *et al.*, 1984b). Since positive signs have been determined by a general triple-resonance experiment (Lubitz *et al.*, 1984b), the monomer model must be rejected for the bacterial RCs. M. Plato and K. Möbius (personal communication) have shown that one may account for these positive signs (as well as for the observed magnitudes) by advanced molecular orbital calculations of special pairs.

C. Magnetic Resonance in Protein Single Crystals

Single crystals are required to maximize the structural information available from magnetic resonance measurements (Gast *et al.*, 1983; Allen and Feher, 1984; Gast and Norris, 1984; Norris and Gast, 1985). The EPR transitions of the triplet state of P^R (Fig. 3) can be determined quite precisely for single RC crystals (Fig. 4). For crystals from *Rps. viridis* and *Rps. sphaeroides* the angles between the crystal surfaces and the triplet x, y, and z axes have been measured (Gast *et al.*, 1983; Gast and Norris, 1984; Norris and Gast, 1985).

EPR studies of the RC protein crystal from *Rps. viridis* with a $P4_32_12$ symmetry (Michel, 1982) revealed that the protein has four magnetically nonequivalent sites and that the crystal has eight proteins per unit cell (Gast *et al.*, 1983; Gast and Norris, 1984; Norris and Gast, 1985). The EPR linewidths of the triplet signals for crystalline RCs are given in Table I, together with the linewidths of the corresponding cation signal for comparison. Although the method is not as sensitive for the cation as for the triplet, the g-anisotropy of the donor cation of *Rps. sphaeroides* has also been published (Allen and Feher, 1984).

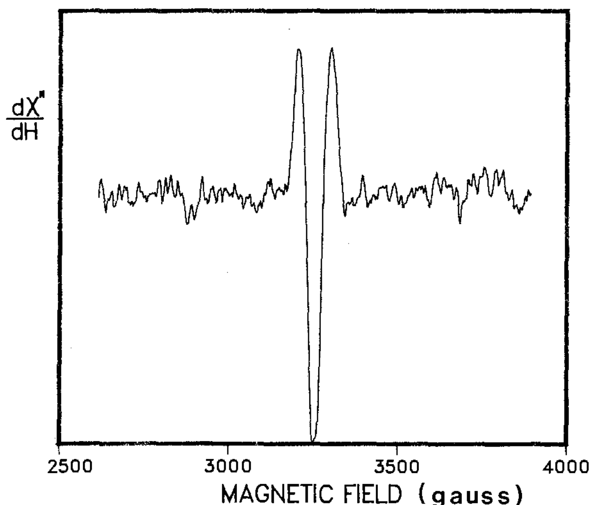


FIG. 3. A typical EPR first derivative spectrum (relative units) of the triplet state P^R in a single reaction center crystal of *Rps. sphaeroides*. The crystal is of the orthorhombic space group (Gast and Norris, 1984). The crystal is aligned so that the EPR magnetic field is along the long growth axis of the crystal. Because of the crystal symmetry only a single pair of lines is observed.

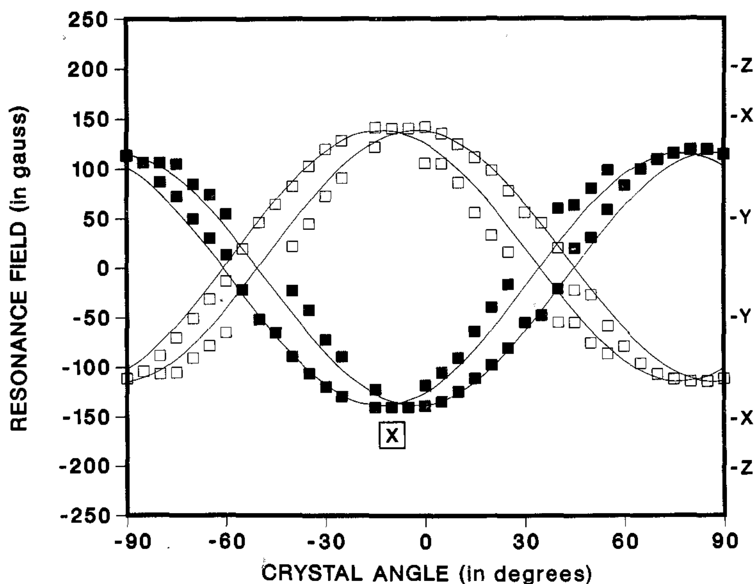


FIG. 4. EPR resonance field splittings for triplet P^R in single crystals of *Rps. sphaeroides* (Gast and Norris, 1984). The positions of the EPR resonance peaks are displayed as a function of the angle between crystal and magnetic field. Negative numbers refer to a reversed field direction. Crystal rotation is about the long growth axis of the crystal so that the applied external magnetic field remains perpendicular to the long axis. Note that even though there are four or eight triplet P^R's per unit cell, at most angles four EPR peaks (open and filled squares) are observed, two resonances ($T|0\rangle$ to $T|-1\rangle$ and $T|0\rangle$ to $T|+1\rangle$) for each angle, consistent with two magnetically inequivalent P^R's per unit cell. The solid lines are calculated. The boxed X indicates a position of the crystal and field where the magnetic field is almost aligned with one of the three triplet axes in the crystal, i.e., the triplet x axis.

TABLE I
Triplet versus Cation EPR Linewidths[a]

Species	EPR linewidth (gauss)	
	Triplet	Cation
Rps. viridis	11.3	11.5
^{2}H *Rps. viridis*	6.8	4.6
Rps. sphaeroides R-26	10.3	9.6

[a] Normal and deuterated crystalline reaction centers.

D. Optically Detected Magnetic Resonance in Photosynthesis

1. FUNDAMENTALS

In EPR, ENDOR, and ESE the magnetic resonance spectrum is obtained by directly measuring the absorption or emission of microwaves. However, in ODMR an optical property of the system is recorded while oscillating magnetic fields of microwaves irradiate the sample in tune with energy differences in the sample. The absorption of microwave photons is usually associated with electron spin flips within triplets or radical pairs. In many cases an external static magnetic field is used to adjust the size of the relevant energy gaps in the sample to match the energy of the microwave quantum. If the microwave energy produces a change in the sample's optical properties by changing the population scheme of the energy levels, then ODMR is possible. In this manner optical absorption can be applied to detect the magnetic resonance spectrum of the radical pair state P^F and the triplet state P^R associated with the photosynthetic process.

2. STANDARD ODMR AND THE TRIPLET STATE P^R

The study of the triplet state P^R is an attempt to learn more about the mechanism of charge separation and the structural nature of the primary donor. Standard ODMR requires unequal populations and decay rates of the three triplet spin sublevels. For this reason, it is performed at low temperatures ~2°K, where the populations of spin sublevels decay only by triplet-to-ground state processes and do not decay by sublevel-to-sublevel population transfer caused by thermal processes known as spin lattice relaxation. Microwaves can induce molecules at 2°K that occupy one of the thermally isolated spin sublevels which decays at one rate to jump to another isolated spin sublevel which decays at a different rate. Consequently, the effective triplet lifetime changes with the application of resonant microwaves. The resulting change in the amount of triplet is then detected by optical means. At higher temperatures the spin relaxation mechanisms result in a Boltzmann distribution between the populations; i.e., the sublevels have very nearly equal populations. Thus, at higher temperatures the sensitivity of standard ODMR, which depends on unequal populations of the triplet sublevels, is strongly decreased, and ODMR is difficult to perform at these higher temperatures.

A very important application of ODMR is absorption detected magnetic resonance (ADMR), in which "clean" singlet-minus-triplet absorption difference spectra are recorded (see Hoff, Chapter 9, this volume,

den Blanken and Hoff, 1982, 1983, and den Blanken *et al.*, 1983). Den Blanken and Hoff find basically similar difference spectra for all bacterial systems and from an analysis of these spectra conclude that on the "optical" time scale the triplet state excitation resides in a monomer half of the special pair dimer.

3. RYDMR OF THE PRIMARY RADICAL PAIR STATE P^F

RYDMR is a version of ODMR that is particularly useful in the detection of short-lived radical pairs such as P^F. The application of resonant (~9 GHz) microwave pulses to the P^F state in an external magnetic field is illustrated in Fig. 5. As in standard ODMR, the microwaves alter the population scheme of the energy sublevels and consequently change the reaction yield of the conversion of P^F to P^R via changes in the spin sublevel kinetics. A corresponding experimental setup is illustrated in Fig. 6. In Fig. 5 the relative triplet yield as a function of external mag-

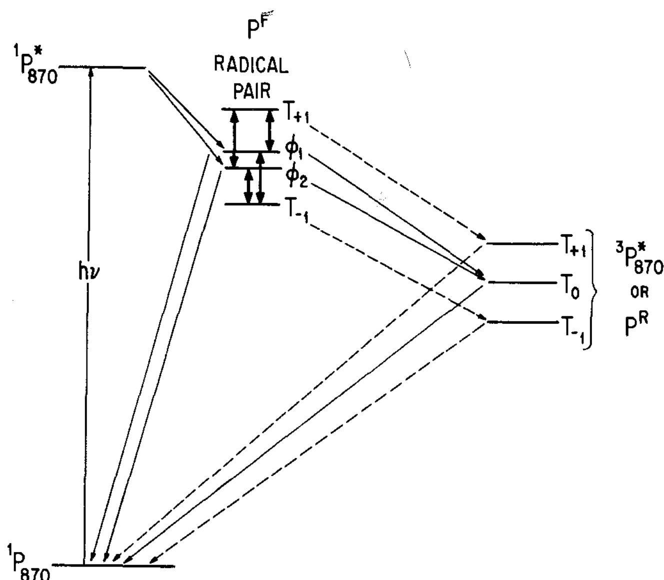


FIG. 5. RYDMR energy level diagram. The single-headed arrows with solid lines represent the decay pathway for P^F without microwaves (represented by double-headed arrows). The microwave transitions are effective only at certain magnetic fields when the radical pair levels $T|+\rangle$ and $T|-\rangle$ match the microwave energy photon, giving rise to spectra such as that in Fig. 6. The pathways represented by dashed lines with single-headed arrows represent the new kinetic pathways that take place in the presence of microwaves. These new routes can be detected by optically monitoring P^F or P^R, which forms the basis for RYDMR–ODMR.

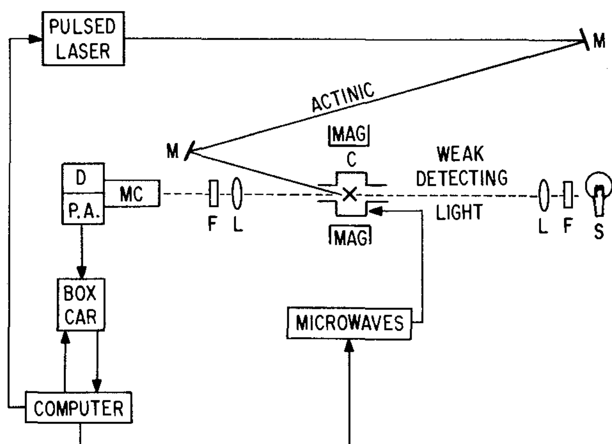


FIG. 6. A simple RYDMR spectrometer. M, Mirror; MAG, external magnet; F, light filter; L, optical lens; S, continuous optical source for measurement of the absorbance of the sample, X, with the beam of light represented by the dashed line; MC, monochromator; D, optical photon detector; P.A., preamplifier. The state P^F is prepared by light from a pulsed laser when a microwave pulse is in the microwave cavity, C.

netic field is monitored by optical absorption spectroscopy. RYDMR signals, i.e., an increase or decrease in the amount of P^F or P^R, are observed with magnetic fields that bring spin sublevels into resonance with the microwave energy as in Figs. 7, 8, and 9. Because P^F is so short-lived compared to the rate of spin lattice relaxation, RYDMR experiments can be performed at room temperature (Ruedin *et al.*, 1972; Frankevich and Pristupa, 1976; Anisimov *et al.*, 1979a,b; Molin *et al.*, 1980; Bowman *et al.*, 1981; Norris *et al.*, 1982).

The interpretation of P^F as a radical pair was initially founded on optical studies of P^F as well as on standard EPR studies of the P^R triplet state (Closs, 1969; Werner *et al.*, 1978; Haberkorn and Michel-Beyerle, 1979; Michel-Beyerle *et al.*, 1979, 1980; Chidsey *et al.*, 1980; Bowman *et al.*, 1981; Boxer *et al.*, 1982a,b; Doktorov *et al.*, 1982; Lersch *et al.*, 1982; Norris *et al.*, 1982; Ogrodnik *et al.*, 1982; Roelofs *et al.*, 1982; Tang and Norris, 1982, 1983). By showing that microwaves affect the state P^F in the manner expected for radical pairs, the RYDMR experiments provide direct evidence for the radical pair nature of P^F, and they demonstrate that $^3P^F$ is responsible for the generation of triplets in the primary photochemical act when the secondary acceptors are prereduced or removed.

Wasielewski *et al.* (1983b) have demonstrated that the lifetime of P^F in *Rps. sphaeroides* in blocked reaction centers could be shortened or length-

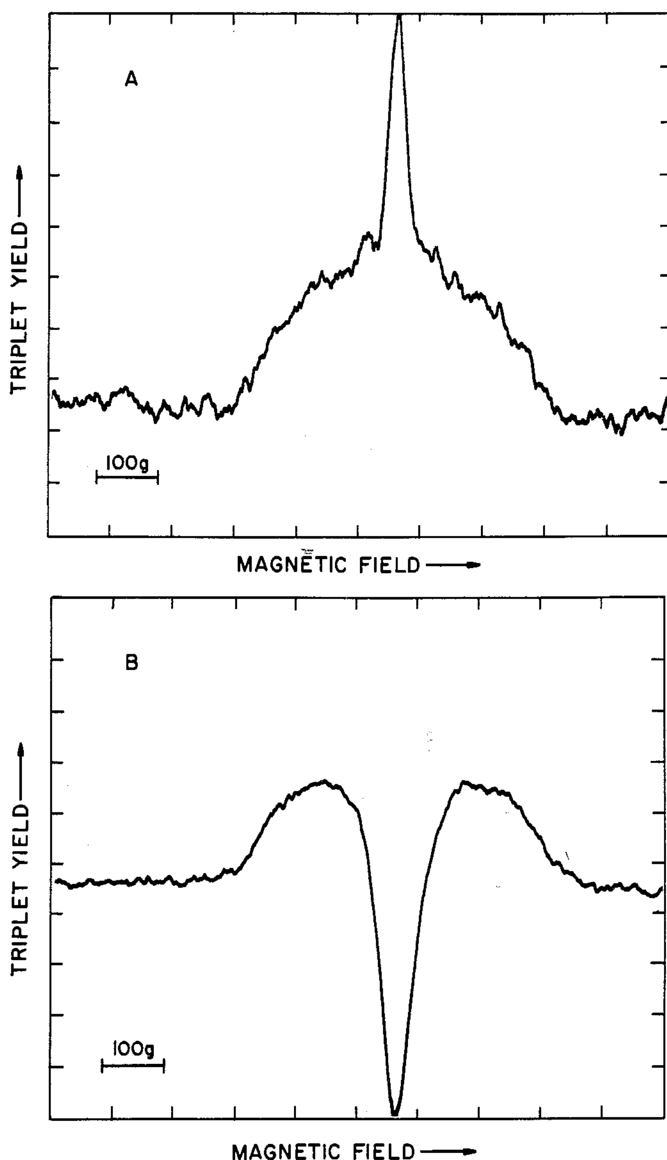


FIG. 7. Typical RYDMR–ODMR spectra. The vertical axis refers to the triplet yield (formation of P^R) in arbitrary units, obtained from measurement of the change in absorbance, and the horizontal axis gives the external magnetic field strength. The microwave power is about 200 W in (A) and about 2000 W in (B) (Bowman *et al.*, 1981).

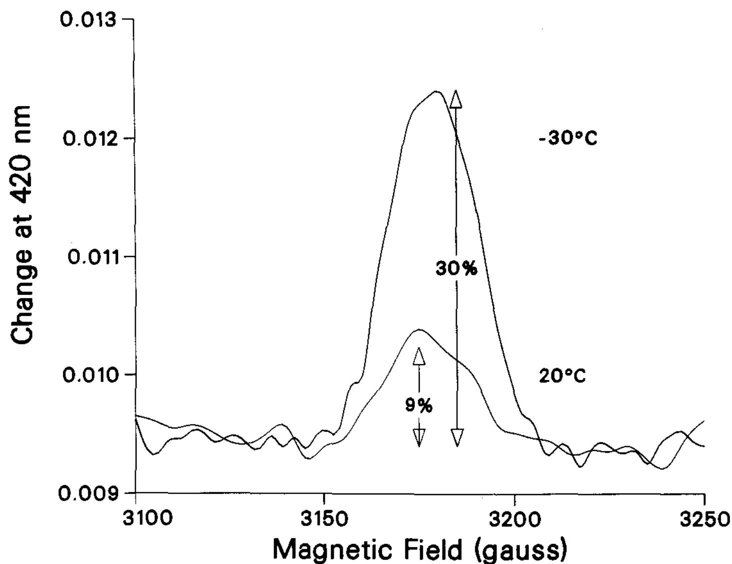


FIG. 8. Low microwave power (270 W) RYDMR spectra at 20° and −30°C of P^R in reaction centers of *Rps. sphaeroides* from which the secondary electron acceptor Q_A had been removed, monitored by the optical absorbance change at 420 nm. The percentages refer to the change in absorbance.

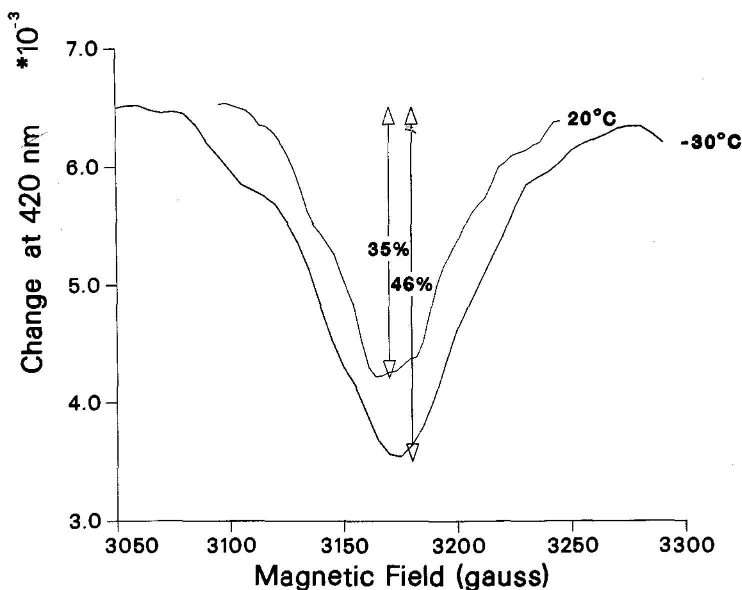


FIG. 9. High microwave power (3.4 kW) RYDMR spectra of reaction centers of *Rps. sphaeroides*. See Fig. 8 for other details.

ened by the application of resonant 9-GHz microwaves. The lifetime of singlet P^F was found to be about 20 ns, whereas the triplet P^F has a lifetime of only about 2 ns. In addition, it was concluded that an isotropic exchange interaction of about 10 gauss accounted for the radical–radical interaction. The anisotropic dipolar interaction in *Rps. sphaeroides* was found to be much larger, around 50 gauss, but this finding is not obviously consistent with the atomic structure emerging from the X-ray diffraction studies of the RC of *Rps. viridis.*

E. Magnetic Field Effects

Another type of magnetic experiment performed requires no oscillating magnetic fields. Instead, it depends on the determination of the relative yield of formation of P^R, measured as a function of the external magnetic field strength as illustrated in Fig. 10. The RYDMR design of Fig. 6 without microwaves is suitable for this experiment. The external magnetic field has been varied from zero to about 50,000 gauss in these

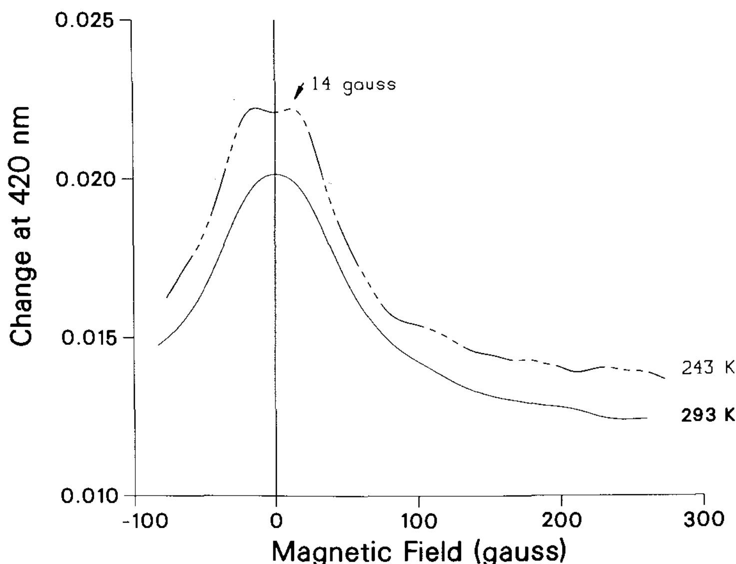


FIG. 10. Typical low-field P^R triplet yield as a function of magnetic field (D. E. Budil and J. R. Norris, unpublished experiment). See Fig. 8 for other details. At very high magnetic fields the triplet yield increases with magnetic field. High-field experiments are important because they are easier to interpret quantitatively than the corresponding low-field experiments (Boxer *et al.*, 1983; Chidsey *et al.*, 1980).

experiments (Blankenship *et al.*, 1977; Hoff *et al.*, 1977; Werner *et al.*, 1978; Blankenship and Parson, 1979; Haberkorn and Michel-Beyerle, 1979; Chidsey *et al.*, 1980; Ogrodnik *et al.*, 1982; Boxer *et al.*, 1983).

V. Concluding Remarks

The structure given in Fig. 1 for bacterial RC should have similarity to the structure of the reaction centers in green plants. However, since Chl *a* does not have a 2-acetyl group (see Seely and Connolly, Chapter 5, this volume), any aggregation of chlorophylls in green plants must be different from that shown by X-ray analysis for BChl *b* in *Rps. viridis,* which involved Mg · · · 2-acetyl interactions. As mentioned already, if the arrangement of the bacterial RC were exactly as shown in Fig. 1, then a dual pathway would exist for the primary charge separation. However, the experimental evidence does not show such a dual pathway of charge separation. Thus, the protein is probably involved in selecting pathway A over pathway B. Consequently, the unknown role of protein in the overall photochemical act must be characterized. The technique of choice for studying the protein involvement in photosynthesis is X-ray diffraction on single crystals of RC proteins (Michel, 1982; Deisenhofer *et al.*, 1984).

For a discussion of primary photochemistry of plants, see van Gorkom (Chapter 10, this volume). Photosystem II, photosystem I, and reactions of green photosynthetic bacteria are discussed by van Gorkom (1985), Rutherford and Heathcote (1985), and Blankenship (1985), respectively.

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4

Methodological Principles of Measurement of Light Emitted by Photosynthetic Systems

JEAN LAVOREL
Association Recherche Biologie Solaire (ARBS)
Centre Energie Nucléaire (CEN)
Cadarache
13115 St. Paul-Lez-Durance, France

JACQUES BRETON
MARC LUTZ
Service Biophysique
Département de Biologie
Centre d'Études Nucléaires de Saclay
91191 Gif-sur-Yvette, France

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ABBREVIATIONS AND SYMBOLS

BChl	Bacteriochlorophyll
BR	Bacteriorhodopsin
CA	Complementary area (of fluorescence)
Car	Carotenoid
Chl	Chlorophyll
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
EPL	Electrophotoluminescence
EPR	Electron paramagnetic resonance
F (F_i)	Fluorescence (intensity related to state i)
I	Light intensity
I	Primary electron acceptor of PSII
L (L)	Luminescence (intensity)
P680	Primary electron donor of PSII
P870	Primary electron donor in (purple) bacterial reaction center
PQ	Plastoquinone
PS	Photosynthesis (or photosynthetic)
PSI, PSII	Photosystems I, II in plant photosynthesis
Q	First PSII electron acceptor (quinone)
Q_B	Secondary PSII electron acceptor (quinone)
RC	Reaction center
RR	Resonance Raman (spectroscopy)
SPC	Single-photon counting
t	Time
TL	Thermoluminescence
Φ_i	Fluorescence yield (related to state i)
τ_f	Fluorescence lifetime

ABSTRACT

Methodological principles related to light emission by photosynthetic systems are explained, with only minor concern for technical details. This chapter should be read as a methodological introduction or guide to other chapters of this book. Fluorescence and luminescence are covered in their classical aspects, but recent developments, such as excitation by short and intense laser pulses or luminescence in the submicrosecond range, are also reviewed. Resonance Raman spectroscopy, which has been less commonly used in photosynthetic studies up to now, is discussed together with its theoretical, although simplified, basis.

I. Introduction

A. *Methodology*

Methodology is the art of planning, designing, and using protocols suited to fulfill precise data acquisition programs. Technology is the collection of technical tools, which one may not use without a thorough

knowledge of their requirements, performances, limits, and inherent risks of artifacts.

In this chapter we shall carefully follow this distinction, placing much emphasis on methodology. Technical details will often be indicated for retrieval in references or summarized in appendices. Intelligent, successful methodology goes hand in hand with good problem-solving ability; i.e., a methodological approach is selected or designed from the known particulars of the object under study in order to verify an intellectual—often implicit—model.

It is difficult to find a satisfactory classification scheme for methods in areas of photosynthetic research. This is essentially because they have evolved into a wide variety of combinations, where correlations among parameters are sought internally—within the same method or family of methods—or externally—between distinct methods. Also, the protocols are becoming increasingly sophisticated; i.e., the preconditioning of the sample, the timing of triggering or measuring events, etc. are taking precedence over the mere direct recording of data. Technical sophistication is also constantly progressing, notably to extend the limits of sensitivity and time resolution.

B. Interaction of Light with Photosynthetic Systems

From the methodological viewpoint, photosynthetic (PS) objects may be characterized by the following properties:

(a) Light is absorbed by pigments, often endowed with photochemical activity; therefore light generally modifies the properties of these objects.

(b) In PS systems, photochemical events trigger or give momentum to a large ensemble of secondary events with a wide spectrum of lifetimes.

(c) PS objects are optically heterogeneous specimens, usually microscopic, densely pigmented particles suspended in a liquid medium.

We shall briefly review here the constraints which the above properties impose on methodology.

The most important property is obviously the photochemical activity. Accordingly, light acts on PS objects as a probe (analytic) or as an energy input (actinic) or both. Strictly speaking, there is never such a thing as purely analytical light, when photoactive pigments are involved. There are only admissible approximations to this situation, which are best quantified by knowing the fraction of reaction centers (RCs) hit per unit of time. The time considered is that of the duration of the measurement. For instance, 1% centers hit per microsecond is a fair approximation to

the analytical condition for a period of a few microseconds, but would mean complete light saturation over a millisecond period! In many instances, the time variation of the fluorescence intensity (F) itself as a consequence of a given amount of analytical light can be used as a simple and quick test.

A unique kinetic property of PS systems is that the zero time of an experiment can be defined with very great precision by giving the sample a "saturating" short actinic light pulse (commonly a few microseconds with a Xe flash lamp down to the picosecond range with pulsed lasers). As a result, all of the discrete systems in the sample can be made to evolve in synchrony. In this respect, the ideal condition is the "single turnover" saturating flash. By this is meant that light is delivered in such a short time and in such a quantity that every RC reacts photochemically once and only once during the flash. Departure from this idealized condition complicates kinetic analysis. Nonsaturation is of little consequence, at least in single-flash experiments, except for some loss in signal strength. However, if the flash duration is not short compared to the reaction center turnover time, "double hits" occur in a fraction of centers.

The above synchrony also requires that the sample be homogeneously illuminated. This again may be only approximately achieved. In an absorbing specimen, light is distributed and absorbed along its propagation direction according to the Beer–Lambert law (i.e., light intensity is an exponential decreasing function of depth). For fluorescence studies, 1–10 μg chlorophyll (Chl)/ml in a 1-cm light path cuvette is not uncommon and ensures fairly homogeneous illumination. Illumination should be homogeneous not only transversely but also laterally (in the plane normal to the light beam direction). Lateral homogeneity will become of increasing concern as more people use laser beams, which are usually narrow with a Gaussian intensity distribution. The effect is discussed by Paillotin *et al.* (1983) and suitable corrections are proposed. Nevertheless, it is advisable to correct the problem experimentally when this is feasible, e.g., by expanding the excitation beam.

When dealing with spectroscopic aspects (e.g., an action spectrum), another problem arises due to the nonhomogeneous microdistribution of pigments in the sample. Comparing the actual sample with one containing the same amount of pigments in solubilized form in the same volume, one can observe quite different spectra. Aside from the well-known spectral shifts due to the different physicochemical environments of pigment molecules, there is a specific effect of inhomogeneous pigment distribution (the so-called sieve effect), which tends to depress the absorption peaks relative to the troughs. The effect can be readily cor-

rected for (see Pulles *et al.,* 1976). Light scattering is known to alter considerably the absorption spectra of PS samples. However, since light emission is related to true absorption of the excitation light, scattering is of secondary importance here.

In modulation methods, the analytic light probes the sample periodically. However, the distinction between "analytic" and "actinic" is not always clear-cut; furthermore, the periodic light beam can be made actinic on purpose (as in flash sequence experiments). In such instances, it is seen that light is performing a frequency selection over the kinetic components of the system. Components with lifetimes shorter than the flash period will be directly observed during the flash interval, whereas those with lifetimes longer than the flash period will only appear indirectly, by modulating the characteristics (e.g., amplitude) of the flash response. The experiment is or can be set up in such a way that only one of the above two classes of kinetic components will be selectively monitored.

II. Fluorescence

A. *Why Measure Fluorescence?*

This question is best answered by a glance at Table I, the result of a survey of some 200 papers published between 1979 and 1983; the topics to which Chl fluorescence has been applied as a methodological tool are listed in order of decreasing frequency. This ordering more or less indicates the current relative preeminance of topics or their popularity. Referring to the question above, it can be said, considering the very large number of studies to which fluorescence gives access, that at present it really is the universal probe of PS events.

To put some order in, if not classify, the bewildering number of methods or variants related to the use of fluorescence in PS, one may choose the primary characteristics of any fluorescence emission: yield, lifetime, spectroscopy, and polarization. This is not strictly satisfactory as there is naturally much overlap between these categories, if only because their characteristics are mutually related—e.g., yield and lifetime or polarization and lifetime. In addition, as mentioned above, much variation is introduced by the way in which one (pre)treats the sample. That includes the analytic/actinic character of light as well as its wavelength and its time dependence (constant or periodic). Methodological aspects are also covered in reviews by Lavorel and Etienne (1977) and Govindjee and Jursinic (1979).

TABLE I
A Survey of Topics Studied by Fluorescence[a]

Topics	%
1. Light harvesting complex (and phosphorylation)	13
2. Regulation of energy distribution, spillover	6*
Slow quenching (P → S)	
F lifetime	
Energy transfer	
3. Q heterogeneity, redox titration	5.5
4. Mg^{2+} effect	5*
PSI fluorescence	
5. α and β units of PSII	4.5
6. State I–state II	3.5*
Q_AQ_B gate	
Herbicides and Q binding	
Stacking of thylakoids	
Orientation, polarization	
7. P680, donors, S states	3
8. Membrane electrostatics	2.5*
Phycobilins	
9. F spectroscopy	2*
Antenna heterogeneity	
Carotenoid quencher	
P^+Q^- recombination	
C550	
PQ pool	
Induction	
Uncouplers	
Mutants	

[a] This survey is based on about 200 papers published during the period 1978–1983. It does not claim to be exhaustive. (*), The percentage applies to each member of the group.

B. Yield

Since $F = \Phi I$ (F, fluorescence intensity; Φ, yield; I, light intensity), we find in this category all applications where F is monitored as a relative measure of Φ. This is often done, and it stems from the proposal many years ago by Duysens and Sweers (1963) that, in higher plants and algae, Φ under usual illumination conditions is simply and uniquely related to the state of PSII traps: when these traps are "open" (photochemically active) F is low, and when they are "closed" (photochemically inactive) F is a maximum. Mechanistically, this is related to the redox state of the photosystem II (PSII) acceptor, Q. Excited Chl may deactivate radiatively (and nonradiatively as well) or by transferring its energy to the RC; the latter acts as a quencher (hence the denomination Q) when Q is oxidized or as a nonquencher (mostly) when Q is reduced. The relationship, though, is seldom linear: because of exciton migration between PSII units (Joliot and Joliot, 1964), the differential of F per closed PSII center ($d\Phi/dQ^-$) is less when only a few centers are closed than when they are nearly all closed.

There is an even simpler relationship between F and the functioning of PSII centers (which also applies to bacterial systems but not to PSI),

since F and the photochemical activity are complementary. The exact *complementarity* relationship is

$$\Phi_p + \Phi_f + \Phi_d = 1 \quad (1)$$

where the Φ's are yields and the subscripts stand for photochemistry (p), fluorescence (f), and nonradiative dissipation (d). This relationship was verified by Delosme *et al.* (1959). It can be shown that, as a consequence of Eq. (1), the complementary area is a valid measure of the number of active PSII centers (see Fig. 1 and Appendix A). This was first demonstrated independently by Malkin and Kok (1966) and Murata *et al.* (1966).

The complementary area (CA) affords a quick and easy means of measuring the PSII activity. It is commonly used in relation to speculations about the so-called α and β PSII centers (see Melis and Duysens, 1979). The CA determination is straightforward, which does not mean that there are no pitfalls. Figure 1 indicates two potential ones: the true position of F_0 and the asymptotic level F_m. The latter is the most serious source of error.

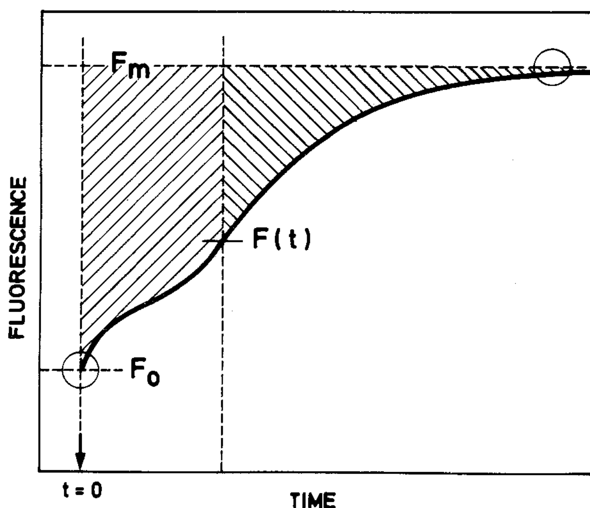


FIG. 1. Scheme representing the induction rise of chlorophyll *a* fluorescence determining the complementary area (CA). F_0, $F(t)$, F_m: fluorescence intensity levels at time zero of illumination, at time t, and at the asymptotic limit, respectively. The complementary area is shown by hatching; at time t, it is composed of two parts representing the fraction of the total pool already filled (hatching slanting upward to the right) or still to be filled (hatching slanting downward to the right). Circles show areas in the curve where methodological or theoretical problems may arise (see text).

(a) The expected meaning of CA is based on some kind of "isolation" of PSII, kinetic or otherwise. For instance, it is obvious that CA has an altogether different meaning in a system where the asymptotic condition is a steady-state electron flow through PSI of nonnegligible rate compared to the mean rate during the F rise. A high enough actinic intensity should ensure adequate kinetic isolation for PSII [with respect to the 20-ms limiting step on the oxidizing side of the plastoquinone (PQ) pool].

(b) One should always bear in mind the assumption implicit in the model, namely that the only cause of variation of F is photochemical quenching. Conditions exist where one may suspect that this is not the case (see Appendix A).

In nonstandard situations, one should check the applicability of CA by a simple test. The direct expression of CA

$$\int_{t=0}^{\infty} (F_m - F)\, dt \tag{2}$$

should be invariant with the exciting light intensity (see, e.g., Telfer *et al.*, 1983, and Appendix A).

We mentioned above that F and the Q^- concentration are not linearly related. If they were, the simplest type of F rise (with DCMU) would be exponential, whereas it is actually sigmoidal. Sigmoidicity has been used as a simple qualitative test for exciton migration between PSII units (Diner and Wollman, 1979; Jennings *et al.*, 1980). Other semiquantitative conclusions drawn from the F rise may be mentioned. Under identical conditions, $t_{1/2}$ of the F rise (with DCMU) is inversely related to the PSII antenna size (Telfer *et al.*, 1983). Similarly, F_m/F_0 can be used as an index of "spillover," i.e., energy transfer from PSII to PSI (see Malkin and Fork, 1981).

The so-called F induction, with its "OIDPSMT pattern" (see Briantais *et al.* and Govindjee and Satoh, this volume), is a more complex phenomenon than the F rise considered so far. It is now clear that there is more than photochemical quenching in these variations. An interesting means of showing the part played by Q quenching has been used by Krause *et al.* (1983). They obtained instantaneous isolation of the PSII reactions by rapid injection of DCMU during the induction course; the immediate F rise then measures the amount of Q that was present at the moment of the injection. An alternative method is to perform a light intensity jump (Bradbury and Baker, 1981).

In all the above methods, the exciting light has a constant intensity. Modulated analytic light was first used by Duysens and Sweers (1963) to

demonstrate the antagonistic effects of PSI and PSII on the Q/Q^- system (see also Duysens, Chapter 1, this volume). Modulation followed by lock-in detection is a standard technique for extracting a small signal from noise (essentially by narrowing the observation bandwidth). In addition, in PS systems it offers the possibility of mixing lights of different wavelengths and intensities. If F is excited by both a constant light of intensity I and a modulated one (i), it can be resolved into two components. The modulated signal $\tilde{F}$—the only one seen by the measuring instrument—is:

$$\tilde{F} = \Phi(I + i)i = \Phi(I)i \tag{3}$$

(when $i \ll I$). In other words, the effect of I on Φ can be demonstrated directly without the calculations that the direct approach would imply. This method is also frequently used for studying so-called state I–state II effects (see, e.g., Hodges and Barber, 1983; Briantais *et al.,* Chapter 18, this volume).

The best noise rejection is obtained with the modulation method when the bandwidth is very narrow. This practically means steady-state measurements because the instrumental time constant is the reciprocal of the bandwidth. A compromise must be accepted—a larger bandwidth—when the modulation method is applied to kinetic studies. For instance, the opposite of the F rise, i.e., the spontaneous reoxidation of Q^- (in darkness), can be monitored by a weak modulated beam following flash preillumination of the sample (Lavergne and Etienne, 1980). Modulation frequencies in the kilohertz range are commonly used. Lasers or solid-state light sources (light-emitting diodes) can be modulated at much higher frequencies if required.

C. *Lifetime*

The methodology related to the *in vivo* Chl fluorescence lifetime (τ) is less generally applied because of its difficulty and cost. As it is discussed in Chapter 7 of this volume by Moya *et al.,* we shall give only a cursory introduction here (for a recent review, see also Gratton *et al.,* 1984).

Electronic excitation in Chl, as in any molecule, has a definite and constant probability per unit time to be emitted as fluorescence, in competition with other processes such as photochemistry. The fluorescence lifetime (τ), which is the average residence time of excitation within the assembly of chlorophyll molecules, is therefore a built-in "chronometer" which indicates the rate of trapping by the RC, and thus its efficiency. Knowing τ is important not only per se as a fundamental parameter but also, in practice, because in many instances it helps to resolve ambiguities

in the interpretation of Φ. For instance, an apparent decrease of Φ might result either from quenching or from a decrease in absorption cross section; only in the former case is τ expected to decrease in parallel with Φ.

τ is determined by two methods:

(a) In phase fluorimetry the exciting light is modulated at a high frequency—typically in the range 10–100 MHz—and since F is similarly modulated, τ is calculated from the phase angle φ between F and I or alternatively from the degree of modulation of F. A component is best detected when the modulation frequency is of the order of magnitude of $1/\tau$. When several species emit simultaneously, the φ and modulation dependencies on the τ_i's are more complex, but the problem can still be solved by successive determinations at several frequencies.

(b) In the pulse method one measures directly the decay of F resulting from a short light pulse (nanoseconds to picoseconds), which requires very fast recording instruments. Here again a component cannot be well resolved unless the pulse width is shorter than τ. In general, because the pulse width is finite, τ is calculated by deconvolution since the signal appears as a convolution product of an instrument function with the fluorescence function: $\exp(-t/\tau)$. With several species emitting, the deconvolution operation can discriminate several exponential components (in practice not more than three; see Appendix B). Two distinct technologies have been implemented in the pulse method: the streak camera and single photon counting (SPC) with time-to-amplitude conversion. The former technology allows the recording of ultrafast events. In the early phase of its development, it was associated with the use of rather intense laser pulses—a topic we shall discuss below (Section II,F). The latter inherently needs weak probing pulses. The idea is to build the F decay curve from the statistics of random events. From many exciting pulses ($>10^4$), the time elapsed between excitation and emission of a fluorescence photon is recorded. The F decay is therefore the frequency of arrivals as a function of time. Probability laws impose a low excitation rate per pulse so that a negligible fraction of multiple F photons are recorded.

D. Spectroscopy

Fluorescence spectroscopy is a method in itself, but it is also frequently combined with other methods, for instance, measurements of polarization and lifetime. Little needs to be said here concerning methodology and technology, which are relatively standard. One important variant is

low-temperature fluorescence spectroscopy. Lowering the temperature below 100°K has several consequences: transitions to states which require a sizable activation energy may no longer occur, including some stabilization steps following charge separation as well as exciton transfer. As the higher molecular vibrational levels are less populated, the absorption and emission bands become narrower and easier to distinguish from one another (see, e.g., Schreiber *et al.,* 1979). Low-temperature spectroscopy has become a standard method for studying the fluorescence emission associated with PSI and, by comparing it to the PSII emission, for monitoring the state I–state II transitions (see, e.g., Fork and Satoh, 1983). Technical details concerning fluorescence spectroscopy (in particular at low temperatures) may be found in Appendix C.

E. *Polarization*

Linearly polarized light has been rather extensively used to characterize the fluorescence of PS systems. As polarization provides information about the orientation of molecules, it has helped solve questions concerning long-range and short-range order. In the first case, it is the chromophore orientation with respect to the membrane plane which is of interest, while in the second the relative orientation between adjacent pigments and the depolarization by exciton transfer are considered.

Polarization of fluorescence, together with linear dichroism, has led to the realization that all pigments are to a large extent oriented relative to the plane of the PS membrane (Geacintov *et al.,* 1974; Breton and Vermeglio, 1982). These measurements require an oriented sample, and a variety of orientation techniques have been used: flow in narrow tubing (Morita and Miyazaki, 1971), air-drying of membranes on a flat surface, a magnetic field (Geacintov *et al.,* 1972; Breton, 1974), an electric field (Sauer and Calvin, 1962; Gagliano *et al.,* 1977), and embedding of the specimen in a matrix of deformable material such as gelatin, polyvinyl alcohol, or polyacrylamide (Abdourakhmanov *et al.,* 1979). At present we favor magnetic field orientation for intact chloroplasts, which preserves the native environment, and polyacrylamide gel deformation for smaller chlorophyll–protein complexes. Both techniques offer the added advantage of being suitable for low-temperature spectroscopy (Haworth *et al.,* 1982b).

Orientational information is best obtained by recording the polarized emission spectrum (Lavorel, 1964). In general, the Chl *a* dipoles emitting at long wavelengths are oriented closer to the membrane plane than the shorter-wavelength emitters (Breton, 1975), while at low temperatures at least five emitters with different orientations have been detected

(Garab and Breton, 1976; Kramer and Amesz, 1982). With isolated Chl–protein complexes, which are mechanically oriented, it is possible to analyze the orientation of an emitting dipole with respect to the dimensions of the particle. This has been achieved with a variety of PSI and PSII particles (Ganago *et al.*, 1983; Tapie *et al.*, 1984) and has been important in assigning emission bands to subsets of functional pigments. Similarly, polarization excitation spectra of chloroplasts at low temperatures have revealed several bands, allowing determination of the orientation of absorption dipoles relative to their corresponding emitting dipoles and to the thylakoid membrane (Kramer and Amesz, 1982).

Because excitation energy is rapidly transferred among a large number of pigment molecules, one can expect depolarization effects to take place; accordingly, measuring the extent of F depolarization of Chl *in vivo* should yield an estimate of the number of transfer steps during the exciton lifetime (see Knox, 1968). This approach has often been applied (Arnold and Meek, 1956; see also Wong and Govindjee, 1981). However, it must be stressed that the results are, in general, ambiguous, as the angular factor of the transition moments in successive transfers will determine the depolarization. For instance, many transfers between parallel dipoles do not cause depolarization. Moreover, a suspension of membrane fragments in which the pigments are oriented with respect to the membrane plane cannot be treated as a true isotropic pigment solution because of a photoselection effect that was first discussed by Breton *et al.* (1973) (Figs. 2a and 2b). True depolarization by energy transfer

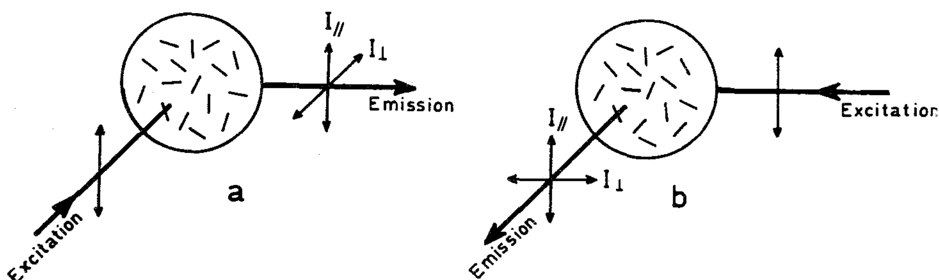


FIG. 2. Scheme representing two different types of measurements of polarized fluorescence on an oriented membrane. The small bars represent dipoles lying at random within a plane; excitation energy is transferred among them. The directions of excitation and observation are at right angles. In (a), excitation is along the normal to the membrane; the two polarized components of fluorescence (‖ and ⊥) reveal the orientation of dipoles with respect to the plane. In (b), excitation is along the plane of the membrane; fluorescence polarization gives a measure of the relative order between dipoles. (After Breton and Geacintov, 1979.)

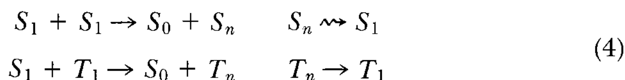
between mutually oriented chromophores should therefore be investigated on an oriented sample in which F is viewed along the normal to the membrane plane, as shown in Fig. 2b (Becker *et al.*, 1976). By using this geometry, some degree of local order among neighboring pigments has been detected for Chl *in vivo*. This order increases for pigments that absorb at longer wavelengths (Garab and Breton, 1976). It has been suggested that exciton–exciton annihilation, which shortens the singlet exciton migration range, could be used to probe the mutual orientation of Chl molecules over shorter distances (Breton and Geacintov, 1979).

The technique of F polarization is also important for investigating the relative orientation between chromophores in small Chl–protein complexes. It is of great interest for analyzing the organization of pigments in the antenna complexes in both green plants (Knox and van Metter, 1979; see also Govindjee and Satoh, Chapter 17, this volume) and photosynthetic bacteria (Breton *et al.*, 1981; Bolt and Sauer, 1981; Kramer *et al.*, 1984). It has only occasionally been used for isolated reaction centers because other, more direct photoselection techniques are available (Breton and Vermeglio, 1982).

F. *Intense Laser Pulses*

The advent of pulsed lasers has led to new possibilities for analyzing the fluorescence properties of photosynthetic systems. Compared to noncoherent light sources, pulsed lasers have two important characteristics: the short duration of the laser pulse (typically 10^{-6} to 10^{-13} s) and the high density of excitation which can be achieved on the target material. The spatial coherence of the beam, which is maintained even after an optical path of several tens of meters, may be used to introduce long time delays between successive pulses (1 ns is equivalent to a 30-cm light path). However, the very high instantaneous photon fluxes which can be achieved in these laser pulses can lead to deactivation processes which are not normally encountered under excitation with more conventional light sources—including sunlight! This is especially true for ps pulse excitation, for which the role of such processes has not always been sufficiently recognized in the early measurements of fluorescence lifetimes. In addition to the normal photochemistry which occurs at the level of the RC and which involves charge separation and stabilization processes, several nonlinear intensity effects must be taken into account when intense laser pulses are utilized to excite PS systems. They were reviewed recently (Geacintov and Breton, 1982; see also van Grondelle and Amesz, Chapter 8, this volume) and only a brief account will be given in the following.

During an intense laser pulse, the rate of arrival of photons may be such that the molecules excited during the first part of the pulse have not yet returned to the ground state when photons from a later portion of the pulse are still arriving. This process—known as *ground state depletion*—gives rise to an apparent decrease of Φ. It is also well known that stimulated emission may occur in many dyes under appropriate excitation conditions. Observing that lasing as well as excited state absorption could occur in dilute chlorophyll solutions under laser excitation, Hindman *et al.* (1978) suggested that such processes take place *in vivo* and could account for the marked decrease of Φ (quenching) observed when PS systems were excited with intense laser pulses. This possibility was critically examined by Geacintov *et al.* (1979), who concluded that the effects mentioned above could not be held responsible for this sort of quenching observed *in vivo*. The quenching has finally been attributed to exciton–exciton annihilation processes (for a review, see Breton and Geacintov, 1980), in which a singlet exciton S_1 collides with either another singlet exciton or a triplet exciton T_1:



$$\begin{aligned} S_1 + S_1 &\rightarrow S_0 + S_n \qquad S_n \rightsquigarrow S_1 \\ S_1 + T_1 &\rightarrow S_0 + T_n \qquad T_n \rightarrow T_1 \end{aligned} \tag{4}$$

Here S_n and T_n represent higher excited states which rapidly decay back to the first excited state and S_0 represents the molecule in its ground state. According to Eq. (4), a singlet exciton is lost on every encounter.

In the early phases (1973–1976) of development of picosecond laser techniques aimed at measuring directly the decay of *in vivo* chlorophyll fluorescence, it was necessary to use rather intense pulses ($>10^{14}$ photons/cm^2 per pulse) in order to detect measurable decay kinetics. Furthermore, trains of picosecond pulses (pulse spacing typically 5–10 ns), which constitute the normal output of mode-locked lasers, were frequently used instead of single pulses isolated from the train with a Pockels cell. Under such excitation conditions, the measured τ of *in vivo* Chl was about an order of magnitude shorter than that measured by more conventional techniques.

Once the importance of exciton annihilation processes was fully recognized, measurements of τ in the low-intensity regime were attempted with the streak camera. Although the results were now in qualitative agreement with those obtained with phase fluorimetry or single-photon counting (see Moya *et al.*, Chapter 7, this volume), there were several stringent intrinsic limitations (poor linearity, low dynamic range) with the early types of streak cameras. The present development of streak cameras with low jitter (Knox and Mourou, 1981) and/or repetitive scan-

ning (synchroscan) will improve the characteristics of these instruments; they should now be well adapted to measurements in the 20–200 ps time range. At present, SPC, as used in several laboratories (Gulotty *et al.*, 1982; Haehnel *et al.*, 1982, 1983), seems to provide the most accurate results obtained by nonphase techniques (see Moya *et al.*, Chapter 7, this volume). However, because of its present limitations, this method does not allow full characterization of decay times shorter than 50–100 ps; this constitutes a barrier to our understanding of energy transfer in PS membranes. Because of the potential interest of this field, it seems important that measurements with a resolution of about 5 ps be achieved. In this respect, the development of high-repetition-rate streak cameras and the measuring of up-conversion techniques together with the use of continuously mode-locked lasers should allow significant progress.

Although exciton annihilation processes have seriously plagued the field of τ measurements for *in vivo* Chl, they have also been put to use for probing some important parameters characterizing energy transfer in PS systems (Geacintov and Breton, 1982). As the energy of a single picosecond pulse increases above 10^{12}–10^{13} photons/cm^2, Φ begins to decline. In chloroplasts, the dependence of the yield on the flash energy can be fitted with equations describing singlet–singlet annihilations taking place over a very large number of Chl molecules encompassing several RCs (Paillotin *et al.*, 1979). Thus, in chloroplasts with closed PSII RCs, the diffusion range of the singlet exciton appears to be limited more by its lifetime than by any topological barrier that might prevent its migration. A similar conclusion has also been drawn in the case of photosynthetic bacteria (Campillo *et al.*, 1977). This problem was recently reconsidered by Bakker *et al.* (1983) for bacteria; in this study the initial state of the RC was controlled and the final state was monitored following a picosecond flash. The main conclusion of this careful study was that the singlet exciton is essentially delocalized over the entire surface of the chromatophore (see van Grondelle and Amesz, Chapter 8, this volume).

Nanosecond laser pulses have been extensively used to monitor absorption changes. Because of their duration, such pulses can induce both singlet–singlet and singlet–triplet annihilations. The relative importance of these two effects depends on the intensity of the laser pulse, the intrinsic singlet lifetime, the intersystem crossing rate, and the relative values of the bimolecular annihilation coefficients (Geacintov and Breton, 1982). In addition, when the RC is open, the duration of the pulse is such that photochemistry, initiated in an early portion of the pulse, can lead to an increase of Φ which will be probed by a later portion of the pulse (Monger and Parson, 1977; Sonneveld *et al.*, 1979).

For microsecond laser pulses, only singlet–triplet annihilations can play a significant role, but triplets that are created (^{3}Chl, ^{3}Car) may have different quenching efficiencies, depending on their location in the antenna or in the RC (Monger and Parson, 1977; Breton *et al.*, 1979; Mathis *et al.*, 1979; Maroti and Lavorel, 1979). Such effects have been observed for the quenching of the various bands characterizing the low-temperature emission spectrum of chloroplasts (Geacintov and Breton, 1982).

The exciton annihilation technique also allows determination of the number of energy-transferring pigments within a given isolated Chl–protein complex. Based on singlet–triplet annihilations, this parameter has been determined as shown in Fig. 3 for antenna preparations from

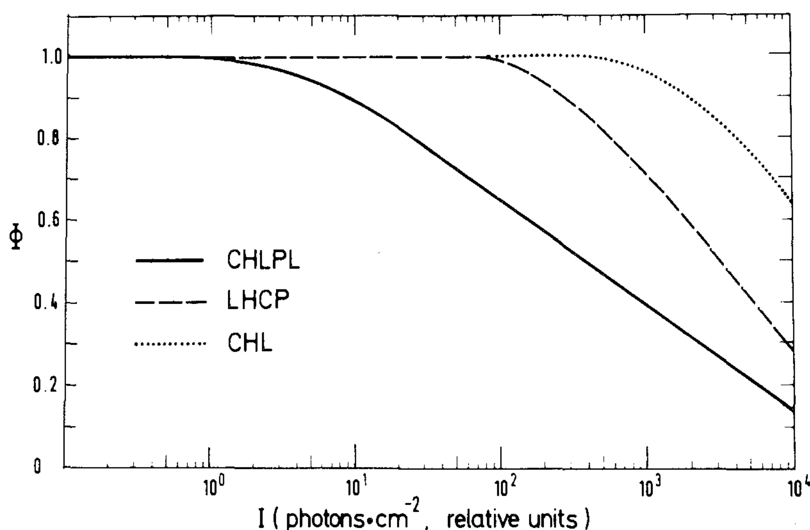


FIG. 3. Fluorescence yield, Φ, of chlorophyll *a* at room temperature as a function of the number of photons absorbed per pigment molecule in spinach chloroplasts (CHLPL), light-harvesting Chl *a*/*b* complex (LHCP), and free pigment (CHL). Excitation, 637 nm; 1-μs dye laser pulse. The horizontal scale (logarithmic) is a relative function of the number of photons absorbed per pigment molecule (*I*, incident laser pulse intensity) since the absorption cross sections of CHLPL and LHCP at 637 nm are approximately the same. The onset of fluorescence quenching for chloroplasts occur at an intensity roughly 100 times lower than for LHCP; as there are six chlorophyll molecules per LHCP, this indicates that the exciton in chloroplasts can diffuse over a large number of pigment molecules, which facilitates bimolecular exciton annihilation. For free pigments (CHL), the chlorophyll molecules are probably too far apart for this mechanism to occur; the fluorescence quenching observed at the highest intensity is attributed to ground state depletion and/or excited state absorption effects. (After Breton and Geacintov, 1980.)

green plants (Breton and Geacintov, 1980). More recently, the same approach—but using singlet–singlet annihilations—was used to determine the number of connected bacteriochlorophylls (BChls) in various antenna preparations from PS bacteria (van Grondelle *et al.*, 1983).

Finally, double laser pulses separated by a variable delay can be used to probe the F induction processes. This approach was pioneered by Mauzerall (1972), but the use of nanosecond laser pulses which create triplet quenchers has led to some ambiguities in the interpretation. This question was recently reinvestigated with pairs of picosecond pulses, the probe pulse being delayed by up to 100 ns (Deprez *et al.*, 1983). It has been demonstrated that F of dark-adapted chloroplasts rises in 28 ± 4 ns, a time which correlates well with the decay of the photooxidized primary electron donor of PSII, $P680^+$.

III. Luminescence (Delayed Fluorescence)

A. Why Measure Luminescence?

Luminescence (L) is the visible sign of a minor imperfection in the primary photochemical charge separation (see Arnold, Chapter 2, Jursinic, Chapter 11, and Sane and Rutherford, Chapter 12, this volume). It is generated by charge recombination, mostly within PSII and bacterial RC. The information one may gather from studying L depends considerably on the time scale of observation; while submicrosecond and microsecond L is intimately related to the transfer of charges within or close to the RC, millisecond (and higher range) L depends on back-flow of electrons from more distant components of the PS apparatus. Luminescence L is less extensively used than fluorescence in PS studies, although we can witness recent remarkable developments, for instance, to characterize PSII and its associated components. The reason that L is less popular than F is probably not that its potentialities are ignored, but that it is technically somewhat more difficult to implement L measurements, even in their simplest modes. Even measuring the very slow components of L (≥ 1 s) requires some minimal mechanical contrivance in order to strictly separate the actinic from the observation periods in the measuring process; besides, as L is a rather weak signal, high detection sensitivity and total absence of stray light are required. Evidently, monitoring the F induction rise is far easier. General information on L methodology can also be found in recent reviews (Malkin, 1977; Govindjee and Jursinic, 1979).

Two groups of L measurement methods stand out in recent develop-

ments. One is thermoluminescence (TL), which, more clearly than room-temperature L, has given new insights into the stability and energetics of states associated with charge storage on both sides of the PSII photoreaction (Inoue, 1981; Demeter *et al.*, 1982; Rutherford *et al.*, 1982). The other problem for which methods are now available is the temporal connection between the F decay and the onset of L; in other words, is L under continuous illumination a sizable fraction of the apparent F? That the ultrashort L is not a negligible fraction of the total chlorophyll emission is an important issue: first, because L on this time scale (ns) might give independent clues to the path of charges during the stabilization time of light energy conversion, and second, because relatively simple methods (see Section III,C) are now available for monitoring it (Shuvalov and Klimov, 1976; van der Wal *et al.*, 1982).

Several general properties of L must be taken into account in order to understand how the methods work.

1. The emission spectra of L and F are essentially identical, and, for all practical purposes, L is very small compared to F (the two phenomena are dissimilar in nature: L is easily saturated while F is a continuously increasing function of I). Therefore, if the sensitivity of the apparatus is high enough to monitor L properly, it will enter saturation during the actinic period because of the high intensity of F. Saturation is in general not harmful to the equipment, but the measuring unit takes time to recover (usually several tens of milliseconds) and to reoperate properly. This problem is usually solved by turning off the signal path mechanically or electronically during the actinic period.

2. L is by nature extremely polyphasic; the lifetime spectrum extends from 10^{-7} to more than 10 s. This means that in order to characterize its decay completely, its amplitude would have to be followed over many decades. In practice, during kinetic studies one is restricted to a narrow range of amplitudes and lifetimes because of the choice of bandwidth and the sensitivity of the instrument.

3. Because L is inherently a low-level signal, it is prone to noise contamination. In time-resolved applications, this means that averaging is mandatory. Recall that, as a rule, if N decays are averaged, the expected noise reduction is by a factor of $\sqrt{N}$ compared to a single sample. For noise reduction through averaging to be effective, it must operate on N identical samples. That means that one must check for drifts caused by the sample or the instrument. For phosphoroscope-type experiments, averaging is simply and approximately performed by setting an appropriate time constant to the instrument (an analog time constant is equivalent to high-frequency noise filtering).

B. Phosphoroscope

According to the frequency selection principle discussed above (Section I,B), the phosphoroscope method is of interest for the study of relatively slow phenomena, when the envelope or average of the L emission is monitored. In addition, F and L may be monitored with the same experimental setup.

The technique is simple: during a phosphoroscope cycle, the actinic light is turned on while the measuring device is off in the first period; in the next period, the positions are reversed (actually, a short blank period separates the successive light and dark phases). The sequence might, e.g., be defined as follows (Satoh and Katoh, 1983): 2 ms light/0.5 ms blank/2 ms dark/0.5 ms blank. This sort of timing is easily achieved with mechanical devices (rotating blades or drum, spinning disk); the speed of the on–off transitions ultimately depends on the dimension of the actinic light beam, more precisely on the cross section of the light beam in the plane of the chopping device—hence this speed can be high when a continuous laser beam is used.

The phosphoroscope method has been criticized on the ground that $L(t)$ is a complex function of the past history of the sample; thus at a given time, contributions from previous cycles should also be present—a *convolution* effect. Note that this is true only when the phosphoroscope is used in a time-resolved fashion. Even then, the objection is only valid if the actinic light is not saturating and the phosphoroscope cycle is in the range of the RC turnover time.

C. Time-Resolved Luminescence

We know now that luminescence lifetimes span many decades, down to the nanosecond range, where they encounter the theoretical limit—the natural lifetime of "prompt" F—and where distinction between F and L becomes operationally meaningless. Measuring ever shorter L components has been the most difficult technical problem in L methodology. This idea is basically the same as with the phosphoroscope: the time resolution depends on how fast the actinic light can be turned off and the photodetector turned on. Technical considerations (electronic transient noise) have set a practical limit of 0.1–1 μs for the blind period between the extinction of actinic light and the first data which may safely be recorded (van der Wal *et al.*, 1982). Because sensitive detectors, such as photomultiplier tubes, and their associated circuitry have limited dynamics, it is not possible simply to start L measurement when F has completely decayed, since the perturbation it has induced in the measur-

ing system may take too long to subside. Gating off the tube and using a tailless actinic pulse is a better—actually the only—solution. Fast turning off of the actinic light is achieved in practice only by relying on properties of pulsed lasers (down to a few picoseconds), as the tail of electronic flashes is still too strong when measuring Chl luminescence several milliseconds after the flash maximum. Photomultipliers are the only optoelectronic devices which can be turned on and off in the nanosecond time range with relative ease. The principle (see, e.g., Rosetto and Mauzerall, 1972) is to pulse one or several dynodes at the first stage of the dynode chain to a negative potential with respect to the photocathode (Fig. 4). In this state, any photoelectron emitted in the vacuum at the photocathode is promptly returned to it; the photomultiplier is effectively turned off. How rapidly this can be done depends primarily on stray capacitance around the pulsed dynodes. Practical implementation of this principle is not, however, straightforward (see van Best, 1977). No commercial apparatus exists.

For millisecond luminescence, mechanical devices such as electromechanical shutters in some cameras solve the problem very easily.

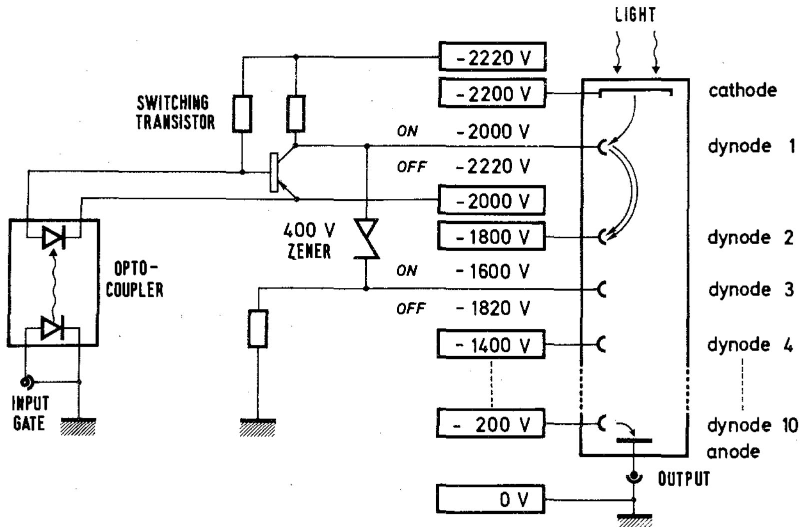


FIG. 4. **Scheme of a photomultiplier electronic gate. A positive voltage pulse (input gate) drives the switching transistor to saturation, which renders dynode 1 20 volts more negative than the photocathode, thereby repelling the photoelectrons; a similar action is relayed through the Zener diode to dynode 3 (relative to dynode 2) in order to improve the effect. The opto-coupler isolates the high-voltage photomultiplier stages from the input pulse equipment. (Adapted from van Best, 1977.)**

Even in a restricted time window, an L decay often displays a polyphasic behavior, as a semilogarithmic plot readily demonstrates. Such types of decays are now commonly analyzed by a variety of programs (see Appendix B) in terms of several exponential components, usually not more than three. This trend is a natural consequence of the now commonplace digital storage of data, allowing their straightforward communication to a computer for analysis. Also, the volume of data implied in such operations is small enough to be handled directly by dedicated, local minicomputers and even microcomputers.

Van der Waal *et al.* (1982) report a minimum time resolution that may be achieved with advanced electronic technology; L components with lifetimes around 5 ns are detected in the photosynthetic bacterium *Rhodospirillum rubrum,* the earliest reliable data points being about 3 ns after a 30-ps frequency-doubled, mode-locked Nd–Yag laser pulse at 532 nm.

The designation *recombination luminescence* (see Shuvalov and Klimov, 1976) applied to these fast emissions, observed when secondary electron transport is blocked, is slightly misleading as there is no reason to doubt that this phenomenon is a luminescence in the usual sense of the word, although it is monitored under special circumstances and is characterized by a very short lifetime (see van Gorkom, Chapter 10, this volume).

This type of luminescence was discovered (Shuvalov and Klimov, 1976) as a particular outcome of experiments in phase fluorimetry (see Section II,C and Moya *et al.,* Chapter 7, this volume), although its existence was suspected for other reasons. A priori, one might not expect to detect L with the phase method, due to its relative weakness relative to F. This belief proved to be too pessimistic. In purple bacteria where the primary acceptor Q had been prereduced (Fig. 5), an L component with a lifetime of about 100 ns and amplitude up to 30% relative to F was readily demonstrated by this method (Godik and Borisov, 1979; see also Haehnel *et al.,* 1983). Various types of evidence point to a distinctive nature and origin of recombination L compared to F. For instance, in the framework of the so-called radical pair mechanism, the separated charge pair is localized for a while within a complex which oscillates between a singlet and a triplet state. The proportion of singlet in this complex can be modified by applying a magnetic field, the result being stimulation of recombination L (Hoff, 1981; van der Wal *et al.,* 1982).

D. Thermoluminescence

The states resulting from the charge separation in PSII can be stabilized at subzero temperatures. Then, by heating the sample at a constant rate, a relatively well-characterized "glow curve" with distinct peaks can

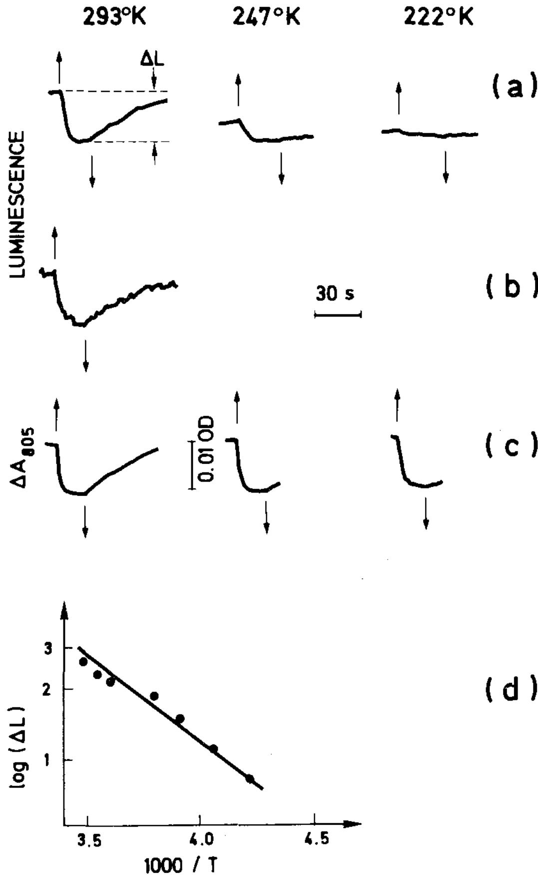


FIG. 5. "Recombination luminescence" in reaction centers of the photosynthetic bacterium *Chromatium minutissimum* under reducing conditions as a function of temperature. The total "fluorescence" (prompt F + recombination L) was excited with a modulated (8.4 MHz) He–Ne laser beam at 632.8 nm and recorded at >900 nm as either the dc (a) or ac (b) component of the signal. (Comparison of both components allows an estimation of the L lifetime.) Actinic light (↑, on; ↓, off) was at >720 nm through a phosphoroscope-type attachment allowing F measurements 0.4 ms after each actinic flash in the cycle. Actinic light oxidizes the primary donor P870 (as indicated by the absorption change traces in c), thereby decreasing the L amplitude in the total emission. In (d), an Arrhenius plot of the L amplitude (ΔL) shows an activation energy of 0.12 eV for recombination. (Adapted from Shuvalov and Klimov, 1976.)

be recorded. Each peak is generated when the appropriate thermal energy kT (k, Boltzmann constant; T, absolute temperature) is available from the ambient medium; this thermal energy should be related somehow to the activation energy of some step along the recombination path. Although the method is not very complicated, only a few groups have used it extensively. However, it has produced a wealth of results in recent years, related mainly to the donor side (Inoue and Shibata, 1982) or the acceptor side (Rutherford *et al.*, 1982) of PSII (see Sane and Rutherford, Chapter 12, this volume).

Obviously, the succession of glow peaks along the thermogram should correspond to the succession of phases (presumably slow ones) of the L decay at room temperature. This has been clearly demonstrated by Desai *et al.* (1982), who use an interesting variant of the method (also see Vass *et al.*, 1980); TL proper and L decay at constant temperature are used in combination to check that the light sum of a given kinetic component is invariant throughout the time–temperature regime (Fig. 6). Such invariance properties are given too little attention in general.

Two kinds of information may be obtained from TL: qualitative and quantitative. By qualitative, it is meant that TL constitutes a kind of "fingerprint" of the system, which, having been suitably pretreated or modified, is frozen in a particular configuration of states. In this man-

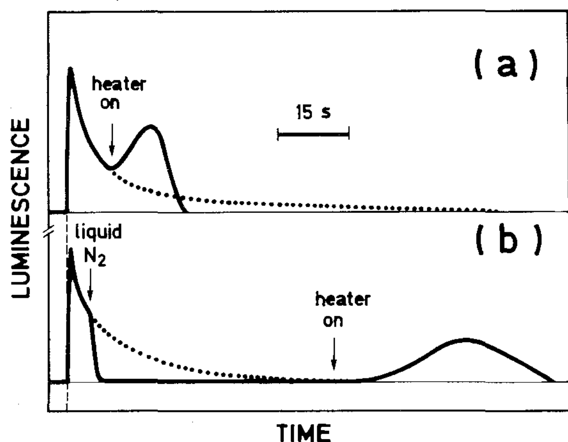


FIG. 6. Equivalence of thermoluminescence and the slow component of luminescence in the green alga *Chlorella.* L was recorded 2.5 s after excitation at 25°C. In (a), where indicated, the sample was heated to 60°C, resulting in a glow peak at about 48°C. In (b) the sample was first brought to liquid nitrogen temperature and then treated as in (a). Dotted lines represent control L decay. Note that the equivalent light sums in these experiments approximately match. (Adapted from Desai *et al.*, 1982.)

ner, peaks belonging to PSII (the majority) and PSI have been separated, and in the former group a correspondence has been attempted with the various "*S* states" of PSII. The protocol is a variant of the Joliot–Kok scheme: *n* flashes are given at some temperature, the sample is rapidly frozen, and TL is measured. By this method, lower temperature limits for the various *S*-state transitions (Kok's model) were determined.

After the discovery of TL (see Arnold, Chapter 2, this volume), the Randall–Wilkins theory was the only *de facto* framework for quantitative interpretation of the glow curve. The basic idea is certainly correct, but without doubt the theory is too simple (Lavorel *et al.*, 1982). Also, the fact that it sometimes yields unreasonable parameter values has cast doubt on its validity (Tatake *et al.*, 1981). The theory was designed for solid-state phenomena; it relates the characteristics of the peak (notably its temperature T_m) to a supposed trap depth and other universal constants. It has been adapted to standard absolute rate theory, where the related quantities are translated into activation enthalpy and entropy. A first step toward improvement was taken by Vass *et al.* (1980), who, instead of relying only on T_m values, used a computer program to decompose the whole thermogram in terms of individual Randall–Wilkins bands, which were then analyzed in the framework of the same theory. Another notable improvement was achieved after the recognition by Devault *et al.* (1983) that the kinetics of TL are determined not only by its immediate precursors ($P680^+I^-$), but by other species as well (Q_B and Kok's *S* states), where the electron and hole are trapped during cooling (see also Vass *et al.*, 1981). Therefore, the thermodynamic parameters of the whole reverse path must enter the picture, which explains how the unreasonable results mentioned above arose. Still, this theory is simplified, as the authors admit; in particular, it should include the possibility of competitive nonradiative side paths. Vass and Demeter (1984) have taken this into consideration, although their scheme does not include precursors other than the immediate ones. Another questionable assumption in the above calculations is that the activation step can be considered an equilibrium; a detailed scheme avoiding this restrictive assumption while allowing for its possibility would appear preferable. Concerning the possibility of side reactions, experimental observations are much needed; if such side reactions are negligible, then the thermogram should display some invariant properties. In particular, the light sum should be independent of the heating rate (within limits); if not, it would be possible to localize competitive side reactions either at the recombination step or at other more distant steps, depending on where the invariance breaks down—for the whole curve or for specific peaks.

Technical details of this method may be found in several papers

(Laufer and Inoue, 1980; Vass *et al.*, 1981; Desai *et al.*, 1982; Rutherford *et al.*, 1982). There are essentially three factors which greatly affect the quality of TL glow curves:

1. The heating rate β. The phenomenological equation:

$$dL = (L'_t + \beta L'_T)\, dt \tag{5}$$

where $L'_t = \partial L/\partial t$ at constant T, $L'_T = \partial L/\partial T$ at constant t, and $\beta = dT/dt$, shows how a high heating rate amplifies the peak's amplitude; on the other hand, it tends to merge closely spaced bands into a single one and to increase the thermal gradient inside the sample, which also tends to mix adjacent bands. A compromise is around 1/10 degree per second.

2. The sample thickness. The sample should be as small and as optically thin as possible to ensure its thermal and illumination homogeneity (obviously, at the cost of sensitivity). Chl concentrations of 0.1 to 1 mg/ml are commonly used.

3. The freezing rate. This problem is the most serious one and has probably led some workers to avoid using this method for fear of poor definition of the experimental conditions. Cooling is inherently slow, at least as compared to the lifetimes involved in the PSII primary events, hence at the start of the heating phase the state of the system is not well defined. A technological improvement in this area is much needed. For the moment, one may refine the protocol (as described by Demeter *et al.*, 1982) to bring the system to a low temperature where the process under study is still known to occur, give it a suitable light treatment, and rapidly cool it afterward.

E. Orientation and Polarization Effects

Electrophotoluminescence (EPL) attracts attention because it is directly related to an important property of the L precursor, which we now see as an electric dipole oriented along the normal to the membrane plane. Such dipoles are thought to give rise collectively to the thylakoid transmembrane field. In turn, the PSII dipole should be sensitive to both this light-induced field and a field established externally.

The question arises whether EPL is basically a "yield" effect or a "substrate" effect, i.e., whether the radiative recombination yield is specifically enhanced by the electric field, the total recombination yield being unaffected, or whether the field directly enhances the rate of recombination, thereby depleting the luminescence substrates (see Lavorel *et al.*, 1982). After earlier conflicting evidence, results by de Grooth and van Gorkom (1981) apparently showed the latter to be the case. They also

described a calibration procedure whereby the L recombination yield comes out to about 3%. The fact that the apparent F can be stimulated by an electric field is another argument in favor of the idea that L is a nonnegligible part of the total *in vivo* Chl emission.

Chloroplasts may be oriented in a strong magnetic field ($\sim 10^4$ gauss) (Section II,E) to obtain information on the orientation of the emitting Chl dipole with respect to the membrane geometry. This method was applied by Farkas *et al.* (1981); the observed polarized L, in agreement with F data, showed the Q_y axis of the emitting Chl to be oriented parallel to the membrane plane. In this experiment, L was transmitted through a Perspex light pipe, since the photoelectron amplification by the photomultiplier is easily affected by the presence of a magnetic field.

IV. Resonance Raman Scattering

A. Light Emitted by Molecules Contains Vibrational Information

Useful information may be expected from vibrational spectroscopy of the pigments and charge carriers in the PS membrane at the (sub)molecular level. This applies to the structures and conformations of these molecules *in situ,* their interactions with their host sites in the membrane, and molecular interactions between adjacent molecules within the same host site.

In classifying light emission spectroscopy according to its vibrational information, one may use the now classical distinction between relaxed fluorescence (F in the preceding sections), resonance fluorescence, and resonance Raman (RR) scattering (Friedman and Hochstrasser, 1974) (Fig. 7). Resonance fluorescence and RR scattering occur from a high vibronic excited state of a molecule before energy redistribution takes place; hence, they reflect the same physical process, in both cases implying, in particular, a phase relationship between incoming and outgoing photons. They can be distinguished from each other by the reemission lifetime, which is characteristic of either the molecular excited state (resonance fluorescence) or the incident radiation (RR), whichever has the narrower linewidth. Because of their generally broad electronic bandwidths, large molecules in a condensed state usually fall in the RR category when excited by monochromatic (e.g., laser) light. With relaxed or "ordinary" F, intramolecular energy transfer occurs, implying a dephasing between the incident and emitted photons, and the emission is generally broadened because of energy redistribution. The vibrational sub-

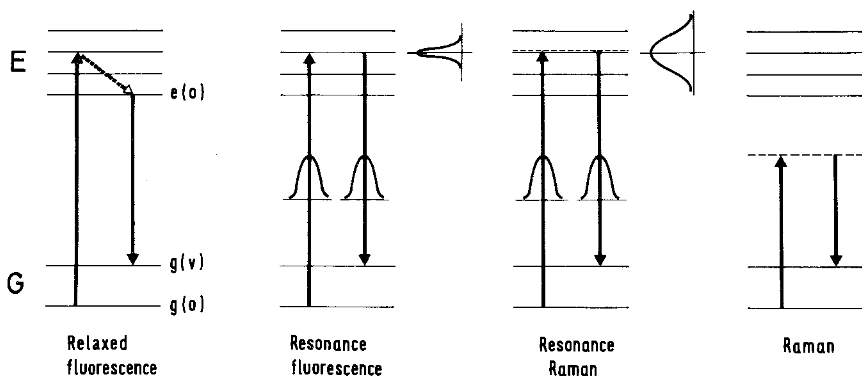


FIG. 7. Energy diagrams for fluorescence and Raman emissions by a molecule. g, ground electronic state; one of its excited vibrational sublevels is indicated as g(v); e, manifold of vibroelectronic excited states. The relative linewidths of the excited molecular states and of the incident and reemitted light are indicated by the bell-shaped curves. In the case of Raman scattering, the dashed line corresponds to the "virtual" (very short-lived) excited state reached by the molecule during the scattering process. Differences in energy between the reemitted photons (downward arrows) and the incoming photons (upward arrows) or the emitting excited state correspond to energies of vibrational modes of the molecule in its ground electronic state. These diagrams illustrate the so-called Stokes processes, which involve light absorption by molecules in their ground vibrational states.

levels of relaxed F are generally too blurred to be really useful in a vibrational study [Shpolsk'ii and site selection spectroscopy (Rebane and Avarmaa, 1982) are based on stringent environmental conditions generally not met *in vivo*]. In practice, RR scattering, where photons are reemitted before intramolecular energy transfer occurs, is the only general method for observing vibrational properties of PS molecules *in vivo* or in complexes extracted from the membrane.

B. Properties of Resonance Raman Scattering

RR scattering is a high-resolution vibronic spectroscopy in which the intensity of the scattered light depends on matching of the excitation wavelength with an electronic transition of the molecule. While the frequencies of the vibrational modes observed are those of the ground or lowest electronic states, the intensities of the bands largely depend on the properties of the higher electronic state(s). It is customary to distinguish RR from "ordinary" Raman scattering. This distinction, however, is operational rather than physical (Fig. 7). Raman scattering is observed when the energy of the incident photons is significantly lower than that

of the lowest electronic transition of the molecule. In this case, the scattering cross sections of the various Raman-active vibrational modes of a molecule are very small and are all of the same order of magnitude. Thus, in a complex molecular assembly excited far from any vibronic transition, the Raman-active transitions of the chemical species present all contribute significantly to the Raman scattering, and most regions of the spectrum tend to become overcrowded and relatively useless. By contrast, the resonance effect promoted by excitation close to a vibronic transition of a single molecular species selectively enhances scattering from that species, by a factor of up to 10^6, and, more precisely, scattering from those vibrational modes involving atoms participating in the vibronic transition. For instance, Chl excited in the far-red or Soret bands yields an RR spectrum showing only vibrations of the conjugated part of the phorbin ring and adjacent bonds, but not of the phytol chain (Fig. 8). RR spectra of such chromophores can be selectively observed from material as complex as whole cells (Lutz, 1975).

The purple membrane of halobacteria (Govindjee and Ebrey, Chapter 14, this volume) is an almost ideal object for RR studies of the primary light-driven events, using retinal of bacteriorhodopsin (BR) as a probe. It contains a highly concentrated chromophore, the RR scattering cross section of which can be very high (Myers *et al.*, 1983). The situation is less

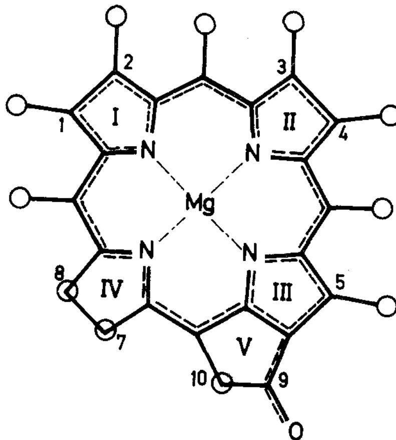


FIG. 8. Chlorophyll *a* as seen by resonance Raman spectroscopy (Soret band excitation). Significant contributions to the spectra arise from the conjugated part of the phorbin skeleton and from the ketone carbonyl in position 9. Note that the vinyl C=C group in position 2 is not seen as significantly conjugated to the phorbin π electron system. Circles indicate nonconjugated atoms or atomic groups which contribute to the vibrational properties of the Raman-active modes.

favorable for most other PS prokaryotes and eukaryotes, which generally contain more than one type of chromophoric molecule. However, in most cases a proper choice of the excitation wavelength makes it possible to selectively tune the RR experiment to each of these pigments, which then constitute independently observable Raman probes. The relatively high concentration of the antenna pigments, with their extensive sets of highly allowed transitions, makes it difficult to observe other membrane chromophores, which occur in much lower concentrations. However, these chromophores, which include the RC pigments and electron carriers that absorb in the visible region, can be studied in isolated complexes derived from the membrane (Lutz, 1984). RR spectroscopy has also produced very specific information on simple *in vitro* systems, in particular on isolated molecules such as chlorophylls, cytochromes, ferredoxin, and plastocyanin (Lutz, 1984).

C. Technical Constraints

The application of RR spectroscopy to PS membranes and complexes encounters several difficulties, two of which, namely photo- and thermal degradation and fluorescence, are generally encountered in studies of biological materials. First, Raman scattering is a low-probability, low-yield process even in resonance conditions, and therefore requires high illumination densities. Second, the Raman photons are easily "drowned" in relaxed fluorescence photons emitted either by the pigments investigated or by other molecular species present. More specific to the PS membrane is the photosensitivity of many of its chromophores, which easily undergo sensitized photooxidations. Contrary to what might be expected, problems of spurious fluorescence and of photodegradation are generally worse in purified complexes than in the intact chloroplast or cell. The choice of an excitation wavelength is often directed not only by the need to obtain high selectivity, but also by the necessity to avoid excitation of unwanted fluorescence. This is why no RR spectra of Chl have yet been produced by excitation in their lowest, strongly radiative electronic transitions (Q_y).

An elegant technique for rejecting F while detecting Raman scattering is to make use of the shorter lifetime of the Raman process ($\sim 10^{-14}$ s) than of the F process by illuminating the sample with laser pulses shorter than the F lifetime and by adequate gating (van Duyne *et al.*, 1974). Studying the K(630) intermediate of the bacteriorhodopsin cycle, Braiman and Mathies (1982) avoided the fluorescence of the initial species BR(570) by measuring RR scattering after the BR fluorescence had already decayed.

Keeping the heating and photooxidation phenomena to a minimum requires that, for a given number of detected Raman photons, the number of excitation photons, and thus also reabsorption of both incident and scattered photons, be kept to a minimum. Various experimental setups and geometries have been used for this purpose. Specifically, the effects of thermal degradation have been minimized by various means, including sample flowing or spinning (Carey, 1982; Lutz, 1984). Cooling the sample to a cryogenic temperature not only improves the spectra by sharpening the bands and allows the observation of certain reaction intermediates (Braiman and Mathies, 1982; Lutz *et al.*, 1982a), but also very efficiently protects the sample against illumination damage (Lutz, 1975). Finally, elimination of photooxidation requires thorough degassing to remove oxygen (Lutz, 1974).

D. Information Obtained from Resonance Raman Spectra

RR provides information on molecular geometry and conformation. Examples of such information on PS systems are the out-of-plane conformations of the vinyl groups of Chl *a* and Chl *b* in the antenna of higher plants and algae (Lutz, 1975, 1977), the in-plane conformations of most of the keto and acetyl carbonyls of BChl *a* in the soluble protein from the green sulfur bacterium *Prosthecochloris aestuarii* (Lutz *et al.*, 1982b), and the specific cis conformation of the carotenoids bound to the bacterial RC (Lutz *et al.*, 1978). Specific details on molecular structures can also be obtained. An example is the demonstration that the Schiff base linkage of retinal to the protein in BR is protonated and that deprotonation occurs upon illumination (Lewis *et al.*, 1974). RR also gives information on close-range, ground state intermolecular interactions such as H bonds. The bonding networks of antenna (bacterio)chlorophyll in the membrane and in isolated pigment–protein complexes (Lutz, 1977, 1984; Lutz *et al.*, 1982b) (Fig. 9) and those of pigments bound to the bacterial RC (Lutz, 1981) have been described by RR spectroscopy.

An invaluable tool of the vibrational spectroscopist is isotopic substitution. Selective modification of certain atomic masses in a molecule permits the identification of vibrational modes involving motion of these atoms and forms a basis for assigning the observed Raman bands to specific vibrational modes. Isotopic substitution in (B)Chl (Lutz, 1984) and in retinal (Mathies, 1982) has permitted such mode assignments. The additional observable parameters yielded by isotopic substitution led to the assignment of a 13-*cis*, rather than an all-*trans* configuration to the M(412) intermediate of the BR cycle (Braiman and Mathies, 1980).

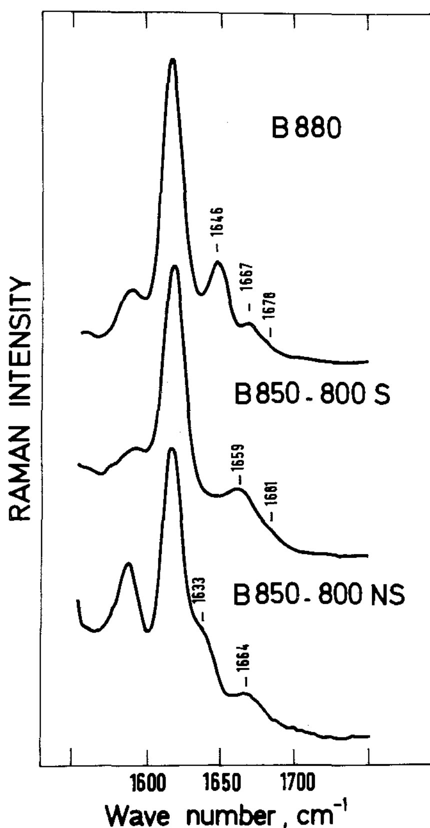


FIG. 9. Stretching modes of conjugated carbonyls in resonance Raman spectra of bacteriochlorophyll *a* bound to antenna complexes from purple sulfur (S) and nonsulfur (NS) bacteria. These complexes are seen to differ by the binding properties of their BChls. Resonance Raman spectra show a structural similarity between B880-type complexes (data not shown), but clearly distinguish between B850-800 complexes of sulfur and nonsulfur bacteria (B. Robert and M. Lutz, unpublished, 1983.)

Environmental interactions have also been characterized by isotopic substitution (e.g., Lewis, 1982).

Because of the dependence of the RR scattering intensities on excited electronic states of the molecule, information about these states can be obtained by studying variations of the intensities, and also of the depolarization ratios of the Raman bands with the excitation wavelength (Sonnich-Mortensen and Hassing, 1980). Unusual excitation profiles for several RR bands of Chl *b* in crystals of a water-soluble Chl *a*/*b*–protein complex were interpreted in terms of environmental vibronic perturba-

tions of these molecules in the Soret region (Lutz *et al.*, 1984). Excitation profiles allowed Myers *et al.* (1983) to discuss the possibility of isomerization of BR in its lower electronic excited states.

In essence, Raman scattering from nonrandom samples contains orientational information and, in particular, permits measures of order parameters in uniaxially oriented systems (Jen *et al.*, 1977). This information is often easier to extract from RR than from ordinary Raman data (Nakajima *et al.*, 1980). Polarized RR spectroscopy is thus of great potential interest in studying the orientation of pigments within the PS membrane, as suggested by studies on carotenoids in liquid crystals (Nakajima *et al.*, 1980) and in lipid bilayers (van de Ven *et al.*, 1984).

Several other specific applications of *in vivo* RR spectroscopy of PS pigments have appeared (Lutz, 1984). Among these, monitoring of membrane potentials through RR spectra of Car, because of its high sensitivity, may well become widely used (Koyama *et al.*, 1979a). Time-resolved developments of this method, on the 10-μs time scale, were applied to kinetic studies of light-induced membrane potentials in *Halobacterium halobium* (Szalontai, 1981) and in liposomes containing BR and β-carotene (Johnson *et al.*, 1981).

RR spectroscopy can also be applied to the study of intermediate states of primary processes, provided these states show electronic transitions in the region presently accessible to selective RR studies, i.e., 300–700 nm. RR is an attractive method for merely identifying these intermediate states, because of the large number of independent observable parameters contained in an RR spectrum or, better, in a set of spectra obtained with different excitation wavelengths.

In some simple cases, these intermediates can be trapped and studied by conventional techniques. This applies to the radical states of the primary donor, P870, (Lutz, 1981) and primary acceptor in the RC of purple bacteria (Lutz, 1980). These studies may benefit from the very short lifetime of the RR process in large condensed molecules. For instance, it has been proposed that in the state $P870^+$ the positive charge, which was found to be localized over two BChl molecules in EPR and ENDOR studies (see Parson, 1982), is actually localized at any moment on one BChl only on the time scale of $\sim 10^{-13}$ s to which RR gives access (Lutz and Kleo, 1979).

In other simple cases, the laser beam is used both to populate a given transient state and to monitor its RR spectrum. A photostationary equilibrium is reached and the transient may then be studied by conventional techniques. A recent example is the observation of the Car triplet in the RC of the purple bacterium *Rhodopseudomonas sphaeroides* (Fig. 10) (Lutz

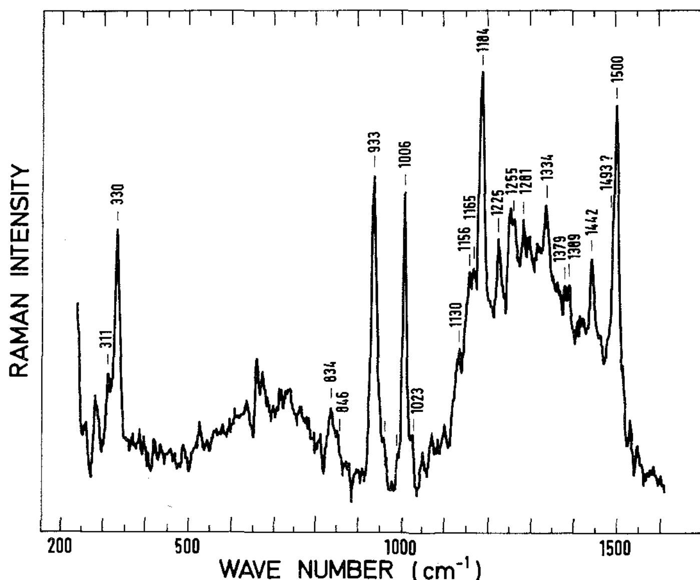


FIG. 10. Resonance Raman spectrum of the triplet state of the carotenoid spheroidene molecule present in reaction centers of *Rps. sphaeroides* obtained at 60°K by using a single continuous-wave laser beam (pump-probe technique). Excitation wavelength, 545 nm. Vibrational modes giving rise to the observed bands involve stretching of chain C=C bonds (1500 cm^{-1}), in-plane bending of chain CH groups, in-plane rocking of methyl groups, and stretching of chain C—C bonds (1184 cm^{-1}), in-plane rocking or stretching of C—CH_3 bonds (1006 cm^{-1}), and out-of-plane wagging of chain CH groups (933 cm^{-1}) (M. Lutz, B. Robert, and B. Szalontai, unpublished, 1984).

et al., 1982a). More sophisticated arrangements involve separate pump and probe lasers (see Callender, 1982).

Steady-state excitation and detection can also be achieved with a flowing sample. A simple, single continuous laser setup can be used, for instance, to obtain RR spectra from photolabile species (Marcus and Lewis, 1977). The time scale can be adjusted by changing the sample velocity and/or the beam diameter (Terner *et al.*, 1979). Spinning cells are also widely used; they require much less material and may be adapted to low-temperature work (Braiman and Mathies, 1982; Carey, 1982).

Further adjustment of the time scale in order to maximize the concentration of a given intermediate and to optimize its detection involves the use of pulsed or modulated laser sources. Although most studies are still made in the microsecond to nanosecond range, the BR cycle has been

investigated in the picosecond domain by combining pulsed laser and sample flow techniques (Terner *et al.*, 1980; Hsieh *et al.*, 1983).

Time-resolved spectroscopy not only yields a large number of structural parameters, but also offers an unusually high degree of freedom. It allows the experimenter to vary the pump and probe laser pulse lengths, pump laser power, delay time between pump and probe pulses, pump and probe wavelengths, etc. It therefore not only permits the identification of transients and the measurement of their lifetimes, but also, as discussed by El Sayed (1979), often allows one to distinguish between the various possible relations among these transients.

E. Future Developments

Many of the foreseeable developments of RR studies in photosynthesis will be made possible by recent technological and methodological improvements. Examples are the use of RR-detected triplet state magnetic resonance (Clarke *et al.*, 1983) and of coherent anti-Stokes resonance Raman spectroscopy (Carey, 1982). The latter method has already been applied to Chl (Hoxtermann *et al.*, 1982) and to BR (Nelson *et al.*, 1979). Because of the spatial coherence of the output beam, it offers the major advantage of easily extracting the vibrational information from spurious emission. Another promising development is that of surface-enhanced RR scattering. Not only does this method combine scattering enhancement by the resonance and surface effects (Cooney *et al.*, 1982), but also a metal electrode may be used to set the electrochemical potential of the system (Cotton and van Duyne, 1982).

Although several natural RR probes exist in the PS membrane, one may foresee the use of artificial RR probes, particularly in treating membrane energization problems (Koyama *et al.*, 1979b) or in studying colorless membrane components (Carey, 1982).

Appendix A: Fluorescence Complementary Area

Referring to Fig. 1, the meaning of the complementary area (CA) may be easily understood in a qualitative way. At $t = 0$, assuming that all PSII RCs are open, the rate of photochemistry is maximal and F is minimal (F_0, while at large t all RCs are (asymptotically) "closed," photochemistry is brought to an end, and F is maximum (F_m). At any finite t, therefore, $F_m - F(t)$ reflects the rate of photochemistry. Moreover, if the relationship between this difference and the rate of photochemistry is a proportional one, it follows—as we shall see below—that the CA (shaded slanting downward to the left in Fig. 1) is also proportional to the amount of substrate photochemically transformed, i.e., to the amount of closed reaction centers in the sample.

We shall give a brief demonstration of the above statement in the framework of a simple phenomenological kinetic scheme. Since CA calculation simply amounts to bookkeeping of

incoming photons and of photons used by photochemistry or otherwise, this approach will be sufficient. We shall consider several cases of different complexity.

1. SIMPLE PHOTOCHEMICAL QUENCHING

Let us assume that the photochemical closing of PSII centers is the only light-induced reaction:

$$Q \xrightarrow{h\nu} Q^- \tag{A.1}$$

(Q, open reaction center; Q^-, closed reaction center). Equation (A.1) refers to the fact that under ordinary light conditions PSII photochemistry (and turnover) is rate-limited by the photoreduction of the primary electron acceptor Q. The rate of the reaction is:

$$-dq/dt = Ik_Pq/(k_F + k_D + k_Pq) \qquad q = q_0 \quad \text{at } t = 0 \tag{A.2}$$

where $q = [Q]$, the k's are rate constants (the subscripts P, F, and D stand for photochemistry, fluorescence, and nonradiative deactivation), and I is the rate of light absorption ($h\nu$/s) in the assumed unit volume containing the sample (for simplicity we assume unit quantum yield of the reaction). Equation (A.2) is rearranged to:

$$-dq/dt = I\{1 - [(k_F + k_D)/k_F][k_F/(k_F + k_D + k_Pq)]\} \tag{A.3}$$

In Eq. (A.3), the ratios of rate constants are related to fluorescence yields:

$$\Phi = k_F/(k_F + k_D + k_Pq) = F/I$$

$$\Phi_m = k_F/(k_F + k_D) = F_m/I$$

where F and Φ are functions of q, i.e., of t. Therefore,

$$-dq/dt = I(1 - \Phi/\Phi_m) = I(1 - F/F_m) = (1/\Phi_m)(F_m - F) \tag{A.4}$$

Taking the integral of Eq. (A.4) yields the fundamental CA relations:

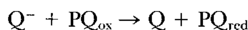
$$-\int_{q_0}^{0} dq = (1/\Phi_m)\int_0^\infty (F_m - F)\,dt \tag{A.5}$$

or

$$q_0 = (1/\Phi_m)\mathrm{CA} \tag{A.6}$$

From Eq. (A.6) it is obvious that CA is independent of I, as was first verified by Malkin and Kok (1966).

To introduce the next case, note that if Q^- is regenerated (e.g., reoxidized by plastoquinone, PQ):



$$Q^- + PQ_{ox} \rightarrow Q + PQ_{red}$$

Eq. (A.1) is no longer valid. In particular, if Q^- is constantly reoxidized by PSI (through PQ), a steady-state condition ($dq/dt = 0$) will soon be reached. Obviously, in such a case CA is not simply related to q as in Eq. (6). What is required for the above relationship is that the $Q \rightarrow Q^-$ step be properly isolated; addition of DCMU, which inhibits Q^- reoxidation by PQ, is the standard way to accomplish this, but it may also be done kinetically if I is large enough compared to the rate of Q^- reoxidation ($t_{1/2} = 200\ \mu$s).

2. PHOTOREDUCTION OF Q AND PQ

Let us assume as above that Q^- is reoxidized by PQ but that the latter is not in turn reoxidized by PSI. In other words, PSII light serves the purpose only of filling the Q − PQ pool with electrons. Kinetic isolation of the PSII-Q-PQ segment is then easily accomplished at moderate I values, since the limiting step between PQ and PSI is slow ($t_{1/2}$ = 20 ms); furthermore, without electron acceptors PSI is not very active. The system is now described by a set of two equations:

$$\begin{aligned} -dq/dt &= Ik_P q/(k_F + k_D + k_P q) + k_0 q q' \\ -dq'/dt &= -k_0 q q' \qquad q' = q'_0 \quad \text{at} \quad t = 0 \end{aligned} \tag{A.7}$$

where $q' = [PQ_{ox}]$ and k_0 is the rate constant of Q^- reoxidation by PQ_{ox}. Setting $s = q + q'$,

$$-ds/dt = Ik_P q/(k_F + k_D + k_P q) \tag{A.8}$$

an equation which can be transformed, like Eq. (A.3), into an expression involving only F, F_m, and ds/dt. Therefore CA now allows quantitation of the total Q − PQ acceptor pool.

Since with DCMU, CA is related only to Q and the latter accommodates only one electron equivalent, the ratio of CA with and without DCMU is a simple measure of the whole acceptor pool in electron equivalents.

3. NONPHOTOCHEMICAL QUENCHING BY PQ_{ox}

As shown by Vernotte *et al.* (1979), PQ_{ox} exerts a limited but nonnegligible quenching effect on PSII fluorescence. Being nonphotochemical, this quenching is different from that considered above. Since it provides another channel of exciton deactivation, it enters the equation differently; e.g., Eq. (A.3) [and similarly Eq. (A.7)] should be modified as follows:

$$-dq/dt = Ik_P q/(k_F + k_D + k_P q + k_Q q') \tag{A.9}$$

where k_Q is the F quenching rate constant related to PQ_{ox}. Again the system must be described by a set of two differential equations as in Eq. (A.7). However, because of the term $k_Q q'$ in the denominator of Eq. (A.9), this system cannot be written as an analytical expression involving F, F_m, and ds/dt, as for Eq. (A.7). Therefore CA cannot be calculated, although it can be numerically computed. Actually, it is found that

$$-ds = (1/\Phi_m)(F_m - F\gamma')\, dt \tag{A.10}$$

where

$$\gamma' = (k_F + k_D + k_Q q')/(k_F + k_D) \tag{A.11}$$

and, like F, is a function of t. Qualitatively, it is seen that quenching by PQ_{ox} causes one to overestimate the PQ pool.

4. QUENCHING IN THE CLOSED STATE

If one assumes that the constant fluorescence F_0 is a "live" PSII fluorescence, i.e., emitted entirely by functional PSII, it represents the excitation which has escaped trapping by the open PSII RC. Then, however, F_m (relative to F_0) is smaller than expected, in view of the high PSII photochemical yield. One therefore may assume that closed centers are (weak) fluorescence quenchers (Butler and Kitajima, 1975) or, alternatively, that only part

of F_0 is "live," the remaining part being "dead," i.e., not subject to any photochemical quenching (Lavorel and Joliot, 1972). The hypothesis of quenching by closed centers also changes the meaning of CA, but in this case the effect is readily calculated by the following differential equation:

$$-dq/dt = I\gamma'' k_{P'} q/(k_F + k_{D'} + k_{P'} q) \tag{A.12}$$

[compare with Eq. (A.2)], where $\gamma'' = k_P/k_{P'}$, $k_{P'} = k_P - k_C$, $k_{D'} = k_D + k_C$, and k_C is the rate constant for F quenching by closed centers. As $\gamma'' > 1$, it is seen that in this case CA underestimates the acceptor pool by a factor $1/\gamma''$.

Appendix B: Methods of Curve Analysis

Fluorescence decay kinetics (see Moya *et al.*, Chapter 7, this volume) illustrates the use of modern methods of objective data analysis, as shown by Gulotty *et al.* (1982), Haehnel *et al.* (1982), Lotshaw *et al.* (1982), Nairn *et al.* (1982), and Telfer *et al.* (1983). The problem is to analyze the decay curve in terms of several exponential components; it is compounded with the need to deconvolute the excitation pulse and instrument functions and to correctly weigh each data point with its probable error. The general mathematical method is that of nonlinear least-squares fitting, described by Levenberg (1944) and Marquardt (1963) and adapted to the specific problem by Laemmli (1970), Grinvald and Steinberg (1974), Tamkivi and Avarmaa (1978), and Turko *et al.* (1983). Textbooks by Bevington (1969) and Kendall and Stuart (1976) should be consulted for proper statistical error evaluation. The same methods of curve fitting may also be applied to L decay (Arata and Parson, 1981).

The contribution of the so-called α and β centers to the F induction rise similarly requires the reduction of data into two exponential components (Melis and Homann, 1978). A complete characterization of parameters in the complementary area (see Appendix A) may be found in Ford *et al.* (1978), Malkin *et al.* (1981), and Malkin and Fork (1981).

Finally, the same general least-squares procedures have been applied to the decomposition of glow curves into individual Randall–Wilkins bands by Vass *et al.* (1980) and Droppa *et al.* (1981).

Appendix C: Techniques of Fluorescence Measurement

A detailed and complete description of a fluorescence setup is given by e.g., Schreiber (1983). Although conventional optics (lenses) are still employed in most cases, they tend to be replaced by fiber optics, which allow compact design and provide protection against accidental misadjustment or other mechanical disturbances (see, e.g., Haworth *et al.*, 1982a).

Fluorescence spectroscopy requires more elaborate equipment, notably when a calibration of the quantum excitation flux must be maintained throughout the spectrum (microcomputers are ideally suited for this) and at low temperatures. These topics are treated in several papers (Govindjee *et al.*, 1979; Rijgersberg *et al.*, 1979; Wang *et al.*, 1980; Sigfridson and Oquist, 1980). The conditions of cooling and how they might affect the F bands are discussed by Harnischfeger (1979).

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5

Fluorescence of Photosynthetic Pigments *in Vitro*

GILBERT RANDALL SEELY
Battelle
Charles F. Kettering Research Laboratory
Yellow Springs, Ohio 45387

JOHN STEPHEN CONNOLLY
Photoconversion Research Branch
Solar Energy Research Institute (SERI)
Golden, Colorado 80401

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ABBREVIATIONS AND SYMBOLS

BChl	Bacteriochlorophyll
BPheo	Bacteriopheophytin
CD	Circular dichroism
Chl	Chlorophyll
CMC	Critical micelle concentration
[M]	Molar concentration of substance M
$^1M^*$	Lowest singlet excited state of M
^3M	Lowest triplet state of M
Pheo	Pheophytin
ϕ_f	Fluorescence yield
SDS	Sodium dodecyl sulfate
τ_f	Fluorescence lifetime
THF	Tetrahydrofuran

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ABSTRACT

Early observations of the physical phenomenon of photosynthesis showed that a low level of fluorescence normally accompanies photosynthesis and that this level is related to the physiological state of the photosynthetic apparatus. For a long time there has been interest in discovering the conditions that determine the level of fluorescence of chlorophyll, and other photosynthetic pigments, *in vitro*. This chapter addresses the problem in two ways. First, the measurable properties of fluorescence are introduced and their uses are briefly discussed. Second, the fluorescence of chlorophyll in various condensed or aggregated systems is discussed in search of systems with fluorescence properties similar to those of chlorophyll *in vivo*. In spite of much work on this subject, there are still some very fundamental questions regarding the fluorescence properties of chlorophyll.

I. Introduction

It is now 150 years since Brewster discovered the fluorescence of chlorophyll (Chl). Since that event, literature on the subject has accumulated to truly staggering proportions. If a reason for this proliferation is sought, it may lie in the recognition that algae and green leaves are weakly fluorescent, and that fluorescence intensity changes accompany changes in the rate of photosynthesis. The compass of this chapter precludes a comprehensive or perhaps even an adequate review of fluorescence of photosynthetic pigments; instead, we set two lesser objectives. First, the various properties of fluorescence are explained and some of their uses discussed. Then the fluorescence of aggregated and heterogeneous systems of Chls is discussed in an attempt to illustrate the value of these properties. The study of Chls in condensed states is motivated by the desire to learn more about the manner of association of the pigment with the thylakoid membrane *in vivo*. Structures of the Chl pigments discussed in this chapter are shown in Fig. 1.

If the purpose of photosynthesis is to convert excited state energy into chemical free energy with the maximum efficiency (see Norris and van Brakel, Chapter 3, this volume), it might seem strange that any light energy at all is "wasted" as fluorescence from the photosynthetic apparatus. However, Bolton *et al.* (1981) have argued from the second law of thermodynamics that for maximal energy storage a certain small loss by fluorescence is unavoidable, and, indeed, part of the fluorescence from plants is believed to result from back electron transfer at reaction centers.

A milestone in the history of Chl fluorescence was the discovery that whereas Chl *a* in dry hydrocarbon and chlorocarbon solvents is almost nonfluorescent, the addition of small amounts of "activators" such as amines, alcohols, or water restores fluorescence to its normal range of

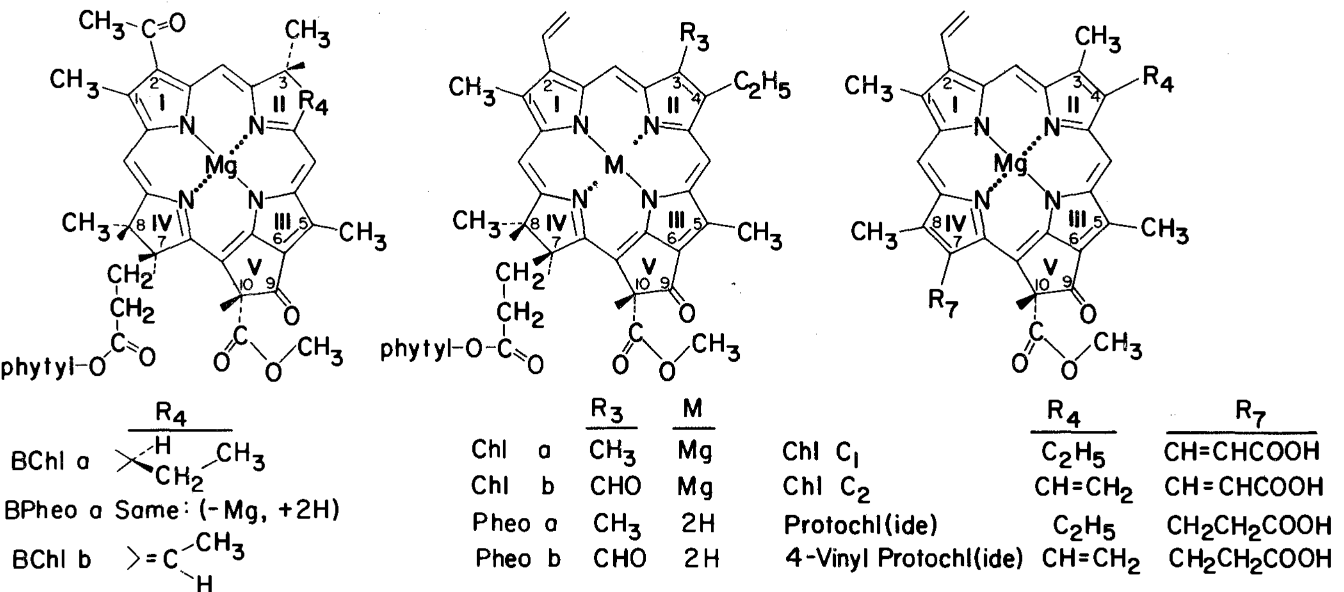

FIG. 1. Structures of naturally occurring chlorophyll pigments mentioned in the text. The conventional numbering of the rings and of the substituted carbon atoms is indicated.

values in polar solvents (Livingston *et al.*, 1949; Evstigneev *et al.*, 1950). It was thus recognized that in dry nonpolar solvents Chl *a* exists as nonfluorescent dimers and oligomers, and that the addition of basic substances which ligate its Mg dissociates the aggregates into monomeric pigment, which is fluorescent.

II. Physical Processes and Their Significance

A. Absorption of Light: Energy Levels

Absorption of a photon raises a pigment molecule to its lowest singlet excited state or to one of its many higher excited states. Usually, energy in excess of its lowest excited state is quickly dissipated into heat. Simplified energy level diagrams of Chl *a* and BChl *a* are given in Fig. 2 with their conventional term designations. Recent and quite thorough quantum mechanical calculations for these molecules have been described by Petke *et al.* (1979, 1980), and these references and Shipman (1982) should be consulted for previous treatments and for more detailed interpretations of the spectra. In spite of many calculations there still remain differences of opinion in the literature as to the location of the second (S_2) excited state of Chl *a* and the composition of the Soret band in the blue and near-ultraviolet.

The energy of the lowest excited state of Chl *a* varies a little with the refractive index of the solvent, from about 15,130 cm^{-1} (661 nm) in ether to about 14,820 cm^{-1} (675 nm) in aniline (Seely and Jensen, 1965). The positions and relative intensities of the absorption bands are further affected by the dielectric constant of the solvent, by its ability to form hydrogen bonds to the carbonyls of Chls, and by whether the Mg is penta- or hexacoordinated.

The energies of the triplet state levels are speculative, except for the lowest level of Chl *a* from which phosphorescence has been observed (Krasnovskii *et al.*, 1974; Mau and Puza, 1977; Dvornikov *et al.*, 1979). For Chl *a* the triplet level T_3 probably lies close to the singlet level S_1 (Petke *et al.*, 1979), and for BChl *a* T_2 is probably close to S_1 (Petke *et al.*, 1980).

B. Singlet State Decay Processes

There are always at least three options for decay of the lowest singlet excited state. With reference to Fig. 2, these are *fluorescence,* in which the molecule returns to the ground state by emission of a quantum of light,

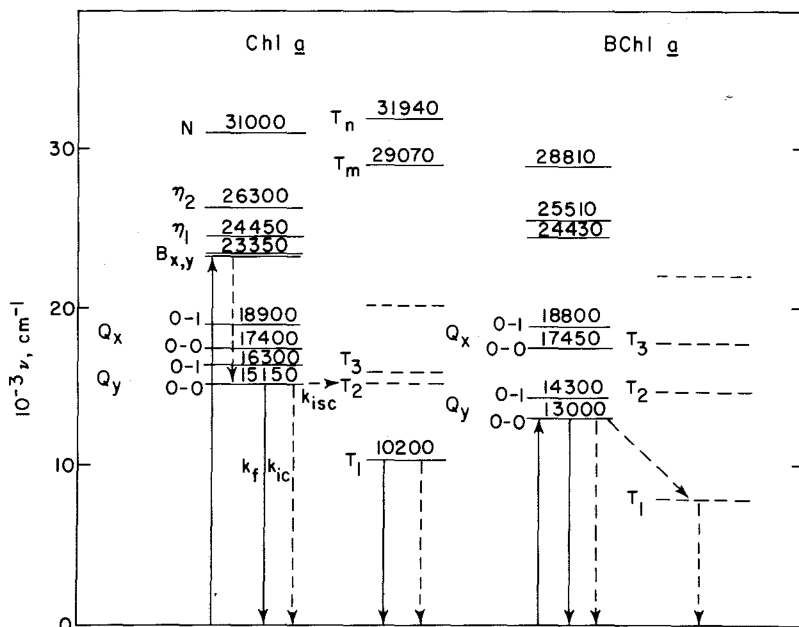

FIG. 2. Energy level diagrams for Chl *a* and BChl *a* in ethyl ether, based on energy levels cited or referenced in Petke *et al.* (1979, 1980). Arbitrary but conventional designations are given for the levels, but the true orbital nature of some of them is likely to be more complex than suggested here. Energy levels are given in reciprocal centimeters; solid lines denote known spectroscopic states; dashed lines are levels calculated by Petke *et al.* (1979, 1980) in lieu of observed states. Solid lines also denote transitions with absorption or emission of a photon, dashed lines those without. Vibrational levels of the ground state have been omitted. The lowest singlet excited states, S_1 and S_2, of Chl *a* and BChl *a* are represented by Q_y and Q_x on the diagram, each with its conspicuous vibrational envelopes designated 0–0 and 0–1. The *x* and *y* designate orientation of the transition moment vectors approximately along axes between rings II and IV, and I and III, respectively (see Fig. 1).

internal conversion, in which the energy of the molecule is converted into vibrational energy of the ground state, and *intersystem crossing* to the triplet manifold with eventual descent to the lowest triplet excited state. If the three rate constants (all s^{-1}) are k_f, k_{ic}, and k_{isc}, respectively, the quantum yield of fluorescence is

$$\phi_f = k_f/(k_f + k_{ic} + k_{isc}) \quad (1)$$

Likewise, the quantum yield of triplet state formation is

$$\phi_T = k_{isc}/(k_f + k_{ic} + k_{isc}) \quad (2)$$

The radiative decay rate k_f is related to the absorption and fluorescence spectra by Eq. (3) (Strickler and Berg, 1962):

$$k_f = 2.880 \times 10^{-9} n^2 \langle \nu_f^{-3} \rangle_{av}^{-1} \int \varepsilon(\nu)\, d \ln \nu \tag{3}$$

in which n is the refractive index of the medium, $\varepsilon(\nu)$ is the molar absorptivity at wave number ν, and

$$\langle \nu_f^{-3} \rangle_{av}^{-1} = \int f(\nu)\, d\nu \Big/ \int f(\nu) \nu^{-3}\, d\nu \tag{4}$$

where $f(\nu)$ is the fluorescence intensity spectrum in quantum units as a function of ν.

For dilute Chl *a* and *b*, in solvents such as ether and toluene, fluorescence and intersystem crossing account for at least 90% of the singlet decay (Bowers and Porter, 1967; Dzhagarov, 1970). For pheophytin (Pheo) *a* and BChl *a*, however, internal conversion accounts for at least half of singlet state decay (Gurinovich *et al.*, 1967; Connolly *et al.*, 1982b). For carotenoids, decay is almost entirely through internal conversion; the triplet state is not formed in appreciable yield by direct excitation.

If certain other substances are present in solution along with Chls, two bimolecular pathways for deactivation of singlet excited states may become available: transfer of energy to a molecule with an excited state of lower energy (see van Grondelle and Amesz, Chapter 8, this volume), and transfer of an electron to or from the other substance (see Norris and van Brakel, Chapter 3, this volume). Both result in quenching of the fluorescence of the originally excited molecule; the former may result in fluorescence of the energy acceptor molecule (*sensitized fluorescence*), the latter in a transitory ion pair, which may under certain circumstances result in permanent chemical change.

Transfer of energy among antenna pigments, ultimately to reaction centers, is crucial to photosynthesis and is discussed in other chapters of this volume (see, e.g., Briantais *et al.*, Chapter 18, Govindjee and Satoh, Chapter 17, Fork and Mohanty, Chapter 16, and Amesz and Vasmel, Chapter 15).

Transfer of energy between two molecules usually follows the law derived by Förster for dipole-induced dipole interactions (Förster, 1948; Ketskeméty, 1962; Knox, 1975; Kawski, 1983):

$$k_{tr}\,(\mathrm{s}^{-1}) = \frac{9000 \ln 10 \kappa^2}{128 \pi^5 n^4 N \tau_0 R^6} \int_0^\infty \frac{f(\nu)\varepsilon(\nu)\, d\nu}{\nu^4} \tag{5}$$

In Eq. (5) the integral is the overlap between the normalized donor fluorescence $f(\nu)$ and the molar absorptivity of the acceptor $\varepsilon(\nu)$ on a wave number (ν) scale, R is the distance between the energy donor and the acceptor, N is Avogadro's number, τ_0 ($= 1/k_f$) is the radiative lifetime of the donor, and κ^2 is an orientation factor. This relation is valid whether or not the donor and acceptor are the same kind of molecule, and the donor may be of triplet multiplicity. The dependence on R^6 means that the rate of transfer varies enormously with separation between the molecules, but it also varies considerably with the mutual orientation (κ^2) of the molecules. Equation (5) is often cast in the simpler form

$$k_{tr} = (R_0/R)^6/\tau_f \tag{6}$$

where τ_f [$= 1/(k_f + k_{ic} + k_{isc})$] is the observed lifetime in the absence of acceptors, and R_0 is the distance, characteristic of the donor–acceptor pair, at which the probability of transfer is equal to that of decay. Estimates of R_0 for transfer between Chl *a* molecules average around 60 Å (Kawski, 1983; see also van Grondelle and Amesz, Chapter 8, this volume), so at the much smaller separations maintained between Chls in the photosynthetic unit, energy transfer is very fast indeed.

Energy transfer as expressed by Eq. (5) or (6) accurately describes the process as it occurs between two molecules, but tells us very little about the migration of energy in an ensemble of molecules. The theory has been extended to describe the case where a donor is embedded in a field of randomly located and oriented acceptors (for a review, see Knox, 1968) and to the very important case (for photosynthesis) where an acceptor trap is surrounded by a field of randomly located donors (Bojarski and Domsta, 1971).

The transfer of an electron from a singlet excited state molecule to an acceptor, or from a donor to an excited state molecule, may occur if free energy decreases in the process. In effect, the energy of the absorbed photon must overcome both the activation energy of electron transfer and the endothermicity of the reaction between ground state molecules. The theory behind the rate of such transfers and its relation to electrochemical potentials and solvent rearrangement energies have been extensively discussed (Marcus, 1964).

Quenching by electron transfer in fluid media can usually be treated as a second-order reaction. The fluorescence yield in the presence of quenchers is given by

$$\phi_{fQ} = k_f/(k_f + k_{ic} + k_{isc} + k_Q[Q]) \tag{7}$$

where [Q] is the quencher concentration. In terms of the yield of unquenched fluorescence ϕ_f and the lifetime τ_f, this becomes

$$\phi_f/\phi_{fQ} = 1 + \tau_f k_Q[Q] = 1 + K_{sv}[Q] \quad (8)$$

For Chl *a*, τ_f is roughly 5×10^{-9} s (see Table I), and the diffusion-limited rate constant is roughly $10^{10}\,M^{-1}\,s^{-1}$, so that the Stern–Volmer constant K_{sv} is approximately 50 M^{-1} at the most. Values of this order of magnitude are commonly reported (Livingston and Ke, 1950; Seely, 1969; Kapinus *et al.,* 1982; Natarajan and Blankenship, 1983).

Electron transfer, like energy transfer, takes place not strictly on contact but over a range of separations, by a process generally called tunneling (DeVault, 1984). Since it depends on orbital overlap, the rate of electron transfer varies more strongly with molecular separation than energy transfer and is negligible beyond 15–20 Å (Potasek and Hopfield, 1977; Ke *et al.,* 1979; Jortner, 1980; Miller *et al.,* 1982).

C. Fluorescence Spectra

The spectra of Chls, like those of most molecules, represent only transitions from the lowest singlet excited state to the ground state. Chls are among the molecules which obey the so-called mirror image relationship between absorption and fluorescence spectra, by which the vibrational structure of the first singlet excited state in absorption is reflected in the vibrational structure of the fluorescence. A more precise formulation is to state that there exists a frequency ν_0 such that for all frequency differences $\Delta\nu$, the normalized absorbance and fluorescence quantum intensities $A(\nu_0 + \Delta\nu)$ and $F(\nu_0 - \Delta\nu)$ are related by Eq. (9).

$$\frac{A(\nu_0 + \Delta\nu)}{\nu_0 + \Delta\nu} = \frac{\nu_0^2 F(\nu_0 - \Delta\nu)}{(\nu_0 - \Delta\nu)^3} \quad (9)$$

Obedience to this relation implies that the molecular configuration and vibrational frequencies change little on excitation (Birks and Dyson, 1963).

A relation between the profiles of the absorption and emission bands in the region of overlap was derived by Stepanov (1957) from the laws of radiation and thermodynamics. For validity, it requires configurational similarity in ground and excited states and thermal equilibration of energy among sublevels before emission. It is conveniently expressed in the form

$$\frac{f(\omega)}{\varepsilon(\omega)} = D(T)\omega^3 e^{-h\omega/kT} \quad (10)$$

where $\varepsilon(\omega)$ and $f(\omega)$ are the molar absorptivity and the normalized fluorescence spectrum in energy units as functions of frequency ω ($= \nu c$), h and k are Planck's and Boltzmann's constants, c is the speed of light, and $D(T)$ is a function of T, the absolute temperature. Plotting the left-hand side against ω, or ν, should give a straight line whose slope can be used to calculate a temperature T. Temperatures so derived for Chl *a* by Singhal and Hevesi (1971) were somewhat higher than ambient in most solvents, perhaps because thermal equilibration was not complete.

From the Stepanov equation a relation can be derived between the Stokes shift $\bar{\nu}_a - \bar{\nu}_f$, or difference in wave numbers between absorption and fluorescence band maxima, and the bandwidth b (Kazachenko, 1965).

$$\bar{\nu}_a - \bar{\nu}_f = \frac{b^2hc}{8kT \ln 2} \quad (11)$$

This relation is obeyed quite well for Chl *a* in solution at 20°C and is useful for correlating absorption and fluorescence band components (Litvin and Stadnichuk, 1979; Stadnichuk and Shutilova, 1980; Kusumoto *et al.*, 1983a).

The fluorescence spectrum of Chl *a* in ether can be represented quite well as the sum of three Gaussian bands centered about 666.7, 687, and 722.5 nm in ether (Fig. 3). Fluorescence bands such as these are broad because they are envelopes of spectra of Chl *a* in all the microenvironments available to it. At sufficiently low temperatures, each Chl *a* molecule becomes frozen into a particular site with narrowly defined energy levels. If fluorescence is excited at ca. 5K with a sharply defined laser frequency, it consists of a continuum with a number of sharp lines superimposed upon it, as in Fig. 4 (Litvin *et al.*, 1969; Avarmaa, 1974; Bykovskaya *et al.*, 1980; Hála *et al.*, 1981). The frequencies of the lines differ from the laser excitation frequency by amounts equal to normal vibrational frequencies of the Chl. The sharp lines are explained as zero-phonon lines, the intensities of which are determined by the number of Chls in sites which absorb the laser excitation wavelength (Hála *et al.*, 1985).

Although certain carotenoids transfer singlet state energy to Chls in photosynthesis, they are not themselves conspicuously fluorescent substances. The reason became clear when it was realized that the excited state responsible for the strong blue absorption bands of polyenes, and for their yellow color, is not the lowest-energy excited singlet state (Hudson and Kohler, 1974). Rather, the lowest-energy state is one of the same symmetry, termed ^1A_g, as the ground state, to and from which transitions are strongly forbidden by symmetry selection rules. This state in

TABLE I
Fluorescence Lifetimes and Quantum Yields at Room Temperature

Pigment	Solvent type[a]	Concentration (*M*)	Degassed	τ_f (ns)	Method[b]	Remarks[c]	Refs.[d]	ϕ_f	Refs.[d]
Chl *a*	H	$\geq 10^{-7}$	Yes	6.44	SPC	a	7	0.35	14,16
		$\geq 10^{-6}$	No	6.54	SPC	b	19		
	P	$\geq 10^{-7}$	Yes	6.07	SPC	a	7		
		?	No	5.1	PFM	c	2–4	0.30–0.33	3,16,20
		?	Yes	6.1		d	11	0.36	11
		$\geq 10^{-6}$	No	5.56 ± 0.22	SPC	b,e	19		
		$\geq 10^{-6}$	No	6.1–6.2	SPC	b,f	19		
	CH	$\geq 10^{-6}$	No	4.6–5.5	SPC	b,g	19		
	A	$\geq 10^{-7}$	Yes	5.50 ± 0.05	Both	a	7,18		
		10^{-5}	Yes	6.3		d	6	0.22	5,20
		$\geq 10^{-6}$	No	5.2–5.8	SPC	b,h	19		
Chl c_1	P	$\geq 10^{-7}$	Yes	6.10	SPC	a	9		
Chl c_2	P	$\geq 10^{-7}$	Yes	6.50	SPC	a	9		
Chl *b*	P	2×10^{-5}	No	3.6		j	1,11	0.11–0.12	10,12,17,20
		?	No	3.9	PFM	c,j	2–4	0.16–0.17	3,4,16
	A	?	No	5.9–6.3		c,j	2,4	0.06–0.10	12,16,20
Pheo *a*	H	7×10^{-4}	Yes	8.13		d	21		
	P	?	Yes	7.3		j	1,11	0.18	10,13,17,20
		?	?	5.3–5.6		j	17	0.23–0.25	10

Pheo *b*	P	?	?					0.14	10
BChl *a*	H	$\leqslant 10^{-6}$	No	3.5–3.6	SPC	k	8	0.24–0.26 (m)	8
	P	$\leqslant 10^{-6}$	No	2.9–3.2	SPC	k	8	0.18–0.20 (m)	8
	CH	$\leqslant 10^{-6}$	No	2.5–2.6	SPC	k	8	0.18–0.21 (m)	8
	A	$\leqslant 10^{-6}$	No	2.3–2.6	SPC	k	8	0.13–0.15 (m)	8
BPheo *a*	P	$\leqslant 10^{-6}$	No	2.5	SPC	k	8	0.13 (m)	8
	A	8×10^{-4}	Yes	2.0 ± 0.2	PSL	k	15	0.09 (m)	15
BChl *b*	P	$\leqslant 10^{-6}$	No	2.5	SPC	k	8		

[a] Solvent types refer to the coordination number of Mg in BChl *a* (8). H, Hexacoordinating solvents (e.g., pyridine, THF, dioxane); P, pentacoordinating solvents (e.g., acetone, acetonitrile, diethyl ether, most simple hydrocarbons); CH, chlorinated hydrocarbons (e.g., CH_2Cl_2, $CHCl_3$, CCl_4); and A, alcohols or alcohol-containing mixtures, irrespective of Mg coordination number.

[b] SPC, Single-photon counting; PFM, phase fluorometry method; Both, SPC and PFM; PSL, picosecond laser.

[c] Remarks: a, reabsorption artifacts and oxygen quenching minimized; b, small reabsorption artifacts; measured lifetimes could be ~0.2–0.4 ns longer in the absence of oxygen quenching; c, first direct measurement; d, significant reabsorption artifacts likely due to high pigment concentration; e, mean and standard deviation of measurements in 12 hydrocarbon solvents; f, 6.10 ns in acetonitrile, 6.21 ns in cyclooctane; g, 4.58 ns in CCl_4, 5.50 ns in $CHCl_3$; h, 5.2 ns in methanol and ethanol, 5.75 ns in 2-propanol; differences may be significant; j, reabsorption artifacts likely but cannot be assessed; k, negligible quenching by dissolved oxygen due to short pigment lifetime; and m, calculated from the relation $\phi_f = \tau_f/\tau_0$.

[d] References: (1) Avarmaa *et al.* (1977); (2) Brody (1957); (3) Brody (1960); (4) Brody and Rabinowitch (1957); (5) Broyde and Brody (1967); (6) Butler and Norris (1963); (7) Connolly *et al.* (1982a); (8) Connolly *et al.* (1982b); (9) J. S. Connolly, E. B. Samuel, and S. P. Berg (unpublished, 1983); (10) Dvornikov *et al.* (1979); (11) Dzhagarov (1972); (12) Forster and Livingston (1952); (13) Gurinovich *et al.* (1968); (14) Hindman *et al.* (1978); (15) Holten (1976); (16) Latimer *et al.* (1956); (17) Livingston (1960a,b); (18) Müller *et al.* (1965); (19) Natarajan *et al.* (1984); (20) Weber and Teale (1957); (21) Yuen *et al.* (1980).

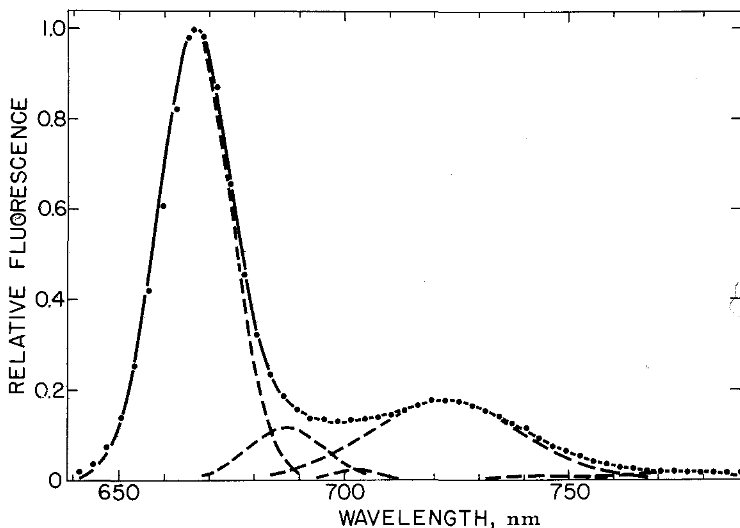

FIG. 3. Fluorescence spectrum of Chl *a*, 5.4×10^{-5} *M* in ether, measured from the front face of a 1-mm cuvette, in quantum units. The spectrum is resolved into three major bands with peak positions (and bandwidths) of 666.7 (19), 687 (20), and 722.5 (40) nm, and three minor components at 702.5 (10), 745.5 (20), and 776.5 (40) nm which together contain less than 4% of the total emitted photons. The components are plotted as dashed traces, their sum as a solid trace, and observed normalized fluorescence intensities as dots. Reabsorption is probably responsible for the deviation of observed intensities from the solid trace in the region around 660 nm.

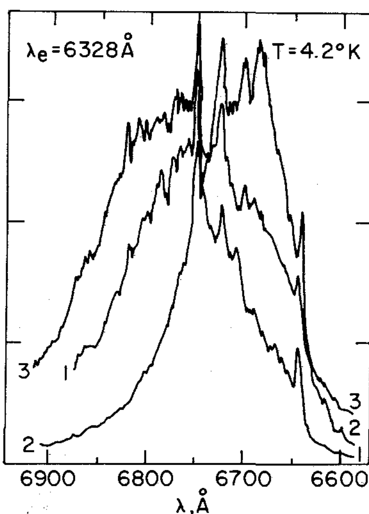

FIG. 4. Luminescence spectra of Chl *a* upon excitation at 4.2°K with the laser line 6328 Å: (1) in toluene, 10^{-5} *M*; (2) in diethyl ether, 10^{-7} *M*; (3) in pyridine, 10^{-5} *M*. (From Avarmaa, 1974.)

carotene has been detected by Thrash *et al.* (1977) in the Raman excitation spectrum upward of 17,230 cm^{-1} (580 nm). β-Carotene does appear to emit a very weak green fluorescence near 18,500 cm^{-1} (540 nm), which probably comes from the ^1A_g excited state (Lyalin *et al.*, 1963; Cherry *et al.*, 1968; van Riel *et al.*, 1983). Thrash *et al.* (1979) have suggested that the excited ^1A_g state is an intermediary in the transfer of energy to Chls *in vivo.*

D. Fluorescence Lifetimes

Notwithstanding the many advances in research on porphyrins and Chls over the past 20 years, there remain many gaps in our understanding of the spectroscopy, photophysics, and photochemistry of the natural Chls and Pheos. In some cases (e.g., fluorescence properties of the bacterial pigments) limitations of available instrumentation have, until recently, precluded reliable studies. In other instances (e.g., fluorescence lifetimes and quantum yields of Chl *a*), earlier results have often been accepted without question or reexamination. In most cases, the influence of solvent and local molecular environment on these fundamental properties has not been examined in any systematic way.

It is not possible in this brief review to present an exhaustive compilation of the published fluorescence data on these molecules. Instead, we have selected those values which we deem to be the most reliable with respect to minimization of artifacts due to self-absorption, aggregation, and quenching by dissolved oxygen. Thus, the lifetime values we list here (Table I) represent, for the most part, the results of measurements taken at very low pigment concentrations in deoxygenated solvents. The same cannot be said for the quantum yield data, which are much more sparse.

There appear to have been only a few cases in which the effect of varying pigment concentration in the low concentration range has been studied. Müller *et al.* (1965) used the phase-shift technique in one of the most careful and definitive early studies of Chl *a*, and they observed a trend of increasing measured τ_f with increasing pigment concentration over the range $\sim 10^{-7}$–10^{-4} *M*. Thus, at [Chl *a*] $\sim 2 \times 10^{-7}$ *M* in degassed 95% ethanol, they found $\tau_f = 5.5$ ns, increasing to ~6.5 ns at [Chl *a*] $\sim 5 \times 10^{-5}$ *M*. Similar results have been obtained for Chl *a* in methanol (Kaplanová and Čermák, 1981) and for Chl *a* and Pheo *a* in pyridine (Yuen *et al.*, 1980).

For molecules with small Stokes shifts, it is well known that with increasing concentration emission spectra are red-shifted and fluorescence quantum yields are reduced by reabsorption of the emitted light (Birks,

1970). The effects of self-absorption on fluorescence quantum yields have been reviewed by Demas and Crosby (1971), and a method for correcting emission spectra has been presented by Christmann *et al.* (1980). Birks (1970) has noted that observed lifetimes can be longer at higher pigment concentrations (provided there is negligible self-quenching) because the residence time of a fluorescent photon in the sampled solution volume is lengthened by successive reabsorption and emission. Hence, the magnitude of the effect can be quite sensitive to the geometry of the experiment.

Connolly *et al.* (1982a) showed, specifically for the case of Chl *a* in diethyl ether, that the effect of concentration on measured lifetimes can be diminished by using thin (2 mm) optical paths and front-face detection. However, the artificial lengthening of observed lifetimes cannot be eliminated experimentally except by using very low pigment concentrations, i.e., concentrations for which the measured sample absorbance at the red (Q_y) wavelength maximum is $\leqslant 0.05$ in the same cell path lengths used in the lifetime measurements. Moreover, extrapolation of values obtained at high concentrations (even $\sim 10^{-6}$ M for Chl *a*) to infinite dilution still tends to give lifetimes that are appreciably (i.e., $\gtrsim 10\%$) longer than those measured with very low pigment concentrations (Connolly *et al.*, 1982a).

Effects of dissolved oxygen on fluorescence lifetimes of these pigments have also been largely neglected. Müller *et al.* (1965) made careful comparisons of the lifetimes obtained over a wide pigment concentration range in aerated and thoroughly degassed 95% ethanol. Connolly *et al.* (1982a) made similar measurements in diethyl ether and calculated the bimolecular rate constant (k_q) for O_2 quenching, using the relation:

$$k_q = \frac{(1/\tau_f) - (1/\tau_f^0)}{[O_2]} \tag{12}$$

where τ_f is the lifetime observed in aerated solution and τ_f^0 is the value for deoxygenated solution *at the same Chl concentration.* They calculated for 95% ethanol that k_q is about 40% of the diffusion-limited rate constant ($\sim 9 \times 10^9\ M^{-1}\ s^{-1}$), but for diethyl ether k_q is only about 3% of the diffusion limit ($\sim 2 \times 10^9\ M^{-1}\ s^{-1}$). The reasons for this wide difference are not at all clear. Several quenching mechanisms are possible—i.e., enhanced intersystem crossing to the triplet (^{3}Chl *a*), spin-allowed energy transfer to give both ^{3}Chl *a* and singlet ($^1\Delta_g$) O_2, enhanced spin–orbit coupling, and electron transfer quenching—but the available data do not permit the sorting out of these processes on a quantitative basis. Energy transfer seems to be precluded because of the additional energy

(~3300 cm^{-1}) required to populate both the Chl *a* triplet state and the $^1\Delta_g$ level of molecular O_2 (Connolly *et al.*, 1982a). More data are needed, in a variety of solvents, to determine the mechanism of O_2 quenching of Chl singlet states.

Until recently, the fluorescence data available on the bacterial pigments were quite sparse. Connolly *et al.* (1982b) made a systematic study of the fluorescence lifetimes of BChl *a* in 15 solvents spanning a range of coordination properties and hydrogen bonding. They showed that the lifetime of singlet excited BChl *a* (^{1}BChl *a*) in any given solvent is roughly a factor of two shorter than the lifetime of ^{1}Chl *a**. A similar relation obtains for bacteriopheophytin (BPheo) *a* and Pheo *a*. These differences most probably reflect faster internal conversion in the lower-energy excited singlet states of the bacterial pigments, since intersystem crossing ($^1S_1^* \rightarrow T_1$) rates appear to be similar, at least in BChl *a* compared to Chl *a* (Janzen *et al.*, 1981). Of greater significance is the trend of τ_f of BChl *a* with solvent (Connolly *et al.*, 1982b). The data indicate that τ_f of BChl *a* is longest (3.5–3.6 ns) in nucleophilic solvents in which the central Mg ion is hexacoordinated, i.e., in pyridine, tetrahydrofuran (THF), and *p*-dioxane. Evans and Katz (1975) have demonstrated that the visible absorption spectrum of BChl *a* in the 560–620 nm region (the Q_x band) can be used to establish the coordination of magnesium. Specifically, pentacoordinated Mg species absorb near 580 nm, while hexacoordinated forms are red-shifted as far as 610 nm. Using this criterion, Cotton and van Duyne (1979) concluded that in THF, as in pyridine, the central Mg ion is also hexacoordinated, and a similar conclusion can be drawn from the absorption spectrum of BChl *a* in *p*-dioxane (Connolly *et al.*, 1982a). Further confirmation that the wavelength of the Q_x band reflects the degree of Mg coordination of BChl *a* has been obtained by Cotton and van Duyne (1981) using resonance Raman spectroscopy.

In solvents in which the BChl magnesium is pentacoordinated (e.g., ether, acetone, acetonitrile) τ_f is reproducibly shorter (3.–3.2 ns), and it is shorter still in (wet) hydrocarbons and halogenated hydrocarbons (2.6–2.9 ns). In very dry hydrocarbon solvents, Chls have a pronounced tendency to aggregate due to intermolecular carbonyl–Mg interactions (Cotton *et al.*, 1978; Katz *et al.*, 1978). However, trace amounts of water disaggregate the pigments by ligating the central Mg ion. Based on the criterion of the absorption maximum of the Q_x band, it seems clear that in the case of BChl *a* the Mg coordination number is five in wet toluene, benzene, hexane, $CHCl_3$, and CCl_4. The shorter lifetimes observed in toluene, benzene, and hexane (as compared with ether, acetone, and acetonitrile) may thus be due to the presence of trace amounts of water

required to maintain the pigment in the monomeric form. In $CHCl_3$ and CCl_4 the short lifetimes of ^{1}BChl *a** probably reflect faster intersystem crossing enhanced by the heavy atoms of the solvent.

In aliphatic alcohols the picture changes substantially. The τ_f's of BChl *a* are significantly shorter, especially in ethanol and methanol. Using the criterion of the absorption maximum of the Q_x band, Connolly *et al.* (1982b) inferred that the central Mg ion of the pigment is hexacoordinated in methanol, ethanol, and 1-propanol, but not in 2-propanol, an observation first noted by Evans and Katz (1975). Irrespective of the Mg coordination number, the τ_f of BChl *a* is shorter in alcohols than in the first two types of solvents listed. This effect is attributed to H bonding of the solvent to the macrocycle (Connolly *et al.*, 1982b). Thus, the lifetime enhancement due to hexacoordination of Mg appears to be more than offset in aliphatic alcohols.

It is not possible to draw any conclusions about variation of fluorescence quantum yield with solvent, beyond those inferred by Connolly *et al.* (1982b) for BChl *a*. For that pigment, the trend of calculated ϕ_f (0.13–0.26) parallels the trend of observed τ_f (2.3–3.6 ns). Since the quantum yield of intersystem crossing to the lowest triplet state is only about 0.3 (Connolly *et al.*, 1982b; Tait and Holten, 1983), it can be inferred that internal conversion is much more efficient in the bacterial pigments ($\phi_{ic} > 0.4$) than in the green-plant analogs ($\phi_{ic} \leq 0.05$) (Gurinovich *et al.*, 1968).

E. Quantum Yield

Measurements of the quantum yield give information about the relative importance of the three deactivation processes in various media, and about the nature of interaction of fluorescent species with quenching substances. For Chl *a* in aprotic solvents such as ethyl ether, toluene, acetone, and pyridine, reported values for ϕ_f are in the range 0.30 to 0.35 (Latimer *et al.*, 1956; Weber and Teale, 1957; Brody, 1960; Dzhagarov, 1972; Hindman *et al.*, 1978), but in H-bonding solvents such as ethanol and methanol the yield falls to about 0.22 (Table I). For Chl *b* in most solvents ϕ_f is lower, around 0.10–0.12. Dissolved oxygen reduces ϕ_f just as it does τ_f, and ϕ_f's determined in its absence are about 7% greater than those determined in the presence of air (Drozdova and Krasnovskii, 1967).

There are two kinds of quenching: static and dynamic. In the case of dynamic quenching, there is no significant interaction between the quencher and the fluorescer in the ground state; reaction in the excited state is controlled by mutual diffusion, and quenching is expressed by

the Stern–Volmer relation, Eq. (8). In the case of static quenching, a complex forms between the ground state of the fluorescer and the quencher, and ϕ_f is proportional to the concentration of fluorescer not so bound. Mathematically, this is represented by

$$\phi_f/\phi_{fQ} = 1 + K_A[Q] \quad (13)$$

which is identical in form to Eq. (8). However, $K_A = [Chl{\cdot}Q]/[Chl][Q]$ for Chl and quencher Q, where the numerator is the concentration of ground state complex, and [Q] is now the concentration of uncomplexed quencher. If, as would often be the case, there is dynamic as well as static quenching, the reduction in fluorescence yield will follow the quadratic relation:

$$\phi_f/\phi_{fQ} = (1 + K_{sv}[Q])(1 + K_A[Q]) \quad (14)$$

Deviation from linearity in a plot of ϕ_f/ϕ_{fQ} against quencher concentration is a good indication of static quenching instead of, or in addition to, dynamic quenching.

Another characteristic of static quenching is that the τ_f is unchanged, even though the fluorescence yield may be much reduced. When both static and dynamic quenching are present, the τ_f falls more slowly, i.e., at higher quencher concentrations than ϕ_f (Natarajan *et al.*, 1984).

Static quenching the Chl fluorescence is less common than dynamic quenching in homogeneous systems, but examples are to be found with strongly π-complexing acceptors such as tetracyanoquinodimethane (Kreslavskii *et al.*, 1981). There are more examples of static quenching of Chls in heterogeneous systems, as will be noted in Section III, and, of course, quenching of Chl fluorescence in the normal course of photosynthesis is a purely static process.

F. Polarization

A symmetry-allowed nondegenerate electronic transition to a singlet excited state has associated with it a vector, defined in the coordinate system of the molecule, whose magnitude is related to the oscillator strength of the transition. The electronic transition is excited most strongly by light whose electric vector is directed parallel to this transition moment vector, and not at all by light polarized perpendicular to it. If light is radiated from the lowest excited state as fluorescence, it will be polarized preferentially parallel to the direction of the transition moment vector of that state. The relation between the excitation and fluorescence polarizations therefore provides information about the average correlation in space between absorbing and emitting transition moment

vectors if they are different, and is commonly quantified by the degree of polarization p:

$$p = (I_{\|} - I_{\perp})/(I_{\|} + I_{\perp}) \tag{15}$$

where $I_{\|}$ and $I_{\perp}$ are the intensities of fluorescence polarized parallel and perpendicular to the exciting light polarization. A better measure is the degree of anisotropy, r, because the denominator is proportional to the total fluorescence intensity (Knox, 1968).

$$r = (I_{\|} - I_{\perp})/(I_{\|} + 2I_{\perp}) \tag{16}$$

If molecules are raised to an excited state higher than the first by absorption of polarized light, there will in general be a finite angle between the transition moment vector of the initially excited state and that of the lowest excited state from which fluorescence appears. The value of p will be less than its maximum possible, and is related to the angle (α) between the vectors by Eq. (17):

$$p = (3 \cos^2 \alpha - 1)/(\cos^2 \alpha + 3) \tag{17}$$

p ranges from $+0.5$ for vectors parallel to -0.3 for vectors perpendicular. The variation of p with wavelength of excitation is often employed, along with circular dichroism (CD) and magnetic CD, to dissect absorption spectra of Chl pigments into discrete electronic transitions, and to estimate their orientations with respect to the lowest excited state (Goedheer, 1955; Sevchenko *et al.*, 1967; Ebrey and Clayton, 1969; Houssier and Sauer, 1969).

The above argument has tacitly assumed that molecules are immobilized during the time between absorption and emission of light. This is approximately true in frozen media and in viscous solvents such as castor oil, in which these measurements are usually made. In the usual organic solvents, however, Chls and similarly small molecules tumble freely during the lifetime of the lowest excited state, and fluorescence is effectively depolarized. More precisely, depolarization is related to the viscosity of the medium η and the molecular volume V by the Perrin equation:

$$r_0/r = 1 + kT\tau_f/\eta V \tag{18}$$

in which r_0 is the value of r in a rigid medium. A somewhat more complex formula has been derived for planar aromatic molecules like Chls (Valeur and Weber, 1978). This relation is useful, for example, for measuring the hydrodynamic volume of a particle or macromolecule to which a fluorescer is attached, or for investigating microviscosity in het-

erogeneous systems. It has also been used to calculate τ_f's (Gurinovich *et al.,* 1968; Goedheer, 1973).

Probably the most critical use of fluorescence polarization is to detect nonradiative transfer of energy between identical molecules in rigid or viscous media. Even in rigid media, transfer of energy once or twice from one molecule to a neighbor in a random system virtually erases memory of the original polarization. The dependence of p or r on fluorescer concentration has been calculated in a number of ways, and these have been reviewed by Knox (1968). A plot of r versus concentration provides the usual experimental means of determining R_0, which appears as a parameter in these relations (Knox, 1968).

In cases where energy is transferred to traps in which fluorescence is quenched, first p decays as concentration is increased, then ϕ_f, then τ_f (Losev and Zen'kevich, 1968; Seely, 1970). Fluorescence polarization is also useful in investigating the structure of Chl aggregates (Zen'kevich *et al.,* 1978).

III. Fluorescence of Aggregated Systems

A. Introduction to Aggregated Systems

Surely no single substance has been examined in so many environments and states of association and aggregation as Chl *a*. The reason for this is not hard to guess: after the distinctive spectral properties of Chls in living cells were recognized, it was found that these properties were preserved to some extent in extracts made by grinding leaves in water, whereby the Chls were confined to particles containing proteins and lipids. Since Chls dissolved in lipids or surfactants are fluorescent, it came to be thought that the fluorescence of leaves might be due to solution of Chls in the lipids. Although it is now well established that all or almost all of the Chls are present in protein complexes in the living cell, the precise relation of thylakoid lipids to these chromoproteins is still not clearly understood. In the early decades of the present century, work with Chls in proteins, lipids, micelles, and emulsions was already beginning. Extension to the other systems reviewed here was natural and direct.

Why a stable aromatic molecule like Chl *a* should be fluorescent is no mystery: the laws of wave mechanics and radiation theory account for it perfectly well. The question is rather why a molecule should not fluoresce, in the absence of any ostensible reaction in its lowest singlet ex-

cited state. Chl *a* is one of the substances whose fluorescence is largely quenched in concentrated solutions, and even in dilute solutions when by some means the molecules are associated into dimers or higher aggregates. Our brief survey of fluorescence in aggregated systems begins with a discussion of concentration quenching.

B. Concentration Quenching

In view of the ability of dense Chl arrays to transfer singlet state energy to reaction centers with high efficiency, the observation of quenching of fluorescence at high concentration in solution is both unexpected and puzzling. To be sure, concentration quenching is well known in dye solutions, and is reliably imputed to the presence of nonfluorescent dimers, which trap and quench excited singlet state energy. Although many kinds of dimers of Chls are known, they appear not to be present in the concentrated solutions in polar solvents where quenching is measured.

In recent years a number of attempts have been made to account quantitatively for Watson and Livingston's (1950) data for quenching of Chl *a* fluorescence in ether (Beddard and Porter, 1976; Gutschick, 1978; Dalton, 1980; Bojarski, 1982). The most reasonable construction is probably that of Beddard and Porter (1976), who attributed quenching to transfer of energy not to dimers but to "statistical pairs" of molecules, which interact too weakly to affect their spectrum much, but strongly enough to quench fluorescence. An alternative conclusion was drawn by Bojarski (1982), who found that a small (~1%) probability of loss of excited state energy on transfer, rather than quenching by pairs, better accounted for the results. There remains a need for data of high accuracy in a variety of media to decide this question.

In fluid solvents such as ether, dynamic quenching is possible at high enough concentrations; in viscous liquids and solid solutions this possibility can be disregarded. Concentration quenching of Chl fluorescence has been examined in cholesterol and other films (Porter and Strauss, 1966), castor oil (Losev and Zen'kevich, 1968), and lecithin (Kelly and Porter, 1970; Kelly and Patterson, 1971). In no case was spectral evidence of dimers, or of fluorescence from other than the monomeric Chl, reported. Nevertheless, in castor oil and lecithin the reduction of the lifetime of the excited state lagged well behind the reduction of fluorescence intensity as the concentration of Chl was increased. This indicates that much of the quenching is static, and owing to absorption of light by nonfluorescent Chl associations.

The process which results in fluorescence quenching in weakly inter-

acting Chls has not been established. Beddard and Porter (1976) wrote of nonfluorescent excimers; others have gone a step further to postulate partial or complete charge transfer from one Chl to another, partly on the ground that it is energetically feasible (Kelly and Patterson, 1971; Gutschick, 1978; Seely, 1978). Since no radical-ion intermediates have been detected, Seely (1978) suggested instantaneous internal conversion triggered by collapse of the ion pair. If so, the quenching process resembles primary photochemical reactions in photosynthetic reaction centers, the rigid structure of which, however, prevents immediate back transfer of the electron. See Parson and Ke (1982) and Norris and van Brakel (Chapter 3, this volume) for a discussion of primary photochemistry of photosynthesis.

C. Chlorophyll Aggregates and Films

1. AGGREGATES IN DILUTE SOLUTION

It is well recognized that Chl *a* in dry hydrocarbon or chlorocarbon solvents at room temperature, and in wet hydrocarbon solvents below room temperature, exists largely in dimeric or higher associations (Cotton *et al.*, 1978; Katz *et al.*, 1978). Because these aggregates have sometimes been suggested as models of Chl association *in vivo* (Katz *et al.*, 1979), there is great interest in their ability to fluoresce, i.e., to possess singlet excited states with lifetimes sufficient for chemistry. In dry hydrocarbons Chl *a* is practically nonfluorescent, but fluorescence can be "activated" by the addition of small amounts of polar substances, such as alcohols, amines, and ether, as we have already noted (Livingston *et al.*, 1949). The activated fluorescence belongs to monomeric Chl *a* molecules ligated through Mg to one or two molecules of the additive.

It is not clear whether anhydrous Chl *a* dimers and oligomers fluoresce at low temperatures, because of doubt as to whether water or other polar impurities are truly absent. Amster (1969) reported weak fluorescence bands at 686 and 724 nm for Chl *a* in CaH_2-dried 3-methylpentane at 77°K, assigned to dimer and higher aggregate, respectively; for Chl *b* the low-temperature fluorescence peak was at 723 nm. Since both solutions show weak, apparently monomer fluorescence at room temperature, the presence of water or other "activator" cannot be ruled out.

The evidence for fluorescence of Chl *a* dimers and oligomers in wet hydrocarbons at low temperature is much better. Fong and Koester (1976) characterized a dimeric complex (Chl $a \cdot H_2O)_2$ in methylcyclo-

hexane–pentane solution which at 121°K has an absorption peak at 702 nm ("A700") and a fluorescence peak at 720 nm. A minor fluorescence band at 682 nm was also seen; the similarity of these positions to those reported by Amster (1969) is to be noted. The properties of A700 show some analogy to those of P700 *in vivo*.

Kooyman *et al.* (1977) identified four fluorescence bands from Chl *a* in wet *n*-octane at 4.2°K, at 669, 687, 725, and 750 nm, whose intensities varied with concentration. These bands were assigned to the monomer hydrate, Chl $a{\cdot}H_2O$, the dihydrate Chl $a{\cdot}(H_2O)_2$, the dimer (Chl $a{\cdot}H_2O)_2$, and the polymer (Chl $a{\cdot}H_2O)_n$, respectively. The second assignment was made by analogy with the fluorescence in octane with added pyridine, where a single band at 686 nm was attributed to the complex Chl $a{\cdot}$(pyridine)$_2$. The same assignment, to Chl $a{\cdot}(H_2O)_2$, was made for the fluorescence band near 680 nm in *n*-pentane–methylcyclohexane (Clarke *et al.*, 1979) and in *n*-octane (Clarke *et al.*, 1982a) at 2°K. Bands at 680 and 730 nm in ethanolic toluene were similarly assigned to Chl $a{\cdot}$(ethanol)$_2$ and (Chl $a{\cdot}$ethanol)$_2$ (Clarke *et al.*, 1979). Fluorescent dimers of Chl *b* at 700 nm in pentane–methylcyclohexane at 77°K and of Pheo *a* and *b* at 713 and 704 nm in octane were reported by Kooyman *et al.* (1979).

It has long been recognized that the microcrystalline Chl *a* hydrate, (Chl $a{\cdot}2H_2O)_n$, which absorbs at 743 nm (Jacobs *et al.*, 1957), fluoresces weakly around 760 nm at low temperature (Bystrova and Krasnovskii, 1968). Fong *et al.* (1982) reported that the decay of this fluorescence at 125 or 90°K in pentane–methylcyclohexane was biphasic with lifetimes of 0.6 and 3.5 ns. Its intensity was only about 1% of that of monomeric Chl *a*. They also reported a delayed fluorescence attributed to a charge recombination process.

Aggregated forms of Chls are also produced when polar organic solutions are diluted with water. Here, as in nonpolar solvents, the aggregated species are nonfluorescent or nearly so at room temperature (Love, 1962; Journeaux *et al.*, 1969). However, aggregates tend to consolidate on aging, and species formed at first may be more fluorescent than the more stable and strongly interacting species formed later (Zen'kevich *et al.*, 1978). For the unique case of Chl–dioxane aggregates in water, ϕ_f has been measured for a number of pigments, e.g., Chl *a*, 5.3×10^{-3} at 693 nm; protochlorophyll, 8.5×10^{-4} at 641 nm; and 4-vinylprotochlorophyll, 1.5×10^{-3} at 642 nm (Zen'kevich *et al.*, 1979). Mixed pigment aggregates have been investigated in the dioxane system, and sensitized fluorescence by energy transfer from one pigment to another has been observed (Zen'kevich *et al.*, 1977).

In none of these examples of Chl aggregates formed from dilute solutions is there evidence of strong fluorescence at room temperature, though some strengthening occurs on cooling.

2. CONCENTRATED SOLUTIONS AND FILMS

Since weakly interacting Chl pairs are apparently able to deactivate singlet excited states rapidly in polar solvents, it is surprising that some strongly interacting Chl associations in nonpolar solvents are fluorescent. Litvin and associates have studied these systems most extensively, finding that as solvent is evaporated from a solution of Chl *a* in ether to yield loose, and then compact, films, the absorption and fluorescence spectra become dominated by ever more red-shifted bands (Litvin *et al.*, 1964). The spectra of such films are sensitive to exposure to vapors of polar solvents such as water, methanol, and pyridine. With the aid of difference spectra (Litvin and Sineshchekov, 1967) and second-derivative spectra (Litvin *et al.*, 1975), at least 15 correlated absorption–fluorescence band pairs were identified at 77°K in alkane hydrocarbon solutions at various concentrations (also see Litvin and Sineshchekov, 1975). In some cases (the 675, 686, and 694 nm fluorescence bands) fluorescence yields are large, comparable to monomer fluorescence, even at room temperature. In most cases fluorescence increased considerably with lowered temperature. These systems appear extremely complex and it is not possible to say what kinds of associations lead to fluorescent species.

A related system of interest is Chl dissolved in polystyrene foils. Here, as in other films, fluorescence is largely quenched by the presence of weakly interacting associations, but there is evidence of fluorescence by aggregated Chl *a* in bands at 685 and 730 nm (Wong *et al.*, 1978). Comparison of ϕ_f and τ_f established that fluorescence was 85% quenched by the static process at concentrations too low ($\leq 1.5 \times 10^{-3}$ M) for energy transfer to be important (Godik *et al.*, 1981). Transfer of energy from Chl *b* and to Chl *a* was also studied in this system (Šiffel and Vavřinec, 1980). Polystyrene is a rigid but poor solvent for Chls, and it appears that nonspecific quenching associations are likely to form as the cast films dry.

Evidence for fluorescent aggregates of Chls in polar solvents at room temperature is sketchy, but rather strong far-red fluorescence of aggregates has been observed at low temperature and high concentration in ethanol and in pyridine (Stensby and Rosenberg, 1961; Brody and Brody, 1962; Brody, 1968).

D. *Micelles and Macromolecules*

1. MICELLES

Somewhat more realistic models of Chl association issue from the observation that Chls are associated with lipids and proteins *in vivo*. Interest in the behavior of Chls in micelles is related, rather, to the use of surfactants such as sodium dodecyl sulfate (SDS), Triton X-100, and digitonin in the resolution of thylakoid membranes.

The most plausible picture of a micelle today is a rather disorderly bundle of surfactant molecules, with hydrophilic groups on the outside by the aqueous phase and hydrophobic parts concentrated toward the center (Menger, 1979). If the surfactant is charged, the micelle will normally be surrounded by a Stern layer of adsorbed counterions and by a more diffuse Gouy–Chapman cloud of counterions (Fendler and Fendler, 1975).

Two concepts of importance in the appreciation of micellar equilibria are the critical micelle concentration (CMC) and the aggregation number (n). The former is the surfactant concentration [S] below which micelles are unstable with respect to molecularly dispersed surfactant. The aggregation number is the average number of surfactant molecules per micelle, and is often considered independent of surfactant concentration. These quantities define the average number (s) of solute molecules such as Chl occupying a micelle by Eq. (19):

$$s = n[\mathrm{Chl}]/([\mathrm{S}] - \mathrm{CMC}) \quad (19)$$

Among the surfactants tested, the most popular for solubilizing Chls has been the polyoxyethylene derivative Triton X-100 (Massini and Voorn, 1968). Chl *a* solubilized in Triton micelles is fluorescent and the fluorescence is partially polarized, indicating that the solute is in a viscous environment. However, if $s > 1$ the fluorescence becomes depolarized owing to energy transfer between pigment molecules in the same micelle (Zen'kevich *et al.*, 1972; Csatorday *et al.*, 1975). At somewhat higher concentrations fluorescence becomes largely quenched, owing to transfer to weakly fluorescent aggregates, as in concentrated solutions (Zen'kevich *et al.*, 1972; Lehoczki and Csatorday, 1975; Lehoczki *et al.*, 1975; Il'ina and Borisov, 1981). Since micelles concentrate Chls from solution, these effects are seen at much lower overall concentration than in homogeneous solutions. The fluorescence of Chl *a* aggregates at high occupation numbers is weak, but there is evidence for a band in the 725–730 nm region in Triton micelles (Szabad *et al.*, 1974; Lehoczki *et al.*, 1975) with a τ_f of about 0.7 ns (Il'ina and Borisov, 1981). Thus, the

fluorescence of Chl *a* in micelles much resembles that in viscous homogeneous solutions at the same local concentration.

2. ADSORPTION TO PROTEINS

Since Chls in nature occur as complexes with proteins, which are fluorescent, complexes with nonphotosynthetic proteins might seem ideal models. Indeed, complexes of Chls with animal proteins, especially serum albumins and milk protein, have been prepared and have displayed a variety of properties. Generally, these complexes show evidence of Chl aggregation and of fluorescence of aggregated species, particularly at low temperatures.

Turning first to fluorescence of albumin complexes, Vacek *et al.* (1977) reported a complex of Chl *a*, Pheo *a*, and bovine serum albumin with three fluorescence bands at 77°K: 670.5, 711, and 726.5 nm. Szalay *et al.* (1981, 1982) described an interesting complex of human serum albumin, lecithin, and Chl *a*. The complex contains typically three albumin molecules, and each albumin contains three interacting Chl *a*'s. The red absorption peak is at 676 nm, and the CD spectrum reveals interaction between the Chls. The room temperature fluorescence peak is at 690 nm but the intensity is weak. Detergents, such as digitonin and SDS, disrupt the Chl interaction. If Chl *a* in dioxane is added to bovine serum albumin solution, the interaction between Chl *a* and dioxane is preserved in the complex with protein (683 nm absorption band, 688 nm fluorescence; Tombácz and Vozáry, 1980).

Giller and associates have prepared a number of complexes between milk lipoproteins and Chls *a* and *b* and sometimes carotene (Sapozhnikov *et al.*, 1966; Giller, 1968). There is spectral evidence for considerable Chl interaction in these complexes. The room temperature fluorescence of Chl *a* complexes has two bands, a stronger one around 675–680 nm and a weaker one around 725–735 nm; at 77°K the fluorescence band around 730 nm is the stronger (Giller *et al.*, 1970). Recently, second derivative spectroscopy and Gaussian deconvolution have revealed many components in the absorption of fluorescence spectra of these complexes (Giller, 1980). Dinant and Aghion (1975) prepared similar complexes with milk lipoproteins, and could demonstrate energy transfer from Chl *b* to Chl *a*.

Complexes between derivatives of Chl and BChl and the proteins apomyoglobin and apohemoglobin have been prepared, and characterized by their fluorescence spectra, lifetimes, and anisotropies (Wright and Boxer, 1981; Clarke *et al.*, 1982b; Kuki and Boxer, 1983).

Davis *et al.* (1985) prepared a complex between Chl *a* and plasto-

cyanin, in which the Chl *a* appears monomeric but is totally without fluorescence. This suggests an interaction between Chl *a* and the Cu of the protein, lending to quenching by energy transfer or perhaps electron transfer.

3. ADSORPTION TO POLYMERS

Proteins are complex polymeric macromolecules of nonrepeating structure, but few of them have evolved specifically for the purpose of binding Chls or similar molecules. There would appear to be better opportunities for selective and regulated binding of Chl in the use of synthetic macromolecules of defined and repeating structure, but few such studies have been done, at least partly because of serious solubility problems.

It has, however, been possible to prepare complexes of Chls with poly(4-vinylpyridine) which are soluble and stable in dry nitromethane (Seely, 1967). In these complexes Chls emit normal monomeric fluorescence, which is partially polarized because of their limited rotational mobility (Seely, 1970) and is quenched at high Chl densities (Seely, 1967, 1971). The quenching is associated with transfer of energy to spectrally detectable associations of weakly interacting Chls. Fluorescence sensitized by energy transfer could be measured in these systems when two or more kinds of pigment were present together, such as Chl *b* and Chl *a*, or Chl *a* and BChl *a* (Seely, 1976a,b).

Chung *et al.* (1982) discuss energy transfer and fluorescence of Chl *a* and *b* bound to poly(dimethylsiloxanes), substituted at intervals with γ-picoline residues, as a function of polymer concentration in ether. Hála *et al.* (1985) compared the low-temperature fluorescence of pheophorbide covalently bound to a lysine–alanine copolymer in dimethylformamide to that of the free pheophorbide.

E. Partially Oriented Systems: Layers and Vesicles

1. MONOLAYERS

Up to now, the fluorescence of more or less randomly oriented Chl aggregations has been discussed. Chls in their native state, however, exist in membrane structures that give every indication of being well oriented. Of artificial systems with some degree of orientation, Chl in monolayers is perhaps the simplest conceptually, though not the easiest to work with experimentally. The techniques for casting Chl monolayers, measuring their surface pressure–molecular area characteristics,

and transferring monolayers from a water surface to a solid support were developed some time ago and were reviewed by Ke (1966).

It was noticed early that fluorescence of Chl monolayers was almost completely quenched (Langmuir and Schaefer, 1937). Fluorescence reappears if the Chl is diluted with miscible surfactants such as oleyl alcohol, phytol, and galactolipids, but not with an immiscible diluent such as stearyl alcohol (Tweet *et al.*, 1964a). Chl fluorescence is, therefore, subject to concentration quenching in monolayers, as in other systems, and also to concentration depolarization by energy transfer (Trosper *et al.*, 1968; Costa *et al.*, 1972). Energy transfer from Chl *b* to Chl *a* has been observed (Costa *et al.*, 1972), as has limited transfer from carotene to Chl *a* (Sineshchekov *et al.*, 1972). Fluorescence is also quenched by energy or electron transfer to Cu Pheo *a* (Tweet *et al.*, 1964b), or to lipophilic quinones (Gaines *et al.*, 1965; Costa *et al.*, 1972).

Gonen *et al.* (1981) made the interesting observation that Chl *a* in monolayers diluted 1 : 1 with hexadecane was still fluorescent, whereas at 1 : 10 dilution with oleyl alcohol fluorescence was undetectably weak. They proposed that hexadecane, by lying flat on the water surface, hinders the mutual approach and rotation of Chl molecules.

Because of their low intensities, fluorescence spectra of Chls in monolayers, diluted or not, are hard to characterize accurately. Gaines *et al.* (1964) reported bands near 710 and 680 nm in undiluted and diluted monolayers, respectively. Aoshima *et al.* (1975) detected bands at 685, 695, and 715 nm from undiluted layers of Chl *a* and at 667 and 685 nm from Chl *b*. The fluorescence of layers diluted with hexadecane was resolved into three bands near 670, 682, and 690 nm, which varied with compression (Gonen *et al.*, 1981). At low temperature (77°K), the fluorescence of Chl *a* in monolayers intensifies, and peaks become prominent at 682–685, 700, and 720–724 nm (Litvin and Gulyaev, 1964). As in films, a number of bands appear at longer wavelengths at high compression and low temperature. Chl monolayers thus resemble films and concentrated solutions in that the fluorescence of aggregated species is favored over that of monomers.

2. BILAYERS AND VESICLES

If monolayers represent an introduction of the effects of orientation in artificial systems, bilayers in addition represent a better model of the thylakoid membrane, minus the protein complement. Bilayers are commonly realized in two configurations, an "open" one in which a lipid membrane (BLM) spans an aperture between two aqueous phases (Tien, 1968) and a "closed" one in which an unsymmetrical bilayer encloses a

small aqueous volume (vesicle). Vesicles, like micelles, form spontaneously in water but from a different kind of amphiphile. Amphiphiles that form vesicles have smaller polar groups, and larger nonpolar groups than those that form micelles; they include naturally occurring lipids (Fendler, 1980; Kunitake *et al.*, 1984).

Fluorescence of open bilayers has been little studied. The fluorescence spectrum of Chl *a* in dioleyl lecithin bilayers has a band near 680 nm, normal for a lecithin medium, and concentration quenching sets in at around 10 mole % Chl *a* (Alamuti and Läuger, 1970). Bilayers cast from extracts of green alga *Chlorella,* probably held together mainly by galactolipids, show strong Chl fluorescence and more efficient transfer of energy from Chl *b* to Chl *a* than in homogeneous solutions at the same concentration (Strauss and Tien, 1973).

Vesicles, at least some of those made with lecithins, undergo a phase change between gel and liquid crystal forms near ambient temperature. When vesicles containing Chl *a* are cooled below the transition temperature, the fluorescence yield drops, sometimes abruptly (Colbow, 1973; Lee, 1975; Brody, 1982). The abrupt drop is apparently due to diminished solubility of Chl in the gel phase, leading to segregation of nonfluorescent aggregates (Eigenberg *et al.*, 1982). As in other systems, Chl fluorescence is subject to concentration quenching in vesicles. The quenching is ascribed to energy transfer to statistical pairs, as in concentrated solution (Beddard *et al.*, 1976; Luisetti *et al.*, 1978). The fluorescence intensity in vesicles is therefore a complex function of Chl concentration and temperature.

Although the absorption band structure of Chl *a* changes with concentration and temperature, the fluorescence appears to be essentially that of the monomer (Brody, 1982; Lee, 1975). However, vesicles containing Pheo *a* show a fluorescence band near 705 nm, associated with an aggregate absorption band at 695 nm (Luisetti *et al.*, 1979).

3. LIQUID CRYSTALS

The partially ordered environment of liquid crystals provides a unique medium for studying the orientation of Chl in systems of macroscopic size. In principle, at least, the orientations of transition moments and the effect of orientation of intermolecular interaction can be deduced from absorption, fluorescence, and CD spectra. Frąckowiak *et al.* (1977a,b, 1981, 1982), Frąckowiak (1978), and Bauman and Wrobel (1980) have examined the spectra of Chl *a*, Chl *b*, Chl *c*, and BChl *a* in a nematic mixture of *p*-ethoxy- and *p*-methoxybenzylidene–*p*-butylaniline as a function of electrical potential across a thin layer (20 μM) of sample.

The intensity of absorption and of fluorescence, parallel and perpendicular to the liquid crystal axis, varies with potential in a complicated way that depends on the manner of association of pigment with the medium (Bauman and Wrobel, 1980). Changes in the emission band and corresponding absorption band are different in magnitude and sometimes direction, for reasons which are not entirely clear, but which may have to do with stronger dipolar interaction between Chls and liquid crystal molecular domains in the excited state than in the ground state (Frąckowiak, 1978). There was little or no evidence of Chl aggregation in this system; the Chl *a* fluorescence band is located at about 681 nm (Frąckowiak *et al.*, 1981), Chl *c* at 641 nm, and BChl *a* at 800 nm (Bauman and Wrobel, 1980).

F. Systems of Adsorbed Pigments

Though lacking the physiological verisimilitude of protein and vesicle systems, adsorbates of Chls to various substrates do promise a degree of control over the manner of pigment binding and association lacking in the former. Two types of substrates have been examined: mineral particles, and plastic particles and films.

Chl *a* adsorbed to polycaprolactam and Pheo *a* adsorbed to polystyrene are monomeric and fluorescent, but only at low surface densities (Cellarius and Mauzerall, 1966; Nekrasov *et al.*, 1966). At higher surface densities aggregates form which effectively quench singlet state energy transferred to them, though photochemistry may persist up to monolayer coverage (Cellarius and Mauzerall, 1966; Kapler and Nekrasov, 1966). However, in several systems there is evidence of fluorescence from aggregated pigments, especially at higher surface densities.

It has been noticed that pretreatment of particle surfaces or coadsorption of solutes mitigates the self-quenching of adsorbed Chl. For example, coadsorption of Triton X-100 increases both the fluorescence intensity and the ratio of 670–680 nm (monomer) to 720 (aggregate) fluorescence of Chl *a* on silica (Galutva and Nekrasov, 1980). Coating polycaprolactam particles with bovine serum albumin or lecithin has marked disaggregating effect on subsequently adsorbed Chl *a* (Nekrasov and Mamleeva, 1978). Even at a low Chl *a* density on protein-coated particles, a fluorescence band near 730 nm accompanies the monomer peak at 675 nm, and at higher concentrations other fluorescent aggregates are evidenced by a gradual shift of the maximum to 690 nm.

In a further extension of these observations, Seely *et al.* (1982) reasoned that if plastic particles were softened with hydrophobic diluents, and if surfactants which could ligate the Mg of Chl were coadsorbed to

the particles along with Chl, the latter would be anchored by its phytyl group inserted into the viscous particle surface, while being kept largely in a monomeric or dimeric state. Under such conditions, the bimolecular processes that lead to fluorescence quenching might be retarded, and fluorescence might persist even to high Chl surface densities. These expectations were realized with Chl adsorbed to polyethylene particles swollen with hydrocarbon diluents, and it was furthermore found that the absorption and fluorescence spectra of Chl *a* were characteristic of the basic surfactant adsorbed with it (Seely *et al.*, 1982; Kusumoto *et al.*, 1983a,b; Seely and Senthilathipan, 1983). As a model of the photosynthetic unit, the viscous polymer substrate and the ligating surfactant mimic the role of the protein in spacing the Chls and restraining their motion. Systems based on these particles have an abundant and varied photochemistry, often unlike that in simpler systems like micelles, which is presently under investigation.

G. Conclusions

One is struck by the bewildering variety of fluorescent forms of Chl that can be evoked by placing it in various environments, as sketched in this review. Certainly they include species spectrally similar to those recognized in living cells. Although there is little doubt that the spectra of Chl *in vivo* are affected by interaction between pigment molecules, the experience with model systems suggests that the most lifelike artificial species are those in which the interactions between pigments are moderated by interactions with other kinds of molecules.

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II

Light Absorption, Prompt and Delayed Emission *in Vivo*

6

Absorption and Fluorescence Emission by Intact Cells, Chloroplasts, and Chlorophyll–Protein Complexes

NORIO MURATA
Department of Regulation Biology
National Institute for Basic Biology
Okazaki 444, Japan

KIMIYUKI SATOH
Department of Biology
Faculty of Science
Okayama University
Okayama 700, Japan

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ABBREVIATIONS AND SYMBOLS

A_1	Primary electron acceptor of photosystem I
A_2	Secondary electron acceptor of photosystem I
Chl	Chlorophyll
Chl *a*	Chlorophyll *a* with wavelength of maximum absorbance indicated
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
F	Fluorescence intensity
kD	Kilodalton
LHC	Light-harvesting Chl *a*/*b*–protein complex
P680	Reaction center chlorophyll *a*, primary electron donor of photosystem II

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P700	Reaction center chlorophyll *a*, primary electron donor of photosystem I
Pheo	Pheophytin
PQ	Plastoquinone
PS	Photosystem
Q_A	First quinone electron acceptor of photosystem II
RC	Reaction center

ABSTRACT

This chapter describes analyses of the absorption and fluorescence spectra of chlorophyll *in situ*. The recent development of a detailed understanding of these spectra has been made possible mainly by two techniques: computer analysis of the spectra to identify several absorption and fluorescence components, and biochemical separation of chloroplasts into several chlorophyll–protein complexes. By use of these techniques, each of the fluorescence components can be ascribed to an emission from one species of chlorophyll, or chlorophyll-like pigment molecules which are bound to the chlorophyll–protein complexes. Yield changes in the fluorescence components can now be understood on the molecular basis of chlorophyll–protein complexes.

I. Introduction

Chlorophyll (Chl) *a* is ubiquitous in organisms displaying oxygenic photosynthesis, being the only pigment that is essential for light absorption and energy trapping. This has been verified by the fact that efficient photosynthesis can be performed by mutants of higher plants (Highkin and Frenkel, 1962) and green algae (Goodenough and Staehelin, 1971) containing Chl *a* but lacking Chl *b*, and by mutants of red algae (Nichols and Bogorad, 1960) and cyanobacteria (van Baalen, 1965) containing Chl *a* but no or reduced amounts of phycobiliproteins.

Recent biochemical research on manipulated membrane proteins has made it possible to isolate pigment–protein complexes in relatively intact states. Thus, photosystem I (PSI) and photosystem II (PSII) are each known to have a photochemical reaction center (RC) complex and a light-harvesting pigment–protein complex. The RC complexes I and II contain Chl *a* and carotenoids but no other accessory pigments (Satoh and Butler, 1978; Mullet *et al.*, 1980a). The pigment compositions and the modes of pigment–protein interactions in the light-harvesting protein complexes of PSII vary greatly with the type of plant (Glazer, 1983). The complex from higher plants (Briantais *et al.*, Chapter 18, this volume) and green algae (Govindjee and Satoh, Chapter 17, this volume) contains Chl *a* and *b* and carotenoids and is called the light-harvesting

Chl *a*/*b*–protein complex II (LHC II). The complex from brown algae and diatoms contains Chl *a* and *c* and carotenoids (Barrett and Anderson, 1980). Red algae and cyanobacteria (Fork and Mohanty, Chapter 16, this volume) contain unique light-harvesting pigment proteins such as phycobiliproteins. RC I, RC II, LHC II, and the Chl *a*/*c* protein complex are hydrophobic (Thornber, 1975) and are embedded in the thylakoid membranes (Anderson, 1975). The phycobiliproteins, on the other hand, are water-soluble and form aggregates called phycobilisomes of about 30 nm diameter, which are attached to the surface of the thylakoid membranes (Gantt and Conti, 1966; Gantt, 1981).

The light-harvesting Chl *a*/*b*–protein complex of PSI (LHC I) in higher plants (Mullet *et al.*, 1980a,b) and green algae (Wollman and Bennoun, 1982) has been identified. The corresponding light-harvesting pigment–protein complexes in brown algae, diatoms, red algae, and cyanobacteria have not yet been studied thoroughly. LHC I (which may be composed of two subcomplexes) seems to be tightly bound to RC I, forming a highly organized complex which corresponds to the so-called PSI complex.

The photosynthetic pigments, except carotenoids, when extracted from the photosynthetic membranes with polar organic solvents, emit fluorescence with high quantum yields and characteristic spectra (see Seely and Connolly, Chapter 5, this volume). But not all pigments in the pigment–protein complexes fluoresce. In LHC I and LHC II, for example, the transfer of excitation energy from Chl *b* to Chl *a* is so efficient that fluorescence is emitted from Chl *a* only, with none from Chl *b*. In isolated thylakoids, intact chloroplasts, and also intact cells, the energy transfer from LHC II to RC II is so efficient that the fluorescence of LHC II can hardly be observed. At room temperature, the fluorescence of intact cells and isolated chloroplasts of higher plants and green algae is attributable mainly to PSII (Duysens and Sweers, 1963), since the fluorescence from PSI is very weak. Fluorescence from phycobiliproteins is often observed in red algae and cyanobacteria, because of less efficient energy transfer within the phycobilisomes and from them to RC II. In these organisms, the fluorescence from phycobiliproteins becomes comparable to and is usually higher than that from RC II.

The fluorescence yields of the RC complexes are affected by changes in various factors, such as energy trapping, excitation energy transfer between the pigment–protein complexes, and the absorption cross section. Changes in the fluorescence yield are, in some cases, regarded as indicators of the rates of transfer of excitation energy within the photosynthetic pigment systems (Papageorgiou, 1975).

II. Absorption and Fluorescence Emission Spectra of Intact Cells, Isolated Chloroplasts, and Thylakoid Membranes

A. Room Temperature Spectra

Isolated chloroplasts and thylakoid membranes display essentially the same absorption and fluorescence spectra as intact cells, since the Chl–protein interaction is not altered during their isolation. However, densely pigmented intact cells and chloroplasts have absorption spectra which are usually distorted by the flattening effect (Duysens, 1956; Das *et al.*, 1967) that depresses peak heights . Also, their fluorescence spectra are greatly affected by self-absorption within such cells and chloroplasts. This decrease in the fluorescence intensity appears greatest in the short-wavelength region of the Chl fluorescence. The most dramatic self-absorption effect is often observed in leaves, in which densely pigmented chloroplasts are concentrated in small volumes; the fluorescence spectrum of leaves frequently differs from that of chloroplasts isolated from them. Thus, detailed study of the absorption and fluorescence spectra requires fragmentation of the chloroplasts and thylakoids into small membrane fragments so that the absorption within them becomes low enough that appreciable distortions are not produced (French *et al.*, 1972).

Since the light energy absorbed by LHC II is efficiently transferred to RC II, and then emitted as red light from the lowest excited state of Chl *a*, study of the fluorescence and absorption spectra has been focused on their red region. The peak position of the absorption spectrum of Chl *a* in the thylakoid membranes varies among plants from 672 to 679 nm. Compared with the red peak of Chl *a* in polar organic solvents, it is shifted by 10–15 nm toward longer wavelengths. This red shift may be produced by the Chl *a*–protein interaction, which is not yet fully understood with regard to Mg coordination by electron donors of the protein side chains.

The fluorescence spectrum of Chl *a* in the thylakoid membranes at room temperature show a peak at 683–685 nm and a broad shoulder at about 730 nm. When the distortion of the fluorescence spectrum due to self-absorption is minimized by fragmenting the membranes, the peak is located at relatively constant wavelength, 683 nm, regardless of the variation in the peak position of the absorption spectrum due to the plant origin. The red shift of the fluorescence peak is 15–20 nm, which is greater than that found with the absorption spectrum. The Stokes shift (shift of peak position between the absorption and fluorescence emission

spectra) ranges, in appearance, from 5 to 10 nm. This variation originates mainly from the variation in the peak position of the absorption spectrum.

B. Low-Temperature Spectra

The absorption spectra of isolated chloroplasts and thylakoid membranes of algae and higher plants at low temperatures show a complicated structure, especially in the red region. A broad band, observed at room temperature, can be resolved into several components by sharpening of the individual spectral bands. These components have been analyzed and their relation to pigment systems and pigment–protein complexes has been examined by differentiation (Brown and French, 1959; Butler and Hopkins, 1970) and curve deconvolution (Brown, 1972; French *et al.*, 1972; Brown and Schoch, 1982) of the spectra.

Cooling intact cells, isolated chloroplasts, and thylakoid membranes causes their fluorescence emission spectra to change and the total fluorescence yields to increase (Brody, 1958; Litvin *et al.*, 1961; Cho *et al.*, 1966; Cho and Govindjee, 1970; Rijgersberg *et al.*, 1979). At 77°K three emission peaks at about 685, 695, and 715–740 nm are observed with a variety of organisms displaying oxygenic photosynthesis (Bergeron, 1963; Govindjee, 1963; Kok, 1963). The former two bands are referred to as F685 and F695, irrespective of their exact peak locations. F685 and F695 are prominent emissions at 77°K in intact cells, chloroplasts, and thylakoid membranes from almost all species of algae and higher plants (Murata *et al.*, 1966b). The long-wavelength band is very broad, and its exact peak location varies from 715 nm (e.g., in the cyanobacterium *Anacystis nidulans*) to 740 nm (in some higher plants) with the plant species and their growth conditions. As will be described later, it is composed of two emissions with peaks at 715–725 nm and 735–745 nm, which are referred to as F720 and F735, respectively. As reviewed by Goedheer (1972) and Lavorel and Etienne (1977), F685 and F695 are emitted from Chl *a* in PSII, but F720 and F735 come from Chl *a* in PSI.

In addition to the emissions mentioned above, a band at about 680 nm, F680, is clearly seen as a shoulder at temperatures below about 34°K (Fig. 1). This band is absent in a barley mutant that lacks LHC II and is less pronounced in a tobacco mutant containing less Chl *b* than the wild strain (Rijgersberg *et al.*, 1979).

F685, F695, and F735 have characteristic temperature profiles. The increase in fluorescence on cooling is most pronounced in the region between 275 and 100°K in F735, between 100 and 50°K in F695, and between 50 and 4°K in F685 (Krey and Govindjee, 1966; Rijgersberg *et*

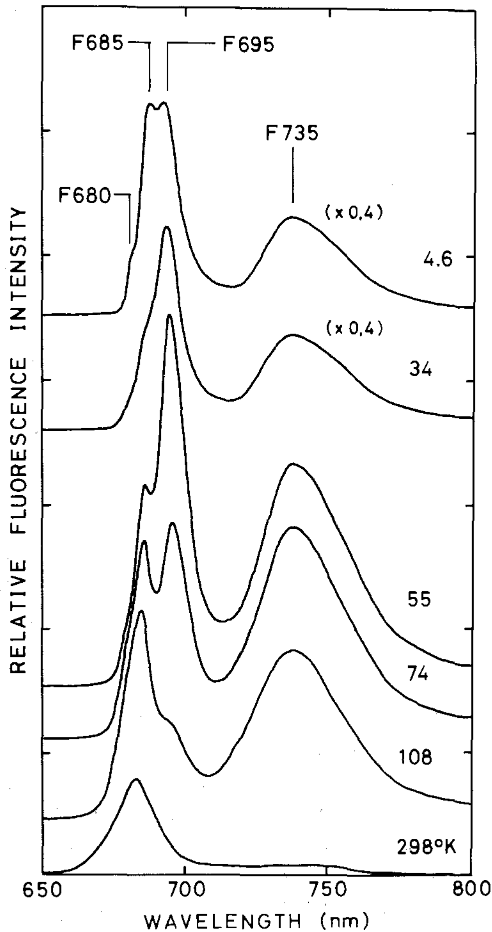

FIG. 1. Fluorescence emission spectra of spinach chloroplasts at various temperatures. Note that the spectra measured at 4.6 and 34°K are recorded at lower amplification and that the spectra are displaced vertically. (From Rijgersberg *et al.*, 1979, except for the 298°K spectrum, which is presented on an arbitrary scale.)

al., 1979). These temperature effects are observed in intact cells, thylakoid membranes, and Chl–protein complexes, but not in Chl dissolved in organic solvents (Avarmaa *et al.*, 1977). Although they can be partly explained in terms of changes in the overlap integral between emission and absorption spectra, causing changes in the efficiency of energy transfer between various pigments and pigment–protein complexes (Rijgersberg, 1980; Amesz and Rijgersberg, 1981), they are not yet well understood.

C. Analysis of Absorption and Fluorescence Emission Spectra

Curve-fitting analysis indicates that the absorption and fluorescence spectra of Chl *a* in an organic solvent are each composed of one major and one minor symmetric component, both with a half-bandwidth of 15–20 nm (Sugiyama and Murata, 1978; also see Seely and Connolly, Chapter 5, this volume). This suggests that a monomeric form of Chl *a* displaying uniform interaction with the surrounding molecules can almost be approximated by a single symmetric component. When the peak positions of absorption and fluorescence spectra of Chl *a* in an organic solvent are compared, the Stokes shift is found to be about 4 nm (see, e.g., Sugiyama and Murata, 1978), which is smaller than the apparent Stokes shift in the thylakoid membranes.

The absorption bands of Chl *a* in the thylakoid membranes of chloroplasts and intact cells, however, are broad, asymmetric, and red-shifted. The absorption spectrum of Chl *a in situ* is produced by the overlapping of some absorption components with shifts of different degrees to longer wavelengths. These components could be detected by differentiation of the spectrum. Brown and French (1959) analyzed the absorption spectrum by the first derivative method and introduced the concept of a "biological form of chlorophyll" in order to distinguish the Chl molecules having different peak wavelengths. By analysis of the fourth derivative of the absorption spectrum at 77°K, Butler and Hopkins (1970) observed six Chl *a* forms and two Chl *b* forms (also see Litvin and Sineshchekov, 1975).

Quantitative analysis of the absorption spectrum with respect to the Chl forms was done by French *et al.* (1972) by computer simulation of the spectrum with several symmetric components. Comparing the results for thylakoid membranes from different plant varieties, they found four universal Chl forms, which displayed about the same peak positions and the same bandwidths (about 10 nm at 77°K) but were present in different proportions, thus producing the wide variation of maximum wavelengths and spectral shapes. They named these forms Ca662, Ca670, Ca677, and Ca684 according to their peak wavelengths at 77°K. Two minor components at longer wavelengths, Ca691 and Ca704, were also found.

The curve-fitting method can be used to separate the fluorescence spectrum at room temperature into symmetric components with a dominant one located at 683 nm and minor ones at 674, 692, and 706 nm (Sugiyama and Murata, 1978). Comparison of the fluorescence components with the Chl forms of the absorption spectrum suggests that the

fluorescence components at 683 and 674 nm are emitted from the absorption components at 681 and 672 nm, respectively, at room temperature (corresponding to Ca677 and Ca670 at 77°K) with a Stokes shift of about 2 nm. The fluorescence components at 692 and 706 nm at room temperature may correspond, respectively, to F695 and F705 (see below) at 77°K.

Analyses of fluorescence emission spectra at 77°K by the curve-fitting method (Litvin *et al.,* 1979; Nielsen *et al.,* 1979; Brown and Schoch, 1982) suggested six components, F680, F685, F695, F705, F720, and F735 with half-bandwidths of about 10, 10, 10, 15, 20, and 25 nm, respectively. While F685, F695, F720, and F735 are discernible as distinct peaks in the spectra, F680 and F705 can be recognized by the curve-fitting analysis. Analysis of the fluorescence lifetime as a function of wavelength (Moya and Garcia, 1983) suggests the presence of both F720 and F735, which display lifetimes of about 3.3 and less than 1 ns, respectively. Fluorescence polarization spectra (Garab and Breton, 1976) indicated the presence of F705, F720, and F735 in the fluorescence spectrum at 77°K. By the same method, Kramer and Amesz (1982) observed several emission components at 4°K in spinach chloroplasts: F680, F685, F695, F735, and other components near 710, 730–735, and 760 nm.

III. Absorption and Fluorescence Emission Spectra of Chlorophyll–Protein Complexes

A. Absorption Spectra

The absorption spectra of the Chl–protein complexes appear to be affected by the detergents used to solubilize the complexes from the thylakoid membranes (Satoh and Butler, 1978; Il'ina *et al.,* 1981; Brown, 1983a). The detergents seem to alter the Chl–protein interactions in the complexes. However, mild detergents, such as digitonin (Satoh and Butler, 1978) or digitonin plus deoxycholate (Picaud *et al.,* 1982; Brown 1983b), can be used to preserve the absorption spectra in their native states as shown in Fig. 2.

Analyses of the absorption spectra at 77°K by using the fourth derivative (Satoh and Butler, 1978) and curve-fitting (Brown and Schoch, 1981; Brown, 1983b) indicate that the PSI complex contains Chl *a* components with peaks at about 662, 668, 679, 687, 695, and 705 nm, and RC II at 660, 670, 677, and 683 nm. LHC II contains Chl *a* components at 660, 670, 677, and 684 nm and Chl *b* components at 640 and 649 nm.

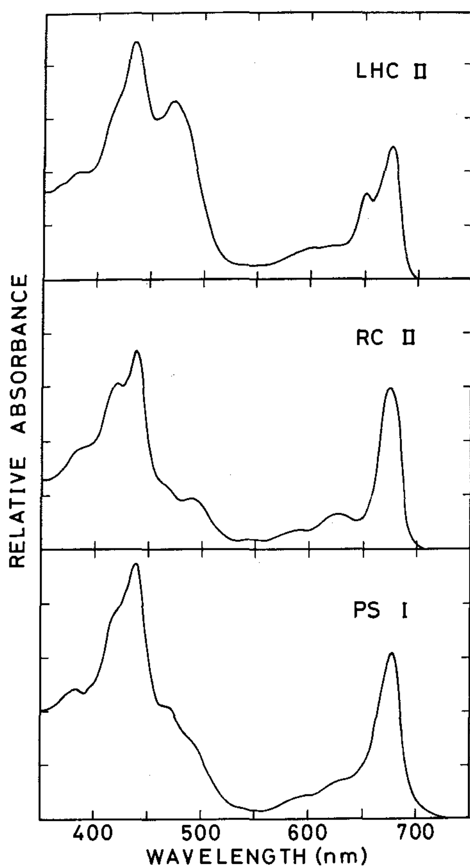

FIG. 2. Absorption spectra at room temperature of the three major Chl–protein complexes prepared from spinach thylakoids with digitonin. LHC II, the light-harvesting Chl *a/b* protein of PSII; RC II, the photochemical reaction center complex II; PS I, the PSI complex. (From Satoh, 1982.)

When the absorption spectra of the three Chl–protein complexes are added in proportions corresponding to their abundance in the solubilized materials, the sum nearly matches the spectrum of the thylakoids, indicating that each spectrum represents the absorption of the native state of each Chl–protein complex (Brown, 1983b).

B. Fluorescence Emission Spectra

The fluorescence emission spectra of membrane fractions and isolated Chl–protein complexes vary greatly with the detergents used. But care-

ful selection of the type and amount of detergent has made it possible to isolate pure Chl–protein complexes which retain the Chl–protein interactions of the intact states as reflected by the fluorescence emission spectrum. Even these mild detergents, when applied to solubilize the thylakoid membranes, alter the fluorescence spectrum, since they appear to interrupt the excitation transfer between the pigment–protein complexes. A successful fractionation of the thylakoid membranes into the Chl–protein complexes is shown in Fig. 3, which compares the fluorescence emission spectra of thylakoid membranes and digitonin-solubilized Chl–protein complexes (Satoh and Bulter, 1978).

1. PHOTOSYSTEM I

Photosystem I of higher plants and green algae contains RC I and LHC I, which are tightly bound to form the PSI complex in the thylakoid membranes. The PSI complex, isolated from higher plant chloroplasts by digitonin (Satoh and Butler, 1978; Satoh, 1979) or Triton X-100 (Mullet *et al.*, 1980a), displays a prominent emission band, F735, but no F685 or F695. F720 is also present but is hidden in the long-wavelength band (Mullet *et al.*, 1980a). It has been suggested that F720 and F735 are emitted from Ca695 and Ca705, respectively, of the PSI complex (Butler *et al.*, 1979; Mullet *et al.*, 1980a).

The existence of LHC I has been proposed (Butler and Kitajima, 1975; Butler and Strasser, 1977), and supporting evidence has been provided by detergent fractionation of the PSI complex (Mullet *et al.*, 1980a; Haworth *et al.*, 1983) and by study of its emergence during chloroplast development (Mullet *et al.*, 1980b). Removal of LHC I from the PSI complex by detergent treatment causes the peak of fluorescence emission at 77°K to shift from 735 to 722 nm. This suggests that RC I and LHC I emit F720 and F735, respectively. In fact, the isolated LHC I from pea chloroplasts fluoresces at about 730 nm (Haworth *et al.*, 1983). A reconstitution study (Ikegami, 1983) supports the idea that F735 is emitted from antenna Chl *a* of PSI. The relation of LHC I to LHC II and its possible functions in energy collection, regulation of energy distribution between two photosystems, and photoprotection have been discussed by many investigators (Anderson *et al.*, 1983; Haworth *et al.*, 1983; Ortiz *et al.*, 1984).

When the Chl content of RC I is reduced to only about 10 Chl molecules per primary electron donor, P700 (Ikegami, 1976; Vacek *et al.*, 1977; Ikegami and Ke, 1984), it emits F705 at 77°K. Ikegami and Ke (1984) proposed that F705 originates from the electronically excited state of the primary electron acceptor, A_1 (probably a special form of Chl *a*), which would be produced by charge recombination between $P700^+$

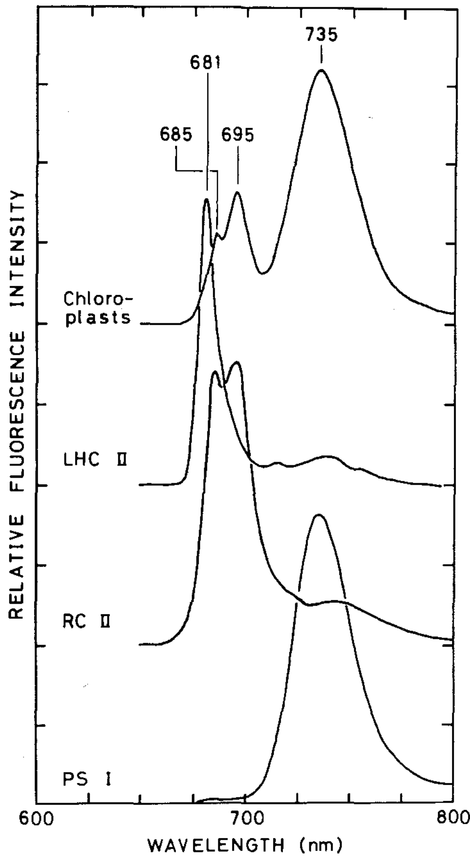

FIG. 3. Fluorescence emission spectra at 77°K of spinach chloroplasts and of the three major Chl–protein complexes shown in Fig. 2. (From Satoh, 1979, 1982.)

and A_1^-. In some preparations of the PSI complex, F705 seems to appear at 690–694 nm at room temperature (Ikegami, 1976; Telfer *et al.*, 1978).

An emission near 695–700 nm in PSI preparations has been reported by several authors (Thornber, 1975; Brown, 1976). However, this emission seems to differ from F705 of RC I and F695 of RC II; it is observed at room temperature under oxidizing conditions, where the F705 and F695 are supposed not to be observed.

2. PHOTOSYSTEM II

Photosystem II of higher plants and green algae contains RC II and LHC II, which are assembled in the thylakoid membranes. The so-called PSII particles are membrane fragments consisting of lipid membranes in

which RC II and LHC II are embedded (Dunahay *et al.*, 1984). In these preparations, F685 and F695 are distinct emissions at 77°K (see reviews by Goedheer, 1972, and Lavorel and Etienne, 1977; Gasanov *et al.*, 1979; Bricker *et al.*, 1983). In PSII particles completely devoid of PSI contamination, no F735 or F720 is observed (Bricker *et al.*, 1983). The small hump in the region around 720–760 nm (Fig. 3) originates from the satellite vibrational bands of F685 and F695 (Rijgersberg and Amesz, 1980).

Isolated RC II contains about 50 Chl *a* and 10 β-carotene molecules per reaction center Chl *a*, P680 (Satoh, 1983), and emits F685 and F695 at 77°K. Although F695 becomes insignificant during purification in some RC II preparations, use of 1,10-phenanthroline, which specifically enhances F695 (Satoh, 1974), clearly shows that all RC II preparations display this emission (Satoh, 1980).

RC II is composed of five kinds of polypeptides of molecular masses ranging from 7 to 50 kD (Satoh, 1983). Two of them are Chl-containing polypeptides of about 47 and 43 kD (Delepelaire and Chua, 1979; Camm and Green, 1983), which emit F685 and F695, respectively (Nakatani, 1983; Yamagishi and Katoh, 1983; Nakatani *et al.*, 1984; Tang and Satoh, 1984). F695 from the isolated 47-kD polypeptide retains its characteristic temperature dependence (Nakatani, 1983; Yamagishi and Katoh, 1983) and sensitivity to 1,10-phenanthroline (Tang and Satoh, 1984). In addition to F695, the 47-kD polypeptide shows a weak emission at 685 nm (Yamagishi and Katoh, 1983; Tang and Satoh, 1984). Based on the fluorescence and absorption anisotropy of RC II, Breton (1982) proposed that F695 is derived from electronic excitation of pheophytin (Pheo) *a*, which is produced by charge recombination between $P680^+$ and $Pheo^-$. The emitter of F685 seems to be Ca677 in RC II, although further study is necessary to confirm this.

Isolated LHC II emits F680 at 77°K (Anderson *et al.*, 1978; Satoh and Butler, 1978; Rijgersberg *et al.*, 1979). The most plausible candidate for the emitter of F680 is Ca677, since Ca670 is located too far from the fluorescence band. Although the fluorescence yield of the isolated LHC II is about 10 times higher than those of the PSI complex and RC II (Satoh, 1982), F680 in intact thylakoids and PSII particles is insignificant (see Section II,B). This is interpreted by an efficient energy transfer from LHC II to RC II in the thylakoid membranes and PSII particles, as noted earlier.

An emission near 695 nm has been observed in the aggregated form of LHC II which is produced on addition of Mg^{2+} (Arntzen and Ditto, 1976) or by its insertion into liposomes (Ryrie *et al.*, 1980; Larkum and Anderson, 1982). The contribution of this emission in intact thylakoids and PSII particles is, if present at all, very small.

IV. Fluorescence Yield

A. Absolute Yield and Lifetime

The quantum yield of fluorescence is defined as the number of emitted quanta divided by the number of absorbed quanta. For Chl *a* dissolved in polar organic solvents, it is about 30% (Förster and Livingston, 1952; Latimer *et al.*, 1956; see Seely and Connolly, Chapter 5, this volume). In contrast, the fluorescence yield of Chl *a* in intact cells and thylakoids at room temperature is about 5%, when it is maximized by the use of strong light or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to keep the acceptor quinone (Q_A) in the reduced form (see, e.g., Murty *et al.*, 1965; Murata *et al.*, 1966b; Beddard *et al.*, 1979). Since the Chl *a* fluorescence is emitted mainly from PSII, this value approximates the fluorescence yield of PSII. The quantum yield of PSI fluorescence, calculated from its lifetime, is about 0.5% (Beddard *et al.*, 1979).

Analysis of picosecond fluorescence kinetics at room temperature (Haehnel *et al.*, 1982) suggests that there are three exponential components with lifetimes of about 0.1, 0.4, and 2 ns. The 0.1-ns component seems to originate from PSI, and the 0.4- and 2-ns ones from PSII. The 2-ns component becomes dominant in the presence of DCMU, suggesting that it is emitted from RC II in which Q_A is reduced.

For a further discussion of lifetimes of fluorescence, see Moya *et al.* (Chapter 7, this volume).

B. Yield Changes

The *in vivo* Chl fluorescence at room temperature (i.e., F685) shows a complex time course after the onset of continuous excitation light (Kautsky *et al.*, 1960; see an earlier review by Govindjee and Papageorgiou, 1971). This phenomenon, called the Kautsky effect, is ubiquitously observed in organisms which perform oxygenic photosynthesis and also in isolated intact chloroplasts (Krause, 1974; Satoh and Katoh, 1980). It is now well known that the change in fluorescence yield is caused by various factors such as quenching at the RCs, quenching by stimulation of internal conversion, a change in excitation transfer, and a change in absorption cross section of PSI and PSII (see reviews by Papageorgiou, 1975; Lavorel and Etienne, 1977; and Briantais *et al.*, Chapter 18, and van Gorkom, Chapter 10, this volume).

The time-dependent change in Chl *a* fluorescence in isolated thylakoids at room temperature is rather simple (Malkin and Kok, 1966; Murata *et al.*, 1966a). During illumination with continuous excitation light, the F685 of dark-adapted chloroplasts increases from a minimum

level at the onset, F_0, to a final maximum level, F_m; the variable part of the fluorescence during the induction period is termed F_v, i.e., $F_v = F_m - F_0$. The fluorescence components at 77°K of intact cells and isolated thylakoids show similar monotonous increases from F_0 to F_m (Murata, 1968). (Also see Chapter 4 by Lavorel *et al.*, for a general discussion, Chapter 18 by Briantais *et al.*, for higher plants; Chapter 16 by Fork and Mohanty, for red algae and cyanobacteria; Chapter 17 by Govindjee and Satoh, for Chl *b*- and Chl *c*-containing algae.)

1. QUENCHING DEPENDING ON THE REDOX STATES OF REACTION CENTERS

In a study on the two light effect on Chl *a* fluorescence at room temperature (i.e., F685), Duysens and Sweers (1963) correlated the fluorescence yield with the redox state of the electron acceptor of photochemical reaction II. This acceptor, called Q according to their terminology, has been identified as plastoquinone bound to RC II (van Gorkom, 1974) and is now called Q_A. The fluorescence yield is high when Q_A is reduced and low when it is oxidized. Butler (1972) proposed that the fluorescence is also quenched by the oxidized form of P680 ($P680^+$).

At 77°K, F695 and F685 display very similar time-dependent increases due to reduction of Q_A under continuous light, although F_v/F_m is a little smaller in F685 than F695 (Murata, 1968). As mentioned in Section III,B, Breton (1982) has proposed a mechanism by which the Q_A-dependent yield change in F695 can be successfully interpreted. According to this mechanism, F695 is emitted from the excited state of Pheo *a* which is produced by charge recombination between $Pheo^-$ *a* and $P680^+$ when Q_A is reduced and thus unable to oxidize $Pheo^-$ *a*. An efficient uphill excitation transfer from the emitter of F695 to that of F685 is suggested by the yield change in F685. F680 seems to follow a similar time-dependent yield change (Rijgersberg *et al.*, 1979), suggesting that the excitation energy can be transferred from RC II to LHC II. At room temperature, at which F695 is not observed, the yield change in F685 is an indirect indicator of the redox states of Q_A and P680. The variable parts of F735 and F720 of PSI also display time-dependent yield changes which are very similar to those of F695 and F685, but with much lower values for F_v/F_m (Murata, 1968). This is regarded as evidence for the excitation transfer (spillover) from PSII to PSI (Murata, 1968; Butler, 1978). Time-dependent increases in F685 and F695 (but not in F680) under continuous illumination were observed in intact cells and isolated thylakoids cooled to 5°K (Rijgersberg and Amesz, 1978; Rijgersberg *et al.*, 1979). These findings indicate that the photochemical reaction of PSII takes place to reduce Q_A even at such low temperatures.

The yield change in F705 from PSI was first observed by Ikegami (1976) and then by Telfer *et al.* (1978). Ikegami and Ke (1984) showed that the yield of F705 depends on the redox states of P700, A_1 and A_2 (the secondary electron acceptor of PSI); it becomes maximal when A_1 is oxidized and P700 and A_2 are reduced. This result led the authors to propose, as noted earlier, that F705 originates from the charge recombination between A_1^- and $P700^+$ when A_2 is reduced and thus unable to reoxidize A_1. It is not known whether F720 and F735 follow the yield change in F705 through excitation transfer between their emitters.

2. QUENCHING BY ΔpH

Quenching of the fluorescence by the energized state of thylakoid membranes was observed by Murata and Sugahara (1969) and Wraight and Crofts (1970). As this quenching is suppressed by uncouplers of photophosphorylation, it is probably induced by the energized state of thylakoid membranes. Telfer *et al.* (1975) observed its induction by the endogenous cyclic electron transport through PSI. The finding that it occurs in all three major emission components at 77°K, F685, F695, and F735 (Murata and Sugahara, 1969), suggests that the internal conversion is stimulated by the energized state.

According to studies by Briantais *et al.* (1979) and Krause *et al.* (1982), the fluorescence quenching is produced by an increase in the intrathylakoid pH. They suggested that the fluorescence decline from the maximum *P* to the intermediate minimum *M* in the Kautsky effect in intact chloroplasts is caused by the same type of quenching. However, this does not agree with the hypothesis of Satoh and Katoh (1980) and Satoh (1981) that the light-dependent activation of the electron transport through ferredoxin-$NADP^+$ reductase produces the fluorescence decline in the Kautsky effect (see Govindjee and Satoh, Chapter 17, this volume, for further discussions).

3. CATION EFFECTS IN ISOLATED THYLAKOIDS

Homann (1969) and Murata (1969b) independently discovered that F685 at room temperature in isolated thylakoids increases on addition of 5–10 m*M* Mg^{2+}. At 77°K, an increase in F685 and F695 and a decrease in F735 are observed, which indicates that the cation regulates the light energy distribution between the two photosystems (Murata, 1969b). The main action of cations has been shown to be suppression of the excitation transfer (spillover) from PSII to PSI (Murata, 1969b; Briantais *et al.*, 1973; Mohanty *et al.*, 1973; Butler, 1978). However, the cations also seem to have another effect on the absorption cross sections of PSI and PSII (e.g., Wong *et al.*, 1981).

A very similar effect on the room- and low-temperature fluorescence is observed with other divalent cations at 5–10 m*M* (Murata *et al.,* 1970), monovalent cations at 100–150 m*M* (Murata, 1971a; Gross and Hess 1973), and trivalent cations (Mills and Barber, 1978). However, at low concentrations divalent and monovalent cations have antagonistic effects on the fluorescence (Gross and Hess, 1973; Vandermeulen and Govindjee, 1974). A quantitative analysis by Mills *et al.* (1976) suggested that monovalent cations competitively inhibit the divalent cation-induced fluorescence increase, and that the competition may be due to binding to the same site of the thylakoid membranes.

The cation-induced light energy redistribution seems to be related to the cation-induced conformational change of thylakoid membranes studied by light scattering (Murata, 1971b) and electron microscopy (Murakami and Packer, 1971; Gross and Prasher, 1974). The current concept of the structural aspects of the cation-induced light energy redistribution (Barber, 1980; Anderson, 1981) is as follows; in the presence of a sufficient amount of cations, grana membranes stack due to neutralization of their surface charge, and the PSI complex moves to unstacked regions and thus becomes segregated from the stacked membranes in which RC II and LHC II are localized. This leads to virtual loss of the excitation transfer from PSII to PSI. In the absence of cations, the electrostatic repulsion between the PSI complexes forces them to become homogeneously distributed within the whole thylakoid membranes. As a result, PSI and PSII complexes become completely mixed, and the excitation spillover from PSII to PSI is facilitated.

Henkin and Sauer (1977) observed a dual effect of Mg^{2+} on the fluorescence yield at room temperature; saturation of the increase occurred at 0.5 m*M* Mg^{2+} in the F_0 level but at 2.5 m*M* Mg^{2+} in the F_m level (see also Wydrzynski *et al.,* 1975). Jennings (1984) suggested that Mg^{2+} stimulates the energy transfer from LHC II to RC II at 0.5 m*M* and inhibits the spillover from RC II to PSI at 2.5 m*M* (see also Wong and Govindjee, 1981). For further discussion, see Briantais *et al.* (Chapter 18, this volume).

4. STATE I–STATE II TRANSITIONS IN INTACT CELLS AND ISOLATED THYLAKOIDS

Light-dependent redistribution of light energy between the two photosystems in intact cells was observed independently by Murata (1969a) with a red alga, *Porphyridium (P.) cruentum,* and by Bonaventura and Myers (1969) with a green alga, *Chlorella pyrenoidosa.* When the organisms are illuminated with light preferentially absorbed by PSI, they enter

state I, in which more light energy is delivered to PSII and less to PSI than before; this was proved by the observation that F685 and F695 become higher and F720 and F735 become lower on preferential illumination of PSI (Murata, 1969a). Illumination of the organisms with light preferentially absorbed by PSII produces a reverse effect on the fluorescence emission components, and thus the light energy distribution; the resultant state is called state II. This phenomenon, called the state I–state II transition, is observed ubiquitously in oxygenic photosynthetic organisms including higher plant leaves (Kitajima, 1976), green algae (Wang and Myers, 1974), diatoms (Shimura and Fujita, 1973), red algae (Murata, 1970; Ried and Reinhardt, 1977; Ley and Butler, 1980), and cyanobacteria (Duysens and Talens, 1969; Mohanty and Govindjee, 1973) as well as intact chloroplasts (Krause, 1974, 1977; Telfer *et al.*, 1975).

Bennett (1977) found that an endogenous protein kinase phosphorylates LHC II in isolated thylakoid membranes of higher plants when plastoquinone (PQ) is reduced, and suggested that this LHC II phosphorylation is related to the state I–state II transition in higher plants and green algae (Bennett, 1980; Bennett *et al.*, 1980). A current concept of the mechanism of state I–state II transition is as follows: (1) light preferentially absorbed by PSII reduces PQ; (2) the reduced PQ activates a protein kinase and LHC II is phosphorylated (Allen *et al.*, 1981; Horton *et al.*, 1981; Telfer *et al.*, 1983a); (3) the phosphorylated LHC II, which is more negatively charged than the unphosphorylated form, moves from the grana to the intergrana region of thylakoids (Andersson *et al.*, 1982; Kyle *et al.*, 1983); (4) more of the light absorbed by LHC II is delivered to PSI and less to PSII (Steinback *et al.*, 1982; Bennett, 1983; Krause and Behrend, 1983); and (5) light preferentially absorbed by PSI oxidizes the reduced PQ and deactivates the protein kinase, and then phosphatase releases phosphate from the phosphorylated LHC II (Kyle *et al.*, 1983), resulting in the movement of LHC II back to the grana region.

Horton and Black (1981), in their study on fluorescence kinetics at room temperature, concluded that the phosphorylation of LHC II in pea thylakoids increases the absorption cross section of PSI and decreases that of PSII, but does not affect the spillover. This is consistent with the mechanism mentioned above in which only LHC II moves from the grana to the intergrana region of thylakoids. However, Telfer *et al.* (1983b) inferred that phosphorylation increases the spillover in a medium containing low concentrations of Mg^{2+}, suggesting that both LHC II and RC II move together at least under some conditions.

The state I–state II transition of *P. cruentum*, however, is not related to protein phosphorylation (Biggins *et al.*, 1984). Moreover, the spillover

rather than the absorption cross section is regulated by the state I–state II transition in this alga (Ley and Butler, 1980; Biggins, 1983). Thus, although the state I–state II transition occurs in plants containing LHC II as well as in those containing phycobiliproteins as the light-harvesting complex of PSII, the underlying molecular mechanisms seem to be different.

For a further discussion of state changes, see Briantais *et al.* (Chapter 18) for chloroplasts, Fork and Mohanty (Chapter 16) for red algae and cyanobacteria, and Govindjee and Satoh (Chapter 17) for green algae, in this volume.

V. Conclusions

Fluorescence emission spectra of intact cells, isolated chloroplasts, and thylakoid membranes measured at low temperatures show six emission bands. At 77°K, F685, F695, F720, and F735 are distinct in most cases, whereas F680 and F705 can be recognized by a curve-fitting analysis of the spectra. F685 is observed from room to low temperatures, whereas all the other components appear to be distinct at low temperatures.

Derivative and curve-fitting analyses of the absorption and fluorescence emission spectra in the red region have made it possible to identify emitters of the fluorescence components. Comparison of the absorption and fluorescence spectra with regard to their analyzed components suggests that F680, F685, F720, and F735 are emitted, respectively, from Ca677 in LHC II, Ca677 in RC II, Ca695 in RC I, and Ca705 in LHC I. F695 seems to be emitted from the excited state of Pheo *a* in RC II, which is produced by recombination of $P680^+$ and $Pheo^-$ *a* (Breton, 1982), and F705 seems to be emitted from the excited state of A_1 in RC I, which is produced by recombination of $P700^+$ and A_1^- (Ikegami and Ke, 1984).

Similarity between the yield changes in F685 and F695 with the redox state of RC II suggests very efficient excitation transfer between the emitters of F685 and F695. Yield changes in F720 and F735, depending on the redox state of RC II, are regarded as evidence for excitation transfer (spillover) from PSII to PSI. It is not known whether the yield change of F705, which depends on the redox state of RC I, affects F720 and F735. F685 at room temperature is regarded as an indirect indicator of the redox state of RC II, since the redox state affects F695, which influences F685 through excitation transfer.

Several factors other than the redox states of the primary electron acceptors and donors regulate the yields of the fluorescence compo-

nents. Cations such as Mg^{2+} increase F685 and F695 and decrease F720 and F735 by suppressing excitation transfer (spillover) from RC II to the PSI complex. Phosphorylation of LHC II decreases F685 and F695 and increases F720 and F735 by facilitating excitation transfer from LHC II to the PSI complex. Formation of ΔpH across the thylakoid membranes or an increase in intrathylakoid pH decreases F685, F695, F720, and F735, presumably by stimulating the internal conversion in PSI and PSII.

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7

Lifetime of Excited States and Quantum Yield of Chlorophyll *a* Fluorescence *in Vivo*

ISMAEL MOYA
PIERRE SEBBAN
Laboratoire de Photosynthèse
Centre National de la Recherche Scientifique (CNRS)
91190 Gif-sur-Yvette, France

WOLFGANG HAEHNEL
Lehrstuhl für Biochemie der Pflanzen
Ruhr-Universität Bochum
D-4630 Bochum 1, Federal Republic of Germany

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ABBREVIATIONS AND SYMBOLS

BChl	Bacteriochlorophyll
BPheo	Bacteriopheophytin
Chl	Chlorophyll
F_0	Minimum level of antenna Chl fluorescence (open PSII reaction centers)
F_{max}	Maximum level of antenna Chl fluorescence (closed PSII reaction centers)
FWHM	Full width at half-maximum
LHC I (II)	Light-harvesting complex: Chl *a*/*b* protein of photosystem I (II)
Pheo	Pheophytin
PSI (II)	Photosystem I (II)
PSU	Photosynthetic unit
P680	Primary electron donor of PSII
P700	Primary electron donor of PSI
P*	Lowest electronic singlet state of the primary donor of bacteria
P^F	Primary charge separation state of bacterial reaction centers
Q_A	First quinone acceptor of PSII
R.	*Rhodospirillum*
RC	Reaction center
Rps.	*Rhodopseudomonas*

ABSTRACT

Fluorescence lifetime (τ) and quantum yield (Φ_f) measurements probe the dynamics of energy migration and storage in photosynthetic organisms. Early measurements, with chlorophyll (Chl)-containing organisms, showed an almost parallel increase in both the average τ (from 0.5 to 2 ns) and the total Φ_f when photosystem (PS) II reaction centers (RCs) are closed. This finding is at the basis of a model of well-connected photosynthetic units (PSU). The introduction of picosecond excitation pulses has enabled photon timing techniques to resolve the room temperature fluorescence emission into at least three kinetic components: the fast (50–100 ps), middle (400–750 ps), and slow (1–2 ns) components. Although the origin of the three components is far from clear, a model of loosely connected PSU is sustained. Several hypotheses for the assignment of these components are discussed, including alpha and beta heterogeneity of PSII, light-harvesting complex (LHC) II, and PSI emissions and a charge recombination mechanism for the origin of variable fluorescence.

In chromatophores of purple bacteria, the (prompt) τ is correlated with the Φ_f and increases from 50 to 250 ps when the RCs become closed. These short τ values involve a particular antenna organization. However, when the primary quinone electron acceptor is reduced before illumination, a 5-ns component is detected which corresponds to the charge recombination between the primary oxidized electron donor ($P890^+$) and reduced electron acceptor, $BPheo^-$. This delayed fluorescence component is superimposed on the prompt fluorescence emission. Multiexponential fluorescence decay kinetics in reduced isolated bacterial RCs are discussed in terms of several intermediary states for the relaxation of $P890^+$ $Bpheo^-$.

I. Introduction

The absorption of light by green plants and photosynthetic bacteria is primarily due to the excitation of pigment molecules from their ground state to higher excited states. Photons absorbed initially by chlorophyll (Chl) or bacteriochlorophyll (BChl) as well as other pigments, depending on the type of organism considered, furnish energy to derive electrochemical processes which lead to the storage of the energy (see Norris and van Brakel, Chapter 3, this volume).

Higher excited singlet states are rapidly converted by internal conversion to the lowest excited singlet state, S_1 (see Shipman, 1982). Some of the energy of the S_1 state is lost via fluorescence emission. Thus fluorescence monitors the concentration of the excited state S_1. This chapter deals only with the fluorescence emitted from Chl *a* and BChl *a*, which has been used for a long time to study the primary processes of photosynthesis (see reviews by Govindjee *et al.*, 1967; Govindjee and Papageorgiou, 1971; Lavorel and Etienne, 1977; Govindjee and Jursinic, 1979). During recent years the availability of instruments with picosecond time resolution has permitted detailed analysis of the fluorescence lifetime (τ) parameters and the process of energy migration, transfer, and conversion.

A. Definition of the Mean Lifetime

Following the absorption of photons of a short light pulse, the initial number (N_0) of the excited states S_1 may decrease by an array of simultaneous and consecutive processes. In a simple scheme where S_1 decays by several parallel first-order processes, the decay of the excited state with time is represented by the following equation:

$$-dN/dt = N_0(k_f + k_i + k_t + k_p) \quad (1)$$

where k_f, k_i, k_t, and k_p represent the rate constants of fluorescence emission, radiationless deactivation by internal conversion, intersystem crossing, and photochemical conversion, respectively.* The time course of the excited states $N(t)$ can be calculated as

$$N(t) = N_0 \exp(-t/\tau) \quad (2)$$

where the lifetime is defined by

$$\tau = 1/(k_f + k_i + k_t + k_p) \quad (3)$$

* The reader should note that different authors use different symbols. For example, k_i is k_{ic}, and k_t is k_{isc} in Chapter 5 by Seely and Connolly (this volume).—editors.

The fluorescence intensity at any moment is proportional to the number of excited states. Its decay, $F(t)$, which equals $k_f N(t)$, follows first-order kinetics. The natural or intrinsic lifetime τ_0 (= $1/k_f$) is the lifetime when fluorescence is the only deactivation pathway. The fluorescence yield Φ_f is related to these lifetimes by

$$\Phi_f = \tau/\tau_0 \tag{4}$$

The fluorescence decay is often more complex than described above, in which case a mean lifetime $\bar{\tau}$ can be calculated from the general expression:

$$\int_0^\infty [tF(t)]\, dt \Big/ \int_0^\infty F(t)\, dt \tag{5}$$

In photosynthetic material the fluorescence decay is assumed to result from the superposition of several (N) exponentially decaying processes. For such a model the mean lifetime is more easily estimated from the lifetime. τ_i and the initial fluorescence intensity A_i of all the individual processes i by

$$\bar{\tau} = \frac{\Sigma_{i=1}^{N} A_i \tau_i^2}{\Sigma_{i=1}^{N} A_i \tau_i} \tag{6}$$

The antenna pigments in higher plants and algae are located in at least six complexes: light-harvesting Chl *a*/*b* protein of PSII (LHC II), light-harvesting Chl *a*/*b* protein of PSI (LHC I); the peripheral Chl *a* complex of PSII (called CP 43), the peripheral Chl *a* complex of PSI, reaction center II complex (called CP 47), and reaction center I complex (P700-containing complex). These proteins may form larger complexes. Such an organization suggests the possibility of very complex fluorescence emission kinetics. Butler and Kitajima (1975; see Butler, 1978) proposed a "tripartite" model for the functional organization of the antenna pigments, dividing them into three groups: LHC II, the peripheral Chl *a* and reaction center complex of photosystem II (termed PSII), and the same for photosystem I (PSI). This tripartite model seems to represent a reasonable first approximation to the actual situation for energy transfer in higher plants and algae.

B. Methods for Lifetime Measurements

Fluorescence lifetime measurements of photosynthetic material with ps resolution are now possible because of the recent progress in laser technology, synchrotron radiation facilities, and detection systems for weak fluorescent signals (also see Lavorel *et al.*, Chapter 4, this volume).

For an exhaustive review of time-resolved fluorescence spectroscopy in biochemistry and biology, see Cundall and Dale (1983). We shall describe below the techniques which have been used for fluorescence lifetime measurements in photosynthetic systems: direct pulse fluorimetry, single-photon counting, and phase fluorimetry.

1. DIRECT PULSE FLUORIMETRY

The total fluorescence decay induced by a single pulse of a few ps duration is directly recorded with a streak camera (Campillo *et al.*, 1976). For a signal-to-noise ratio sufficiently low to resolve kinetics with ps lifetimes the pulses must have about 10^{16} photons/cm^2. However, Campillo *et al.* (1976) demonstrated that at intensities exceeding 10^{13} photons/cm^2 additional nonradiative deactivation occurs by exciton–exciton annihilation. This annihilation seems to be possible because of the presence of a large number of antenna pigments in the system. The recent development of the synchroscan camera (Wittmershaus *et al.*, 1985) overcomes the need for high-energy laser pulses by improving the sensitivity through signal-averaging techniques and the use of high-repetition mode-locked lasers.

2. SINGLE-PHOTON TIMING

In this technique one monitors the delay between the excitation laser pulse and single fluorescence photons detected with a photomultiplier (PM). The histogram of the delays accumulated in a multichannel analyzer represents the fluorescence decay. The advantages of this technique are the use of low-energy excitation laser pulses (thus avoiding excitation annihilation problems), excellent sensitivity, and the possibility of a multicomponent analysis of the decay. The time resolution is limited by the full width at half-maximum (FWHM) of the so-called instrumental function (i.e., the response of the measuring setup to a very short excitation), which can range from 200 to 400 ps for fast PMs. Channel plate PMs may improve the instrumental function up to 50 ps (Yamazaki *et al.*, 1985). A numerical deconvolution of the fluorescence decay with the instrumental function allows resolution of lifetimes as short as 30–50 ps. A disadvantage is that several minutes are needed to accumulate, during repetitive flashes, a decay at low excitation pulse intensities.

3. PHASE FLUORIMETRY

The fluorescence emission is delayed with respect to the exciting light pulse. If the exciting light is modulated at a frequency ν close to $1/\tau$, a

phase shift and a damped intensity of the modulated fluorescence are observed. The fluorescence lifetime can be deduced from the phase lag (φ) or from the relative modulation (m). For a single-exponential model φ and m are related to the lifetime τ by

$$\tan \varphi = 2\pi\nu\tau \quad (7)$$

$$m = [1 + (2\pi\nu\tau)^2]^{-1/2} \quad (8)$$

With suitable frequencies, the time resolution in the differential mode can be of a few ps (Moya *et al.*, 1981). As with single-photon timing one has to assume a model, e.g., a multiexponential decay, for the analysis. A mixture of N independent components can be resolved, in principle, by the determination of m and φ at N different frequencies (Weber, 1981).

The resolution of this method has recently been improved through the utilization of mode-locked lasers (see Gratton *et al.*, 1984). They provide a large number of upper harmonics of the fundamental frequency of repetition, and allow the detection of energy transfer mechanisms by comparison of the apparent lifetime values deduced from φ or from m. An essential advantage of phase fluorimetry is the short time required for the measurement of φ and m, which favors the method for studies involving lifetime and quantum yield changes in the millisecond range. Single-photon timing and phase fluorimetry are fully complementary and could be associated in the same fluorescence instrument for time-resolved measurements.

II. Early Measurements

A. *Contributions of Early Lifetime Measurements to Photosynthetic Concepts*

The earliest measurements of the fluorescence lifetime of Chl *in vivo* were made independently by Dmitrievsky *et al.* (1957), using the phase method, and by Brody and Rabinowitch (1957), using the direct pulse method. The latter authors pointed out that the quantum yield of fluorescence directly measured by Latimer *et al.* (1956) for the green alga *Chlorella* was lower than the ratio τ/τ_0 [see Eq. (4)]. This was explained by the existence of weakly fluorescent or nonfluorescent Chl *a*, which did not contribute to the fluorescence yield deduced from the lifetime measurements but did affect the directly measured fluorescence yield.

Another complexity is the apparent contradiction between the four- to fivefold increase of the fluorescence intensity as the reaction centers (RCs) become closed, and the high quantum yield (0.9) for photochemistry; the latter implies an increase in Φ_f by a factor of 10 in terms of the simple model described in Eq. (1). Two interpretations for this discrepancy are: (1) an increased k_i in closed reaction centers compared with open ones, and/or (2) the existence of a portion of Chl *a* whose fluorescence emission is largely independent of the state of RCs. Fluorescence from disconnected Chl *a* molecules has been excluded (see later), and the fluorescence yield of PSI is too small to explain the discrepancy. Alternative suggestions will be discussed below.

During the period from 1960 to 1970 one of the major questions dealt with was the extent of energy transfer among photosynthetic units (the entity of antenna pigments physically or statistically associated with an RC). The two extreme models being discussed were the "puddle" model, with independent units, and the matrix or "lake" model, in which several reaction centers shared a common pool of antenna molecules (see van Grondelle and Amesz, Chapter 8, this volume). Phase fluorimetry was the method which provided the best resolution at the time, but unfortunately it could not resolve the multiexponential decays which we now know to exist. During that time Tumerman and Sorokin (1967), Müller *et al.* (1969), and Briantais *et al.* (1972) observed an almost linear increase in the fluorescence intensity as a function of the average lifetime, which increased from 0.5 ns with open RCs to 1.5–2 ns when the RCs were closed. Although this relationship would have suggested the lake model (Tumerman and Sorokin, 1967), it was consistent with a partial lake or "connected" organization of the antenna (Moya, 1974), and in agreement with the conclusions derived by Joliot and Joliot (1964) from the nonlinear relationship between the steady-state rate of oxygen evolution and the number of open PSII RCs.

Later, Borisov and Godik (1970, 1972) reported, in photosynthetic bacteria, the separation between an extremely fast decay with a variable lifetime of 10–100 ps and a kinetic component with a long lifetime (nanosecond range) which was not affected by the state of the RC ("dead" fluorescence).

After the early work during 1957–1967, remarkable progress in the study of the energy transfer became possible with the introduction of mode-locked lasers (Merkelo *et al.*, 1969), the optical Kerr gate (Seibert *et al.*, 1973), and the streak camera (Campillo *et al.*, 1976), which allowed direct measurement of fluorescent kinetics with ps time resolution.

B. Exciton Annihilation Measurements

In early studies of directly monitored fluorescence kinetics induced by high-intensity picosecond laser pulses (Seibert *et al.*, 1973; Beddard *et al.*, 1975; Kollman *et al.*, 1975; Paschenko *et al.*, 1975; Shapiro *et al.*, 1975, Yu *et al.*, 1975), the τ's were found to be considerably shorter than those observed by either the phase shift or the single-photon timing method (for reviews, see Campillo and Shapiro, 1978; Govindjee and Jursinic, 1979; Breton and Geacintov, 1980; Pellegrino and Alfano, 1982). Campillo *et al.* (1976) and Mauzerall (1976) demonstrated that τ and Φ_f decreased at energies of picosecond pulses greater than 10^{13} photons/cm^2. They attributed this effect to singlet–singlet annihilation, which provided an additional deactivation pathway for the excited states:

$$S_1 + S_1 \rightarrow S_n + S_0 \tag{9}$$

Here S_n may be the first excited singlet state S_1, a higher excited singlet state, or the ground state, S_0. This bimolecular mechanism implies an encounter between the excited states and therefore depends on (1) the exciton migration in photosynthetic membranes and (2) the structure of the photosynthetic apparatus. Information on these two aspects can be derived from the integrated Φ_f as a function of the intensity of the exciting picosecond laser pulse. The Φ_f begins to decline at laser energies providing only one hit per 2000–3000 Chl molecules. This is a considerably higher value than the 200–300 Chl molecules per RC. Mauzerall (1976) used Poisson statistics and an isolated photosynthetic unit model to explain the high-intensity effects. However, it seems that the model of Swenberg *et al.* (1976), which assumes free energy migration between photosynthetic units, and the model of Paillotin *et al.* (1979), which limits the free exciton migration to confined domains of several photosynthetic units, explain the data more successfully.

The recent models of den Hollander *et al.* (1983) and Paillotin (1984) use a Pauli master equation (see, e.g., van Grondelle and Amesz, Chapter 8, this volume) to describe the dynamics of excitons, the dependence of Φ_f, and the number of closed RCs in terms of the number of photons absorbed per photosynthetic unit. Bakker *et al.* (1983), using a similar approach, deduced a rate constant of 3–6 $\times$ 10^{11} s^{-1} for the energy transfer between neighboring antenna molecules in *Rhodospirillum* (R. *rubrum* and *Rhodopseudomonas* (Rps.) *capsulata* and found that 14–17 and 30 units, respectively, were connected in these bacteria, probably by the B800 antenna complex (an 800-nm absorbing BChl complex).

For detailed discussions of the analyses of the photosynthetic apparatus by exciton annihilation, the reader may consult the reviews of

Geacintov and Breton (1982), Mauzerall (1982), Swenberg (1982), and van Grondelle and Amesz (Chapter 8, this volume).

C. Moderate Intensity Direct Decay Measurements

Barber *et al.* (1978) used single 530-nm picosecond excitation pulses of moderate intensity to avoid the singlet annihilation processes. Using a streak camera, they found, in isolated chloroplasts, decays distinctly different from monoexponential kinetics, with an average τ of 0.4 ns at low concentrations of monovalent cations, which became 1.6 ns after the addition of 10 m*M* $MgCl_2$. These values were in agreement with those obtained earlier by the phase-shift method (Moya *et al.*, 1977). Later, Beddard *et al.* (1979) and Searle *et al.* (1979) resolved two exponential components in chloroplasts. The former authors used single-photon timing to measure the *in vivo* Chl *a* fluorescence and reported values of 410 and 1400 ps for the two τ components. The yield of the slow component was found to increase with increasing Φ_f.

III. Single-Photon Timing Experiments

A. Three Exponential Deconvolution

Besides excitation with low-intensity picosecond pulses, single-photon timing measurements provide the advantage of an amplitude dynamic range exceeding three orders of magnitude. Since the instrumental function of 150–300 ps is not negligible when compared to the τ of a fluorescence decay component, the actual decay time of fluorescence is obtainable only by numerical deconvolution. One has to assume a decay law $I(t)$ (intensity of fluorescence as a function of time) which contains adjustable parameters, e.g., the amplitudes A_i and the lifetimes τ_i of a sum of N exponential decays:

$$I(t) = \sum_{i=1}^{N} A_i \exp(-t/\tau_i) \tag{10}$$

Most authors use the nonlinear least-squares method to minimize the reduced χ_i^2, defined by

$$\chi_i^2 = \frac{1}{n-u} \sum_{i=1}^{n} \frac{[F_i(t) - C_i(t)]^2}{F_i(t)} \tag{11}$$

where u is the number of unknown parameters, n is the number of data points, and $F_i(t)$ and $C_i(t)$ are the ith points of the fluorescence data and

of the calculated convolution, respectively. The excellent signal-to-noise ratio, which is proportional to the square root of the number of counts per channel, has made it possible to analyze the fluorescence kinetics by the multicomponent decay law. A correct model shows no systematic deviations in the plot of the difference between the actual decay and the best fit of the convoluted model, and the χ_i^2 would be near unity as expected for Gaussian noise.

Problems which may reduce the resolution of the above approach have been reported by Catterall and Duddel (1983); they consist of the following. (1) The larger width of the instrumental function; the width limits the analysis of kinetic components with lifetimes which are longer than about one-fifth of its FWHM. (2) The lower signal-to-noise ratio; this ratio determines the ability to detect systematic deviation from the best fit of a two- or three-exponential decay. (3) The "color effect" (Müller *et al.,* 1969) due to the higher photon energy of the excitation than the fluorescence, which may introduce an additional delay; this is usually taken into account by the fitting procedure with an additional parameter for a time shift of the fluorescence relative to the excitation. (4) The narrow time window of the measurement; this window must be wide enough to resolve a slow component superimposed on much faster components. (5) The fluorescence produced by the preceding pulses, which may contribute to the measured time course if the time between the pulses is not long enough for complete relaxation of a slow component; at a pulse distance of 12 ns of the mode-locked laser this can be included in the analysis of the signal (Haehnel *et al.,* 1982), or a cavity dumper can be used to reduce the pulse frequency, thus eliminating the problem (see Haehnel *et al.,* 1983).

B. Measurements in Chloroplasts of Higher Plants and in Green Algae

A three-exponential model was first used by Haehnel *et al.* (1982) to describe the fluorescence kinetics, measured with a single-photon timing apparatus, in chloroplasts of higher plants and in intact green algae. These authors studied the dependence of Φ_f and τ of the individual components as a function of increasing excitation intensities. Similar but quantitatively different results were reported soon thereafter by Gulotty *et al.* (1982). The three-exponential analysis was used during investigations of the effect of Mg^{2+} addition (Nairn *et al.,* 1982), the redox state of the first stable electron acceptor of PSII (Karukstis and Sauer, 1983a,b), the phosphorylation of LHC II (Haworth *et al.,* 1983), and plant devel-

opment (Karukstis and Sauer, 1983c). (For a review of the fluorescence decay kinetics, see Karukstis and Sauer, 1983d.)

Table I summarizes results of a three-component analysis and Fig. 1 shows the dependence of the lifetime and yield of the three components, in green algae, with increasing fraction of "closed" RCs (Haehnel *et al.*, 1983).

The results can be summarized as follows: (1) When most of the RCs are open, two main components with τ's of 100 and 500 ps and relative Φ_f's of 0.3 and 0.7 are observed. These components are usually called the fast and the middle components. (2) As the RCs are closed, a slow component with a lifetime of 1.4 ns (with mainly open centers) and 2.2 ns (closed centers) increases in amplitude to account for more than 65% of the total maximal fluorescence yield (F_{max}); this change parallels a decrease in the Φ_f of the fast component, which becomes close to zero at F_{max}. Thus, the complementarity between the yield of photochemistry and the Φ_f of the slow component seems to be fulfilled. (3) The middle component shows only a slight increase in both τ and Φ_f as the RCs are closed, thereby contributing an almost constant portion to the total Φ_f.

The fast and slow components were assumed by Haehnel *et al.* (1983) to be mainly due to fluorescence emission from the Chl *a* antenna of PSII with open and closed RCs, respectively. Photosynthetic units in a "separate package" would show fluorescence decay components with distinct τ's, one for units with open RCs and another for units with closed RCs. The opposite dependence of the yield of the slow and fast τ components on the state of the RC is consistent with a mechanism of largely separated PSII units (Haehnel *et al.*, 1982, 1983). However, the increase in the τ of the slow component, from 1.4 ns (almost all RCs open) to 2.2 ns (closed RCs), is evidence for a limited possibility for energy transfer (Haehnel *et al.*, 1983).

The middle τ component has been ascribed by Haehnel *et al.* (1982, 1983) to LHC II because it was preferentially excited by light at 652 nm (absorbed by Chl *b* in LHC II), exhibited an almost constant Φ_f, and peaked at $\lambda < 685$ nm. However the increase of τ of the middle component from 0.5 to 1.1 ns is not yet understood.

The mean lifetime $\bar{\tau}$, calculated from the deconvoluted parameters of the three exponential components, shows an almost linear increase with the total fluorescence yield by a factor of about 4 (Haehnel *et al.*, 1982). This result is in agreement with earlier phase shift measurements on the green algae *Chlorella* (see e.g., Briantais *et al.*, 1972). However, the τ parameters produced from the deconvolution show only a small increase on closing the RCs (Haehnel *et al.*, 1983), and it was concluded that

TABLE I
Decay Components of the Fluorescence Kinetics in Chlorella and Chlamydomonas[a,b]

		Lifetimes (ns)			Preexponential factors (%)			Yield (rel. units)			
Species	F	τ_1	τ_2	τ_3	α_1	α_2	α_3	Φ_1	Φ_2	Φ_3	Total yield
Chlorella vulgaris	$F \geqq F_0$[c]	0.13	0.50	1.4	47	52	1	5.1	21.6	0.9	27.6
	F_{max}	0.10	1.2	2.2	12	43	45	0.6	33.9	65.4	=100[d]
Chlamydomonas reinhardtii	$F \geqq F_0$[c]	0.11	0.61	1.4	52	44	4	5.4	25.7	5.1	36.2
	F_{max}	0.06	1.1	2.3	43	27	30	2.5	29.5	68.0	=100[d]

[a] From Haehnel *et al.* (1983).

[b] All data calculated on the basis of a triple-exponential model function. At F_{max} a biexponential model function is sufficient, however, to describe the experimental decay. The data given represent average values from several experiments.

[c] Decay components calculated from experiments carried out with a photon density of 3.5×10^{11} photons/cm^2.

[d] Arbitrary reference value.

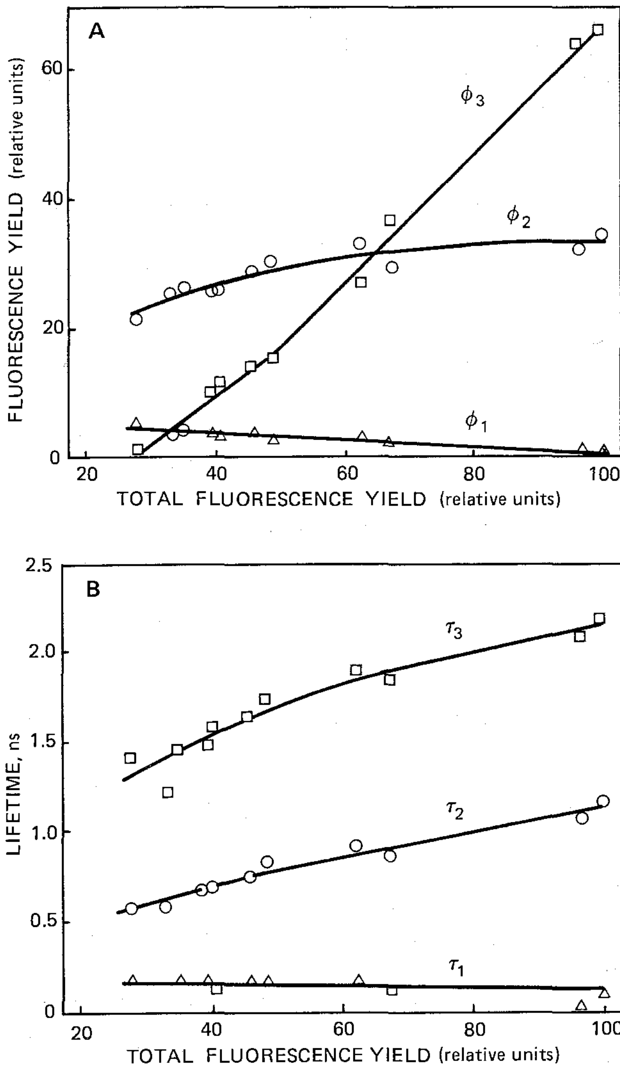


FIG. 1. (A) Yields (Φ) of the components of Chl *a* fluorescence kinetics obtained by the single-photon counting method in the green alga *Chlorella (C.) vulgaris* as a function of the total fluorescence yield. Wavelength of analysis, 682 nm. The total fluorescence yield was increased by increasing the concentration of diuron (DCMU) up a maximum of 20 μM and then by further addition of up to 10 m*M* hydroxylamine. Addition of DCMU alone resulted in a total fluorescence yield of 67%. (B) Lifetimes (τ_1, fast; τ_2, medium; τ_3, slow) of the components of Chl *a* fluorescence kinetics of *C. vulgaris* as a function of the total fluorescence yield. (From Haehnel *et al.*, 1983.)

energy transfer, measured by the increase in τ of the slow component, was rather limited. On the other hand, the proportionality between the average τ and the total Φ_f, which was at the basis of a well-connected model of PSII units, seems rather fortuitious in view of the deconvolution into three exponentials.

However, it has recently been shown that the middle component is almost unchanged in a barley mutant lacking LHC II (Karukstis and Sauer, 1984) and that the slow component is still present in a mutant lacking PSII RCs (Green *et al.*, 1984). Thus, the ideas mentioned above may have to be modified.

Butler *et al.* (1983) suggested a different explanation for the three components, based on the proposal of Melis and Homann (1978), that PSII consists of two distinct types of units, α and β. The α units represent interconnected groups of PSII units which can transfer energy among themselves, whereas the β units are associated with separate PSII units, which cannot transfer energy to other PSII units. It was proposed that the middle τ component may originate from the antenna associated with PSII β units, while the fast and slow components originate from PSII α units in their open and closed states, respectively.

The fluorescence of the antenna of PSI has recently been considered. It has been proposed (Butler *et al.*, 1983) that part of the fast component arises from PSI. The wavelength dependence of the lifetime of the fast component suggests superposition of a component originating from PSI, with a τ of about 80 ps and peaking near 700 nm (Yamazaki *et al.*, 1985), and another component from PSII antenna pigments, with a τ of about 180 ps and peaking near 685 nm (Holzwarth *et al.*, 1984).

C. The Delayed Fluorescence Hypothesis

There are several similarities between the electron acceptor side of the RC complex of PSII and that of photosynthetic bacteria. In PSII the primary electron acceptor is a pheophytin (Pheo) molecule and the secondary electron acceptor, Q_A, is a bound plastoquinone (see van Gorkom, Chapter 10, this volume), whereas in photosynthetic bacteria Pheo is replaced by bacteriopheophytin (BPheo) and plastoquinone by ubiquinone (see Norris and van Brakel, Chapter 3, this volume). The reactions of charge stabilization in PSII are:

$$(\text{P680* Pheo } Q_A) \rightarrow (\text{P680}^+ \text{ Pheo}^- \, Q_A) \rightarrow (\text{P680}^+ \text{ Pheo } Q_A^-) \quad (12)$$

Klimov *et al.* (1978) and Shuvalov *et al.* (1980) observed that PSII-enriched particles, at a low redox potential, emit fluorescence with a τ of 4.3 ns. Furthermore, the time course of this slow component was found

to parallel the disappearance of the radical pair state ($P680^+$ $Pheo^-$). Therefore the slow lifetime component of PSII, which represents the major part of the variable Chl *a* fluorescence, has been interpreted as delayed fluorescence ($h\nu'$) originating from recombination of the charges in closed RCs:

$$(P680^+\ Pheo^-\ Q_A^-) \rightleftharpoons (P680^*\ Pheo\ Q_A^-) \rightarrow (P680\ Pheo\ Q_A^-) + h\nu' \quad (13)$$

K. Sauer's research groups has adopted this interpretation of the origin of the slow τ component, exhibited by isolated thylakoids and intact algae (see e.g., Karukstis and Sauer, 1983d). The delayed emission may originate from the PSII RC or, ultimately, from the Chl *a* antenna after back transfer (see Jursinic, Chapter 11, this volume). However, it is not understood why the τ of the slow component in chloroplasts is twice as short as that found by Klimov *et al.* (1978) in isolated PSII particles and why it is still present in a barley mutant lacking PSII RCs (Green *et al.*, 1984). This is therefore an open question.

IV. Wavelength-Resolved Lifetime Measurements

A. Room Temperature Measurements

The wavelength dependence of the fluorescence τ at room temperature is very small, in spite of the obvious kinetic heterogeneity of the fluorescence decay after ps pulse excitation. The molecular species responsible for the fluorescence emission at room temperature in higher plant chloroplasts and in green algae exhibit almost the same emission spectra. However, Yamazaki *et al.* (1985) recently reported ps time-resolved fluorescence spectra, in the green algae *Chlorella,* of both the PSII antenna (peaking at 685 nm) and the PSI antenna (emission observed at 690–730 nm in the initial time region 0–180 ps). These results are in agreement with multiexponential decays at several emission wavelengths reported by Haehnel *et al.* (1983) and Magde *et al.* (1982).

B. Low Temperature Measurements

It is well known that lowering the temperature to 77°K induces large modifications in the fluorescence emission spectra of chloroplasts compared with the room temperature emission band, which exhibits a single peak at 685 nm (thought to originate from the PSII antenna complex). The stimulation of fluorescence intensity which appears under such low-temperature conditions produces a band at 696 nm (perhaps due to the

PSII RC complex) and another band at 730 nm (due to a long-wavelength absorbing form of Chl *a*, C705). (See Murata and Satoh, Chapter 6, and Govindjee and Satoh, Chapter 17, this volume.) Butler and Norris (1963) reported the τ for F730 to be 3.1 ns in bean leaves. Mar *et al.* (1972) measured the τ for F730 and F685 as 2.3 and 1.4 ns, respectively, in the green alga *Chlorella*, a result that was discussed further by Hervo *et al.* (1975).

Moya *et al.* (1981) provided complete τ spectra at 77°K for spinach chloroplasts (Fig. 2); large variations appear in the τ spectra, which originate from the overlapping of several fluorescence emissions with different lifetimes. Moya and Garcia (1983) used a new approach for the analysis of the fluorescence τ spectra, using several of the harmonics which constitute the output of a mode-locked laser. The fluorescence emission spectra recorded under high-frequency modulated light provide, in addition to the time-integrated spectrum (i.e., at zero frequency), two independent spectra which are the imaginary and real parts of the spectrum at the frequency of the harmonic. By repeating the experiment with n harmonics, $2n + 1$ equations (from the different spectra) can be obtained. Such a set of equations can be resolved by analytical methods (Weber, 1981) or fitted by a model of Gaussian components, using a least-squares method (Moya and Garcia, 1983).

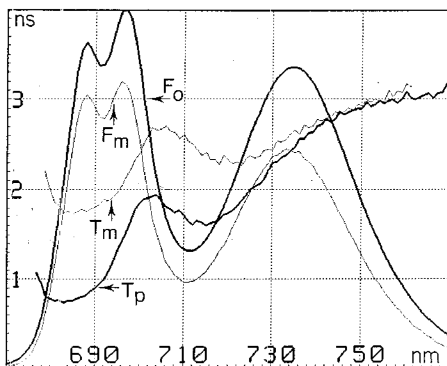


FIG. 2. Fluorescence emission spectra of spinach chloroplasts at 77°K (closed reaction centers). F_0, Intensity of the emission upon excitation with unmodulated light, arbitrary units; F_m, intensity of the emission at 58 MHz (same units as F_0); T_p, averaged lifetime spectrum deduced from the phase shift; and T_m, averaged lifetime spectrum deduced from the relative "modulation" (see text). Note the strong wavelength dependence of the averaged lifetime spectra. (From Moya *et al.*, 1981).

Moya and co-workers demonstrated that in chloroplasts with closed PSII RCs, the so-called PSI band was the superposition of a component peaking at 723 nm with a τ ranging from 0.5 to 1 ns, attributed to the antenna core of PSI, and a second component peaking at 737 nm with a τ of 3 ns. At least three components were required to fit the PSII emission: two main bands located at 685 nm (0.3 ns) and 696 nm (1 ns), and a long τ component (4 to 6 ns) presumably located at 685–695 nm (Moya *et al.,* 1981). Only the fast emissions at 685 and 696 nm seem to be related to changes in the redox state of PSII RCs (Moya, 1979). Average τ values of the same order of magnitude have been reported by Wong *et al.* (1981).

Photon-counting fluorescence decay measurements at 77°K have been performed by Reisberg *et al.* (1982), who claimed that up to five components were present. Three of these were predominant at wavelengths <690 nm and appeared to correspond to the three components seen at room temperature (Haehnel *et al.,* 1982). A 3-ns component was found at wavelengths >730 nm, in addition to a 100-ps rise component, which was related to energy transfer from the bulk antenna to C705. A similar rise component at the onset of the 730-nm fluorescence was seen earlier by Campillo *et al.* (1977a,b) and Pellegrino *et al.* (1983). This has been thoroughly studied by Wittmershaus *et al.* (1985), who reported a shorter delay of 16 ps between the rise of F685 and F730. Such a delay seems too small to be consistent with a simple energy transfer mechanism from the species emitting at 685 nm to that emitting at 730 nm. It seems to lie within the mechanism of PSI.

Avarmaa *et al.* (1979) carried out 4°K fluorescence studies by using single-photon counting, and found τ's of 1, 2.5, 4, and 3.2 ns for the emissions at 681, 685, 696, and 730 nm, respectively.

V. Lifetime Measurements in Subchloroplast Particles

A. Light-Harvesting Chlorophyll a/b Protein (LHC II)

These proteins, which bind more than 50% of the total Chl in higher plants and green algae, act as the main antenna for light absorption. LHC II can transfer energy to the RC complex of PSII. Although the detailed mechanism of energy transfer is not known, it is suggested that exciton delocalization may exist within a single Chl–protein complex, followed by slower (tens of ps) Förster inductive resonance transfer between Chl–protein complexes (see below and van Grondelle and Amesz, Chapter 8, this volume).

The fluorescence τ of the isolated complex is expected to be longer than that of the complex when it is located in chloroplasts, because of the absence of natural quenching by energy transfer to the PSII RC. A fluorescence τ of 4 ns has been reported by Searle and Tredwell (1979). Phase fluorimetric measurements of Il'ina *et al.* (1981) showed that an aggregated state is formed in the presence of 10 mM $MgSO_4$ with a τ of 0.6 to 1 ns, whereas a more or less "monomeric" form, with a τ of about 4.1 ns, is stabilized in the presence of a detergent. These results are in agreement with those obtained by Nordlund and Knox (1981). However, Lotshaw *et al.* (1982) found two τ components (1.2 and 3.3 ns) even in monomeric species.

Lifetime spectra were measured by Moya and Tapie (1984) for both the aggregated and monomeric forms of LHC II. At 77°K the average τ strongly depended on the emission wavelength. Three components were resolved, peaking at 680 (F680), 685 (F685), and 695–698 (F695) nm. The analysis held for both the monomeric and aggregated species, but in the latter case the relative weights of F685 and F695 were dramatically increased. It is believed that at room temperature a high degree of coupling exists between the three components. At 77°K the uphill transfer is preferentially inhibited: the energy tends to accumulate in the lowest excited state before radiative emission occurs.

B. Photosystem II Subchloroplast Particles

Little information is available on the fluorescence properties of PSII particles. Sauer and Brewington (1978), using digitonin-extracted particles from spinach chloroplasts and a conventional photon-counting instrument, found a single τ component of 0.34 ns when the RCs were open and two decay components of 0.8 and 2 ns when the RCs were closed. However, the detergent digitonin may have altered the physical organization of Chl in such particles, thus limiting the usefulness of this type of preparation. Shuvalov *et al.* (1980) applied the same technique to Triton-solubilized PSII particles containing 30–40 Chl/RC of PSII, P680. In the presence of ferricyanide a τ of 0.42 ns was observed. The addition of sodium dithionite, which reduced the plastoquinone, induced the appearance of a fluorescence component with a τ of 4.3 ns, in addition to a 1.06-ns (prompt) fluorescence component. The 4.3-ns component was interpreted as delayed fluorescence, following Eqs. (12) and (13). Direct fluorescence decay measurements, using a ps laser and a streak camera detection system, on digitonin-extracted PSII particles revealed a τ of about 500 ps at low photon densities. At 77°K this τ increased to 2.5 ns (Searle *et al.*, 1977).

C. *Photosystem I Subchloroplast Particles*

Borisov and Il'ina (1973) were the first to report that the fluorescence τ of isolated PSI particles may be <100 ps. This was confirmed by Searle *et al.* (1977), who also found a τ component <100 ps in isolated PSI particles at room temperature, which became about 1.9 ns at 77°K. Lifetime spectra of PSI particles, isolated by the procedure of Burke *et al.* (1978) and containing about 110 Chl/RC of PSI, P700, were measured by Moya *et al.* (1981) under excitation with 139-MHz modulated light. At room temperature, they found an average τ of <100 ps, but with a marked wavelength dependence. The contribution of an extremely fast τ component (<15 ps) was predominant around 690 nm. The τ spectra of these particles at 77°K were very similar to the long-wavelength spectra of chloroplasts, except that all the lifetimes were 0.5 ns shorter. The decomposition into Gaussian components showed two bands peaking at 720 nm (0.33 ns) and 735 nm (2.5 ns), similar to those already shown in chloroplasts.

PSI particles containing about 10 Chl/P700 have been isolated by Ikegami (1976). Direct fluorescence decay measurements of this preparation at room temperature, using picosecond pulses at 515 nm and streak camera detection, were reported by Kamogawa *et al.* (1983). At a low photon fluence (i.e., less than one excited antenna molecule/P700) two main fluorescence components with lifetimes of 10 and 70 ps were found. Wavelength-resolved measurements showed that the faster component predominated around 690 nm and the slower one at 680 nm. The authors attributed the two kinetic τ components to different kinds of antenna Chl. The 70-ps fluorescence component lasts much longer than the time needed to oxidize P700 (<25 ps), as measured by Kamogawa *et al.* (1981). Therefore, a simple mechanism of energy transfer from short- to long-wavelength pigments does not hold here. In addition, no charge in the kinetic parameters were detected when P700 was oxidized.

VI. Fluorescence Lifetime of Photosynthetic Bacteria

A. *Variable Bacteriochlorophyll Fluorescence from Chromatophores*

The light-harvesting apparatus of the photosynthetic bacteria is simpler than that of plants (see Kaplan and Arntzen, 1982). Only one photosystem is present in these organisms. This allows easier analysis of the

time-resolved fluorescence data. Furthermore, advances in the isolation and purification of the different pigment–protein complexes of the antenna and the RC (see Okamura *et al.*, 1982), whose spectroscopic properties are close to those observed *in vivo,* greatly help in understanding the energy transfer mechanism and the deactivation processes of the excited states of the energy carrier pigments.

In purple bacteria, one or two types of light-harvesting pigment–protein complexes are present. One is the so-called B800–B850 complex, where at least one molecule of BChl 800 (absorbing with a peak at 800 nm) and two molecules of BChl 850 (peak at 850 nm) are present (Cogdell and Crofts, 1978). The other, the B875 complex, contains at least two molecules of BChl 875 (peak at 875 nm) (van Grondelle and Rijgersberg, 1981; see also Amesz and Vasmel, Chapter 15, this volume). Monger and Parson (1977) suggested that the B800–B850 antennas surround the B875 complexes, the latter being directly associated with the RCs.

Borisov and Godik (1970, 1972), working with *R. rubrum* chromatophores and using the phase fluorimetry method, were the first to suggest that the "live" fluorescence, directly associated with the photochemical state of the RCs, could be at least one order of magnitude shorter than the average measured τ of 1 ns (Govindjee *et al.*, 1972). More recently, Sebban and Moya (1983) also showed, by the phase fluorimetry method, that in *Rps. sphaeroides* the functional fluorescence is essentially emitted by the B875 antenna. Its τ, correlated with the Φ_f, varies from 50 to 250 ps during the light-induced transition from the open state (reduced primary electron donor, P) to the closed state (P^+) of the RCs. By picosecond flash experiments, Campillo *et al.* (1977a,b) found a fluorescence τ of 100 ps in chromatophores of *Rps. sphaeroides.* In *R. rubrum* chromatophores, Freiberg *et al.* (1983), using the same method, obtained τ values of 50 and 190 ps at low and saturating light intensities, respectively. Measurements of τ carried out on photoactive complexes from the green bacterium *Chlorobium limicola* by Borisov *et al.* (1977) also give short values (<100 ps).

Fok and Fetisova (1983), Kudzmauskas *et al.* (1983), and Sebban and Barbet (1985) suggested that the fluorescence τ's obtained in photosynthetic bacteria could be taken into account by a semilocalized model of the energy transfer process in the antenna. These authors suggested that the excitation energy could migrate to the RCs by a slow electrostatic process (Förster-type mechanism) between sites, each containing several BChls in strong interaction. The size of these elementary sites seems to be in agreement with the biochemical minimal unit of the B800–B850

complex (four BChls 850 and two BChls B800 per pair of polypeptides) recently proposed by Kramer *et al.* (1984).

In photosynthetic bacteria, Φ_f is directly related to the redox state of the primary electron donor P (see Duysens, Chapter 1, this volume). However, the P^+ state of the RCs, obtained either by saturating light or by the addition of ferricyanide, still remains a quencher of the antenna fluorescence. This was first suggested by Clayton and Clayton (1972) and Godik and Borisov (1977) and was recently confirmed by Sebban *et al.* (1984), who measured a fluorescence τ of about 0.65 ns in chromatophores from an RC-less mutant of *Rps. sphaeroides,* three times higher than that observed for wild-type chromatophores in their closed state (P^+) (200 ps). Thus, to explain these quenching processes in closed RCs, it is necessary to assume an additional deactivation pathway for the excitation coming from the antenna. Van Grondelle and Duysens (1980) suggested that a pigment complex **C** (probably BChl molecules), different from the main antenna, could exist in the vicinity of the RC. The antenna energy should be transferred to the reaction centers via **C.** The enhancement of the rate constant (K_c) of the nonradiative deactivation pathways of **C** when the reaction centers are closed would be responsible for the quenching properties of such RCs. For an F_{max}/F_0 value of 5 observed for the live part of the fluorescence of chromatophores from *Rps. sphaeroides* and the 200-ps τ of chromatophores with the RCs closed, K_c was calculated to be about $(80\ ps)^{-1}$ (Sebban, 1985). This high rate, when compared to that of the main antenna [about $(1\ ns)^{-1}$], must result either from large conformational changes in the vicinity of the RCs when they are closed, or from the influence of the local electrical potential induced during the primary charge separation by the negative charge on the primary quinone electron acceptor (Q_A) or further on the electron transfer chain (Godik and Borisov, 1977).

B. *Nanosecond Delayed Fluorescence in Chromatophores*

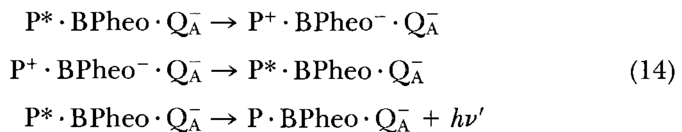
As stated earlier, the functional fluorescence τ observed with chromatophores ranges from 50 to 250 ps. However, under reducing conditions, when the primary quinone electron acceptor (Q_A) is reduced before illumination, a long τ component (several ns) is observed (Shuvalov and Klimov, 1976).

Since 1975, picosecond absorption measurements on isolated RCs have permitted the attribution of electron transfer kinetics to the electron carriers present in the RCs (see Parson and Ke, 1982; Norris and

van Brakel, Chapter 3, this volume). It is now generally accepted that excitation of the primary electron donor P (a dimer of BChls) to its lowest excited singlet state P* is followed, within 5 ps, by transfer of an electron to a molecule of BPheo (Dutton *et al.*, 1975; Kaufmann *et al.*, 1975; Rickley *et al.*, 1975; Netzel *et al.*, 1977; Holten *et al.*, 1978), giving rise to the radical pair state P^F (P^+BPheo^-). It was suggested that the electron transfer to BPheo could involve a molecule of BChl (P800) (Shuvalov *et al.*, 1978a,b; Akhmanov *et al.*, 1980). However, Borisov *et al.* (1983) have given theoretical and experimental evidence against electron localization on BChl (P800).

Under normal conditions, P^F declines to the state $PBPheoQ_A^-$ in about 200 ps. When Q_A is reduced before illumination, the τ of P^F is lengthened to about 12 ns, allowing charge recombination between P^+ and $BPheo^-$ to occur with reexcitation of P.

Thus, the above phenomenon was suggested to be at the origin of the observed nanosecond luminescence in chromatophores under reducing conditions. Klimov *et al.* (1976), working on a complex containing a cytochrome, RC BChl electron donor P890, and electron acceptors PBheo and Q_A from *Chromatium minutissimum*, measured a τ of 6 ns for the recombination luminescence ($h\nu'$) created as follows:



$$\begin{aligned} P^* \cdot BPheo \cdot Q_A^- &\rightarrow P^+ \cdot BPheo^- \cdot Q_A^- \\ P^+ \cdot BPheo^- \cdot Q_A^- &\rightarrow P^* \cdot BPheo \cdot Q_A^- \\ P^* \cdot BPheo \cdot Q_A^- &\rightarrow P \cdot BPheo \cdot Q_A^- + h\nu' \end{aligned} \tag{14}$$

An activation energy of 0.12 eV was measured for the above process. Godik and Borisov (1980) suggested that this delayed fluorescence comes from the BChl antenna after reexcitation by P*. This was confirmed by Sebban and Moya (1983), who observed with *Rps. sphaeroides* chromatophores that the spectrum of the ns component was nearly identical to that of the prompt fluorescence. The above τ figures are in agreement with the earlier work of Godik and Borisov (1979) on chromatophores from *R. rubrum* (5 ± 0.3 ns). The fluorescence τ's from chromatophores observed under physiological conditions (50–250 ps) are very different from those measured at a low redox potential (4–6 ns), when Q_A is reduced before illumination. Also, the delayed fluorescence emission was clearly demonstrated by Godik and Borisov (1979) and by Sebban and Moya (1983) to be an additional component distinguishable from the prompt live fluorescence. Thus, Klimov's hypothesis of the origin of the variable fluorescence does not seem to be valid, at least in photosynthetic bacteria.

The effect of a membrane potential on the delayed fluorescence of

R. rubrum chromatophores was studied by Borisov *et al.* (1980) and Kotova *et al.* (1981), who observed an enhancement of the delayed fluorescence yield and τ, suggesting that the rate constant of charge recombination between P^+ and $BPheo^-$ is increased by such a potential. Thus the charge separation between P^+ and $BPheo^-$ appears to be electrogenic. This was confirmed by van der Waal *et al.* (1982).

The temperature effect on the τ of delayed fluorescence was studied by Godik *et al.* (1982). These authors showed that the τ of delayed fluorescence (4.1 ± 0.5 ns) in *R. rubrum* chromatophores is constant from 200 to 300°K, whereas the Φ_f of delayed fluorescence increases in the same temperature range. These results were interpreted in the framework of a model in which the mobility of the RC proteins in the nanosecond or subnanosecond time range produces an electrostatic stabilization of the ion-radical state P^F ($P^+ \cdot BChl \cdot BPheo^-$) into two isoenergetic substates ($P^+ \cdot BChl^- \cdot BPheo$ and $P^+ \cdot BChl \cdot BPheo^-$).

C. Delayed Fluorescence from Isolated Reaction Center Complexes

Although picosecond absorption measurements on isolated RCs have been available since 1975, it was only in 1982 that their picosecond fluorescence decay kinetics began to be analyzed. Schenck *et al.* (1982), working on RCs isolated from the R26 strain of *Rps. sphaeroides,* were the first to point out the heterogeneity of the delayed fluorescence at low redox potentials. These authors measured an average τ of delayed fluorescence of 6 ns. In addition to a picosecond component due to the direct decay of P^+, Sebban and Barbet (1984), using the phase method with several modulation frequencies, found at least three other components (at low redox potentials). One of them had the same decay time (12 ns) as P^F measured by absorption techniques. Supporting the hypothesis of Godik *et al.* (1982), these authors interpreted the presence of the two other components as reflecting the electrostatic stabilization of P^F by the RC protein motions in the nanosecond time range. Woodbury and Parson (1984), using the single-photon counting method, further characterized the emission from reduced RCs from the R26 strain of *Rps. sphaeroides.* They also found three components for the delayed part of the fluorescence with τ's of 0.7, 3.2, and 11 ns. The intensity of the slowest component depended on both the temperature and the magnetic field in the same way as the absorbance change of P^F. The time constants for the two faster components of the delayed fluorescence were essentially independent of the temperature and magnetic field. These authors suggested that P^F relaxes during its lifetime through three substates, any one of which can decay with the same time constant

(11 ns) as the homogeneous P^F state, seen by absorbance changes. Here also, a mechanism involving movement of proteins, solvents, or chromophores of the RCs is suggested to be at the origin of the relaxation of P^F. However, Woodbury and Parson (1984) noted that these movements should be temperature-sensitive processes, at variance with the behavior of the two faster delayed fluorescence components which they observed.

An important comment must be made here about the results of both Sebban and Barbet (1984) and Woodbury and Parson (1984). In both reports the presence of three components in the delayed part of the fluorescence was postulated to fit the experimental decay curves. However, neither the phase shift method nor the single-photon counting analysis can exclude the presence in this emission of more than three components. If this is true, it would not be physically meaningful to attribute one precise component to one well-defined substate of P^F. In fact, a more complex fluorescence decay from reduced RCs could reflect either a more progressive (almost continuous) stabilization of P^F in each reaction center or only a τ distribution in the analyzed population of RCs. This distribution could reflect different conformational states of the RC proteins leading to different chromophore environments.

In support of the above hypothesis, Kleinfeld *et al.* (1984) found very different kinetics of charge recombination between P^+ and Q_A^- in isolated reaction centers (R26 strain) cooled to 77°K in the dark (i.e., in the PQ_A state) and under illumination (i.e., in the $P^+Q_A^-$ state). They interpreted their results in terms of a movement (about 1 Å) of P away from Q_A during the charge separation, due to structural changes. As a result, the electronic orbital overlap between P^+ and Q_A^- should decrease compared to that in PQ_A, minimizing the probability of charge recombination.

Similar experiments on native RCs, as well as on their subunits, in the ns time range will help in understanding the role of the protein fluctuations in energy stabilization.

VII. General Conclusions

A. Chlorophyll-Containing Systems

Photon-counting experiments in algae and in chloroplasts from higher plants provide clear evidence of the heterogeneity of the room temperature fluorescence emission, but there is no definitive answer to the question of whether all the components resulting from the deconvo-

lution of the observed kinetics have a physical meaning. At least three exponential components are required to fit the data. However, a fluorescence decay containing four components can often be deconvoluted, to produce good fits, by a sum of only three components (in which case the deconvoluted parameters may be meaningless). This is due to the finite information that can be extracted from fluorescence decays in the presence of noise.

Although there is general agreement on the τ components when all RCs are closed, the deconvolution at the F_0 fluorescence level (and at levels intermediate between F_0 and F_{max}) may be uncertain because: (1) the decay kinetics are fast, compared to the width of the instrumental function, and the accuracy of the deconvoluted parameters tends to decrease, and (2) inhomogeneities in sample illumination may occur and can introduce an artifactual mixture of components. Thus, it seems likely that models other than those used thus far may also fit the experimental data. Other aspects of the heterogeneity are also found by means of time-resolved τ spectra at low temperature.

Attempts to interpret the τ parameters in terms of rate constants of energy exchange between subsets of the photosynthetic apparatus have been rather limited. Butler *et al.* (1983) analyzed the multicomponent fluorescence decay obtained by Haehnel *et al.* (1983) in the framework of the so-called bipartite model of PSII, defined earlier by Butler (1978). It is shown that a biexponential decay is expected if the rate constants governing the energy transfer from the PSII antenna to the PSII RCs and back are introduced. It would be fruitful to extend this approach to the "tripartite" model, which must naturally generate a three-exponential fluorescence decay for PSII.

B. Photosynthetic Bacteria

There is general agreement that the fluorescence τ in chromatophores increases from 50 ps to 200–250 ps as the RCs go from an open to a closed state. These results are interpreted in terms of a globular antenna structure. The delayed fluorescence emission with a τ of 4–6 ns, which appears only under low redox potential conditions, seems unrelated to the variable BChl fluorescence. Lifetime measurements on isolated RCs of photosynthetic bacteria provide a useful took for analyzing the mechanisms of energy stabilization. It has been proposed that motion of the RC protein in the nanosecond range plays a role in energy stabilization. Thus, it is now necessary to apply time-resolved techniques to both the protein and the pigment part of the system.

C. Future Prospects

In the technical area several improvements in time resolution can be expected in the near future, including low-jitter microchannel plate photomultipliers, synchronized streak cameras, and multifrequency phase fluorimetry, which could reduce the time uncertainty by as much as one order of magnitude in certain experiments.

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8

Excitation Energy Transfer in Photosynthetic Systems

RIENK van GRONDELLE
Department of Biophysics
Physics Laboratory of the Free University
1081 HV Amsterdam, The Netherlands

JAN AMESZ
Department of Biophysics
Huygens Laboratory of the State University
2300 RA Leiden, The Netherlands

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ABBREVIATIONS AND SYMBOLS

APC	Allophycocyanin
BChl	Bacteriochlorophyll
BChl 800, 850, 875	Bacteriochlorophyll absorbing at about 800, 850, and 875 nm, respectively
B800–850, B875	Bacterial light-harvesting pigment–protein complexes containing BChl 800 and BChl 850 and BChl 875, respectively
Car	Carotenoid
Chl	Chlorophyll
I	Intermediate acceptor, probably (bacterio)pheophytin in PSII and purple bacteria

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k_{DA}	Rate of energy transfer from a donor (D) to an acceptor (A)
k_l	Rate of loss of first excited singlet state (S_1)
k_f	Rate of fluorescence
k_{isc}	Rate of intersystem crossing
k_{ic}	Rate of internal conversion
k_t, k_t^o, k_t^c	Rate of trapping, for open traps (k_t^o) and for closed traps (k_t^c)
k_h	Rate of energy transfer between a pair of neighboring identical molecules
k_1	Overall rate of decay of a single excitation due to losses and trapping
k_2	Overall rate of decay of a pair of excitations due to singlet–singlet annihilation
k_3	Overall rate of decay of a single excitation due to singlet–triplet annihilation
LDAO	Lauryl dimethylamine oxide
LDS	Lithium dodecyl sulfate
P	Primary electron donor in bacterial or plant photosynthesis
PC	Phycocyanin
PE	Phycoerythrin
PS	Photosystem
Q_A	First stable acceptor (quinone) in PSII and purple bacteria
RC	Reaction center

ABSTRACT

Energy transfer among photosynthetic pigments is a fundamental aspect of the photosynthetic process. The major mechanism, dipole–dipole coupling, is described and a number of examples are discussed. In photosynthetic systems two types of transfer should be distinguished: transfer between nonidentical pigment molecules, which is usually down an energy gradient, and transfer between identical long-wavelength pigments that surround and interconnect the reaction centers, where trapping takes place. The time to reach the latter group of antenna pigments can be very short (≤ 1 ps). The energy transfer in the long-wavelength antenna seems amenable to fairly realistic statistical models, and reasonable estimates for the transfer and trapping efficiencies can be obtained.

The second part of this chapter bears on the question of how photosynthetic units are connected and form the so-called domains. Older experiments, especially those that related the observed fluorescence yield to the fraction of closed traps, suggested that many reaction centers share a common pool of antenna molecules. More recent studies of singlet–singlet and singlet–triplet annihilation in high-intensity laser pulses have yielded information about the domain sizes. A general method for analyzing the results of such experiments will be presented and some representative examples will be discussed.

I. Introduction

Energy transfer is the first process that occurs after light absorption by the photosynthetic pigments (see a basic description, e.g., in Govindjee and Govindjee, 1975). It was first shown to occur on a large scale in photosynthetic systems by Duysens (1952). For a historical overview we

refer to Chapter 1 by Duysens in this volume. The transfer of excitation energy leads to a significant reduction in the number of active sites (reaction centers, RCs) needed to convert the excited state into a charge-separated state (in higher plants, about one per 250–300 chlorophylls). Nevertheless, the overall efficiency of energy transfer in most cases exceeds 90%. In general, excitation transfer takes place from pigments absorbing at shorter wavelengths to those absorbing at longer wavelengths, until the excitation reaches a group of more or less identical pigment molecules having the lowest excited state energy, which funnel the excitation into the reaction center (see, e.g., Seely, 1973).

This chapter will first (Section II) deal with the transfer of excitation energy between two pigment molecules, either different or identical. Examples of the first category are the transfer of excitation energy from carotenoid (Car) molecules to bacteriochlorophyll (BChl), from BChl molecules absorbing at higher energy to those absorbing at lower energy in purple bacteria (e.g., BChl 800 → BChl 850), or from chlorophyll (Chl) *b* to Chl *a* in higher plants and green algae. Examples of the latter category are the transfer among BChl or Chl molecules with identical absorption spectra.

In Section III some examples are given of energy transfer pathways in photosynthetic organisms, followed by a description of the energy transfer in a homogeneous domain, consisting of (more or less) identical pigment molecules, and the trapping of excitations by RCs in such a domain. This description will also deal with some statistical aspects, and exact expressions for the trapping probability will be given.

Finally, in Section IV we shall discuss the processes of singlet–singlet and singlet–triplet annihilation that occur after an intense picosecond or nanosecond laser pulse and compete strongly with the normal decay processes such as trapping and fluorescence.

Several reviews on the process of excitation energy transfer in photosynthetic systems have appeared during the past few decades and these can be consulted for complementary information (Duysens, 1964; Borisov and Godik, 1973; Knox, 1975, 1977; Amesz, 1978; Borisov, 1978; Breton and Geacintov, 1980; Pearlstein, 1982; van Grondelle and Duysens, 1982; van Grondelle, 1985).

II. Mechanisms of Energy Transfer

A. *Rate of Energy Transfer; Exciton Model*

The transfer of excitation energy from a donor D to an acceptor A usually manifests itself by quenching of the fluorescence of D and sensitization of the emission of A by photons absorbed by D.

If excited D (D*) normally fluoresces (rate constant k_f^D) or forms a triplet state via intersystem crossing (rate constant k_{isc}^D) or if its excitation energy is converted into heat by internal conversion (rate constant k_{ic}^D), then the lifetime of its excited state τ_l^D and its fluorescence yield ϕ^D are given by (see Fig. 1):

$$\tau_l^D = \frac{1}{k_l^D} = \frac{1}{k_f^D + k_{isc}^D + k_{ic}^D}; \qquad \phi^D = \frac{k_f^D}{k_l^D} \tag{1}$$

where k_l^D represents the total decay rate of the excited state D*. For Chl *a*, $k_l^D \simeq 2.2 \times 10^8\ s^{-1}$, $k_f^D \simeq 6 \times 10^7\ s^{-1}$, and $\phi^D \simeq 30\%$; for BChl *a*, $k_l^D \simeq (2.5\text{–}3) \times 10^8\ s^{-1}$, $k_f^D \simeq 6 \times 10^7\ s^{-1}$, and $\phi^D \simeq 20\text{–}25\%$ (see, e.g., Bowers and Porter, 1967; Connolly *et al.*, 1982a,b). If irreversible energy transfer takes place to A (rate constant k_{DA}) the fluorescence and triplet yield of D will be quenched. The fluorescence of D* will decay rapidly to zero, and the fluorescence of A will rise with a time constant equal to that of the decay of D*. An example of this is given by the red alga *Rhodella violacea,* where the main emission of phycoerythrin was found to decay with τ = 34 ps, identical to the rise time of phycocyanin emission (Holzwarth *et al.*, 1982). Another example is found in phycobiliproteins from cryptomonads, where a decay time of about 10 ps in cryptovilin was accompanied by a rise of about 10 ps in the phycocyanin fluorescence (Hanzlik *et al.*, 1984).

For the fluorescence yield ϕ^D and the excited state lifetime τ^D of D in the presence of A we find:

$$\tau^D = \frac{1}{k_l^D + k_{DA}}; \qquad \phi^D = \frac{k_f^D}{k_l^D + k_{DA}} \tag{2}$$

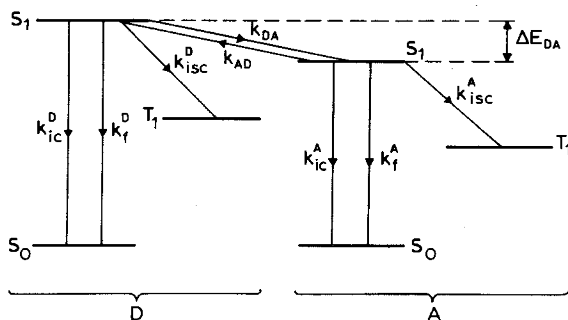


FIG. 1. Diagram illustrating the various decay pathways of the donor D and the acceptor A. The rate constants are explained in the text. *S*, Singlet; *T*, triplet levels.

The transfer process may be taken as truly irreversible only if the energy difference between D* and A*, ΔE_{DA}, is sufficiently large compared to the thermal energy (see Section I,C).

The rate of energy transfer from a donor D to an acceptor A can be calculated from the exciton theory (Kasha, 1963, Förster, 1965; Knox, 1975, 1977; Pearlstein, 1982), which we will describe briefly. In quantum mechanics the ground (S_0) and first excited (S_1) singlet states of D and A are described by normalized wave functions Ψ_A^0, Ψ_D^0, Ψ_A^1, Ψ_D^1, which are the stationary solutions of the time-dependent Schrödinger equation:

$$H_A\Psi_A^0 = E_A^0\Psi_A^0; \qquad H_A\Psi_A^1 = E_A^1\Psi_A^1 \tag{3}$$

in which H_A represents the Hamiltonian of the isolated molecule A. It includes the kinetic energy of the nuclei and electrons and the Coulombic attractions and repulsions between the various nuclei and electrons. Usually the spin of the electrons is also included. A similar set of relations holds for D.

When D and A are interacting molecules, e.g., by Coulomb interaction between the electron clouds of D and A, the Hamiltonian of the total system is given by:

$$H_{DA} = H_D + H_A + V_{DA} \tag{4}$$

where V_{DA} represents the interactions. If one of the two molecules, say D, becomes excited, the excitation will have a finite probability of being found on A after some time. Because Eq. (4) leads to stationary solutions of the total system, the fact that the excitation can occur on both D and A can only be accounted for by taking linear combinations of the locally excited states $\Psi_A^0\Psi_D^1$ and $\Psi_D^0\Psi_A^1$. If, for simplicity, we assume that only two electrons are involved in the transition, the proper antisymmetric initial state wave function (where only D is excited) is given by (Dexter, 1953):

$$\Psi_i = \frac{1}{\sqrt{2}}[\Psi_D^1(1)\Psi_A^0(2) - \Psi_D^1(2)\Psi_A^0(1)] \tag{5a}$$

and for the final state (where only A is excited):

$$\Psi_f = \frac{1}{\sqrt{2}}[\Psi_D^0(1)\Psi_A^1(2) - \Psi_D^0(2)\Psi_A^1(1)] \tag{5b}$$

The number between parentheses after each wave function indicates one of the two electrons involved.

The rate of energy transfer from the initial to the final state is governed by the so-called interaction matrix element, given by:

$$U_{DA} = \langle\Psi_i|V_{DA}|\Psi_f\rangle = U_{DA}^{C} + U_{DA}^{EX} \tag{6}$$

U_{DA} has two terms; the first is the *Coulomb* contribution:

$$U_{DA}^{C} = \langle \Psi_D^1(1)\Psi_A^0(2)|V_{DA}|\Psi_D^0(1)\Psi_A^1(2)\rangle \quad (7)$$

where U_{DA}^{C} represents the Coulomb interaction between the transition charge densities $\Psi_D^1(1)\Psi_A^0(2)$ and $\Psi_D^0(1)\Psi_A^1(2)$. The initially excited electron on D returns to the ground state of D, while simultaneously an electron on A is promoted to one of the excited state orbitals of A. This is schematically shown in Fig. 2.

The second term is the *exchange* contribution:

$$U_{DA}^{EX} = \langle \Psi_D^1(1)\Psi_A^0(2)|V_{DA}|\Psi_D^0(2)\Psi_A^1(1)\rangle \quad (8)$$

In this case the transfer of the excitation is described as the exchange of the excited electron of D with a nonexcited electron of A. The final result is that D has returned to the ground state and A is excited (Fig. 2).

From the expressions for U_{DA}^{C} and U_{DA}^{EX}, their relative contribution to the rate of energy transfer can be estimated. Let us first look at the expression for U_{DA}^{EX} [Eq. (8)]. In principle, this represents the Coulombic interaction between the charge densities $\Psi_D^1(1)\Psi_A^1(1)$ and $\Psi_D^0(2)\Psi_A^0(2)$. The integral in Eq. (8) thus requires the values of Ψ_A^1 and Ψ_A^0 at the positions of electrons 1 and 2, $\vec{r}_1$ and $\vec{r}_2$, respectively. A similar requirement applies to Ψ_D^1 and Ψ_D^0. Because $\vec{r}_1$ and $\vec{r}_2$ represent *different* positions in space, in general the integral in Eq. (8) will be small. In the two-

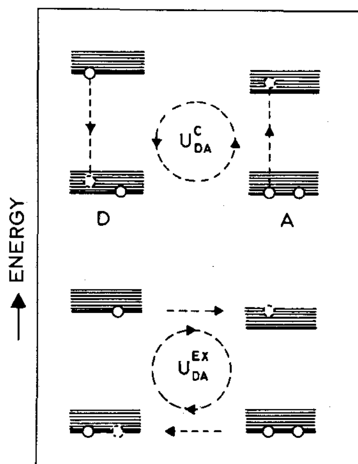


FIG. 2. Schematic representation of the Coulomb (upper) and exchange (lower) contributions to excitation energy transfer.

electron approach V_{DA} is given by (Dexter, 1953; Dörr and Kuhn, 1982):

$$V_{DA} \propto \frac{e^2}{|\vec{r}_1 - \vec{r}_2|} \tag{9}$$

and therefore a significant contribution from U_{DA}^{EX} to the rate of energy transfer is obtained only if D and A are very close.

In contrast, in the expression for U_{DA}^{C} [Eq. (7)] the values of Ψ_A^0 and Ψ_A^1 are required at the *same* point in space, and similarly for Ψ_D^0 and Ψ_D^1. This fact makes the Coulomb contribution to the rate of energy transfer usually the dominant term. This is certainly so if orbital overlap between D and A is small, i.e., if the probability of finding an electron of D in an orbital of A is very low, which occurs if the shortest distance between them exceeds the van der Waals contact distance (~4 Å). Moreover, it follows (see Section II,B) that the Coulomb contribution to the rate of energy transfer can be calculated from the transition dipole strengths of D and A, $\vec{\mu}_D$ and $\vec{\mu}_A$ (e.g., $\vec{\mu}_D = \langle \Psi_D^0 | \vec{\mu} | \Psi_D^1 \rangle$, where $\vec{\mu}$ is the electric dipole operator). Therefore, if optically allowed transitions are involved, and if the donor–acceptor separation is not too small, energy transfer will take place mainly via the Coulomb mechanism.

B. Coulomb Contribution to Excitation Energy Transfer

If optically allowed transitions are involved in the transfer of excitation energy, the interaction between donor D and acceptor A can be approximated by a dipole–dipole interaction and the integral in Eq. (7) is given by (Knox, 1975; Pearlstein, 1982):

$$\begin{aligned} U_{DA}^{C} &= 5.04 \frac{|\vec{\mu}_A| \cdot |\vec{\mu}_D|}{R_{DA}^3} (\cos \alpha - 3 \cos \beta_1 \cos \beta_2) \\ &= 5.04 \frac{|\vec{\mu}_A| \cdot |\vec{\mu}_D|}{R_{DA}^3} \cdot \kappa \end{aligned} \tag{10}$$

where U_{DA}^{C} is given in reciprocal centimeters, the transition dipole strengths $|\vec{\mu}_A|$ and $|\vec{\mu}_D|$ are in debyes (= 10^{-18} esu cm), the dipole separation R_{DA} is in nanometers, κ is an orientation factor in which α is the angle between the two dipoles, and β_1 and β_2 are the angles between each dipole and the vector $\vec{R}_{DA}$ connecting them (Fig. 3).

For BChl *a* typical values of $\vec{\mu}_A$ and $\vec{\mu}_D$ are about 7 debyes and for Chl *a* about 5 debyes (Sauer, 1975; Pearlstein, 1982). If we have a pair of identical interacting BChl *a* molecules at a distance of 1 nm and take $\kappa = 1$, we obtain $U_{DA}^{C} \approx 250\ \text{cm}^{-1}$. In the following we will discuss two extreme cases: (i) strong coupling and (ii) weak coupling.

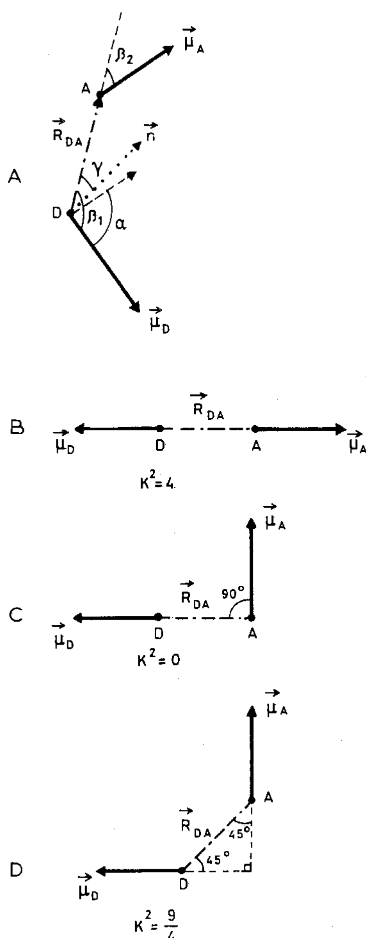


FIG. 3. (A) The donor–acceptor conformation that determines the geometric factor κ in Eq. (10). The angle γ between the normal $\vec{n}$ on the ($\vec{\mu}_D$, $\vec{\mu}_A$) plane and the vector $\vec{R}_{DA}$ connecting D and A is also shown. The lower diagrams illustrate a few simple cases: (B) $\kappa^2 = 4$, (C) $\kappa^2 = 0$, and (D) $\kappa^2 = 9/4$.

1. STRONG COUPLING

Strong coupling occurs if $U_{DA}^{C} \gg \Delta E$, where ΔE is a measure of the bandwidth of the electronic transitions involved (A → A* and/or D → D*) (Kasha, 1963; Förster, 1965). For example, for BChl *a* absorbing at 880 nm, the bandwidth of the Q_y transition at room temperature is about 30 nm, which corresponds to $\Delta E \simeq 400\ \text{cm}^{-1}$.

In the strong coupling case the transfer of excitation energy is a coherent process; i.e., the relation between the phases of the wave functions of the locally excited states $\Psi_D^1\Psi_A^0$ and $\Psi_D^0\Psi_A^1$ is fixed. The excitation oscillates back and forth between D and A and is never more than instantaneously localized. However, starting with the excitation on D, a maximum occurs in the excitation density on A after $t = (4cU_{DA}^C)^{-1}$, where c is the speed of light ($c = 3 \times 10^{10}$ cm s^{-1}), and this defines a quasi-rate constant:

$$k_{DA} = 4cU_{DA}^C \tag{11}$$

We note that $k_{DA} \propto R_{DA}^{-3}$. For the example mentioned above, with $U_{DA} \simeq$ 250 cm^{-1} we find that $k_{DA} \simeq 3 \times 10^{13}$ s^{-1}. It may be clear that for most photosynthetic pigments at ambient temperatures, the width of the electronic transitions does not allow the strong coupling case to occur. Even in the light-harvesting BChl *a* complex of green sulfur bacteria at 4°K the individual absorption bands are still 5 nm (about 100 cm^{-1}) wide (Rijgersberg, 1980).

It has been argued (Knox, 1977) that the time during which an excitation may be considered coherent is actually short ($\lesssim 10^{-13}$ s). This is mainly due to all kinds of dephasing processes (collisions, interaction with intramolecular or with lattice vibrations), which lead to loss of the phase relationship between $\Psi_D^1\Psi_A^0$ and $\Psi_D^0\Psi_A^1$. In that case, after a very short period during which the excitation is delocalized and the strong coupling case holds, the excitation must be considered as localized on either D or A and the transfer process is truly described by the theory developed by Förster (1948, 1949).

2. WEAK COUPLING: THE FÖRSTER EQUATION

In the theory of weak coupling the total rate of energy transfer from D to A is given by (Förster, 1948, 1949, 1965; Dexter, 1953):

$$k_{DA} = k_f^D \left(\frac{R_0}{R_{DA}}\right)^6 \tag{12}$$

in which k_f^D is the rate of fluorescence of the donor D in the absence of A ($k_f^D \simeq 6 \times 10^7$ s^{-1} for BChl *a* and Chl *a*); R_0 is the distance (in nanometers) at which the rate constants for energy transfer to A and for fluorescence are equal, and is given by (Pearlstein, 1982):

$$R_0{}^6 = 8.8 \times 10^{12}\, \kappa^2 n^{-4} \int F_D(\nu)\varepsilon_A(\nu)\nu^{-4}\, d\nu \tag{13}$$

In Eq. (13) $\varepsilon_A(\nu)$ is the molar extinction coefficient, ν is the wave number, $F_D(\nu)$ is the normalized emission spectrum of D ($\int F_D(\nu)\, d\nu = 1$), n is the

refractive index, and the orientation parameter κ is given in Eq. (10). The parameter R_0^6 depends strongly on the overlap of the donor emission spectrum and the acceptor absorption spectrum and is readily calculated from experiments. For example, for Chl *a* $R_0 \simeq 90$ Å ($\kappa^2 = 1, n = 2$) (Knox, 1975; van Grondelle and Duysens, 1982); taking $R_{DA} = 20$ Å, we find for the rate of energy transfer between two Chl *a* molecules: $k_{ChlaChla} \simeq 5 \times 10^{11}\ s^{-1}$. For a number of donor–acceptor combinations of photosynthetic pigments the values of R_0 and k_f are listed in Table I.

Finally, we draw attention to the dependence of R_0^6 on the square of the orientation parameter κ. The maximum of κ^2 is 4; the minimum is 0. Note from Eq. (10) that even with $\vec{\mu} \perp \vec{\mu}_D$, κ^2 is generally not zero. For a random orientation of D and A, κ^2 must be averaged, and we obtain $\overline{\kappa^2} = 2/3$. A few illustrative examples are shown in Fig. 3. Information about κ^2 can be obtained in a number of ways. Measurement of the polarization of the fluorescence of A with respect to the polarized excitation of D yields α (Albrecht, 1961; Breton and Vermeglio, 1982); measurement of the linear dichroism of the chromophores informs us about the angles that $\vec{\mu}_A$ and $\vec{\mu}_D$ make with respect to an axis or a plane of orientation;

TABLE I
Energy Transfer Parameters from a Number of Donor–Acceptor Combinations[a]

Donor	Acceptor	R_0 (Å)	ϕ^D	τ_1^D (ns)	Refs.[b]
Chl *b*	Chl *a*	100	0.12	3.9	a, b, c
Chl *a*	Chl *a*	80–90	0.32	5.1	a, b, c, d
β-Carotene	Chl *a*	≃50	$\lesssim 10^{-5}$	$\lesssim 10^{-3}$	a, e, f, g
BChl *a* 875	BChl 875	90	0.20	3–4	a, h, i
BChl *a* 800	BChl 850	66*[c]	0.20	3–4	j
BChl *a* 800	BChl 800	100*	0.20	3–4	k
Spirilloxanthin	BChl 875	≃75**	$\lesssim 10^{-5}$	$\lesssim 10^{-3}$	a, e, f
Neurosporene	BChl 850	≃50**	$\lesssim 10^{-5}$	$\lesssim 10^{-3}$	a, e, f
PE	PC	60	≃1	≃3	l, m
PC	APC	64	≃0.6	≃2	l, m
APC	Chl *a*	70	0.68	3–4	a, l, m

[a] Förster critical distance R_0, *in vitro* observed donor fluorescence yield ϕ^D, and fluorescence lifetime τ_1^D for a number of photosynthetic donor–acceptor pairs. R_0 values are calculated for $\kappa^2 = 1$. Note that the R_0 values are those for which fluorescence and energy transfer are equally fast. For donor molecules with a low fluorescence yield the actual distance should be considerably smaller for energy transfer to compete effectively with other deactivation processes (Duysens, 1964; Knox, 1975).

[b] (a) Duysens (1952); (b) Bowers and Porter (1967); (c) Connolly *et al.* (1982a); (d) Knox (1975); (e) Dirks *et al.* (1980); (f) Dallinger *et al.* (1981); (g) Song and Moore (1974); (h) Campillo *et al.* (1977); (i) Connolly *et al.* (1982b); (j) van Grondelle *et al.* (1982); (k) Kramer *et al.* (1984); (l) Grabowski and Gantt (1978a); (m) Grabowski and Gantt (1978b).

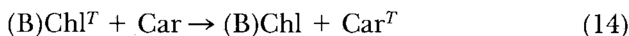
[c] *, R_0 values at 4°K; **, our estimates.

and circular dichroism (Pearlstein, 1982) gives the product sin α cos γ, γ being the angle between $\vec{R}_{DA}$ and the normal to the plane defined by $\vec{\mu}_A$ and $\vec{\mu}_D$ (see Fig. 3A).

C. Exchange Contribution to Excitation Energy Transfer

As noted above, the term U_{DA}^{EX} contributes to excitation transfer only if the Coulomb term is small and the distance is very short. If the energy transfer involves optically forbidden transitions (e.g., singlet to triplet), the Coulomb term is zero, but the exchange term may exist and provide the only mechanism for energy transfer.

An illustrative example is the transfer of the (B)Chl triplet state to a closely associated Car molecule



$$(B)Chl^T + Car \rightarrow (B)Chl + Car^T \tag{14}$$

This transfer process takes about 20 ns in various photosynthetic systems (Mathis, 1969; Breton and Mathis, 1970; Monger *et al.*, 1976; Schenck *et al.*, 1984), and although its rate is slow, the long lifetime of the $BChl^T$ or Chl^T state (at least a few microseconds; Monger *et al.*, 1976; Breton *et al.*, 1979) guarantees a high efficiency. We remark here that this process is quite important to avoid photooxidative damage (see Hoff, Chapter 9, this volume).

The total rate constant for energy transfer according to the exchange mechanism is given by (Dexter, 1953; Razi Naqvi, 1980):

$$k_{DA}^{EX} = \left(\frac{2\pi}{\hbar}\right)^2 \left(U_{DA}^{EX}\right)^2 \int F_D(\nu)\varepsilon_A(\nu)\,d\nu \tag{15}$$

where $\varepsilon_A(\nu)$ is the spectral distribution of the acceptor absorption and $F_D(\nu)$ is the spectral distribution of the donor emission; both functions are normalized to unity on a frequency scale. U_{DA}^{EX} is given by Eq. (8) and $\hbar = h/2\pi$, where h is Planck's constant. U_{DA}^{EX} decreases approximately exponentially with R_{DA}, depends probably on the orientation of D and A, but is independent of the dipole strength of the transitions in D and A. For a donor–acceptor distance of about 4 Å, Dexter (1953) estimated exchange energy transfer to be 100 times less efficient than Förster transfer, assuming both to be allowed (10^{11} s^{-1} versus 10^{13} s^{-1}).

It has been argued that the transfer of singlet excitations from Car to (B)Chl does not occur via the Förster mechanism, due to the extremely low yield of the carotenoid fluorescence, which suggests very rapid internal conversion ($k_{ic} \simeq 10^{12}$ s^{-1}) (Song and Moore, 1974; Razi Naqvi, 1980; Dallinger *et al.*, 1981). Mainly because triplet energy transfer from (B)Chl to Car is known to occur and because the lowest excited state of Car is optically forbidden (Thrash *et al.*, 1979), the exchange mechanism

has been invoked for the transfer of singlet excitations as well. This requires the (B)Chl and Car molecules to be very close.

D. Reversible versus Irreversible Energy Transfer

If the temperature is high enough or the energy difference ΔE_{DA} (see Fig. 1) small enough, back transfer of the excitation from A to D becomes possible. When D and A are both in a thermally relaxed state before transfer occurs, either from D to A or vice versa—we note that this is a prerequisite for the validity of the Förster equation—the ratio of the rate of transfer from D to A, k_{DA}, and the rate of transfer from A to D, k_{AD}, is given by:

$$k_{AD}/k_{DA} = e^{-\Delta E_{DA}/k_B T} \tag{16}$$

where k_B is Boltzmann's constant and T the absolute temperature. If ΔE_{DA} is not known, the ratio k_{DA}/k_{AD} can be calculated from the ratio of the appropriate overlap integrals [see Eqs. (12) and (13)].

If there are N_D donor molecules and N_A acceptor molecules, and if the transfer between the two groups of pigment molecules is sufficiently fast compared to the losses, the excitation density in D and A reaches a steady state during the excitation lifetime and the ratio of the number of excited molecules D (N_D^*) to excited molecules A (N_A^*) is given by:

$$N_D^*/N_A^* = (N_D/N_A)e^{-\Delta E_{DA}/k_B T} \tag{17}$$

It is assumed here that $N_D^* \ll N_D$, $N_A^* \ll N_A$. From this follows the ratio of the fluorescence intensities of D and A:

$$\phi^D/\phi^A = (k_f^D/k_f^A)(N_D^*/N_A^*) \tag{18}$$

Such a relation was indeed observed for the ratio of the two main emission bands at 817 and 828 nm of the light-harvesting BChl *a* complex of green sulfur bacteria (Rijgersberg, 1980). The ratio ϕ^D/ϕ^A does not inform us about the value of k_{DA}, although from the observation of the validity of Eq. (18) in a given system a lower limit for k_{DA} can be calculated.

Apart from a rapid initial decay of D*, which occurs on selective flash excitation of D and represents the rate constant k_{DA}, the fluorescence from D or A shows an equally rapid decay in time. An analogous situation occurs on excitation of A. If ΔE_{DA} is small, the fast initial phases may easily escape detection. This may explain why Holzwarth *et al.* (1982) observed identical decay times for different chromophores (Jung *et al.*, 1980) of a cryptomonad phycobiliprotein.

In conclusion, fluorescence yield measurements allow only an estimate of the rate of energy transfer k_{DA} if the transfer process can be consid-

ered irreversible. As an example we will describe in the next section some of the fluorescence properties as a function of the temperature in the B800–850 light-harvesting complex of the purple bacterium *Rhodopseudomonas* (*Rps.*) *sphaeroides*.

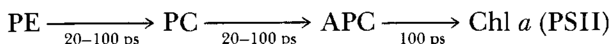
III. Energy Transfer Pathways in Photosynthetic Systems

A. Antenna Systems

A comprehensive discussion of the various pathways, rates, and efficiencies of energy transfer in photosynthetic systems is beyond the scope of this chapter. Only a brief survey of some of the more important systems will be presented.

An important part of the antenna of blue-green algae (also called cyanobacteria) and red algae is contained in the phycobilisomes (see Fork and Mohanty, Chapter 16, this volume). In order of increasing energy of excitation, they contain the phycobiliproteins allophycocyanin (APC), phycocyanin (PC), and in many species, including almost all red algae, phycoerythrin (PE). Phycobilisomes consist of an APC core to which rodlike structures are attached that contain PC and, at the periphery, PE. They may contain several hundreds of these pigments, which occur in a number of spectral forms (Gantt, 1981; Scheer, 1982; Glazer, 1983, 1984).

Measurements of action spectra of the Chl *a* fluorescence have shown that the energy absorbed by the phycobiliproteins is used with an efficiency of more than 90% in photosynthesis (Duysens, 1952). Most of this energy is transferred to photosystem II (PSII) (Cho and Govindjee, 1970b; Grabowski and Gantt, 1978a,b; Kramer, 1984) according to the following scheme:



$$\text{PE} \xrightarrow[\text{20–100 ps}]{} \text{PC} \xrightarrow[\text{20–100 ps}]{} \text{APC} \xrightarrow[\text{100 ps}]{} \text{Chl } a \text{ (PSII)}$$

The transfer times are approximate ones; they were mainly derived from measurements of the fluorescence decay at various wavelengths after a short flash (Porter *et al.*, 1978; Pellegrino *et al.*, 1981; Holzwarth *et al.*, 1982; Hefferle *et al.*, 1983a,b; Wendler *et al.*, 1984; Suter *et al.*, 1984). Recently, time-resolved fluorescence spectra obtained for various species have provided a visible demonstration of these energy transfer steps (Mimuro *et al.*, 1984; Yamazaki *et al.*, 1984).

The absorption and fluorescence properties of smaller phycobiliprotein complexes that contain only a limited number of pigment molecules have led to the concept of "sensitizing" (s) chromophores that rapidly transfer excitation energy to "fluorescing" (f) chromophores of the same

type. The latter are at somewhat lower energy (Teale and Dale, 1970; Jung *et al.*, 1980; Zickendraht-Wendelstadt *et al.*, 1980; Glazer, 1983, 1984). Time-resolved fluorescence and absorption experiments indicate s-to-f transfer times of about 10–30 ps (Kobayashi *et al.*, 1979; Gillbro *et al.*, 1983; Hefferle *et al.*, 1983b; Holzwarth *et al.*, 1983, 1984; Suter *et al.*, 1984; Wendler *et al.*, 1984). From the efficiency of singlet–singlet annihilation (see Section IV), Dagen *et al.* (1984) estimated the transfer time from s to f in α and β subunits of PE to be about 16 ps. It was also shown recently that in some cases, due to energy transfer, a maximum depolarization of the emission or absorption can be observed within a few tens of picoseconds (Gillbro *et al.*, 1983; Hefferle *et al.*, 1983b; Wendler *et al.*, 1984).

At room temperature, the emission spectra of algae and higher plants usually show a broad maximum at 685 nm. The shape of these spectra probably reflects a thermal equilibrium between the various spectral forms of chlorophyll in the two photosystems. On cooling to 77°K or lower, a characteristic emission pattern appears with major peaks at 685, 695, and 720 or 735 nm (for reviews see Amesz and Rijgersberg, 1981; Bose, 1982). These bands are often called F685, F695, and F720 or F735, respectively; F735 is observed in higher plant chloroplasts and F720 in many algae. A number of minor bands can also be distinguished. There is substantial evidence that F685 and F695 are emitted by PSII and F720 and F735 by PSI (Govindjee and Yang, 1966; Murata *et al.*, 1966; Cho and Govindjee, 1970a; Rijgersberg and Amesz, 1980; Kramer *et al.*, 1981).

Since the fluorescence yield of some of these bands is several times higher at low temperature than at room temperature, fluorescence excitation spectra at low temperature provide a sensitive way to probe energy transfer within the pigment systems and to distinguish between the pigments associated with PSI and II (Cho and Govindjee, 1970a,b; Kramer *et al.*, 1981). Such excitation spectra can even be obtained from highly scattering preparations (Kramer, 1984). Figure 4 shows the excitation spectra for F695 and F735 of spinach chloroplasts measured at 4°K (Kramer *et al.*, 1981). Comparison of the absorption and excitation spectra shows that even at 4°K the efficiencies of energy transfer within the two photosystems are close to 100%.

A relatively simple antenna is that of the purple bacteria. The light-harvesting complexes of *Rps. sphaeroides* and related species have been extensively investigated. Two different light-harvesting complexes have been isolated: B800–850 and B875. The first one is characterized by BChl *a* absorption bands at 800 and 850 nm, the second one by a band near 875 nm (see Amesz and Vasmel, Chapter 15, this volume).

Fluorescence of BChl 800 and BChl 850 can be observed at all temper-

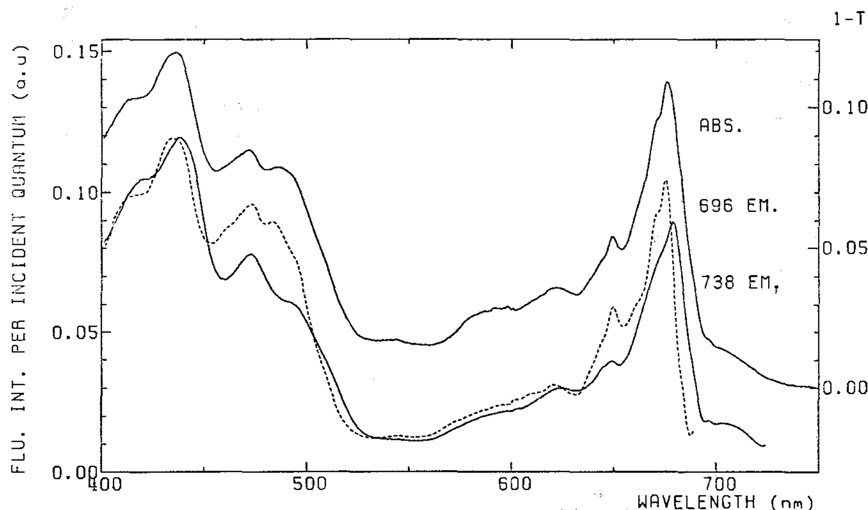


FIG. 4. Excitation spectra (4°K) of fluorescence emitted at 696 nm (F695; dotted line) and at 738 nm (F735) by spinach chloroplasts. ABS, Absorption spectrum. Right ordinate, 1 − transmission; left ordinate, fluorescence intensity in arbitrary units. (From Kramer *et al.*, 1981.)

atures. Between 200 and 300°K the fluorescence yield of BChl 800 relative to that of BChl 850 can be described by Eqs. (17) and (18), indicating a thermal equilibrium between the excitation densities on BChl 800 and BChl 850. This allows an estimate of a lower limit for the rate of energy transfer from BChl 800 to BChl 850 at 300°K of 5×10^{11} s^{-1} (van Grondelle *et al.*, 1982). Below 100°K no BChl 800 emission is observed on BChl 850 excitation. Application of Eq. (1) leads to a direct estimate of the rate of energy transfer from the observed fluorescence yield at 4°K: $k_{800-850} \simeq 3 \times 10^{11}$ s^{-1}. The Car composition of B800–850 is heterogeneous. Two thirds of the Car selectively transfers excitation energy to BChl 850, one third to BChl 800, as indicated by the excitation spectra (van Grondelle *et al.*, 1982) and by measurements of the fluorescence polarization (Kramer *et al.*, 1984). Energy transfer from Car to BChl *a* and from BChl 800 to BChl 850 occurs with almost 100% efficiency and appears to be independent of the Car composition and the temperature (Cogdell *et al.*, 1981; van Grondelle *et al.*, 1982). The mechanism involved in Car-to-BChl *a* transfer may be either dipole–dipole resonance or exchange energy transfer in view of the short distances involved (Razi Naqvi, 1980). To stabilize the initially excited Car molecule, optically forbidden excited states of lower energy (Thrash *et al.*, 1979), specific Car interactions as reflected by an intense Car circular dichroism (Song,

1978; Bolt *et al.*, 1981; Kramer *et al.*, 1984), or even triplet pair states (Rademaker *et al.*, 1980; McGann and Frank, 1983; Kingma *et al.*, 1983, 1984, 1985) may be important.

Measurements of linear dichroism, circular dichroism, fluorescence polarization, and excitation spectra (Bolt and Sauer, 1981; Breton *et al.*, 1981; Kramer *et al.*, 1984) have led to a fairly detailed model for the pigment organization in the B800–850 complex, with a basic unit that contains at least six BChl and three Car molecules (Fig. 5). The BChl 850 molecules are thought to be bound to the central histidine part of the

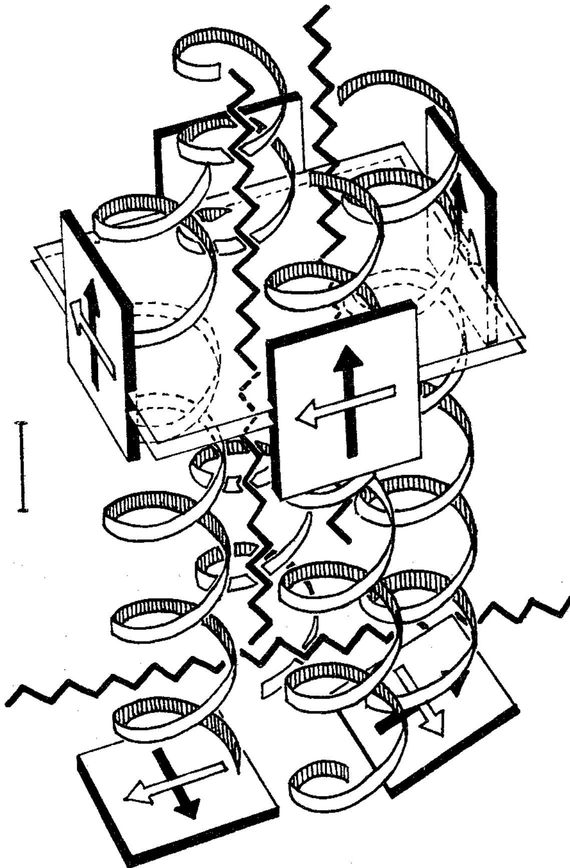


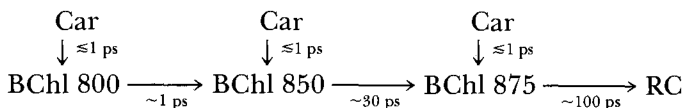
FIG. 5. Schematic model of the B800–850 complex. The upper square boxes are the porphyrin rings of BChl 850 and the lower ones are those of BChl 800. Open arrows, Q_y transitions; solid arrows, Q_x transition moments. Zigzag lines represent carotenoids (spheroidene) and spirals represent the α-helices of the constituent peptides. The plane of the membrane is horizontal; the vertical bar represents 5 Å. (From Kramer *et al.*, 1984.)

central hydrophobic stretch of amino acids (Tadros *et al.*, 1983; Cogdell and Valentine, 1983; Theiler *et al.*, 1984) of the apoproteins of B800–850. These stretches are thought to constitute transmembrane α-helical sections in bacterial antenna complexes. The BChl 850 Q_y transition dipoles are approximately parallel and the Q_x transitions perpendicular to the membrane plane. The Q_y transitions of BChl 850 are arranged in such a way that they form a circularly degenerate oscillator (Breton *et al.*, 1981).

The BChl 800 molecules are probably at the interface between the hydrophobic and the polar regions. The distance to BChl 850 would then be 20–25 Å, which agrees with the measured rate of energy transfer from BChl 800 to BChl 850 (van Grondelle *et al.*, 1982). The Q_y as well as the Q_x transitions of the two BChl 800 molecules are mutually perpendicular, but both are roughly parallel to the membrane plane. The BChl 800–BChl 800 dipole–dipole distance is at most 19 Å, and extensive energy transfer between at least two BChl 800's occurs before transfer to BChl 850 takes place (Kramer *et al.*, 1984; Kramer, 1984).

In the intact system efficient energy transfer occurs from B800–850 to the B875 complex. The B875 complexes surround and interconnect the RCs according to the "lake" or "matrix" model (Monger and Parson, 1977). Together they form a domain that may contain 10–20 RCs and a large number of BChl 875 molecules (see Section IV,C), probably arranged in a two-dimensional regular pattern. After its arrival in B875, the excitation energy stays localized mainly in the B875 domain until it is trapped by the RC or lost by fluorescence, triplet formation, or internal conversion.

Estimated transfer times for the various transfer steps are shown in the scheme given below:



B. Random Walk Model for Energy Transfer and Trapping

In the past, models for the transfer of excitation energy among more or less identical pigment molecules have been obtained by computer simulation (Robinson, 1967; Knox, 1968, 1977; Shipman, 1980), a master equation approach (Knox, 1977; Pearlstein, 1982), and a random walk description (Montroll, 1969; den Hollander and Kasteleyn, 1982). In the model to be discussed below the long-wavelength antenna pig-

ments are assumed to be organized in *domains*, each of which may contain several reaction centers. Within a domain no barriers for excitation transfer exist, but excitation transfer between different domains is assumed to be absent.

Energy transfer in the domain is assumed to be an incoherent "hopping" of the excitation (exciton) among more or less identical pigment molecules, and therefore a random walk description may be applied. Because the random walk description is not very sensitive to the actual choice of the lattice or to the formation of small clusters of antenna pigments (Knox, 1977), a square lattice is chosen to represent the domain. Each domain contains λ RCs that are taken to be regularly distributed over the domain. The number of antenna molecules per reaction center is N and energy transfer takes place to the nearest neighbors only. The RC occupies only a single lattice point, and the rates of excitation transfer to and from the RC are assumed to be the same as those among the long-wavelength antenna pigments. Although it was recently shown that B875 is spectrally inhomogeneous and contains a small proportion of BChl *a* absorbing at longer wavelengths (Borisov *et al.*, 1982; Kramer, 1984), the simple model is probably valid for purple bacteria at room temperature, since in that case the energy differences are small compared to the thermal energy. The same applies to plant PSI and PSII (Knox, 1977; Shipman, 1980).

The transfer and trapping process in a photosynthetic system, according to this model, is governed by two parameters: the probability that the excitation is lost on a single energy transfer step and the probability that an excitation after reaching a trap does escape and resumes its random walk. These probabilities depend on a set of physical rate constants: k_h, k_l, and k_t, where k_h represents the hopping rate, i.e., the rate of transfer between two neighboring antenna molecules [see Eq. (12)], k_l the rate of loss on an antenna [see Eq. (1)], and k_t the rate of trapping by the reaction center. It has been shown that a rigorous mathematical description of this model is possible (den Hollander and Kasteleyn, 1982; den Hollander *et al.*, 1983). We shall discuss only the following simple case.

For a photosynthetic system in which the losses are not too large, i.e., $Nk_l \ll k_h$, the trapping probability f_t is given by the following simple expression (den Hollander *et al.*, 1983):

$$f_t \simeq \left[\frac{Nk_l}{4k_h}\left(\frac{1}{\pi}\ln N + 0.195 + \frac{4k_h}{k_t}\right) + 1\right]^{-1} \tag{19}$$

Two limiting examples of Eq. (19) can be distinguished. The first arises if no escape from the RC is possible, i.e., if trapping is perfect. In that

case $k_t \gg k_h$ and we find:

$$f_t \simeq \left[\frac{1}{\pi}\left(\frac{k_l}{4k_h}\right) N \ln N + 1\right]^{-1} \tag{20}$$

This is the diffusion-limited case. The fluorescence yield is governed by the average number of steps it takes for an excitation to reach the trap and the probability of loss per step. The second extreme case arises if $k_t \ll k_h$ and N is not too large. Then:

$$f_t \simeq \frac{k_t/N}{k_l + k_t/N} \tag{21}$$

which is the expression proposed by Duysens (1979). This is the trap-limited case. The excitation will visit the RC many times before being trapped, and the probability of finding the excitation on the RC is simply N times smaller than the probability of finding it on one of the antenna molecules. Note that k_h no longer occurs in the expression for the trapping probability.

It should be noted here that with a reasonable set of parameters the simple model predicts an approximately exponential decay of the fluorescence (Pearlstein, 1984). It is therefore useful to introduce an overall rate of decay, k_1, which describes the decay of the excitations due to losses and trapping and is given by:

$$k_1 = \frac{k_l}{1 - f_t} \tag{22}$$

where f_t follows from Eq. (19). In principle, k_1 is found directly from the observed quantum efficiency of trapping and the rate of excitation decay in the absence of trapping k_l [$k_l \simeq (3\text{–}5) \times 10^8\ \text{s}^{-1}$ (Connolly *et al.*, 1982b)]. Equation (22) is a consequence of the fact that the sum of all decay probabilities must be equal to one.

Let us give one simple example. For most photosynthetic purple bacteria $N = 50$ and $R_0 = 90$ Å ($\kappa^2 = 1, n = 1$). With $R = 18$ Å (Pearlstein, 1982) and $k_f = 6 \times 10^7\ \text{s}^{-1}$ (Zankel *et al.*, 1968) we find $k_h \simeq 10^{12}\ \text{s}^{-1}$. Taking $k_t = 3 \times 10^{11}\ \text{s}^{-1}$, corresponding to the rate of charge separation in isolated RCs (Parson and Ke, 1982), and $k_l \simeq 5 \times 10^8\ \text{s}^{-1}$, we calculate from Eq. (19): $f_t \simeq 0.92$ and $k_1 = 6 \times 10^9\ \text{s}^{-1}$. The latter corresponds to an overall fluorescence decay time of 160 ps. The experimentally observed values are: $f_t = 0.94$ in *Rhodospirillum (R.) rubrum* (Kingma, 1983) and $k_1 = 2 \times 10^{10}$ to $5 \times 10^9\ \text{s}^{-1}$ for a number of purple bacteria (see Amesz and Vasmel, Chapter 15, this volume).

In practice, fluorescence decay times that are either nonexponential or can be described by a sum of exponentials have been reported for several systems (Campillo *et al.*, 1977; Haehnel *et al.*, 1982; Nairn *et al.*, 1982; Gulotty *et al.*, 1982; Sebban and Moya, 1983; Karukstis and Sauer, 1983a; see Moya *et al.*, Chapter 7, this volume, and Karukstis and Sauer, 1983b, for a review). Three sources of nonexponential decay may be mentioned that are not included in the simple model. The first occurs if a significant fraction of the excitation starts in a peripheral antenna and only slowly reaches the long-wavelength antenna. Such a mechanism has been proposed to account for a strong 500-ps decay component in PSII of green plants (Nairn *et al.*, 1982; Haehnel *et al.*, 1983).

The second occurs if the charge separation leads to a charge-separated state that is energetically not much lower than the excited state. After trapping, the equilibrium distribution will give rise to a significant population of the excited state and the emission decay will show a second phase that is a weighted sum of the loss rate in the antenna and the decay rate of the intermediate state (Godik and Borisov, 1980; van Bochove *et al.*, 1981; Nairn *et al.*, 1982). A simple calculation suggests that the 500-ps decay component observed in PSII may in part be attributed to such an effect. Equation (19) can be adapted by taking a larger value for the escape probability to account for the escape after recombination of the intermediate state (van Grondelle, 1985).

The third cause may be related to the proposed structural heterogeneity of PSII, which may consist of so-called α centers, which are connected via a common antenna, and β centers, which are isolated. The two centers can be distinguished spectroscopically, e.g., by their characteristic fluorescence behavior (Melis and Homann, 1978; Melis and Duysens, 1979; see van Gorkom, Chapter 10, this volume). It was recently suggested that the multiphasic fluorescence decay may be ascribed to a part arising from α centers (fast and slow phases) and a part from β centers (middle phase) (Butler *et al.*, 1983).

C. *Relation between the Fraction of Closed Traps and the Fluorescence Yield*

On capture of an excitation, a reaction center will change its photochemical state from active (or open) to inactive (or closed). Vredenberg and Duysens (1963) were the first to observe that, if the RCs of purple bacteria become closed during illumination, the fluorescence yield increases according to the simple equation:

$$\phi = \frac{\phi_0}{1 - p\bar{x}} \tag{23}$$

In Eq. (23), ϕ_0 is the fluorescence yield with all the traps open, $\bar{x}$ is the total fraction of closed traps, and p is a parameter that reflects the difference in trapping between open and closed traps and depends on the model under consideration (Vredenberg and Duysens, 1963; Knox, 1975; Duysens, 1979; den Hollander *et al.*, 1983). This relation has been observed in chromatophores or whole cells of purple bacteria with their RCs either in the state $P^+Q_A^{(-)}$ or in the state PQ_A^- (Godik and Borisov, 1977; Kingma *et al.*, 1983), where P denotes the primary electron donor and Q_A the first quinone acceptor, and in chloroplasts of higher plants or algae with the PSII RCs in the state PQ_A^- (Joliot and Joliot, 1964; van Gorkom *et al.*, 1978). A hyperbolic relation was also observed with artificial quenchers (Rijgersberg and Amesz, 1980; Sonneveld *et al.*, 1980) and on the formation of quenching Car triplet states in the antennas of purple bacteria (Monger and Parson, 1977) and higher plants or algae (Breton *et al.*, 1979; Sonneveld *et al.*, 1980). For all these cases different values of p were observed because the quenching efficiency was different on each occasion.

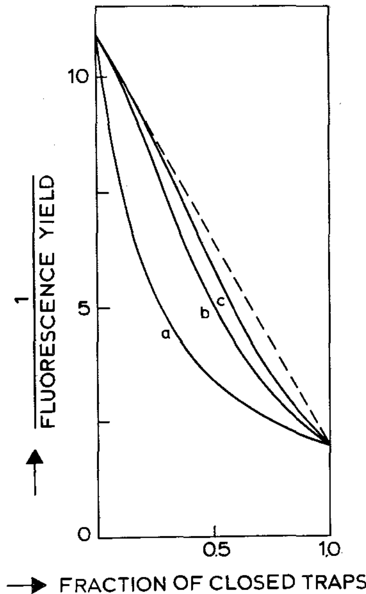


FIG. 6. Plot of the reciprocal of the fluorescence yield as a function of the fraction of closed traps for various values of the number of reaction centers per domain, λ: (a) $\lambda = 1$, (b) $\lambda = 4$, and (c) $\lambda = 10$. The dashed line is the asymptote for $\lambda \rightarrow \infty$ and corresponds to Eq. (23). (Adapted from den Hollander *et al.*, 1983.)

It was noted by Vredenberg and Duysens (1963) that Eq. (23) implies that many RCs share a common pool of antenna molecules, and this was later called the lake or matrix model (Robinson, 1967; Duysens, 1979): if an excitation encounters a closed RC it has a finite probability of diffusing away and being trapped in another, still open, RC.

When the domain contains many traps ($\lambda \gtrsim 10$) the lake model applies. If the domain contains only one trap ($\lambda = 1$), which can be either open or closed, it is easily seen that a linear relation exists between the fluorescence yield and the total fraction of closed traps (the "puddle" or "separate unit" model). For intermediate cases, say $1 < \lambda < 10$, a complicated averaging procedure must be followed to account for the statistical fluctuations occurring in each domain. For a more extensive discussion and the resulting expressions for the fluorescence yield we refer to Paillotin *et al.* (1983) and den Hollander *et al.* (1983). The relations that are obtained in this way for some values of λ are plotted in Fig. 6.

IV. Excitation Annihilation

A. Introduction

Upon the introduction of intense short laser pulses in the study of photosynthetic systems, it gradually became clear that anomalously low yields and short lifetimes were observed in many cases (Mauzerall, 1976; Swenberg *et al.*, 1976). Moreover, at high excitation energies the fluorescence decay appeared strongly multiphasic (for reviews, see Campillo and Shapiro, 1978; Breton and Geacintov, 1980). Two processes are considered to be responsible for these phenomena.

The first process is singlet–singlet annihilation. It occurs if two or more singlet excitations are simultaneously present in one domain, and it is induced by a dipolar interaction between two nearby excited molecules due to which one of the excited molecules returns to the ground state while the other is promoted to a higher excited singlet state. This higher excited state reverts again within 10^{-13} s to the lowest excited singlet state via rapid internal conversion. Thus the net result of this process is the loss of one excitation. The process is described by the following equation:

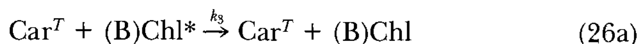
$$\text{(B)Chl}^* + \text{(B)Chl}^* \xrightarrow{k_2} \text{(B)Chl}^* + \text{(B)Chl} \qquad (24)$$

In principle, other products of this reaction should be considered as well, e.g., the loss of both excitations or the formation of one or more triplet states (Swenberg and Geacintov, 1973). The rate constant k_2 in

Eq. (24) is the overall biexcitation decay rate per pair of excitations. It includes the hopping of both excitations in the domain and the actual probability of annihilation once the collision occurs. In fact, the total probability of annihilation, f_a, is found from an expression analogous to Eq. (19) with N replaced by the number of antenna molecules in a domain, N_D. In addition, instead of the probability of escaping from a trap, the probability that the excitons escape on collision must be used (den Hollander *et al.*, 1983). The rate constant k_2 then follows from f_a via:

$$k_2 = 2k_1 \frac{f_a}{1 - f_a} \tag{25}$$

The second process is singlet–triplet annihilation. It is very similar to singlet–singlet annihilation, but now one of the excited states is in an excited triplet state (T). Two classes of triplet states should be considered, as indicated by the following equations:



$$\mathrm{Car}^T + \mathrm{(B)Chl}^* \xrightarrow{k_3} \mathrm{Car}^T + \mathrm{(B)Chl} \tag{26a}$$

$$\mathrm{(B)Chl}^T + \mathrm{(B)Chl}^* \rightarrow \mathrm{(B)Chl}^T + \mathrm{(B)Chl} \tag{26b}$$

The rate constant k_3 is the overall biexcitation decay rate for singlet–triplet annihilation. Because triplet states are relatively immobile compared to singlet states (Swenberg and Geacintov, 1973), k_3 is dominated by the rate of energy transfer for the singlet excitation and the actual probability of annihilation on collision.

In most cases (B)Chl^T states are formed via intersystem crossing from (B)Chl*. This process has a low yield, typically of the order of 10% in chloroplasts and purple bacteria with all the RCs closed. However, once formed, the triplet has a much longer lifetime than the excited singlet state, and therefore in long flashes or flash trains appreciable concentrations may be formed (Breton and Geacintov, 1977). The Car triplet state (Car^T) is obtained via triplet–triplet transfer from (B)Chl^T or, alternatively, via singlet fission of the excited Car (Rademaker *et al.*, 1980; McGann and Frank, 1983; Kingma *et al.*, 1983, 1984, 1985).

B. Singlet–Singlet Annihilation in Relation to the Size of the Domain

It was recognized by Paillotin and colleagues (1979, 1983; Breton and Geacintov, 1980) that excitation annihilation phenomena could be used to extract information about the size of the domain, in addition to the diffusion properties of the excitation. This is due to the fact that the rate

of excitation annihilation depends linearly on the number of pairs of excitations in the domain.

For the case that the RCs are all closed (or altogether absent), Paillotin *et al.* (1979) derived the following expression for the fluorescence yield $\phi(z)$ as a function of the average number of excitations per domain, z, generated by picosecond flash:

$$\phi(z) = \phi_{\max}\left[1 + \sum_{k=1}^{\infty} \frac{1}{k+1} \frac{(-z)^k}{(r+1)\cdots(r+k)}\right] \quad (27)$$

where $\phi_{\max}$ is the fluorescence yield at "low" light intensity (with closed RCs) and r is a parameter that determines the shape of the fluorescence yield curve as a function of pulse intensity [$r = 2k_1/k_2$; see Eqs. (22) and (25)]. From the shape of this curve the parameter r is estimated and thus the relation between the pulse intensity I and z is obtained. From this the number of connected antenna molecules in a domain, N_D, is calculated directly.

Two limiting cases of Eq. (27) are frequently encountered in the literature. The first occurs if $k_2 \gg k_1$. Then $r \rightarrow 0$ and the total fluorescence yield $\phi(z)$ as a function of the pulse intensity is given by (Mauzerall, 1976; Paillotin *et al.*, 1979):

$$\phi(z) = \frac{\phi_{\max}}{z}(1 - e^{-z}) \quad (28)$$

Physically, Eq. (28) implies that annihilation is perfect: if two or more excitations occur simultaneously in a domain, all except one will be annihilated. Equation (28) will be found if the domain is small, if the rate of energy transfer is high, and if the probability of annihilation on collision is large. The size of the domain is found directly by reading the point on the fluorescence vs. pulse intensity curve where, on the average, one excitation per domain is created [$\phi(1)/\phi_{\max} = 0.63$].

The second case arises if $k_2 \ll k_1$; then $r \gg 1$ and we find for $\phi(z)$:

$$\phi(z) = \phi_{\max}\frac{r}{z}\ln\left(1 + \frac{z}{r}\right) \quad (29)$$

Originally Eq. (29) was derived from a continuum kinetic model (Swenberg *et al.*, 1976), with the parameters k_2V or k_2S, where V and S represent the volume and the surface of the domain, respectively. These are related to the parameters that occur in Eq. (29) via the relation:

$$\frac{z}{r} = \left(\frac{z}{2k_1}\right) k_2 = \left(\frac{z}{S}\right)(k_2S)\frac{1}{2k_1} = \left(\frac{z}{V}\right)(k_2V)\frac{1}{2k_1} \quad (30)$$

in which z/S and z/V have the dimensions of photons/cm^2 and photons/cm^3, respectively and k_2S and k_2V the dimensions of surface and volume diffusion coefficients, respectively.

Physically, Eq. (29) implies that the probability of annihilation for a pair of excitations is much smaller than the probability of being lost or trapped. Equation (29) may be expected to be valid if the domain is large, if the rate of energy transfer is low, or if the probability of annihilation on collision is small.

If Eq. (28) is found experimentally the domain size is determined exactly, but only a lower limit for the excitation transfer rate can be calculated. If, on the contrary, Eq. (29) is observed only a lower limit for N_D can be extracted, but a precise number for the excitation diffusion rate is obtained.

C. Experimental Results

1. PHOTOSYSTEMS I AND II

Excitation annihilation in isolated chloroplasts and intact algae has been studied extensively. At all temperatures the shape of the fluorescence yield vs. pulse intensity curve with all PSII RCs in the state PQ_A^- can be fitted with Eq. (29), suggesting a matrix model for energy transfer. Using Eq. (27) to describe the observed fluorescence quenching curves, Paillotin *et al.* (1979) concluded that at room temperature $r \gtrsim 5$ and $\lambda \gtrsim 2$, while $\lambda \gtrsim 4$ below 200°K. An estimate was obtained for the rate of annihilation: $k_2 \lesssim 2 \times 10^8$ s^{-1}, which would correspond to a rate of energy transfer $k_h \simeq 10^{11}$ s^{-1}. At low temperature (77°K) the amount of fluorescence quenching measured at either 735 nm (PSI) or 685 nm (PSII) was found to be the same. Geacintov *et al.* (1977) explained this unexpected result by assuming that the majority of excitations are absorbed by a combined antenna PSII core complex, in which annihilation takes place before either 685-nm fluorescence or transfer to PSI has occurred. However, this scheme appears to conflict with the fact that clearly different excitation spectra have been obtained for PSI and PSII fluorescence (see Fig. 4), suggesting that only a limited fraction of the excitations is shared by the two photosystems (Kramer *et al.*, 1981).

2. PHYCOBILISOMES

In isolated phycobiliproteins of various blue-green and red algae (PE, PC, or APC trimers and hexamers), significant quenching of the fluorescence yield and shortening of the fluorescence lifetime can be observed at high laser pulse energies, probably due to singlet–singlet annihilation

(Doukas *et al.*, 1981; Wong *et al.*, 1981; Hanzlik *et al.*, 1984). For a small subunit of PE containing only four chromophores no excitation annihilation was observed (Dagen *et al.*, 1984).

In intact phycobilisomes the excitations absorbed by PE and PC are rapidly transferred to APC (see Section III,A). Twelve APC trimers are connected in the intact phycobilisome, thus increasing the number of interacting excitations dramatically. Both factors result in a strongly enhanced (approximately 100-fold) exciton annihilation in APC (Searle *et al.*, 1978; Pellegrino *et al.*, 1981).

3. PURPLE BACTERIA

Figure 7 shows that measurement of the fluorescence intensity as a function of the intensity of a picosecond pulse is a sensitive technique for obtaining the number of connected pigment molecules for the B875 complex and for B800–850 prepared with different detergents (van Grondelle *et al.*, 1983). The fluorescence yield vs. pulse intensity curve for B800–850 prepared with LDAO could be fitted with Eq. (29), in agreement with the supposed large size of the complex (Clayton and Clayton, 1972). The results indicated that more than 300 BChl *a* molecules are connected in this complex and that rapid energy transfer occurs among them ($k_h \simeq 10^{12}\ s^{-1}$). However, when the B800–850 complex is prepared with LDS, the fluorescence quenching sets in at a much higher pulse intensity, indicating a relatively small complex containing only about 30 BChl *a* molecules.

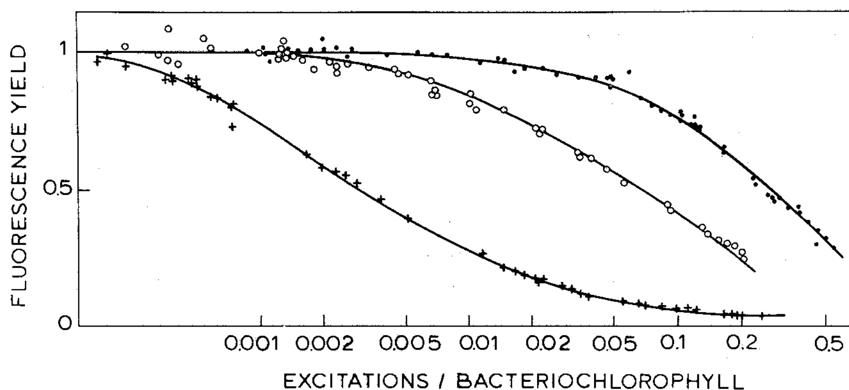


FIG. 7. Fluorescence yield as a function of the intensity of a 30-ps, 532-nm laser flash for several antenna complexes of *Rhodopseudomonas sphaeroides*. (●) B875 prepared with LDS; (○) B800–850 prepared with LDS; (+) B800–850 prepared with LDAO. (Data from van Grondelle *et al.*, 1983.)

In the B875 complex the fluorescence yield vs. pulse intensity curve obeys Eq. (28). The number of connected BChl *a* molecules is about 6–8, in close agreement with the number obtained from electrophoresis measurements (Broglie *et al.*, 1980; Hunter *et al.*, 1982).

Excitation annihilation in chromatophores of *Rps. sphaeroides* mutants was studied by Campillo *et al.* (1977). They concluded that in most of these the fluorescence yield as a function of the pulse intensity could be described by Eq. (28), suggesting large domains that probably contain many RCs.

Figure 8 shows the fluorescence yield as a function of the pulse intensity in *R. rubrum* (Bakker *et al.*, 1983). With all RCs in the state $P^+Q_A^-$, the experiment can be fitted with Eq. (27), using $r \simeq 1$. From this the relation between z, the average number of excitations per domain, and the intensity is obtained, and a domain size $N_D \simeq 900$ can be calculated. If the RCs are all open before the laser pulse, the low-intensity fluorescence yield is about a factor of 3–3.5 lower. Due to the trapping of excitations by the open RCs, the quenching curve is shifted to higher

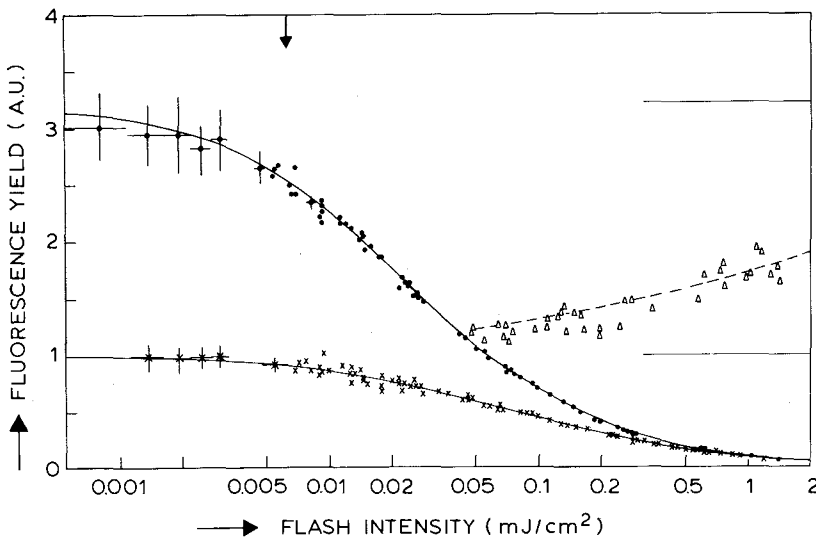


FIG.8. Fluorescence yield as a function of the pulse intensity in *Rhodospirillum rubrum* chromatophores: (●) with all reaction centers in the closed state by continuous background illumination; (x) with all reaction centers open before the pulse. The fluorescence detected with a weak xenon flash 1 ms after the laser pulse in the case where the reaction centers are initially all open is shown by the open triangles (△). The arrow indicates the intensity of the laser flash where there is on the average one excitation per domain. (From Bakker *et al.*, 1983.)

intensity. In the intensity range where more than a few excitations per RC occur, the two curves tend to merge. In the same experiment the fraction of traps closed by the picosecond pulse was monitored by measuring the fluorescence from a weak xenon flash shortly after the intense ps pulse. Comparison of these experiments with the expressions given by den Hollander *et al.* (1983) gave an optimal fit for λ = 14–17.

Applying the random walk model to calculate the probability of trapping relative to annihilation yielded the following set of rate constants: $k_t^o \simeq (3-4) \times 10^{11}\ s^{-1}$, $k_t^c \simeq 10^{11}\ s^{-1}$, and $k_h \simeq (1-2) \times 10^{12}\ s^{-1}$. The value of k_t^o is in reasonable agreement with the time required to reduce the BPheo electron acceptor in isolated reaction centers (Parson and Ke, 1982). The rate of energy transfer between two neighboring BChl 875 molecules implies an intermolecular distance $R \lesssim 15$ Å. Very similar results were obtained with *Rps. capsulata* (Bakker *et al.*, 1983).

D. Singlet–Triplet Annihilation

Paillotin *et al.* (1983) have proposed a general theory to describe singlet–triplet annihilation in photosynthetic systems. This theory is analogous to that of trapping, as outlined in Section III, the only difference being that during the trapping process RCs are converted from a quenching to a non- or less quenching state, while in the present case, during the pulse, quenching centers are formed due to triplet formation.

Most experiments employing triplet states as fluorescence quenchers have led to the conclusion that a matrix model for the antenna pigment organization gives the best description of the results. For several mutants of *Rps. sphaeroides,* Monger and Parson (1977) found that the relation between the concentration of antenna triplet states (either $BChl^T$ or Car^T) and the fluorescence yield followed Eq. (23), in support of the matrix model. In addition, it was observed that singlet–triplet annihilation occurred predominantly in the B875 antenna. They concluded that the most realistic model for the organization of the antenna complexes in *Rps. sphaeroides* is a core of B875 containing several RCs, surrounded by peripheral B800–850. The quenching rate for a carotenoid triplet state was approximately equal to that of an open RC, while $BChl^T$ was thought to trap excitations even five times faster (Monger and Parson, 1977). In contrast, RC triplet states do not quench the antenna fluorescence at all (Holmes *et al.*, 1978).

Similar conclusions were drawn for PSII of plants (den Haan *et al.*, 1974; Breton *et al.*, 1979; Sonneveld *et al.*, 1980). It was concluded that Car triplet states are responsible for the observed fluorescence quench-

ing and that a matrix model explains all the pertinent observations. The rate of quenching of excitations by a Car triplet state was found to be similar to that of an open RC.

V. Concluding Remarks

In this chapter we have presented a survey of the theory of energy transfer between photosynthetic pigments and of the models that can be applied to describe the energy transfer pathways in photosynthetic systems. Although many details had to be omitted, the examples may suffice to demonstrate that fluorescence measurements of all kinds not only have provided a wealth of information about these pathways, but sometimes have even led to fairly detailed structural models for the pigment organization. Together with measurements of circular and linear dichroism, these studies may be expected to yield increasingly detailed and realistic models of the various antenna systems that are found in bacterial and plant photosynthesis.

Although important advances have been made in recent years, there are still many observations that are only poorly understood. A clear example is given by the complicated kinetics of the fluorescence decay in various photosynthetic systems. New statistical theories will have to be developed to understand the time-dependent fluorescence polarization. Now that instrumentation will soon make it possible to enter the subpicosecond region, where even individual "hops" of the excitation may be monitored via the time dependence of the fluorescence spectrum and of the fluorescence polarization, the need for an extended theoretical background for analysis of these measurements will be felt even more forcefully.

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9

Triplets: Phosphorescence and Magnetic Resonance

ARNOLD JAN HOFF
Department of Biophysics
Huygens Laboratory of the State University
2300 RA Leiden, The Netherlands

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ABBREVIATIONS AND SYMBOLS

ADMR	Absorbance detected magnetic resonance
BChl	Bacteriochlorophyll
BPheo	Bacteriopheophytin
Car	Carotenoid or carotene
Chl	Chlorophyll
EPR	Electron paramagnetic resonance
FDMR	Fluorescence detected magnetic resonance
HOMO	Highest occupied molecular orbital
I	Intermediary electron acceptor
ISC	Intersystem crossing

Light Emission by Plants and Bacteria

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LD	Linear dichroism
LUMO	Lowest unoccupied molecular orbital
MODS	Magnetooptical difference spectroscopy
ODMR	Optically detected magnetic resonance
P	Primary electron donor
Pheo	Pheophytin
PS	Photosystem
Rb	Rhodobacter
Rps.	*Rhodopseudomonas*
RC	Reaction center
$T - S$	Triplet minus singlet
X	Primary electron acceptor

ABSTRACT

The physical properties and the role of triplet states in photosynthesis are discussed. Triplet absorbance and phosphorescence spectra of chlorophyll and chlorophyllous pigments *in vitro* and *in vivo* are briefly reviewed, and electron magnetic resonance data, in both zero and high magnetic fields, are discussed. The usefulness of reaction center triplet states as a probe of structural properties is examined and recent developments in the measurement of triplet-minus-singlet absorbance difference spectra are reviewed.

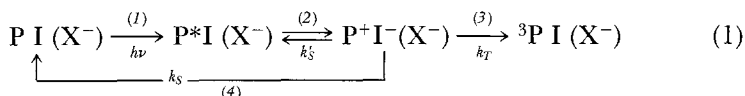
I. Introduction

A. General Background

Lewis and Kasha (1944) first proposed that long-lived metastable phosphorescent states in organic molecules were triplet states. This proposal was soon used to explain energy storage in the primary act of photosynthesis (for pertinent discussions, see Franck, 1957, 1959; Franck and Rosenberg, 1964; Robinson, 1963, 1966; Livingston and Pugh, 1959; Livingston, 1960; Vandermeulen and Govindjee, 1973; Beddard, 1976; Beddard *et al.*, 1977). The concept is attractive: singlet excitation energy is captured in the antenna systems and is trapped in a metastable triplet state by intersystem crossing (ISC). From such a relatively long-lived state there would be ample time to convert the excitation energy into the chemical energy of separated charges. However. experimental results render the involvement of the triplet state unlikely: (1) Chlorophyll (Chl) and bacteriochlorophyll (BChl) triplet states *in vitro* and *in vivo* lie much lower than the first excited singlet state (see Section I,B). This means that a considerable part of the incident photon energy would be wasted as heat. Furthermore, the energy of the triplet state is barely sufficient for the reduction of the stable electron acceptors and is insufficient for the reduction of the transient acceptors that have been discovered in the past decade. A solution to this difficulty would be a

two-photon process (Franck, 1957; Fong, 1974, 1975): the first photon generates a triplet state, and the second one excites this triplet state to the next higher triplet state by resonant energy transfer via a singlet–triplet collision process. This higher lying triplet state, however, has a very short lifetime, and it seems that the original advantage of having the photoreaction proceed from a long-lived triplet state instead of a short-lived singlet state is lost (see, e.g., the contributions of discussants in Franck, 1957). (2) Accurate measurement of the quantum yield of the primary photooxidation of BChl *in vivo* gives a value of 1.02 ± 0.04 (Wraight and Clayton, 1973). This makes a two-photon process unlikely unless there is a pool of long-lived lowest triplet states that persists in the dark for considerable periods of time. This is theoretically extremely unlikely, and experimentally there are no indications of such a long-lived state (Govindjee and Warden, 1977). Even for a one-photon process, the probability of ISC for Chl *in vitro* is much less than one; for example, for Chl *a* in ethanol it is 0.64 (Bowers and Porter, 1967). Although this probability could be raised somewhat for pigments in a regular array of micelles (Robinson, 1966) or in aggregates (Alfano *et al.*, 1985), ISC with a probability of unity does not seem possible. (3) The advent of very fast (picosecond) laser spectroscopy has made it clear that photooxidation of the primary electron donor proceeds in less than 5 ps (Shuvalov and Klevanik, 1983; Borisov *et al.*, 1983; Martin *et al.*, 1986). There is no evidence that ISC in chlorophyllous pigments can occur within such a short time span. (4) Magnetic field effects on the yield of a recombinational triplet state and on the fluorescence (see Section II) and the analysis of the line shape of spin-polarized electron paramagnetic resonance (EPR) signals of primary reactants in bacterial reaction centers (RCs) (Hoff *et al.*, 1977b) favor a singlet precursor for charge separation.

While the considerations above seem to exclude a Chl triplet state as an intermediate in the process of photochemical energy conversion in photosynthesis, the study of these states remains of interest for several reasons. First, Chl and carotenoid (Car) triplet states in antenna complexes and in the RC may act as quenchers of excited singlet states. Second, the formation of carotenoid triplet (^{3}Car) states by triplet energy transfer from ^{3}Chl states protects the photosynthetic organism against attack by singlet oxygen, which can be produced by the reaction of triplet oxygen with ^{3}Chl but not with ^{3}Car (Foote and Denny, 1968; Krinsky, 1968). Third, in prereduced RCs or in RCs which lack the first stable acceptor, triplet states are produced by a recombination reaction



$$\mathrm{P\,I\,(X^-)} \xrightarrow[h\nu]{(1)} \mathrm{P^*I\,(X^-)} \underset{k_S'}{\overset{(2)}{\rightleftharpoons}} \mathrm{P^+I^-(X^-)} \xrightarrow[k_T]{(3)} \mathrm{^3P\,I\,(X^-)} \qquad (1)$$

$$\mathrm{P^+I^-(X^-)} \xrightarrow[k_S]{(4)} \mathrm{P\,I\,(X^-)}$$

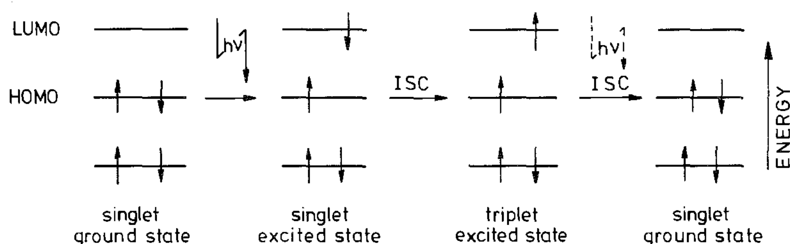


FIG. 1. **Distribution of electrons over the highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbital of singlet and triplet states. ISC, Intersystem crossing.**

where P, I, and X stand for the primary electron donor (Chl *a* or BChl), the intermediary electron acceptor (e.g., pheophytin, Pheo), and the first stable electron acceptor, respectively.

The first evidence for recombination to the triplet state of the primary donor was found by EPR (Dutton *et al.*, 1972) and by nanosecond optical spectroscopy (Parson *et al.*, 1975) of bacterial RCs. It has now been shown that step (*3*) of reaction (1) can take place in all photosystems (see Section V). The recombinational triplet state [see reaction (3)] is a versatile probe of the RC and its primary photochemistry. (For a review of primary photochemistry of photosynthesis, see Parson and Ke, 1982, and Van Gorkom, Chapter 10, and Norris and van Brakel, Chapter 3, this volume). The study of the characteristic parameters of the triplet state ^3P and of its formation yields information on the structure of P and on the organization of P, I, and X. After a brief excursion into the physics of the triplet state, we will focus on some of these investigations in the following sections.

B. Physics of the Triplet State

Optical transitions of aromatic molecules in the visible spectral region are usually $\pi \rightarrow \pi^*$ absorptions of the π-electrons or $\pi \rightarrow \pi^*$ transitions of the lone $2p$ pair of heteroatoms (oxygen, nitrogen, etc.). Depending on the spin pairing of the two unpaired electrons, the excited state is either a singlet state (spins antiparallel) or a triplet state (spins parallel). This is illustrated in Fig. 1. The unexcited molecule is in the singlet ground state; i.e., all electronic orbitals are occupied by a pair of electrons with opposite spins.† This is a consequence of the Pauli exclusion

† It is helpful to think of the spin angular momentum as a little magnet with a north and a south pole, which orients itself in a magnetic field along particular directions (which are given by the quantization rules of quantum mechanics).

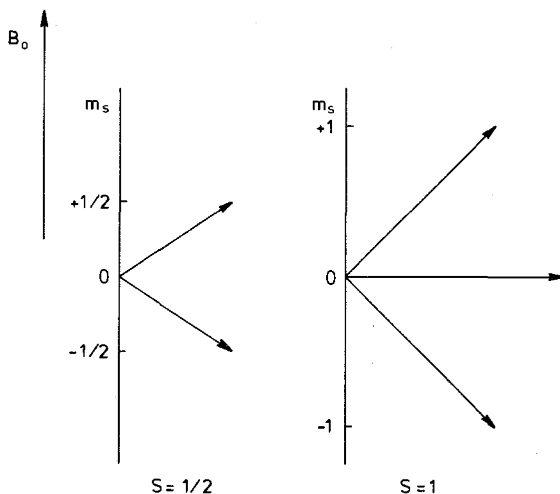


FIG. 2. **Quantized orientations of the spin angular momentum vector S in a magnetic field B_0; m_S is the magnetic spin quantum number (projection of S on the B_0 axis).**

principle, which states that no two electrons can have the same quantum numbers identifying the "state" of the electron with respect to the orbital energy and the spin (and orbital) angular momentum. For a nondegenerate singlet ground state energy level, the orbital angular momentum is zero as there are always two electrons per orbital moving in opposite directions. However, interaction with excited states may admix a usually small amount of orbital angular momentum in the singlet ground state.

The total spin of a molecule is represented by the spin quantum number S, the vectorial sum of all the individual electron spins, which is zero for the singlet ground state.* The total spin angular momentum is given by $\mathbf{S} = \sqrt{S(S+1)}$ in units of $h/2\pi$ (h is Planck's constant). The value of the projections of **S** on an axis of quantization (e.g., an external magnetic field, B_0, Fig. 2) is restricted to $m_S h/2\pi$, with m_S ranging in unit increments from $-S$ to $+S$. The multiplicity is the number of allowed projections and is given by $2S + 1$; it is unity for the singlet ground state. On excitation by a photon of sufficient energy, one of the electrons of the highest occupied molecular orbital (HOMO) may jump to the next higher orbital, the lowest unoccupied molecular orbital (LUMO). During the excitation process the spin state of the excited electron is preserved, because of the law of conservation of angular momentum, so

* A few molecules, including O_2, have a triplet ground state, for which $S = 1$.

that the excited molecule is still in an $S = 0$ singlet (S_1) state and would remain there if there were no coupling between spin and orbital angular momentum. However, the spin moment and the magnetic moment generated by the orbital motion interact magnetically, and through this spin–orbit interaction spin angular momentum of the electrons may be converted into orbital angular momentum without violating the conservation law. This means that there is a certain probability that the spin vector in the LUMO is inverted (this is allowed by the Pauli principle as the unpaired electrons are in different orbitals). The sum of all individual electron spin vectors now adds up to unity, $S = 1$, and the multiplicity is $2 \times 1 + 1 = 3$, i.e., we have a triplet (T_0) state. The probability of the spin inversion or ISC (as it is usually called) depends on the strength of the spin–orbit interaction, which for a single atom is proportional to the nuclear charge Z. As chlorophyllous pigments contain only relatively light atoms, spin–orbit coupling is weak and ISC in the LUMO is a relatively slow process, comparable to that of deexcitation by fluorescence; the yield of ^{3}Chl states *in vitro* is about 65% (Bowers and Porter, 1967).

Once the molecule is in a triplet state it may remain there for a long time (microseconds to milliseconds for chlorophylls, depending on the temperature) as deexcitation to the singlet ground state again involves a "forbidden" spin flip. The $T_0 \rightarrow S_0$ transition is much slower than the $S_1 \rightarrow T_0$ transition, because (1) the electronic orbitals of the initial and the final state are different, and (2) the latter transition presumably occurs through higher-energy triplet states, which makes it easier to dispose of the excess energy as heat. The triplet state may decay directly to S_0 with emission of radiation (phosphorescence) or without radiation. When the energy gap between the S_1 and T_0 states is comparable to kT (k is Boltzmann's constant and T is temperature), the triplet may decay to S_0 via S_1 with emission of delayed fluorescence (Fig. 3).

The energy of the T_0 state is usually appreciably lower than that of the S_1 state. This is because the two unpaired electrons have the same spin quantum number and, according to the Pauli principle, cannot move in the same electronic orbital. On average, the two electrons are farther apart in the triplet state than in the singlet state, hence the energy of Coulombic repulsion is less and the state energy is lower. The decrease in repulsion energy is usually accounted for by introducing the so-called exchange energy, $-J$. For two unpaired spins on one molecule the exchange energy is usually negative ($J > 0$) and the triplet state lies lower than the excited singlet state. This means that the wavelength of phosphorescence emission is longer than that of fluorescence emission.

The three energy levels of the triplet state are nondegenerate. This is

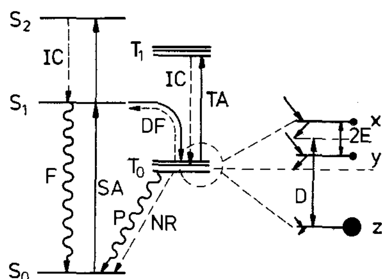


FIG. 3. **Energy level diagram of the singlet and triplet manifold. S_0, singlet ground state; S_1 and S_2, singlet excited states; T_0 and T_1, first and second excited triplet states; *SA* and *TA*, singlet and triplet absorptions, respectively; F and DF, fluorescence and delayed fluorescence, respectively; P, phosphorescence; NR, nonradiative transition; IC, internal conversion. Enlarged T_0 levels (right): *x*, *y*, and *z*, eigenstates of the dipole–dipole interaction; *D* and *E*, zero-field splitting parameters. Downward arrows: to the right, populating probabilities; to the left, decay rates. Filled circles: equilibrium populations.**

a consequence of the fact that the two unpaired electrons behave like little magnets. The two magnetic dipoles exert a force on each other, the magnetic dipole–dipole interaction. The strength of this interaction depends on the relative orientation of the two dipoles and their separation r. The interaction is mathematically represented by a tensor (a matrix with 3×3 elements), $\mathbf{D}$, which is sandwiched between the two spin vectors (the magnetic dipole vectors). The elements of $\mathbf{D}$ are functions of r and of the coordinates of the spin vectors in some molecular coordinate frame. It can be shown that for a particular choice of coordinate frame (which often but not always coincides with that spanned by the molecular symmetry axes) $\mathbf{D}$ is diagonal with elements $-X$, $-Y$, $-Z$ having the property $X + Y + Z = 0$. These three diagonal elements represent the energies of the three triplet sublevels (Fig. 3). Conventionally these levels are called x, y, and z levels; the ordering of their energies depends on molecular structure. Usually, the three (dependent) values of X, Y, and Z are combined in two independent parameters, $D = -\frac{3}{2}Z$ and $E = -\frac{1}{2}(X - Y)$. By convention $|D| > |E|$. These two parameters are called the fine structure or zero field splitting parameters. They can be expressed as averages over the coordinates of the unpaired electron: $D = a\langle(r^2 - 3z^2)/r^2\rangle$, $E = a\langle(y^2 - x^2)/r^2\rangle$, where $a = \frac{3}{4}\gamma_e^2$. Here γ_e is the electronic gyromagnetic ratio, i.e., the ratio between the orbital magnetic moment of the electron and its spin angular momentum; it is commonly written as $g\beta_e$, where β_e is the electronic Bohr magneton and the scalar g the electronic g-factor. For the free electron $g = 2.0023$. Thus, D is a measure of the average squared projection of the distance vector $\mathbf{r}$ on the z

axis and E is a measure of the asymmetry in the x–y plane. For planar aromatic molecules D is expected to be positive.

The application of an external magnetic field pulls the three triplet sublevels still farther apart, as shown in Fig. 4a. The energy of the spin

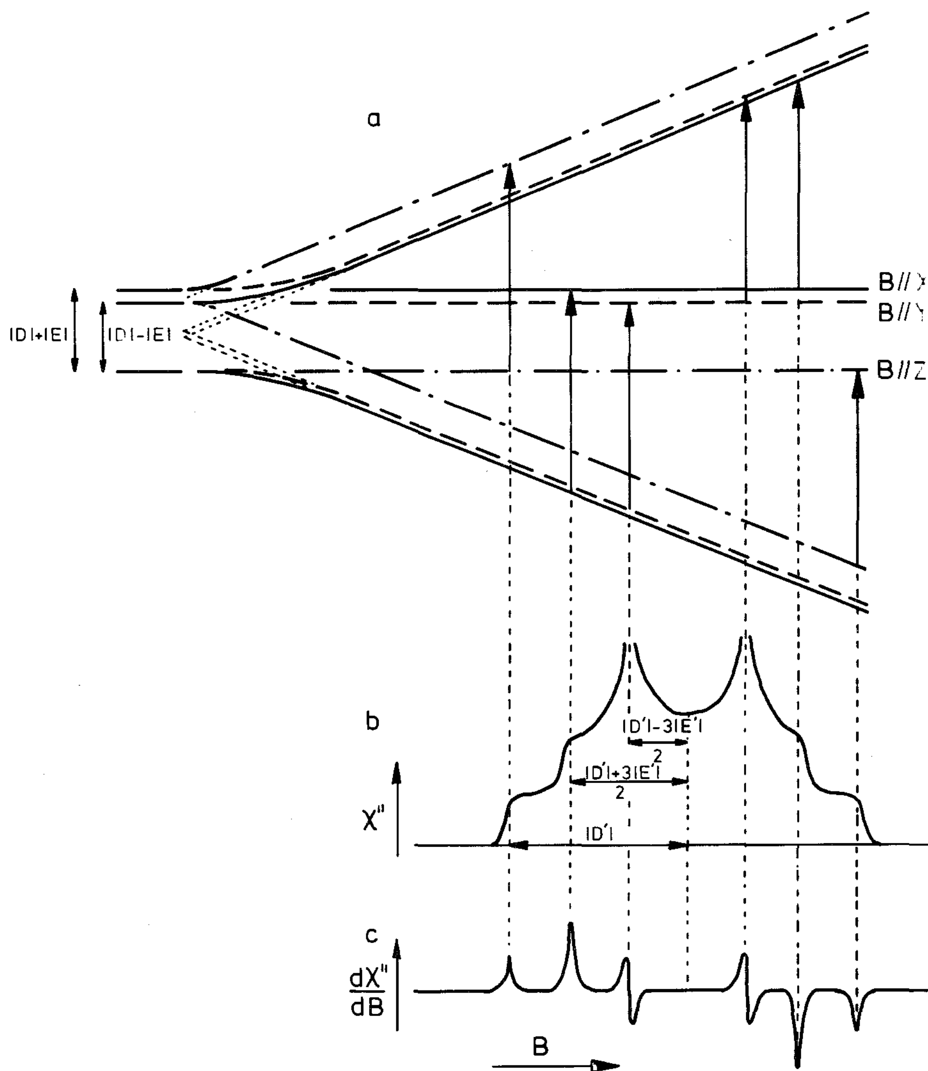


FIG. 4. (a) Triplet levels as a function of magnetic field B. (b) Microwave absorption spectrum χ'' for a randomly oriented system, assuming Boltzmann equilibrium. (c) Derivative $d\chi''/dB$ from (b). $|D|$ and $|E|$, zero-field splitting paramaters; x, y and z, spin axes.

magnet in a magnetic field B_0 is given by $E = g\beta B_0 m_S$, the Zeeman energy, where m_S is the projection of the (quantized) spin vector on the magnetic field axis (Fig. 2). For radicals with one unpaired electron $S = \frac{1}{2}$, the multiplicity is 2 (they are doublet states), $\mathbf{S} = \frac{1}{2}\sqrt{3}$, and $m_S = +\frac{1}{2}$ or $-\frac{1}{2}$. For triplet states, $S = 1$, $\mathbf{S} = \sqrt{2}$, and $m_S = 1, 0$, or -1. Thus, for sufficiently high B_0 the triplet sublevels are separated by $g\beta B_0$ plus or minus a contribution due to the dipolar coupling. The latter is dependent on the orientation of the molecule with respect to the direction of B_0. When one of the dipolar axes (i.e., the axes of the molecular coordinate frame for which $\mathbf{D}$ is diagonal) is parallel to B_0, the energy of the corresponding triplet sublevel remains independent of B_0 (Fig. 4a).

Just as in optical spectroscopy, where one induces a transition between two electronic energy levels by irradiation with an oscillating electromagnetic field ("light"), one can induce transitions between the triplet sublevels by electromagnetic radiation of a suitable, "resonant," frequency. The only difference is that in the absorption of light an electric dipole transition is induced with the electronic component of the field, whereas in the latter case magnetic dipole transitions are induced with the magnetic vector of the field. The resonance frequency depends on the amplitude of B_0. For $B_0 = 0$, the frequencies are given by $(|D| \pm |E|)/h$ and $2|E|/h$. These frequencies are independent of the orientation of the molecule. For $B_0 \gg |D|$ the resonance frequencies depend on the orientation. In Fig. 4a they are shown for the three so-called canonical orientations: $B_0 \parallel x$, $B_0 \parallel y$, and $B_0 \parallel z$, where $\parallel$ means parallel. For a "powder" (i.e., a random system) all orientations are present with a probability given by an angular distribution function, and the resulting range of resonant fields for electromagnetic radiation of *one* particular frequency is indicated in Figs. 4b and 4c.

The branch of spectroscopy discussed above is called electron spin or electron paramagnetic resonance. It is a powerful tool for identifying radical and triplet states, and applications to photosynthetic materials, in both zero and high magnetic fields, will be discussed in the following sections.

The energy separations of the three triplet sublevels that are produced by the magnetic dipole–dipole interaction or by the application of an external magnetic field are small on the energy scale of optical spectroscopy. For example, the $S_1 \leftarrow S_0$ transition* for Chl *a in vivo* is at an energy of about 14,700 cm^{-1} (680 nm), whereas for $B_0 = 330$ mT, the magnetic energy separations are of the order of only 0.3 cm^{-1}. Thus, for optical spectroscopy with ordinary resolution, one can safely neglect the

* By convention, the higher-energy state is mentioned first.

magnetically induced splittings. The absorbance bands of the triplet states, i.e., the transitions $T_1 \leftarrow T_0$, $T_2 \leftarrow T_0$, etc., are usually of lower oscillator strength than the transitions within the singlet manifold, and the bands are much broader (see Section III). One of the characteristic features of the triplet–triplet absorbance spectrum of Chl is the absence of a strong Q_y band. [For descriptions of different absorption bands of the singlet state, see Sauer (1975) and Shipman (1982).] The shape of the phosphorescence spectrum, although much shifted in wavelength maxima of emission, resembles that of the fluorescence spectrum (Section IV). The intensity of the phosphorescence of Chl *in vitro* and especially *in vivo* is low and difficult to detect.

C. Formation of Triplet States

1. INTERSYSTEM CROSSING

During illumination with high-intensity light a large fraction of the RCs may be closed; i.e., the primary electron donor is oxidized. Under these conditions the lifetime of singlet excitations in the antenna Chls is 10-fold or more enhanced, leaving sufficient time for triplet formation by ISC with a yield of 2% in bacterial systems (Monger *et al.*, 1976) and 30% in photosystem (PS) II of plants (den Haan, 1976; Kramer and Mathis, 1980). The Chl triplets may migrate by triplet energy transfer and eventually may be trapped on Car. This trapping protects the organism against attack by singlet O_2. Normally, O_2 is in the triplet ground state. Collision with a ^{3}Chl may convert O_2 into very reactive singlet O_2 by a spin flip-flop process, deactivating ^{3}Chl to the singlet ground state. The energy of ^{3}Car is somewhat lower than that of ^{3}Chl and, in fact, too low to allow the O_2 spin flip (Foote, 1968; Foote and Denny, 1968; Krinsky, 1968, 1979; Boucher *et al.*, 1977).

2. RADICAL RECOMBINATION

Illumination at a low redox potential causes an accumulation of reduced acceptors, eventually generating from the state P I X^- [see Eq. (1)] the singlet state $^1(P^+I^-)$; i.e., the two unpaired electron spins on the individual radicals are aligned opposite directions. This phase relation is changed in time because each spin precesses around an axis set up by local magnetic fields (nuclear hyperfine fields, dipolar fields, etc.). For the two radicals, the directions of the two local fields and their amplitudes will generally differ, so that in due course the spins are no longer oppositely aligned. Thus, there is a time-dependent probability that the two spins are parallel, i.e. the radical pair is in the triplet state $^3(P^+I^-)$. In

general, after time t the pair is in a mixture of the states $^1(P^+I^-)$ and $^3(P^+I^-)$. Electron–hole recombination then leads to one of the singlet states P I or P*I [steps (*2*) and (*4*) in Eq. (1)] or to the triplet state ^{3}P I [step (*3*)] with a probability given by the probability amplitude at time t of $^1(P^+I^-)$ and $^3(P^+I^-)$, respectively. In the absence of an external magnetic field, all three triplet sublevels of the state $^3(P^+I^-)$ are populated with about the same probability, and consequently the populations of the three sublevels of ^{3}P formed by recombination are not much different (Hoff and Gorter de Vries, 1978). However, in an external magnetic field B_0, the two triplet sublevels of $^3(P^+I^-)$ with quantum number $m_S =$ +1 or −1 are separated from the $m_S = 0$ level by the Zeeman energy $g\beta B_0$. The $m_S = 0$ level of $^3(P^+I^-)$ is almost degenerate with the $^1(P^+I^-)$ level. (These two levels are separated by the exchange and dipolar energies, which are much smaller than the Zeeman energy for values of B_0 exceeding 10 to 100 mT.) The large energy gap precludes mixing of $^1(P^+I^-)$ and the $m_S = \pm 1$ levels of $^3(P^+I^-)$ and only the $m_S = 0$ level of $^3(P^+I^-)$ is populated during the spin dephasing. (Note that now the spins precess about a common axis, i.e., in the direction of B_0, and the change in phase relation is affected by the different amplitudes of the projections of the two local magnetic fields on the B_0 axis.) This has two consequences: first, the yield of $^3(P^+I^-)$ and thus that of ^{3}P will be smaller, and second, only the $m_S = 0$ level of ^{3}P will be populated, leading to a characteristic EPR spectrum (Section V). The magnetic field-induced depression of the yield of ^{3}P has been demonstrated experimentally (Blankenship *et al.*, 1977; Hoff *et al.*, 1977c; for a review, see Hoff, 1981), providing a direct demonstration that the precursor of charge separation is a singlet state. (If it were a triplet state, a magnetic field would increase the triplet yield.) In very high magnetic fields the triplet yield increases again, because then the rate of singlet–triplet mixing is enhanced (Chidsey *et al.*, 1980).

At room temperature the triplet state of the primary donor is rapidly transferred to a ^{3}Car state in Car-containing organisms. At cryogenic temperatures, below about 50°K, this transfer is gradually almost completely inhibited in bacteria (Parson and Monger, 1976; Frank *et al.*, 1983; Schenck *et al.*, 1984); in plants it becomes a factor of 2–3 slower at 5°K than at 50°K (Kramer and Mathis, 1980).

3. SINGLET FISSION

If the energy of an excited singlet state is sufficiently high, it may split into two triplet states: $S_1 \rightarrow T_0 + T_0'$, one of the triplet states being located on the excited molecule and the other on an adjacent pigment. If

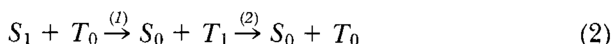
these two molecules are identical, the process is called homofission ($T_0 \equiv T_0'$), otherwise heterofission. Recently, experimental evidence was obtained that singlet fission indeed occurs when photosynthetic bacteria are excited in Car absorption bands (Rademaker *et al.*, 1980; Elfimov *et al.*, 1982; McGann and Frank, 1983; Nuijs *et al.*, 1985).

II. Manifestation of the Triplet State in Photosynthesis

As partly discussed above, the triplet state may manifest itself in a number of ways: (1) triplet–triplet absorbance, (2) phosphorescence, (3) delayed fluorescence, (4) paramagnetism, EPR spectroscopy, (5) quenching of singlet excitations, (6) triplet–triplet fusion, (7) magnetic field effects on fluorescence and absorbance, and (8) protection against oxidation.

The first four items will be discussed in the following sections, the next three are summarized below, and item 8 was already discussed.

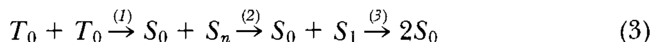
Quenching of excited singlet states is represented by the following sequence of reactions (Rahman and Knox, 1973; Knox and Ghosh, 1975):



$$S_1 + T_0 \xrightarrow{(1)} S_0 + T_1 \xrightarrow{(2)} S_0 + T_0 \qquad (2)$$

Step *(1)* occurs when the fluorescence band of the S_1 state and the absorbance bands of the T_0 state match; step *(2)* is a very rapid internal conversion process in which the excitation energy is lost as heat. The quenching process and applications of its study are discussed elsewhere in this volume (van Grondelle and Amesz, Chapter 8; Lavorel *et al.*, Chapter 4).

Triplet–triplet fusion is represented by the following set of reactions (e.g., see Swenberg and Geacintov, 1973):



$$T_0 + T_0 \xrightarrow{(1)} S_0 + S_n \xrightarrow{(2)} S_0 + S_1 \xrightarrow{(3)} 2S_0 \qquad (3)$$

where S_n is a higher excited singlet state. Step *(2)* is again a rapid internal conversion process, and step *(3)* may be accompanied by emission of (delayed) fluorescence. Step *(1)* is usually fairly slow when the triplet states are randomly generated in a pigment array, since triplet energy migration is governed by short-distance exchange interactions which are usually much weaker than the electric dipole interactions that govern singlet energy transfer. Consequently, delayed fluorescence produced by step *(3)* is weak. However, when triplets are generated by singlet

fission, $S_1 \rightarrow 2T_0$, the fusion process of Eq. (3) may efficiently remove the triplet states.

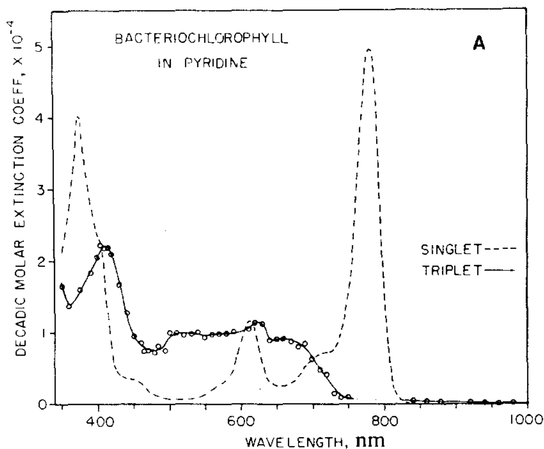
The combined spin vector of the intermediary $T_0 \cdot T_0$ complex resulting from singlet fission may have a value of $S = (-1) + (+1) = 0$, or $S = (+1) + (+1) = 2$. Thus, the complex may be in a singlet or a quintet state. Obviously, only triplet states in complexes having $S = 0$ may fuse to singlet states as in Eq. (3), step (*1*). The probability of finding the complex in the singlet state is dependent on an applied magnetic field (Merrifield *et al.*, 1969; Swenberg and Geacintov, 1973). Hence, the intensity of delayed fluorescence generated by step (*3*) is magnetic field-dependent. A search for such an effect in the green alga *Chlorella* was unsuccessfully undertaken by Stacy *et al.* (1971). More recently, evidence was obtained that singlet fission plus step (*3*) is probably responsible for the observation of a magnetic field effect on the fluorescence of chromatophores of *R. rubrum* on excitation in the Car absorption bands under ambient redox conditions (Rademaker *et al.*, 1980; Elfimov *et al.*, 1982; Kingma *et al.*, 1985a,b).

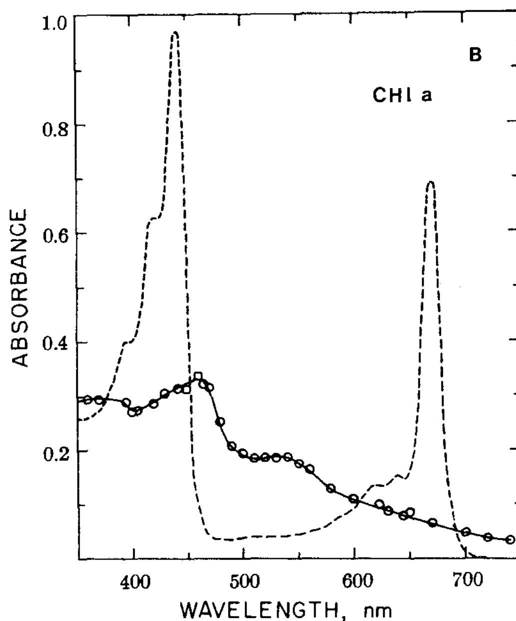
The effect of an applied magnetic field on the fluorescence at low redox potentials is a consequence of steps (*3*) and (*4*) in Eq. (1). As discussed in Section I,C, the radical pair P^+I^- is generally a time-dependent mixture of the singlet state $^1(P^+I^-)$ and the triplet state $^3(P^+I^-)$. An external magnetic field of moderate intensity (<1 T) lowers the probability of the $^3(P^+I^-)$ state, thus raising the yield of delayed fluorescence [the reverse reaction of step (*4*), Eq. (1)] in photosynthetic bacteria (Voznyak *et al.*, 1978; Rademaker *et al.*, 1979) and in *Chlorella* (Rademaker *et al.*, 1979).

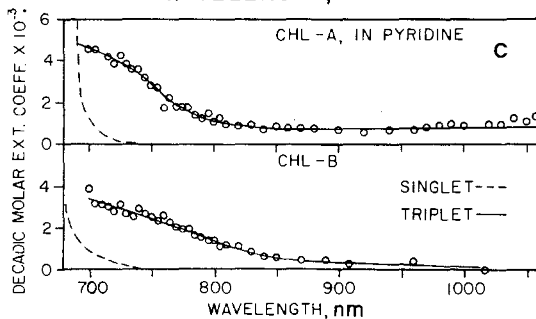
The study of the magnetic field effect on the fluorescence at low redox potentials yields information on the spin dynamics within the radical pair and on the interactions between the radicals. It is a sensitive method for monitoring the degree of reduction of the first stable electron acceptor (Kingma *et al.*, 1983). In very high magnetic fields the fluorescence decreases (Hoff, 1981), which is a corollary of the increase in triplet yield observed in such high fields (Chidsey *et al.*, 1980).

III. Triplet Absorbance Spectra

The $\pi \rightarrow \pi^*$ absorbance spectra of ^{3}Chl *a*, ^{3}Chl *b*, ^{3}BChl *a*, and several pheophytins have been measured by flash spectroscopy by a number of investigators (Linschitz and Sarkanen, 1958; Pekkarinen and Linschitz, 1960; Chibisov, 1969; Holten *et al.*, 1976). In Fig. 5 the spectra of ^{3}Chl *a*, ^{3}Chl *b*, and ^{3}BChl *a* are displayed. The spectra of the triplet states are


BACTERIOCHLOROPHYLL
IN PYRIDINE
A
SINGLET
TRIPLET
DECADIC MOLAR EXTINCTION COEFF, X 10^{-4}
WAVELENGTH, nm


B
CHl a
ABSORBANCE
WAVELENGTH, nm


CHL-A, IN PYRIDINE
C
CHL-B
SINGLET
TRIPLET
DECADIC MOLAR EXT. COEFF. X 10^{-3}
WAVELENGTH, nm

broad and structureless and show a considerable intensity in the wavelength region between the S_1 (Q_y and Q_x) and S_n (Soret) transitions. In all cases the strongest T–T transition lies just to the long-wavelength side of the Soret peak, whereas a second peak lies at the high-energy side of this transition. The T–T absorbance spectra of Chl extend to the infrared well beyond the S_1 transition (Fig. 5c).

The triplet-minus-singlet ($T - S$) absorbance spectra of the ^{3}P triplet states *in vivo* have been determined by flash spectroscopy (Zieger and Witt, 1961; Parson *et al.*, 1975; Shuvalov and Parson, 1981a,b; Setif *et al.*, 1981) and more accurately by an optically detected magnetic resonance (ODMR) method, which is discussed in Section VI.

IV. Phosphorescence

A. Technique of Measurement

The first instrument to measure delayed luminescence (which comprises delayed fluorescence and phosphorescence) was devised by Becquerel (1858). It consisted of two rotating sectored wheels. The first wheel alternately blocked the exciting light and let it pass; the second wheel exposed the sample for observation in the periods when no light illuminated the sample. Thus, only delayed emissions were detected. In one or another form, this scheme is still widely used today (Melhuish, 1964; Longworth, 1968). Usually, frequency selective amplification and phase-sensitive detection are employed to rectify the modulated output of the photodetector. Lifetime measurements are performed by varying the phase relation between the two rotating wheels.

B. Phosphorescence of Chlorophylls in Vitro

Phosphorescence data on Chl and related molecules are scant. The quantum yield of phosphorescence is low (10^{-5} to 10^{-4}) at long wavelengths (900–1000 nm), where the quantum efficiency of photomultipliers is low, and the experiments are marred by emission from impurities, which are often present even in the best purified preparations because of degradation of the pigments. Newer, reliable phosphores-

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FIG. 5. Singlet and triplet absorbance spectra of BChl *a* in pyridine (A), Chl *a* in pyridine (B), and Chl *a* and *b* in pyridine (C). Solid lines with open circles are for triplet, dashed lines for singlet absorption. [(A) and (C) from Pekkarinen and Linschitz, 1960; (B) from Linschitz and Sarkanen, 1958.]

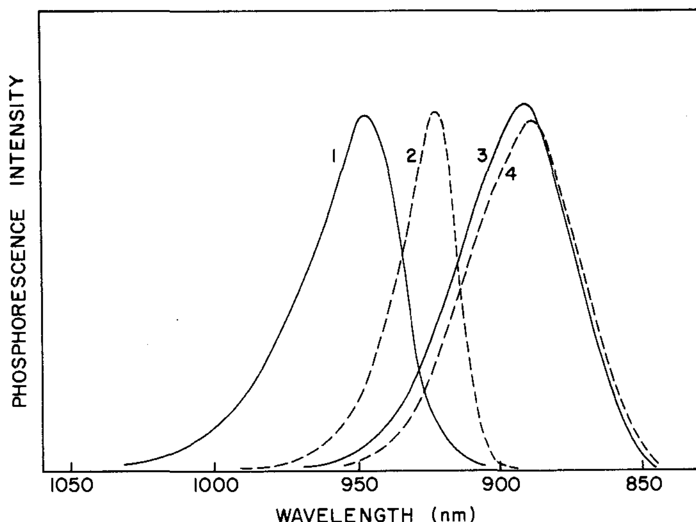


FIG. 6. Phosphorescence spectra in diethyl ether–isopentane–ethanol glass at 77°K of chlorophyll *a* (1), Chl *b* (3), pheophytin *a* (2), and Pheo *b* (4). (From Mau and Puza, 1977.)

cence data are reviewed by Krasnovskii (1982); recent phosphorescence spectra of solid solutions of Chl *a* and *b* and of Pheo *a* and *b* are shown in Fig. 6. Some discrepancies are still present; e.g., the wavelength of the maximum of the phosphorescence band and the lifetimes have varied somewhat in the literature, and a band at longer wavelengths observed by Dvornikov *et al.* (1979) is not reported by others. Generally, however, the data are now consistent (Table I). Note that the wavelength maximum of phosphorescence is quite sensitive to the solvent, and to the presence of ligands as pyridine or ethanol in the case of Mg-containing pigments. The decay rate k_d of Chl triplet states is dominated by nonradiative processes and depends exponentially on the energy gap $\Delta E = E(T_0) - E(S_0)$ between the lowest triplet state and the singlet ground state (Englman and Jortner, 1970; Kleibeuker *et al.*, 1978; Lebedev and Krasnovskii, 1978). Figure 7 is a plot of $\log(k_d \cdot \Delta E^{1/2})$ versus ΔE, from Kleibeuker *et al.* (1978), showing a good correlation. The plot may be used to estimate the energy of the triplet state of BChl *a* and bacteriopheophytin *a* (BPheo *a*) from the measured lifetime. Up to now the *in vitro* phosphorescence of BChl *a*, BChl *b*, BPheo *a*, and BPheo *b* has remained undetected, presumably because of the fast radiationless decay and ensuing low triplet concentration. For BChl *a* in pyridine the nonradiative decay rate at room temperature as determined by laser flash spectroscopy is about 11×10^3 s^{-1} (Pekkarinen and Linschitz, 1960;

TABLE I
Phosphorescence of Chlorophylls and Pheophytins

Pigment	Solvent	Wavelength (nm)	Lifetime[a] (ms)	Reference
Chl *a*	MTHF[b]	960	—	Kleibeuker (1977)
	Diethyl ether	930	2.4–2.8 (80°K) 1.9, 6.2 (4.2°K)	Mauring *et al.* (1980)
	n-Octane	948	—	Mau and Puza (1977)
	Pyridine	985	1.8	Krasnovskii *et al.* (1975)
	Ethanol	960	—	Mau and Puza (1977)
	EPA[c]	950 (970)	1.7 (2.0)	Mau and Puza (1977) Krasnovskii *et al.* (1974)
Chl *b*	MTHF	915	—	Kleibeuker (1977)
	Diethyl ether	890	4.3	Krasnovskii *et al.* (1974)
	n-Octane	882	—	Mau and Puza (1977)
	Pyridine	930	3.1	Krasnovskii *et al.* (1975)
	Ethanol	901	—	Mau and Puza (1977)
	EPA	890	2.1	Mau and Puza (1977)
BChl *a*	Pyridine	—	0.085	Pekkarinen and Linschitz (1960)
Pheo *a*	Diethyl ether	930	0.96 (80°K) 0.4, 1.7 (4.2°K)	Mauring *et al.* (1980)
	Pyridine	940	1.1	Krasnovskii *et al.* (1975)
	Ethanol	932	—	Mau and Puza (1977)
	EPA	922	1.5	Mau and Puza (1977)
Pheo *b*	Diethyl ether	890	—	Krasnovskii *et al.* (1974)
	Pentane	960	—	Krasnovskii *et al.* (1974)
	Ethanol	900	—	Mau and Puza (1977)
	EPA	888	1.6	Mau and Puza (1977)
BPheo *a*	Pyridine	—	0.016	Holten *et al.* (1976)

[a] Measured at 77°K unless otherwise indicated.
[b] MTHF, methyltetrahydrofuran.
[c] EPA, diethyl ether–isopentane–ethanol 5 : 5 : 2.

Connolly *et al.*, 1973). At 1.5°K, the sublevel averaged decay rate is about $9.8 \times 10^3\ s^{-1}$, as detected by ODMR (Section V). The decay of BPheo *a* is determined by flash spectroscopy as $6.3 \times 10^4\ s^{-1}$ (Holten *et al.*, 1976). These values (Fig. 7) yield ΔE(BChl *a*) as $8.0 \times 10^3\ cm^{-1}$ (1250 nm) and ΔE(BPheo *a*) as $8.1 \times 10^3\ cm^{-1}$ (1235 nm). Analogously, k_d(BChl *b*) = $9.5 \times 10^3\ s^{-1}$ (den Blanken and Hoff, 1983a), which yields ΔE(BChl *b*) as $8.1 \times 10^3\ cm^{-1}$ (1235 nm). The estimate of ΔE for ^{3}BPheo *a* agrees with that calculated by Gouterman and Holten (1977) ($7800\ cm^{-1}$) based on tabulations of the energy gap between the S_1 and T_0 states of porphyrins

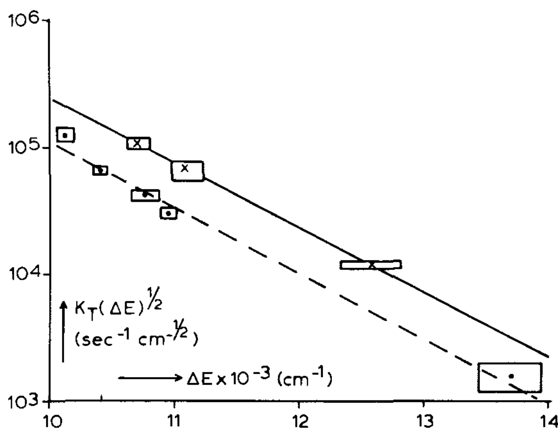


FIG. 7. Energy gap law relating the sublevel averaged triplet decay rate constant k_T to the energy gap $\Delta E = E(T_0) - E(S_0)$ between the first excited triplet state and the singlet ground state. Data points for Mg-containing compounds (dashed line): Chl *a* in ethanol (wavelength maximum of phosphorescence λ_{ph} = 987 nm, k_T = 1250 s^{-1}), Chl *a* in methyltetrahydrofuran (MTHF) (λ_{ph} = 960 nm, k_T = 630 s^{-1}), Chl *b* in ethanol (λ_{ph} = 929 nm, k_T = 410 s^{-1}), Chl *b* in MTHF (λ_{ph} = 913 nm, k_T = 290 s^{-1}), and Mg-porphine in *n*-octane plus ethanol (λ_{ph} = 730 nm, k_T = 14 s^{-1}). Data points for the free-base compounds (solid line): Pheo *a* in MTHF (λ_{ph} = 935 nm, k_T = 1050^{-1}), Pheo *b* in MTHF (λ_{ph} = 900 nm, k_T = 630 s^{-1}), and porphin free base in *n*-octane (λ_{ph} = 795 nm, k_T = 105 s^{-1}). Error limits are indicated by the rectangles. (From Kleibeuker *et al.*, 1978.)

and chlorins. The estimate for ^{3}BChl *a* compares reasonably well with the calculations of Song (1972) and Petke *et al.* (1980), yielding ΔE values of 7100 cm^{-1} and 7880 ± 1200 cm^{-1} (for ethyl-bacteriochlorophyllide *a*), respectively, and with the estimates based on quenching reactions with electron acceptors ($\Delta E > 5100$ cm^{-1}; Connolly *et al.*, 1973), on triplet transfer to β-carotene (ΔE = 8300–9700 cm^{-1}; Connolly *et al.*, 1973; Herkstroeter, 1975), on the photooxidation of BChl *a* (in pentacoordinating solvents 7882 $cm^{-1} < \Delta E \leq 8300$ cm^{-1}, in hexacoordinating solvents 7300 $cm^{-1} \leq \Delta E < 7882$ cm^{-1}) (7882 cm^{-1} is the energy of singlet ($^1\Delta_g$) O_2; Marsh and Connolly, 1984), and on the temperature dependence of delayed fluorescence [for ^{3}BChl *a* and ^3P ΔE = 9400 and 7900 (±500) cm^{-1}, respectively; Shuvalov and Parson, 1981a].

C. Phosphorescence of Chlorophylls in Vivo

Phosphorescence of intact and etiolated leaves and of chloroplasts is extensively reviewed by Krasnovskii (1982). The wavelengths of emission and the lifetimes are shown in Table II together with the assign-

TABLE II
Phosphorescence of Leaves at 77°K

Material	Wavelength (nm)		Lifetime	Assignment	Reference
	Excitation	Emission	(ms)		
Bean leaves	627	870	6.2	Protochlorophyll(ide)	Krasnovskii *et al.* (1977)
	630, 647	920, 970	2.5–3.3	Protochlorophyll(ide)	Krasnovskii *et al.* (1977)
	678	1000	1.5	Chlorophyllide	Krasnovskii *et al.* (1977)
	668	960	1.7	Chl *a*	Krasnovskii *et al.* (1977)
Maize leaves	>640	980	1.9	Chl *a*	Krasnovskii *et al.* (1980)
	>690	995	1.4	Chl *a*	Krasnovskii *et al.* (1980)

ments. The afterglow is considerably stimulated by the addition of dithionite and in maize mutants with an abnormal Car composition, indicating that it originates mainly from antenna Chl triplets. The precise location of the triplet states giving rise to phosphorescence *in vivo* is as yet obscure. Excitation spectra at 77°K show that the phosphorescence arises mainly from pigments absorbing at about 670 nm, i.e., somewhat to the blue of the main absorbance of leaves at about 682 nm (Krasnovskii *et al.,* 1980). This suggests that the phosphorescence is mainly due to disaggregated Chl.

V. Magnetic Resonance in High Magnetic Field

EPR of the triplet states of porphyrins and Chl *in vitro* is a wide field, and the reader may consult Kleibeuker (1977) and Levanon and Norris (1978) for a discussion of this area. In this and the following section we will concentrate on EPR of triplet states *in vivo.*

A. Triplet States of Reaction Center (Bacterio)Chlorophylls

The triplet state of the primary donor, ^{3}P, is almost exclusively populated via the radical recombination mechanism (Section I,C). By virtue of being the photochemical trap, the singlet excited state of P has an especially short lifetime and the probability of formation of ^{3}P by ISC is

negligible. Population of ^{3}P by triplet transfer from antenna ^{3}BChl states in photosynthetic bacteria is possible, but the concentration of ^{3}BChl is very low when P is not oxidized.

The EPR spectrum of ^{3}P is shown in Fig. 8. The spectrum deviates considerably from the triplet EPR spectrum of Fig. 4. The six allowed transitions for which $\Delta m_S = 1$ have the pattern AEEAAE, where A stands for absorption (the higher level has a lower population, as is normally the case for Boltzmann equilibrium) and E for emission (the population of the higher level is higher than that of the lower level, the so-called population inversion); the spectrum is said to be spin-polarized. It can be shown that such a pattern of polarization cannot be the result of ISC in a single molecule (Schaafsma *et al.*, 1976), but it can easily be explained as the result of radical recombination (Thurnauer *et al.*, 1975). In a high magnetic field (i.e., $B_0 \gg D$) recombination to ^{3}P can occur only from the $m_S = 0$ triplet sublevel of $^3(P^+I^-)$, the triplet state of the radical pair P^+I^- (Section I,C,2). Because of the conservation of angular momentum, ^{3}P is then also solely populated in the $m_S = 0$ level.

The first such polarized triplet EPR spectra were observed by Dutton *et al.* (1972) and Leigh and Dutton (1974) in purple bacteria. Subsequently, they were also found in green sulfur bacteria (Swarthoff *et al.*, 1981) and in PSI and II of plants (Frank *et al.*, 1979a; Rutherford and Mullet, 1981; Rutherford *et al.*, 1981a,b; Setif *et al.*, 1982, 1985; Gast *et al.*, 1983). Hence, it is fair to conclude that all photosystems have very similar kinds of charge separation and radical recombination mechanisms. Because of its strong polarization, the spectrum is easily detected. From the spectrum, the absolute values of the zero-field parameters D and E (see Section I,B) and, with proper precautions (Gast and Hoff, 1978), the molecular decay rates k_i ($i = x, y, z$) of the three triplet sublev-

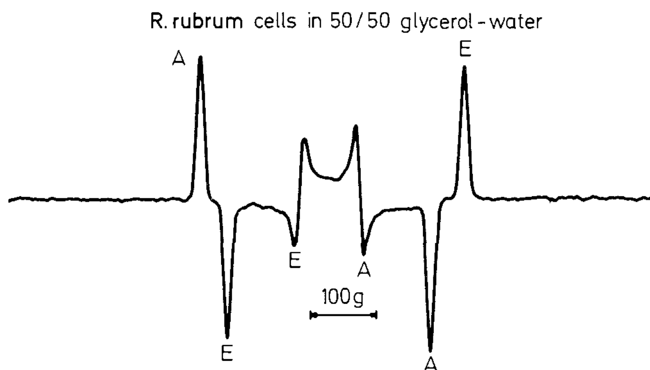


FIG. 8. Triplet EPR spectrum of *Rhodospirillum rubrum* cells at 5–20°K measured with light modulation. A, Absorption; E, emission. (From Uphaus *et al.*, 1974.)

els can be determined. As these parameters are more accurately determined by EPR in zero field than by high-field EPR (Section VI and Tables III and V), we will postpone a discussion of their connection with the organization of the primary donor to Section VI. Here, we only note that D for ^3P is temperature-dependent and increases by about 7% in going from 1.2 to 300°K, as determined by flash EPR (Hoff and Proskuryakov, 1985).

Although from a single EPR spectrum only the absolute values of D and E can be determined, the sign of D can be found by the effect of temperature on EPR peak intensity. An increase in temperature leads to an increase in spin–lattice relaxation, which tends to diminish and eventually abolish the polarization of the EPR peaks in a pattern that depends on the sign of D (Thurnauer, 1979). In this way D of ^3P in bacteria was found to be positive. As noted in Section I,C, this agrees with the triplet state being located on a planar aromatic system. Thus, at least in photosynthetic bacteria, ^3P seems to be rather planar; i.e., either the triplet state is localized on one of the constituent monomeric BChls, or when it is delocalized, the complex of the two BChls is more prolate than oblate (more like a pancake than a beer can). Recent X-ray diffraction data of crystallized reaction centers of *Rps. viridis* show that P is a flat BChl dimer (Deisenhofer *et al.*, 1984). The sign of E of triplet states *in vivo* has not yet been determined.

An important step forward in the characterization of the triplet state was recently made by the recording of triplet EPR spectra of single crystals of reaction centers of *Rps. viridis* (Gast *et al.*, 1983) and *Rb. sphaeroides* (Gast and Norris, 1984). Such spectra allow the determination of the number of magnetically inequivalent "sites" (i.e., primary donor complexes) and their orientation with respect to the crystal axes. In *Rps. viridis* four sites were found having $P_{4_12_12}$ symmetry. To within a few degrees the orientation of the triplet X and Y axes coincides with the N–N axes of the pyrrole rings of *one* of the two BChls making up the primary donor (Norris *et al.*, 1985; J. R. Norris, private communication). This BChl is closest to the primary Bpheo acceptor. For *Rb. sphaeroides* R-26 also, four inequivalent triplet sites were found with orthorhombic symmetry.

Although the pigments in the reaction center crystal of *Rps. viridis* have C_2 symmetry (Deisenhofer *et al.*, 1984), i.e., a rotation of 180° turns one of the BChl of the primary donor into the other, the localization of the triplet state on one BChl (see also section VI,D) seems to indicate that at least in the triplet state, a local asymmetry is present, perhaps induced by the protein environment. Such local asymmetry would also explain that only one of the two possible pigment donor–acceptor chains in the reaction center is active.

B. Triplet States of Antenna Pigments

When the RC is closed by (photo)oxidation of P or by the formation of ^{3}P, the lifetime of singlet excitations in antenna pigments is much enhanced and antenna triplets can be formed with appreciable yield. Frank *et al.* (1979a–c, 1980, 1982, 1983, 1984) observed EPR signals of such triplet states in antenna BChl and RC Car of several photosynthetic bacteria. The $|D|$ and $|E|$ values are summarized in Table III. It is seen that at low temperatures and under reducing conditions ^{3}P predominates, but that at higher temperatures, and in untreated cells, signals presumably due to ^{3}Car appear. The polarization pattern depends on the mode of formation: it is EAAEEA for energy transfer from ^{3}P to the Car in the RC. Because of conservation of angular momentum the transfer must be from the $m_S = 0$ state of ^{3}P to the $m_S = 0$ state of ^{3}Car. The change in pattern compared to ^{3}P indicates that D of ^{3}Car is negative. For direct formation by ISC the polarization pattern is EAEAEA.

The temperature dependence of the concentration of ^{3}Car as measured by EPR (Frank *et al.*, 1983; Hoff and Proskuryakov, 1985) agrees well with that found by optical techniques (Cogdell *et al.*, 1975; Schenck *et al.*, 1984). Apparently above about 40°K the triplet is trapped on Car, whereas below 10°K ^{3}P is predominant. This dependence of the concentration of ^{3}Car on temperature was rationalized in terms of activated triplet energy transfer (Frank *et al.*, 1983; Schenck *et al.*, 1984). Varying the Car composition by changing the growth conditions of the bacteria results in D and E differences, which were attributed to the different chain lengths of the carotenoids (Frank *et al.*, 1982).

In plant materials (isolated chloroplasts and PSI and II particles) antenna triplets have been found by several investigators (Uphaus *et al.*, 1974; Hoff *et al.*, 1977a; Frank *et al.*, 1979a; McLean and Sauer, 1982). These triplets were attributed to antenna Chl *a*, pheophytin *a* (Pheo *a*), or Car triplet states; their $|D|$ and $|E|$ values are shown in Table III.

C. Magnetophotoselection

The powder EPR spectrum of triplet states is strongly anisotropic (Figs. 4 and 8); i.e., at certain field positions well-defined spatial distributions of the molecules are selected. For example, at values of B_0 corresponding to the outermost peaks (see Fig. 4), the molecules are oriented with their dipolar z axis parallel to the magnetic field. Excitation with polarized light then offers the possibility of determining the orientation of optical transition moments with respect to the dipolar axes (Kottis and Lefebvre, 1964; Thurnauer and Norris, 1976, 1977; Boxer and Roelofs, 1979; Frank *et al.*, 1979a; Trosper *et al.*, 1982). The orientation of the

TABLE III

Representative Zero Field Splitting Parameters ($cm^{-1} \times 10^4$) of Triplet States of (Bacterio) Chlorophylls and Carotenoids *in Vivo* and *in Vitro*

Pigment	$\|D\|$	$\|E\|$	Reference
Bacteriochlorophyll			
Rhodobacter (Rb.) sphaeroides			
Strain R-26, cells	187.2 ± 0.2	31.2 ± 0.2	Hoff (1976)
Reaction centers	188.0 ± 0.4	32.0 ± 0.4	den Blanken *et al.* (1982a)
Rhodospirillum (R.) rubrum			
Strain S1, cells	187.8 ± 0.6	34.3 ± 0.3	Hoff (1976)
Strain FRI, cells	187.9 ± 0.6	34.3 ± 0.3	Hoff and Gorter de Vries (1978)
Rhodopseudomonas (Rps.) capsulata			
Strain ATC 23872, cells	184.2 ± 0.6	30.3 ± 0.3	Hoff and Gorter de Vries (1978)
Chromatium (C.) vinosum			
Strain D, cells	177.4 ± 0.6	33.7 ± 0.3	Hoff and Gorter de Vries (1978)
Chloroflexus (C.) aurantiacus			
Reaction centers	197.7 ± 0.7	47.3 ± 0.7	den Blanken *et al.* (1983b)
Prosthecochloris (P.) aestuarii			
Reaction centers	208.3 ± 0.7	36.7 ± 0.7	Vasmel *et al.* (1984)
Rps. viridis[a]			
Cells	156.2 ± 0.7	37.8 ± 0.7	den Blanken and Hoff (1983c)
Reaction centers	160.3 ± 0.7	39.7 ± 0.7	den Blanken and Hoff (1983c)
BChl *a* in methyltetrahydrofuran	230.2 ± 2.0	58.0 ± 2.0	den Blanken and Hoff (1983a)
BChl *b* in methyltetrahydrofuran	221.0 ± 2.0	57.0 ± 2.0	den Blanken and Hoff (1983a)
Chlorophyll			
Photosystem I particles	281.7 ± 0.7	38.3 ± 0.7	den Blanken and Hoff (1983b)
Photosystem II particles	285.5 ± 0.7	38.8 ± 0.7	den Blanken *et al.* (1983a)
Light-harvesting protein	301 ± 2	39 ± 2	McLean and Sauer (1982)
Chl *a* in methyltetrahydrofuran	281.0 ± 6.0	39.0 ± 3.0	Kleibeuker and Schaafsma (1974)
Carotenoids			
Rb. sphaeroides 2.4.1			
Reaction centers[b]	290.0 ± 0.5	44.0 ± 0.6	Frank *et al.* (1980)
Light-harvesting protein[c]	326.0 ± 0.7	36.0 ± 0.7	Frank *et al.* (1982)
Photosystem I particles[d]	388 ± 1.5	38.6 ± 0.1	Hoff *et al.* (1977a)
	383 ± 13	40 ± 13	Frank *et al.* (1979a)
β-Carotene in micelles[e]	333.0 ± 1.0	37.0 ± 1.0	Frank *et al.* (1980)

[a] Contains BChl *b* instead of BChl *a*.

[b] Measured at 10, 100, and 160°K.

[c] Measured at 100°K.

[d] The signals were originally attributed to pheophytin *a* (Hoff *et al.*, 1977) of which $|D| = 339 \times 10^{-4}\ cm^{-1}$ and $|E| = 33 \times 10^{-4}\ cm^{-1}$ (Uphaus *et al.*, 1974).

[e] Measured at 160°K.

TABLE IV
Orientation of Spin Axes and Optical Transition Moments of Reaction Center Triplets[a]

Pigment	Angle in degree with membrane normal[c] x	y	z	Reference
Primary donor BChl				
Rb.				
sphaeroides R-26[b]	90	10–20	70–80	Tiede and Dutton (1981)
Rb. sphaeroides WT[b]	90	15	75	Frank *et al.* (1984)
R. rubrum	90	10–20	70–80	Hales and Das Gupta (1979)
Rps. viridis	50	40	85	Frank *et al.* (1979c)
Rps. palustris	45	45	75	Frank *et al.* (1979c)
Carotenoid				
Rb. sphaeroides WT	40	55	80	Frank *et al.* (1984)

Species	Angle in degree with transition moment[d]: 860 (990[e]) nm			546 nm			850 nm			830 nm			Reference
	x	y	z	x	y	z	x	y	z	x	y	z	
Rb. sphaeroides R-26	5	90	82	65	65	35							Frank *et al.* (1979b)
				60	60	45							Boxer and Roelofs (1979)
Rps. viridis	60	40	65				45	60	60	60	45	60	Trosper *et al.* (1982)

[a] The ordering of the spin axes is chosen such that $\frac{1}{3}|D| + |E| = X$, $\frac{1}{3}|D| - |E| = Y$, and $|D| = -\frac{3}{2}Z$.

[b] For full genus names, see Table III; WT, wild type.

[c] To avoid an unwarranted impression of accuracy, angles are rounded off to the nearest decimal or quintal; i.e., the sum of the squares of the direction cosines need not add up to unity here.

[d] The angles were obtained from magnetophotoselection experiments by spectral simulation. In view of the fact that the peak positions of the lines were not always well-simulated (in a few cases the simulated positions are off by as much as 20%, taking into account the slight differences in scale of the published figures), the solution regions of the angles as given by the authors are not reproduced here.

[e] For *Rps. viridis*.

dipolar axes and/or of the optical transition moments can be related to the membrane normal by studies on mechanically and magnetically oriented systems (Frank *et al.*, 1979b, 1984, Hales and Das Gupta, 1979). The results of several of such investigations are shown in Table IV.

VI. Magnetic Resonance in Zero Magnetic Field

Electron spin (paramagnetic) resonance of the triplet state can also be carried out in the absence of a magnetic field, because the degeneracy of the triplet sublevels is lifted by the dipolar interaction (Section I,C). It can be shown that between the zero-field eigenstates, transitions can be induced by an oscillatory magnetic field of the proper (resonant) frequency (van der Waals and de Groot, 1967). For the systems of interest to us the frequencies lie in the lower microwave range (0.1 to 1 GHz). The transitions can be detected conventionally by detecting the absorption of energy from the microwave field, but in a much more sensitive way by monitoring the effect of resonant microwaves on the optical properties of the sample. This is shown in Fig. 3 where the various schemes of what is commonly called optically detected magnetic resonance are indicated. They all hinge on the fact that in general the three triplet sublevels are unequally populated and depopulated, the individual rates depending on molecular symmetry. If we denote the rates of populating the levels by p_i ($i = x, y, z$) and those of depopulating them by k_i ($i = x, y, z$), then in the absence of spin–lattice relaxation [at liquid helium temperature (~4°K or below)] the steady-state relative sublevel populations are given by $n_i = \text{const}\, p_i/k_i$. Usually, at least one of the n_i's is different from the others. Application of microwaves resonant between levels with different equilibrium populations transfers population from one level to another and eventually leads to a new equilibrium population of all three levels.

The decay rates of the three sublevels are composed of the rates of radiative decay (phosphorescence) and of nonradiative decay by internal conversion. Usually, the phosphorescence (if there is any) is strongly dependent on the symmetry of the sublevel (x, y, or z), and the redistribution of equilibrium population induced by the resonant microwaves leads to a change in the phosphorescence intensity [which is, of course, proportional to the population of the phosphorescing sublevel(s)]. A phosphorescence-detected ODMR experiment on aromatic triplet states was first carried out by Schmidt and van der Waals (1968).

The microwave-induced redistribution of equilibrium population of the triplet sublevels generally leads to a change in the total triplet con-

centration, i.e. $\Sigma\ n_i$ *(microwaves on)* $\neq \Sigma\ n_i$ *(microwaves off)*. This then means that the population of the singlet ground state also changes, as $[S_0] + [T_0] = 1$. (The population of S_1 can be neglected under the usual conditions of illumination.) This in turn leads to a change in S_1 fluorescence and to a change in S_0 absorbance, both of which are proportional to the population of S_0. The first fluorescence-detected ODMR experiment (commonly called FDMR) on photosynthetic triplet states was carried out by Clarke *et al.* (1975), and the first absorbance-detected one (ADMR) by den Blanken *et al.* (1982a). To date, no phosphorescence-detected ODMR of (B)Chl *in vitro* or *in vivo* has been reported, presumably because of the very low quantum yield of phosphorescence.

A. Optically Detected Magnetic Resonance of Bacterial Triplet States

FDMR and ADMR of triplet states of the bacterial primary electron donor have been reviewed recently (Hoff, 1982, 1986). Representative FDMR spectra of whole cells of purple bacteria are shown in Fig. 9. Of the three possible transitions, only two, at frequencies corresponding to the $|D| + |E|$ and the $|D| - |E|$ transitions, were observed. The third transition, at a frequency $2E/h$ (h is Planck's constant) is absent, presumably because of almost equal population of the sublevels. The three bacterial species examined exhibit slight differences in resonant frequency, hence in $|D|$ and $|E|$. The values of the zero-field splitting parameters of these and other bacterial species as measured with ODMR are shown in Table III.

As already mentioned, the decay rates k_i can be measured with precision with ODMR techniques, provided one uses a short pulse of microwaves to perturb the equilibrium population (van Dorp *et al.*, 1975; Hoff and Gorter de Vries, 1978). Methods which depend on saturation of the microwave transitions (Chiha and Clarke, 1978) have been shown to yield unreliable results for the fast-decaying triplet states in bacterial photosynthesis (Hoff and Cornelissen, 1982). Values of k_i obtained with the pulse method for a number of photosynthetic bacteria are shown in Table V.

For whole cells the FDMR technique works very well because of the relatively high yield of fluorescence. The fluorescence yield of the isolated RC proteins, however, is very low and the FDMR signal-to-noise ratio poor (den Blanken *et al.*, 1982b). Detecting the absorbance obviates this difficulty, and it was shown that ADMR spectroscopy increased the sensitivity of ODMR of RCs by several orders of magnitude (den Blanken *et al.*, 1982a; den Blanken and Hoff, 1982). This opened the way to a detailed study of ^{3}P in isolated RCs (see below).

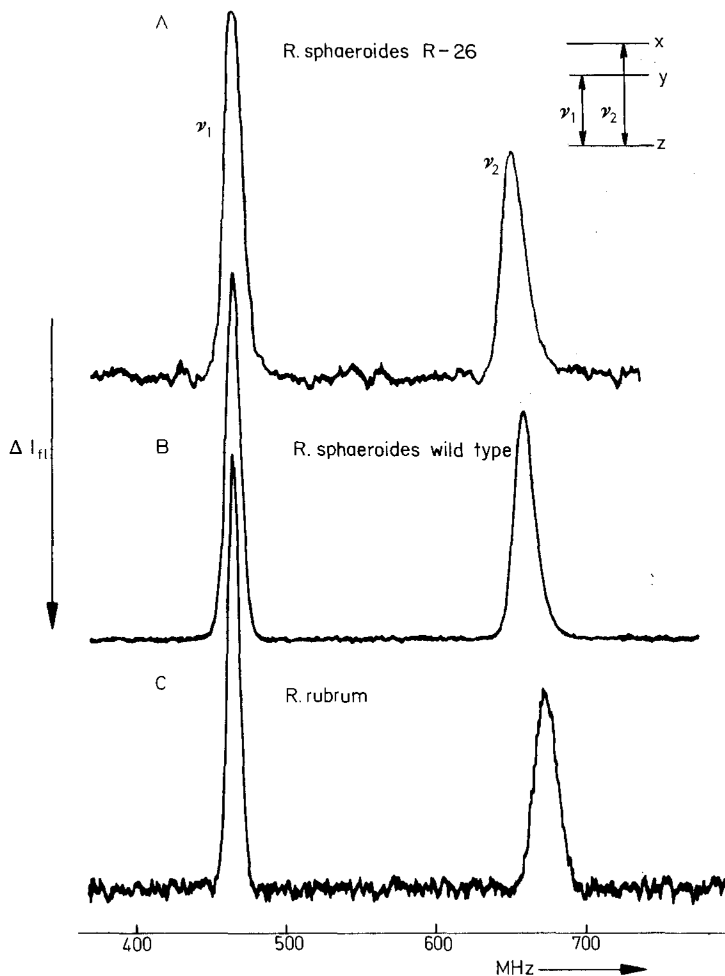


FIG. 9. Fluorescence detected zero-field resonance (FDMR) spectrum at 2°K of whole cells of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* frozen under reducing conditions. Continuous irradiation with blue light, detection at 911 nm. The spectra are single scans. R-26 designates a carotenoidless mutant. Inset: Triplet energy levels in zero magnetic field (see Fig. 3); ν_1 and ν_2 are resonant transition frequencies. (From Hoff, 1976.)

B. Relation between the Triplet Parameters and the Structure of P

As discussed in Section I,B, the values of $|D|$ and $|E|$ are a function of the spatial extent and distribution of the triplet wave function. In general, the larger a π-electron system, the smaller is $|D|$. In fact, for simple

TABLE V
Triplet Sublevel Decay Rates[a]

Species	k_x	k_y	k_z	Reference
Rb. sphaeroides				
Strain R-26, cells	9,000 ± 1,000	8,000 ± 1,000	1,400 ± 200	Hoff (1976)
R. rubrum				
Strain S1, cells	8,000 ± 700	7,200 ± 700	1,350 ± 150	Hoff (1976)
C. aurantiacus				
Reaction centers	12,660 ± 750	14,290 ± 800	1,690 ± 50	den Blanken *et al.* (1983b)
P. aestuarii				
Reaction centers	6,790 ± 500	3,920 ± 300	1,275 ± 100	Vasmel *et al.* (1984)
Rps. viridis				
Cells	<16,000	<16,000	<2,600	den Blanken and Hoff (1983c)
Reaction centers	13,700 ± 900	16,100 ± 1300	2,420 ± 90	den Blanken and Hoff (1983c)
BChl *a* in MTHF	11,950 ± 700	15,900 ± 1300	1,635 ± 50	den Blanken and Hoff (1983a)
BChl *b* in MTHF	12,400 ± 900	14,900 ± 1300	1,300 ± 30	den Blanken and Hoff (1983a)
PSI particles	990 ± 100	1,010 ± 100	92 ± 5	den Blanken and Hoff (1983b)
PSII particles	930 ± 40	1,088 ± 50	110 ± 5	den Blanken *et al.* (1983a)

[a] Data in s^{-1}. For full genus names and abbreviations, see Table III.

structures such as dimeric complexes one can derive exact (but not unambiguous) relations between $|D|$, $|E|$, the k_i's, and the geometry of the dimer components as expressed in the directional cosines of the monomer triplet spin axes with respect to the spin axes of the aggregate (Sternlicht and McConnell, 1961; Hochstrasser and Lin, 1968; Clarke *et al.*, 1977; Bowman and Norris, 1978; Haegele *et al.*, 1978; Hoff, 1982). Attempts to use these relations to elucidate the structure of ^{3}P (Clarke *et al.*, 1977; Haegele *et al.*, 1978) failed, because values of the k_i's used were incorrect (Hoff, 1976; Hoff and Gorter de Vries, 1978; Hoff and Cornelissen, 1982; den Blanken and Hoff, 1983a). Moreover, the values of $|D|$ and $|E|$ and presumably also of the k_i's may change appreciably when the triplet state is slightly mixed with charge transfer states (e.g., of the form $B^+ \cdot B^-$ or $P^+ \cdot B^-$, where B is a BChl monomer) (Kooyman and Schaafsma, 1980). Furthermore, they are also sensitive to the ligand state of the (B)Chls (Kooyman *et al.*, 1977; Clarke *et al.*, 1982; den Blanken and Hoff, 1983a). It therefore seems to be a hazardous enterprise to determine the geometry of a dimeric P complex on the basis of

the triplet parameters without taking the above information into account. Conversely with known geometry (Deisenhofer *et al.*, 1984) one can now attempt to estimate, e.g., the amount of charge transfer in ^{3}P.

C. Optically Detected Magnetic Resonance of Plant Triplet States

FDMR and ADMR of triplets in plant material have been reviewed recently (Schaafsma, 1982; Hoff, 1986). In algae, chloroplasts, and subchloroplast particles a number of different triplets has been found which show a fairly wide range of $|D|$ and $|E|$ values (Table III). The triplets have been variously attributed to RC (^{3}P) triplets, antenna Chl *a* and Chl *b* triplets, Car or pheophytin triplets, etc. From the sign of the FDMR resonances of these triplets, one may obtain information on the mode of energy transfer from the antenna to the trap (Hoff *et al.*, 1977a; Hoff and Gorter de Vries, 1978; Searle *et al.*, 1981). Accurate values of k_i of ^{3}P of PSI and PSII were measured with the ADMR technique (Table V).

It is evident that the zero-field splitting parameters and the k_i's of ^{3}P in PSI and PSII do not deviate much from the monomer values. This, however, may *not* be taken as evidence that P700 and P680 (the primary electron donors of PSI and PSII, respectively) are monomeric Chl *a*. From a theory which relates the triplet parameters to geometry (Section VI,B), it follows that the values of the parameters for the dimer are equal to those for the monomer when the dimer is a plane-parallel structure in which all monomer spin axes are parallel (x to x, y to y, etc.). Moreover, other influences such as ligands and the admixture of charge transfer character may fortuitously cancel the differences between monomer and dimer parameters imposed by structure.

D. Triplet-Minus-Singlet Absorbance Difference Spectra

Consider an ensemble of different types of pigments, of which one particular type can be excited into the triplet state. Microwaves resonant with the zero-field triplet sublevel splittings will generally change the total triplet concentration, hence the singlet ground state concentration, thereby changing the singlet ground state absorbance of the particular pigment. This, however, is not the only change in absorbance of the sample. When neighboring pigments interact with the pigment by electrostatic dipole–dipole interactions, the absorbance spectra of the other pigments in the ensemble are sensitive to the presence of the triplet state. Thus, by modulating the triplet state concentration one may record a triplet-minus-singlet ($T - S$) absorbance difference spectrum

(den Blanken and Hoff, 1982), which contains negative bands due to the photoinduction of the triplet state of the particular pigment, positive bands due to triplet-induced large changes in the electrostatic interactions with proximal pigments, band shifts due to small changes in the interaction with more distant pigments, and also, of course, the triplet–triplet absorptions in the triplet manifold (which reproduce the triplet absorbance spectrum). As the triplet state is less perturbing than the radical pair ion state (for example, the charge-induced Stark effect is absent), one may hope to discern more subtle interactions in the $T - S$ spectrum than in the oxidized-minus-reduced spectrum. The $T - S$ spectrum as measured with ADMR is free from absorbance changes

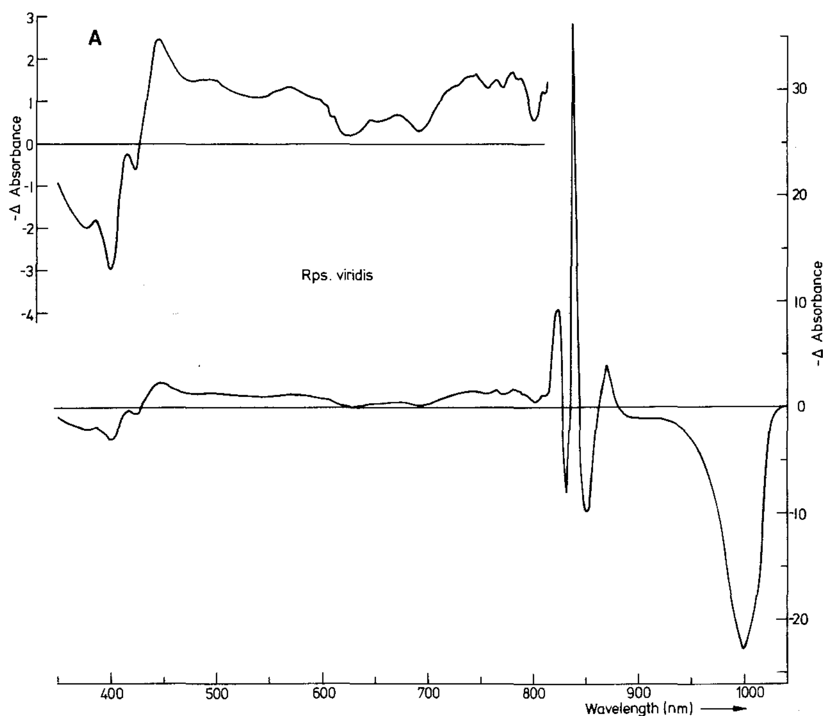


FIG. 10. (A) Triplet-minus-singlet absorbance difference spectrum at 1.2°K of reaction centers of *Rhodopseudomonas viridis* measured with absorbance detected zero-field resonance (ADMR) of the triplet state. The microwave frequency was set at 356 MHz (the $|D| - |E|$ transition). Spectral resolution was 6.4, 2.4, 1.6, and 2.4 nm in the 350–450, 450–790, 790–900, and 900–1050 nm regions, respectively. (B) Enlarged portion of the 790–900 nm region of (A). Circles are actual data points; the noise is less than one diameter. (From den Blanken and Hoff, 1982.)

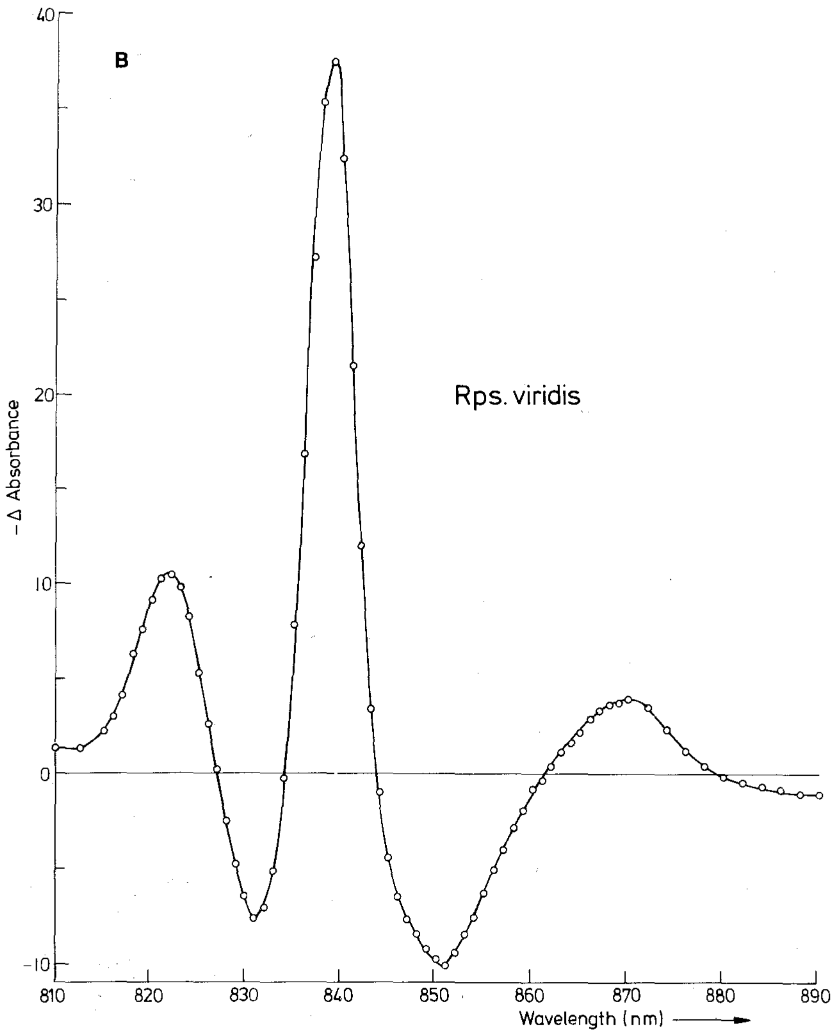


FIG. 10. *(Continued)*

induced by photooxidation and photoreduction of pigments, Stark shifts, etc., as in flash spectroscopy (Setif *et al.*, 1981, 1982).

The high sensitivity of the ADMR technique permits the recording of $T - S$ spectra with unprecedented resolution and accuracy. As a representative example, Figs. 10a and 10b display the $T - S$ spectrum of *Rhodopseudomonas* (*Rps.*) *viridis*, a purple bacterium which contains BChl *b*. One clearly sees the bleaching of the long-wavelength absorption band

of the primary electron donor at 990 nm, the appearance of a band at 838 nm, and band shifts at 827 and 860 nm. Smaller features at shorter wavelengths are due to bleaching of the Q_y and Soret absorption bands and to triplet–triplet absorption. The interpretation of the $T - S$ spectrum in the 830-nm region is ambiguous, as in this region several absorbance bands overlap. It is clear, however, that there is no strong bleaching in this region that is not part of a band shift. This rules out a certain interpretation of the oxidized-minus-reduced spectrum, where a strong bleaching was attributed to the so-called blue (i.e., high energy) exciton component of the primary electron donor (Vermeglio and Clayton, 1976; Rafferty and Clayton, 1978, 1979). This bleaching should be substantially the same in the $T - S$ and the oxidized-minus-reduced spectra, contrary to observation.

The appearing band at 838 nm was attributed to the absorption of one singlet BChl of the primary donor; the other BChl was assumed to contain the localized, ^{3}P triplet state. Because the interaction between one singlet and one triplet BChl in the primary donor dimer is less strong than between the two BChls in the singlet state (which interaction is presumably in large measure responsible for the red shifted 990 nm band), the appearing band of the nontriplet BChl should be close to those of the relatively unperturbed, accessory BChls which absorb in the 830 nm region. Note that the BChl triplet state shows little absorption in the near-infrared. Thus, the ^{1}BChl ^{3}BChl complex of ^{3}P absorbs only weakly around 990 nm, and the $T - S$ spectrum shows a bleaching of this band. The bandshifts at 827 and 860 nm are attributed to the accessory BChls.

The concept of a localized triplet state in ^{3}P of *Rps. viridis* (which was also advanced by Shuvalov and Parson, 1981a,b) is nicely corroborated by the recent EPR experiments on reaction center single crystals (Gast *et al.*, 1983; Norris *et al.*, 1985; J. R. Norris, private communication) (Section V,A).

Den Blanken and Hoff (1983b) and den Blanken *et al.* (1983a) have determined the $T - S$ spectra of PSI and PSII particles. Comparison with the $T - S$ spectra of Chl *a* monomers and dimers *in vitro* gave strong evidence that P700 and P680 are dimeric Chl *a* complexes (Fig. 11).

The interpretation of the $T - S$ spectrum is facilitated by comparing it with linear dichroic $T - S$ [LD-($T - S$)] spectra. A simple technique for recording such spectra with ADMR detection was described recently (den Blanken *et al.*, 1984). It rests on the introduction of a preferred direction in the sample by applying linearly polarized microwaves. The absorbance changes are then detected parallel and perpendicular to this direction, yielding an LD-($T - S$) spectrum, in the same way as in the

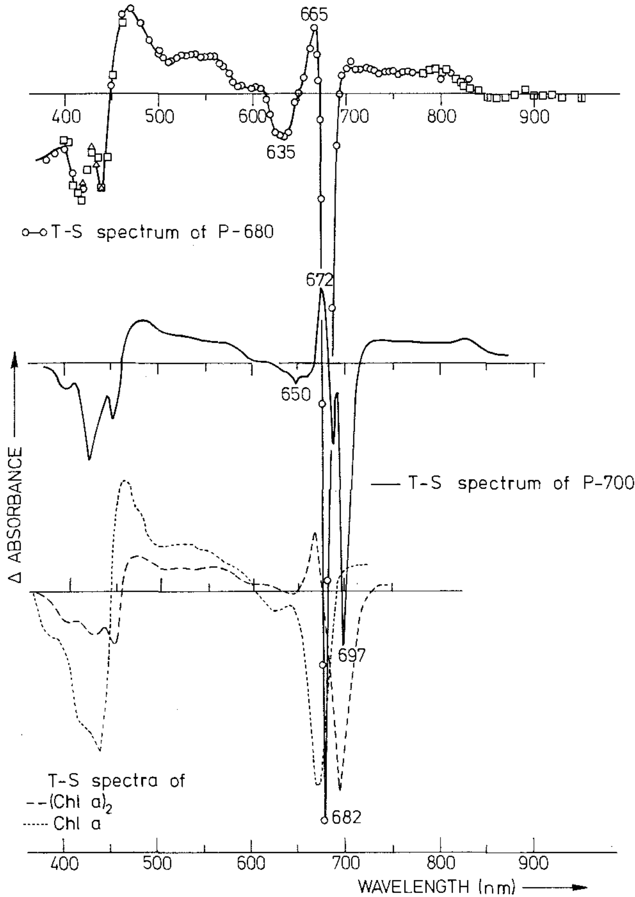
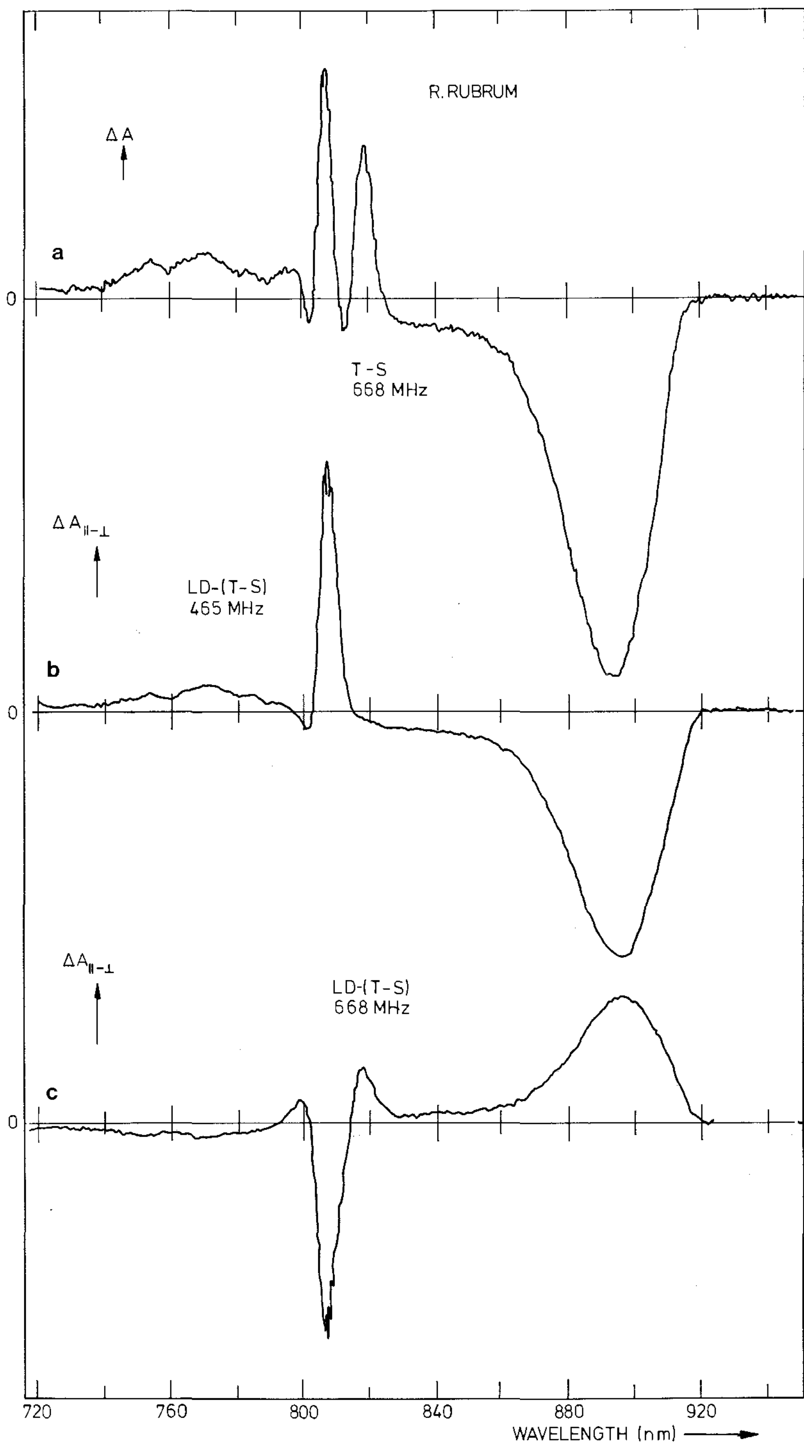


FIG. 11. Triplet-minus-singlet spectra of particles enriched in photosystem (PS) II (top, from den Blanken *et al.*, 1983a), of PSI particles (middle, from den Blanken and Hoff, 1983b), of Chl *a* in pyridine (bottom, dotted line, after Linschitz and Sarkanen, 1958), and of (Chl *a*)$_2$ in methylcyclohexane (bottom, dashed line, from Periasamy and Linschitz, 1979).

more commonly employed photoselection technique. By choosing different microwave transitions between two different sublevels, one selects axial distributions of molecules with the axes of orientation mutually perpendicular (van der Waals and de Groot, 1967). This feature facilitates the interpretation of the LD-($T - S$) spectra and reduces the ambiguity in the determination of the angle between optical transition moments, and between optical transition moments and the spin axes (Hoff *et al.*, 1985a). This is illustrated in Fig. 12, where the $T - S$ and two



R. RUBRUM
ΔA
a
0
T-S
668 MHz
ΔA∥-⊥
LD-(T-S)
465 MHz
b
0
ΔA∥-⊥
LD-(T-S)
668 MHz
c
0
720
760
800
840
880
920
WAVELENGTH (nm)

LD-($T - S$) spectra of *R. rubrum* are displayed, the latter taken at the $|D| + |E|$ and the $|D| - |E|$ ADMR transitions. It is clearly seen that the polarization of the 890-nm band due to the primary electron donor is uniform over its entire width for both microwave transitions, indicating that it is a single band or is composed of bands having parallel transition moments, thus ruling out a composition of two exciton bands. (These must have perpendicularly polarized transition moments, which would behave differently for the two microwave transitions.) A detailed quantitative analysis of the spectra has allowed the determination of the angles between the triplet z and y axes and the transition moments of the bands at 890 nm (bleaching of the primary electron donor), 819 nm (a band shift of an accessory BChl pigment), and 807 nm (a monomer band due to a single BChl in ^3P); i.e., at 890 nm: $\alpha_z = 18°$, $\alpha_y = 72°$; at 819 nm: $\alpha_z = 55°$, $\alpha_y = 41°$; and at 807 nm: $\alpha_z = 21°$, $\alpha_y = 74°$ (Meiburg, 1985).

Up to now, the ADMR technique has been limited to very low temperatures (below 4.2°K). To obtain $T - S$ spectra in the higher-temperature regime, presently 12°K and upward to room temperature, a new technique has been devised, labeled MODS for magnetooptical difference spectroscopy (Hoff *et al.*, 1985b). In this method, the concentration of the triplet state is modulated by a sinusoidally varying magnetic field of weak intensity (Rademaker, 1980). By using MODS it has been shown that at higher temperatures band broadening occurs, which obscures one of the sharp features present around 830 nm (*Rps. viridis*) or 800 nm [*Rhodobacter* (*Rb.*) *sphaeroides*] in the $T - S$ spectrum at 1.5°K (Hoff *et al.*, 1985b). These results make it unlikely that, as proposed earlier, a triplet charge transfer state $^3(P^+B^-)$ is admixed with ^3P at higher temperatures (Shuvalov and Parson, 1981a,b).

VII. Conclusions

Characteristics of the triplet states in photosynthesis provide a highly versatile probe of the structure of the pigment–protein complexes, comprising both the antenna and the RC proper. Up to now most of the information has come from various forms of magnetic resonance and absorbance spectroscopy, alone or in combination, phosphorescence being very weak and as yet of limited information content. With high-field

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FIG. 12. Triplet-minus-singlet ($T - S$) (a) and linear dichroic $T - S$ (LD-($T - S$)) (b and c) spectra of reaction centers of *Rhodospirillum rubrum*. The microwave field was set resonant within the $|D| + |E|$ transition at 668 MHz (a and c) or the $|D| - |E|$ transition at 465 MHz (b).

EPR it has been demonstrated that in all photosystems the triplet state of the primary electron donor is generated by recombination of the primary photoinduced radical pair P^+I^-. The zero-field splitting parameters and decay rates of these triplets are a probe of their molecular configuration and environment, but the interpretation is as yet ambiguous.

Among the magnetic resonance methods, fluorescence and absorbance detected electron spin resonance in zero (external) magnetic field (FDMR and ADMR, respectively) have gained prominence in the past few years. In particular, the technique of monitoring triplet-minus-singlet ($T - S$) absorbance difference spectra by ADMR is a new and promising tool for investigating structural relationships between the RC pigments. It is probably fair to say that this new application has drawn zero-field EPR out of the closet of the specialist and has made it a branch of spectroscopy of general interest to physical biologists. Several papers have appeared in which this technique has been explored (den Blanken and Hoff, 1982, 1983b,c; den Blanken *et al.*, 1983a,b,c, 1984; Vasmel *et al.*, 1984; Hoff *et al.*, 1985a); these may be consulted for more details on the technique and on the spectral information it has offered so far. The ADMR method has recently been extended to determine linear dichroic $T - S$ spectra from which detailed information can be obtained on the structure of the RC pigment–protein complex.

Finally, the new technique of magnetooptical difference spectroscopy (MODS) permits the recording of accurate $T - S$ spectra at higher temperatures, up to 300°K. It is anticipated that for the RC triplet state this technique will supersede conventional flash photolysis spectroscopy, which is much less accurate and more time-consuming.

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Fluorescence Measurements in the Study of Photosystem II Electron Transport

HANS van GORKOM
Department of Biophysics
Huygens Laboratory of the State University
2300 RA Leiden, The Netherlands

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ABBREVIATIONS AND SYMBOLS

Chl	Chlorophyll
C550	Band shift of pheophytin *a*, associated with Q_A reduction
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea, diuron
EPR	Electron paramagnetic resonance
F	Fluorescence intensity
I	Intermediary electron acceptor of PSII (pheophytin *a*), acts between P and Q_A
M	Oxygen-evolving complex
P	Chl *a* which, on excitation (P*), acts as the primary electron donor; also P680
PQ	Plastoquinone
PSII	Photosystem II
Q_A	Traditional primary electron acceptor (plastoquinone)
Q_B	Secondary electron acceptor (formerly B or R), member of the plastoquinone pool bound to the plastoquinone reducing site of PSII
RC	Reaction center
Tris	Tris(hydroxymethyl)aminomethane
ϕ	Fluorescence yield
X†	"Alternative" electron acceptor of unknown identity, acting instead of Q_A, of PSII; known also as Q_2 or X_a
Z	Secondary electron donor of PSII, probably a plastoquinol

† This must not be confused with X in Eq. (1), Chapter 9, which is a generic electron acceptor of all photosystems.—editors.

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ABSTRACT

This chapter deals with the study of changes in the chlorophyll fluorescence yield in photosystem II, as a tool for studying the photochemical reaction and subsequent electron transfer reactions. The first half of the chapter deals with the method as such. It explains how fluorescence yield changes come about, how they are measured, and how to interpret them. It points out that the variable fluorescence has the characteristics of prompt fluorescence as opposed to those of delayed fluorescence. In the second half of the chapter generally relevant results of fluorescence yield studies on system II electron transport are presented. These include electron transport at the electron acceptor side, heterogeneity of system II, and intermediates which act as fluorescence quenchers.

I. Introduction

The pathways of photosynthetic electron transport involve many intermediates, complicated reaction schemes, and various regulation mechanisms. Perhaps the first glimpse into this wonderland was the visual observation of chlorophyll (Chl) *a* fluorescence changes by Kautsky and Hirsch (1931). On illumination of leaves or algae after a period of darkness, the intensity of Chl *a* fluorescence passes through a characteristic sequence of changes before settling to its steady-state value. The kinetics of this so-called fluorescence induction are complex and highly dependent on physiological conditions, as numerous studies have shown. The early observations and conclusions are briefly reviewed in Chapter 1 of this volume by Duysens, who also discusses the *complementarity* between fluorescence and electron transport. Electron transport has been reviewed by Crofts and Cramer (1982).

Normally, the excitation of Chl caused by illumination is trapped by the primary reactions of photosynthesis. But when these reactions cannot take place, e.g., when the primary reactants have not yet recovered from a previous photoreaction, the "traps" are "closed" and the excitation is ultimately lost by fluorescence emission or by conversion into heat. In this sense, Kautsky's discovery did not open the black box of photosynthetic electron transport; the Chl fluorescence merely indicates to what extent light energy goes into the black box or is rejected, and by itself provides no clue to what is taking place inside.

The dependence of the Chl fluorescence yield on the redox state of Q_A, the first stable electron acceptor of photosystem II (PSII), was recognized by Duysens and Sweers in 1963, long before any of the electron transport intermediates in this photosystem could be detected directly (see Fig. 1 in Duysens, Chapter 1, this volume). It is now clear that many electron transfer reactions in the photosynthetic apparatus modify the Chl *a* fluorescence yield, or can be made to do so under certain experi-

mental conditions. The development of sophisticated biophysical techniques for monitoring the intermediates themselves was a prerequisite for further progress, but has not rendered Chl fluorescence yield measurements obsolete. Especially in more or less intact systems, where the concentration of Chl exceeds that of the electron transport intermediates by two orders of magnitude, fluorescence changes may be much easier to measure than more direct indicators such as optical absorbance or electron paramagnetic resonance (EPR). This advantage is particularly obvious in the case of PSII, which is responsible for most of the Chl fluorescence at physiological temperatures in oxygen-evolving organisms. The fluorescence yield of PSI is not only very low but also invariant under most conditions, and has rarely (Ikegami, 1976; Ikegami and Ke, 1984) been used as a probe for electron transfer. In photosynthetic bacteria large bacteriochlorophyll (BChl) fluorescence changes can be observed, but they have not been used much to study electron transport. It was with the purple photosynthetic bacterium *Rhodospirillum rubrum*, however, that Vredenberg and Duysens (1963) first demonstrated a direct relation between fluorescence yield and the redox state of the reaction center (RC). It was found that the light-induced bleaching at 890 nm was quantitatively correlated with the BChl fluorescence yield. This helped to establish that the pigment responsible for the bleaching, P870, is a primary reactant in the bacterial photosystem. When P870 is in the oxidized (bleached) state, a subsequent excitation cannot be used for photosynthesis and the probability that the excitation is lost by fluorescence is increased. Further information on the fluorescence properties of photosynthetic bacteria may be found in reviews by Amesz and Vasmel (Chapter 15, this volume) and Amesz (1978).

In the case of PSII, the useful information gained from a Chl fluorescence yield change is normally not limited by the precision of measurement. Fluorescence measurements have found wide application as a convenient method for detecting PSII electron transfer reactions, and they continue to play an essential role in pioneering studies, pointing the way to new insights in photosynthetic electron transport. The reader should consult chapters on Chl fluorescence of chloroplasts and leaves of higher plants (Briantais *et al.*, Chapter 18), of phycobilin-containing algae (Fork and Mohanty, Chapter 16), and of Chl *b* and Chl *c*-containing algae (Govindjee and Satoh, Chapter 17) in this volume.

II. Methods of Measurement

The usual practice in measuring, e.g., a PSII fluorescence induction curve is to illuminate the sample with continuous light and to measure

the fluorescence simultaneously. Detection of the incident light itself is avoided by using green or blue illumination, which can be easily separated from the red fluorescence by optical filters. Blue illumination is commonly used, but it should be kept in mind that a single spinach chloroplast or *Chlorella* cell may absorb more than half of it and as a consequence the incident light intensity even in dilute samples is intrinsically inhomogeneous by more than a factor of two. Therefore green light, which is less strongly absorbed, is often preferable.

In this type of measurement, the same light source is used to induce both the measured fluorescence and electron transfer. One can also use a separate, much weaker measuring beam, which is nonactinic, meaning that it does not significantly contribute to the induction of photosynthesis by the other, actinic light source. Selective detection of the fluorescence excited by the measuring beam is obtained by modulating its intensity and selecting the modulated part of the fluorescence signal electronically. This method yields a much lower signal-to-noise ratio, especially during actinic illumination, but allows measurement of the fluorescence yield before and after the actinic light period. Often, the actinic light is supplied in the form of single-turnover flashes, i.e., flashes intense enough to activate all RCs and short enough to allow only a single photoreaction in each of them. In this case the photomultiplier will normally be protected from the intense fluorescence during the flash by a shutter or electronic gating circuit, and modulation of the measuring beam would seem pointless. However, it is not, because some fluorescence emission usually continues for quite a long time after illumination, and this delayed fluorescence induced by the actinic light often is not negligible compared to the fluorescence excited by the measuring beam. For a further discussion of methodology, see Lavorel *et al.* (Chapter 4, this volume).

Delayed emission of Chl fluorescence comes about by the reversal of photosynthetic electron transport and shows wide variations of intensity and decay time (Lavorel, 1975; Amesz and van Gorkom, 1978; Jursinic, Chapter 11, and Sane and Rutherford, Chapter 12, this volume). Inevitably, the measuring beam used to probe the fluorescence yield induces delayed fluorescence as well. Does that mean that the measured fluorescence yield actually is that of a somewhat arbitrary mixture of prompt and delayed fluorescence, dependent, among other things, on the modulation frequency used? In recent years this question has become increasingly difficult to answer, especially since the photoreduction of Q_A is now known to proceed via at least one intermediary electron acceptor, I, which is a pheophytin *a* molecule (Klimov and Krasnovsky, 1982;

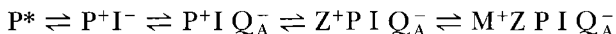
Nuijs *et al.*, 1986). The presence of Q_A^- thus can no longer be assumed to prevent the primary photochemical reaction.

However, the consequences of this notion for the traditional interpretation of Chl fluorescence yield changes in terms of the Q hypothesis and the complementarity principle are negligible, as will be explained in the next section.

III. Is the Variable Fluorescence Prompt or Delayed Emission?

The excited state resulting from light absorption by an antenna pigment is transferred rapidly from molecule to molecule (Knox, 1975; Pearlstein, 1982; van Grondelle and Amesz, Chapter 8, this volume). Now and then it hits upon an RC chlorophyll, P, where it may lead to electron transfer. Inevitably, some excitations are lost on the way, as can be observed by fluorescence measurements on a picosecond time scale (Moya *et al.*, Chapter 7, this volume). Such emission, lasting only about 400 ps or less, is real "prompt" fluorescence (however, see Breton, 1983).

Once the excited state has led to electron transfer, a sequence of secondary electron transfer reactions is initiated which serves to stabilize the charge separation, i.e., to decrease the probability of back reaction (for a general discussion, see Parson and Ke, 1982). In PSII this process involves at least the following reactions:



$$P^* \rightleftharpoons P^+I^- \rightleftharpoons P^+I\,Q_A^- \rightleftharpoons Z^+P\,I\,Q_A^- \rightleftharpoons M^+Z\,P\,I\,Q_A^-$$

in which P* is singlet excited P, Q_A^- is a plastosemiquinone anion (van Gorkom, 1974), Z^+ is a plastosemiquinone cation (Dekker *et al.*, 1984a; O'Malley and Babcock, 1984), and M denotes the oxygen-evolving complex, where probably four manganese atoms are oxidized from a valence of 3 to 4 in successive photoreactions of PSII (Dekker *et al.*, 1984b). The reaction chain decreases the equilibrium concentration of excited Chl in a stepwise fashion (for a more detailed discussion, see van Gorkom, 1985). Consequently, the fluorescence intensity is also greatly decreased, but some reexcitation of Chl via these equilibria continues as long as an oxidized electron donor and a reduced electron acceptor are present. The fluorescence resulting from reexcitation of Chl by reversed electron transport is responsible for the experimentally observed *delayed fluorescence*.

Delayed fluorescence is easily distinguished from prompt fluorescence by its lifetime, activation energy, and yield. Since the lifetime of the

excited state is intrinsically limited to about 10 ns, any longer-lasting emission is obviously delayed. The intensity of delayed fluorescence, in contrast to that of prompt fluorescence, increases with temperature due to the activation energy of reversed electron transport. The yield of delayed fluorescence is very low; even when all charge separations eventually are reversed, e.g., when electron transport beyond Q_A is inhibited, the concomitant steady-state intensity of delayed fluorescence is still about seven times less than the minimum intensity of prompt fluorescence caused by the same illumination (de Grooth and van Gorkom, 1981). This is probably due to the fact that an irreversible recombination to the triplet state of P cannot be prevented when the primary radical pair P^+I^- is formed by reversed electron transport (van Gorkom, 1985; see also Hoff, Chapter 9, this volume).

An approximately fivefold increase in fluorescence yield is observed on reduction of Q_A. This is due to emission which does not have the characteristic properties of delayed fluorescence. Neither its decay rate (2-ns lifetime; see Moya *et al.*, Chapter 7, this volume) nor its yield is obviously lower than might be expected if no electron transfer could take place at all (Thielen and van Gorkom, 1981c) and its yield does not increase with temperature (e.g., Mathis, 1984). When Q_A is in the reduced state, the primary radical pair P^+I^- can probably still be formed (Klimov and Krasnovsky, 1982). The enhanced fluorescence must then be ascribed to excitations produced by the back reaction $P^+I^- \rightarrow P^*$, just like delayed fluorescence (Klimov *et al.*, 1977). In this case, however, the back reaction seems to produce only the singlet excited state of P and not the triplet state. This indicates that P^+I^-, generated by P* in the singlet state, always reacts back to P* before it can lose its singlet character (van Gorkom, 1985). The singlet-to-triplet conversion via the radical pair P^+I^- may be expected to require about 10 ns (Hoff *et al.*, 1977). Since this process (for a review, see Hoff, 1981) initially proceeds as a quadratic function of time, it would be negligible if P^+I^- reacted back to P* in less than a few hundred picoseconds. This seems reasonable since the excitation is known to visit several PSII centers within its 2-ns lifetime (van Grondelle and Amesz, Chapter 8, this volume) and probably spends at least half this time in the antenna (van Gorkom, 1985).

The characteristic properties of delayed fluorescence thus arise not at the moment of charge separation, but rather at the moment when the radical pair loses its pure singlet character. For convenience, the terms "prompt" and "delayed" fluorescence may be defined accordingly. For the practical purpose of fluorescence yield studies it is not relevant that, in the presence of Q_A^-, the excited state transiently generates a radical pair now and then. The variable fluorescence of PSII may still be consid-

ered as prompt fluorescence, and real delayed fluorescence excited by the measuring beam, even when no modulation is used, does not significantly contribute to the measured fluorescence yield.

IV. Quantitative Aspects

Most electron transport studies employing fluorescence yield measurements start from the Q hypothesis of Duysens and Sweers (1963) (see circles in Fig. 1). When Q_A is in the reduced state excitations generated in the PSII antenna cannot be trapped in a stable form and the maximum fluorescence yield Φ_m is observed (unless other quenching processes are introduced). When all Q_A is oxidized the trapping of excitations results in a minimum fluorescence yield, Φ_0, which is five times lower than Φ_m. The value of five is typical for isolated chloroplasts of higher plants, but significant differences between species and between culture conditions occur and Φ_m/Φ_0 ratios from three to seven have been reported. Smaller ratios are usually observed in isolated membrane fragments, and may also result from technical imperfections in the measurement ("false light" due to part of the incident light being read as fluorescence or to fluorescent or phosphorescent material in the sample or in components of the apparatus).

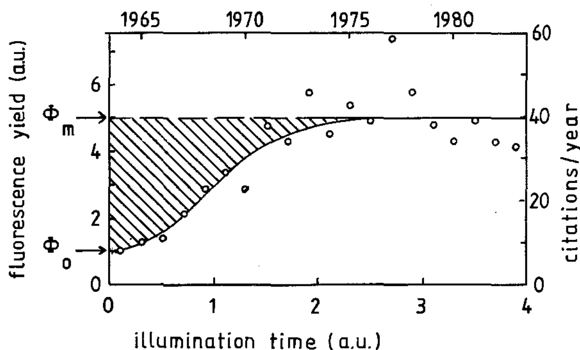


FIG. 1. Chlorophyll (Chl) fluorescence induction on photoreduction of the electron acceptor Q_A of photosystem II. The photoreduction of Q_A (in dark-adapted chloroplasts in the presence of DCMU) causes an approximately fivefold, sigmoidal rise of the Chl fluorescence yield, from Φ_0 to Φ_m. The hatched area, the "integrated fluorescence deficit" or "complementary or induction area," is a measure of the amount of Q_A. Arbitrary units (a.u.). Circles (upper and right-hand scale): the citation frequency of Duysens and Sweers (1963), as recorded by the Science Citation Index, illustrates that the Q hypothesis has become neither obsolete nor too trivial to cite.

The first requirement for a quantitative interpretation of fluorescence yield changes is accurate knowledge of the signal amplitudes corresponding to Φ_m and Φ_0 under the conditions of measurement. Prolonged dark adaptation of a chloroplast suspension usually results in complete oxidation of Q_A^-; otherwise, addition of some ferricyanide is adequate for obtaining Φ_0. A reliable value of Φ_m is more difficult to obtain, because under normal conditions Q_A^- is too rapidly reoxidized by plastoquinone (PQ). This reoxidation can be prevented either by reduction of the PQ pool, or by blocking its binding site on the PSII RC with herbicides such as DCMU (diuron). The former method is complicated by some direct fluorescence quenching by PQ (Vernotte *et al.*, 1979; see Section VII), which also disappears on reduction. The latter method leaves a reoxidation of Q_A^- in seconds, which is due to back reaction with an oxidized electron donor in PSII (Bennoun, 1970). This reaction can be prevented by the addition of hydroxylamine, which keeps the endogenous electron donors in the reduced state. It should be mentioned that both methods normally cause some reduction of Q_A in the dark, as will be discussed in Section VI. Therefore, the initial fluorescence yield observed at the onset of illumination under these conditions is normally higher than Φ_0.

Once Φ_0 and Φ_m are known, the fluorescence yield can be used to estimate the fraction of centers in which Q_A is in the reduced state. In general, however, the fluorescence yield is not a simple function of the Q_A^- concentration, due to the heterogeneous antenna properties of PSII (Section V). The extent of the *fluorescence deficit,* the difference between the observed fluorescence yield Φ and the fluorescence yield reached when all Q_A is reduced, Φ_m, is a linear measure of the rate of excitation trapping and hence presumably of electron transport (Delosme *et al.*, 1959; see also Duysens, Chapter 1, and Lavorel *et al.*, Chapter 4, this volume).

Bennoun and Li (1973) have shown that the complementarity between fluorescence and electron transport holds in the presence of DCMU and hydroxylamine; the rate of hydroxylamine oxidation is proportional to the fluorescence deficit $\Phi_m - \Phi$. The amount of Q reduced and hydroxylamine oxidized by a certain period of illumination is therefore proportional to the fluorescence deficit, $\Phi_m - \Phi$, integrated over that period (see Lavorel *et al.*, Chapter 4, this volume). The *complementary area,* the fluorescence deficit integrated over the complete induction curve from Φ_0 to Φ_m (see Fig. 1), is proportional to the total number of PSII RCs.

The complementary area is a very convenient tool, and since 1966 (Murata *et al.*, 1966; Malkin and Kok, 1966) it has often been used to

determine the fraction of "closed" PSII centers or the relative number of electron acceptors available to PSII under a given set of conditions. It should always be kept in mind that the fluorescence deficit actually is proportional to the rate at which excitations are "consumed" in the process of Q_A^- accumulation, and the quantum efficiency of part of this process affects the proportionality constant. Excitation losses occurring before electron transfer to Q_A, if proportional to the measured fluorescence, do not spoil the complementarity (see Section III). However, the back reaction by reversed electron transport from Q_A^- to P^+ decreases the quantum efficiency of the measured Q_A^- accumulation without a corresponding increase of the fluorescence yield; as pointed out in Section III, the yield of the delayed fluorescence associated with the back reaction is negligible. Initially, the back reaction proceeds with a rate constant of about 5000 s^{-1} (120-μs half-time) (Haveman and Mathis, 1976), but the P^+ concentration is rapidly lowered by secondary electron transfer from Z and M (and from an artificial electron donor, if present). The total amount of charge pairs lost by back reaction during this stabilization process is a function of the rate constants of all the electron transfer reactions involved and must be rather sensitive to the conditions of the experiment. Often it can be measured by illumination with single-turnover flashes (see Section II), taking care that the flash duration is much shorter than the half-time of the most rapid back reaction (which normally is about 120 μs for $P^+Q_A^-$, but see Section VII). Usually, such flashes fail to produce a stabilized charge separation in at least 10% of the PSII RCs under more or less physiological conditions. This fraction contributes to the complementary area of fluorescence induction, and it is clear that complementary areas measured under different conditions may contain substantially different contributions from the back reaction, especially if these conditions modify the secondary reactions at the oxidizing (electron donor) side of PSII. Inhibition of electron transport in that region can be detected and studied conveniently by fluorescence induction measurements.

At this point it may be concluded that the quantitative interpretation of Chl fluorescence yield changes meets with serious complications. It is in the discovery of these complications, on the other hand, that the power of the method becomes clear. Fluorescence yield measurements allow the study not only of Q_A itself, but also of other photosynthetic electron transport reactions if they influence the reduction level of Q_A or involve an intermediate which directly dissipates Chl excitations. In the following sections the main results of such applications will be briefly summarized.

V. Kinetics of Q_A Photoreduction; Heterogeneity

Fluorescence measurements have revealed various kinds of heterogeneous behavior at the level of the primary electron acceptor. It is not surprising that some heterogeneity in kinetic or thermodynamic parameters appears in most quantitative studies on PSII. This photosystem oxidizes water to oxygen once every four photoreactions and reduces PQ to plastoquinol once every two photoreactions. In between, the oxidizing and reducing power is stored in PSII, which thus can be in any of eight different states. Moreover, PSII contains two redox components of unknown function, cytochrome (Cyt) *b*-559 and the component responsible for EPR "signal II_s," which may cause further heterogeneities. Fluorescence measurements indicate, however, that in addition to this multiplicity of interconvertible states, the existence of two structurally and functionally different types of system II, called α and β, must be assumed.

The distinction between α and β centers was originally made by Melis and Homann (1975), who observed that the fluorescence induction curve in the presence of DCMU is biphasic. The last 15% or so of the fluorescence rise occurs with slow, exponential kinetics. This slow phase usually accounts for one-third of the complementary area.

Figure 2A shows the fluorescence (dashed line) and area kinetics of tobacco chloroplasts. Figure 2B is a logarithmic plot of the same data. The main, fast phase is nonexponential, which is attributed to energy transfer between the α centers. Its initial slope corresponds to a rate constant three times larger than that of the slow phase. Homogeneous illumination is important in this experiment: on illumination with blue or red light, for which the transmittance of a single chloroplast may be less than 50%, the two phases become mixed. The precise level of Φ_m is somewhat arbitrary, because at longer times the fluorescence usually does not remain exactly constant. This difficulty primarily affects the estimation of the rate constant of the slow phase, which is not a serious handicap for most purposes. When the β phase is attributed to separate PSII units, one can choose the Φ_m level such that strictly exponential kinetics are obtained for most of this phase, a method which in practice yields quite reproducible results (Thielen and van Gorkom, 1981a).

It was shown by Melis and Duysens (1979) that both phases are accompanied by the absorbance changes characteristic of the reduction of Q_A to Q_A^-, i.e., the reduction of PQ to a plastosemiquinone anion (van Gorkom, 1974). The fraction of Q_A associated with the β phase was rather large, and an analysis of the quantum yields under the same experimental conditions led to the conclusion that the quantum yield of

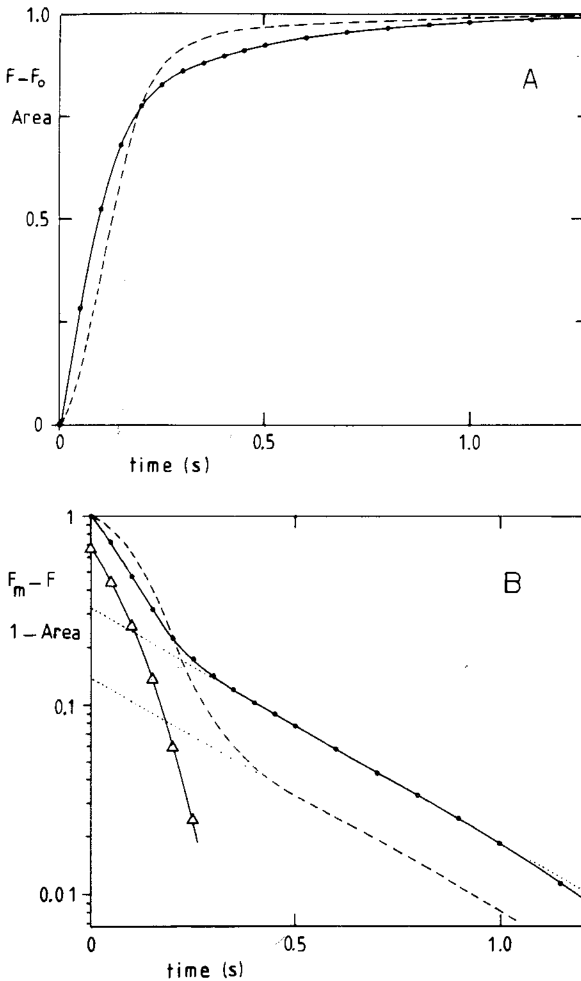


FIG. 2. (A) Chlorophyll fluorescence induction (dashed line) on illumination of dark-adapted tobacco chloroplasts in the presence of DCMU. The solid line indicates the kinetics of the integrated fluorescence deficit, the "area growth." Fluorescence intensities (F, F_0, F_m) are equivalent to yields (Φ, Φ_0, Φ_m) when expressed in relative units, if the intensity and wavelength of illumination are kept constant. (B) A logarithmic plot of the same data reveals that the area grows biphasically; the fast, nonexponential phase (marked with triangles; slow phase subtracted) is attributed to PSIIα, the slow exponential phase to PSIIβ. (From Thielen and van Gorkom, 1981c.)

Q_A reduction during the β phase is even higher than during the α phase (Thielen and van Gorkom, 1981c). Thus, the β phase is slow not because of any inefficiency but because fewer quanta are channeled toward β centers; PSII occurs in two forms with a threefold different antenna size. The existence of two different types of system II regarding antenna properties is further supported by the observation that excitation transfer between PSIIβ units does not seem to occur, whereas between the α units no barrier to excitation transfer can be detected. Moreover, in a tobacco mutant the light-harvesting Chl *a*/*b* protein was found to be associated with system IIα only (Thielen *et al.*, 1981).

Not only are the antenna properties different, but the recovery of the two induction phases as a function of dark time after complete photoreduction of both $Q_{A\alpha}$ and $Q_{A\beta}$ in the presence of DCMU proceeds with clearly different kinetics. The α phase is restored more rapidly (Melis and Homann, 1976). This was earlier known as the "reprise" effect (see, e.g., Lavorel and Etienne, 1977). Additional differences between the recovery kinetics of the α and the β induction phase were shown by Thielen and van Gorkom (1981c).

The midpoint potentials of $Q_{A\alpha}$ and $Q_{A\beta}$ appear to be widely different. The β induction phase disappears below about 120 mV (Horton, 1981; Thielen and van Gorkom, 1981b), while the α phase usually titrates in two waves near 0 mV (Q_H) and near −250 mV (Q_L) (Cramer and Butler, 1969; Golbeck and Kok, 1979; Horton and Baker, 1980; Horton and Croze, 1979; Malkin and Barber, 1979). The observed relative amplitudes of Q_H and Q_L quenching are quite different in different reports, and so are the interpretations of this additional heterogeneity. Recent titrations of fluorescence lifetime components (Karukstis and Sauer, 1983a,b), also in view of the possible relation of these components to the α–β heterogeneity (Butler *et al.*, 1983), seem to have added to the confusion. Thielen and van Gorkom (1981b) found that only Q_L was associated with the reduction of $Q_{A\alpha}$ if no other semiquinones were present. This interpretation agrees best with other thermodynamic properties of PSII (van Gorkom, 1985). Other authors attribute only the high-potential wave (Q_H) to Q_A and the low-potential wave to a different electron acceptor: a further reduction of Q_A^- to Q_A^{2-} (Malkin and Barber, 1979) or the elusive "alternative" acceptor (Bouges-Bocquet, 1980; Diner and Delosme, 1983) which will be denoted *X* here.

The existence of *X* was postulated to explain a fluorescence quenching remaining after photoreduction of Q_A (Joliot and Joliot, 1979), which was particularly obvious after hydroxylamine treatment at low pH (Joliot and Joliot, 1981a) (*X* being denoted Q_2 in those studies), and—independently—to explain the observed photooxidation of P under con-

ditions where Q_A was already in the reduced state (Eckert and Renger, 1980) (X being denoted X_a in this case). The charge pair P^+X^-, which appears to be the main source of delayed fluorescence in the submillisecond time domain (Meiburg *et al.*, 1984), does not span the thylakoid membrane and its function is still a matter of speculation. Fluorescence yield measurements by Joliot and Joliot (1981b) have shown that the flash-induced accumulation of the state P X^- is inefficient and subsequent reoxidation of X^- is rather insensitive to DCMU, at least in hydroxylamine-treated chloroplasts with hydroxylamine as an electron donor. If these conclusions apply under other conditions as well, the functioning of X instead of Q_A under certain circumstances should be detectable by fluorescence induction measurements: the complementary area should become very large or the fluorescence might not rise at all. It has been suggested (van Gorkom, 1985) that the phenomenon of ΔpH-dependent quenching (Krause *et al.*, 1983; Briantais *et al.*, Chapter 18, this volume) can be explained in this way, since a large ΔpH appeared to induce the functioning of X in all of PSII (Renger *et al.*, 1977), whereas normally it seems restricted to PSIIβ (Meiburg *et al.*, 1984). Further research is needed to clarify the possible implications of these findings, and as long as no direct method for measuring X/X^- is available, Chl fluorescence yield measurements will remain an indispensable tool in its study.

VI. Reoxidation of Q_A^-; Binary Oscillations

Electron transfer from Q_A^- to the PQ pool takes place at a specific PQ binding site; when a PQ molecule has received one electron, it remains bound to the site as a plastosemiquinone anion, until a subsequent photoreaction of the same PSII RC allows its further reduction to plastoquinol and release (Velthuys, 1982). The semiquinone is now usually called Q_B^-; in earlier literature B^- or R^- was usually used. Q_B^- is spectroscopically hardly distinguishable from Q_A^- (van Gorkom *et al.*, 1982), except that it causes a much smaller electrochromic shift of the absorption bands of pheophytin (C550 is one of those shifts) (Lavergne, 1984; Schatz and van Gorkom, 1985). The only simple way to detect electron transfer from Q_A^- to Q_B is to measure the concomitant decrease of the Chl fluorescence yield.

Plastoquinone and plastoquinol are rapidly exchanged with other molecules of the pool and also with herbicides such as DCMU (if present), which thereby inhibit electron flow from Q_A^- to the pool (Velthuys, 1981b, 1982). The semiquinone Q_B^-, however, is not released

from the binding site within minutes. Exchange with other molecules is still possible, albeit at a reduced rate, but only in the quinone form via the equilibrium with Q_A^-, while the extra electron remains located on the same PSII RC:

$$Q_AQ_B^- \rightleftharpoons Q_A^-Q_B \rightleftharpoons Q_A^- + Q_B$$

With this mechanism, an increase of the Chl fluorescence yield due to electron transfer from Q_B^- to Q_A can be induced by the addition of DCMU (Velthuys, 1981b) or by the reduction of the PQ pool (Thielen and van Gorkom, 1981b). In prolonged darkness Q_B^- is reoxidized to some extent and subsequent illumination by a series of single-turnover flashes leads to an alternately high and low Q_B^- concentration (a "two-electron gate"). This oscillation can be detected by measuring the amplitude of the fluorescence increase induced by the addition of DCMU as a function of the number of flashes fired before DCMU addition (see Fig. 3). In fact, this observation led Velthuys and Amesz (1974) to postulate the existence of Q_B^-. The small amplitude of the oscillation, less than 25% of the variable fluorescence, is due to the presence of a significant amount of Q_B^- remaining after dark adaptation and to the nonlinearity of the fluorescence yield with the fraction of Q_A reduced. This is clearly illustrated by the (unpublished) experiment shown in Fig. 4, in which the

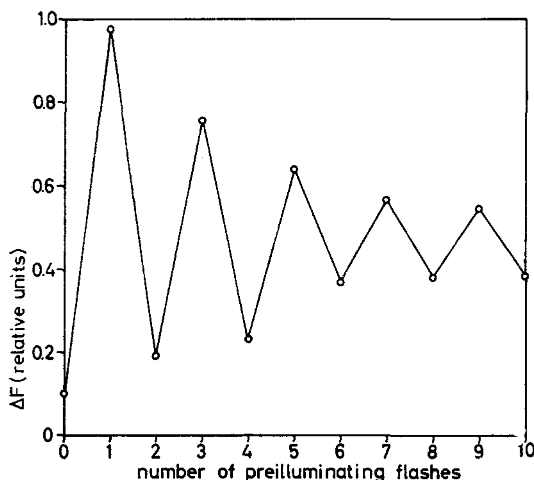


FIG. 3. Chlorophyll fluorescence (ΔF) observed after illumination of dark-adapted spinach chloroplasts by a series of single-turnover flashes and subsequent DCMU addition. The units correspond to $(\Phi - \Phi_0)/\Phi_0$. Oxygen evolution was inactivated by Tris treatment and an artificial electron donor was present. (From Velthuys and Amesz, 1974.)

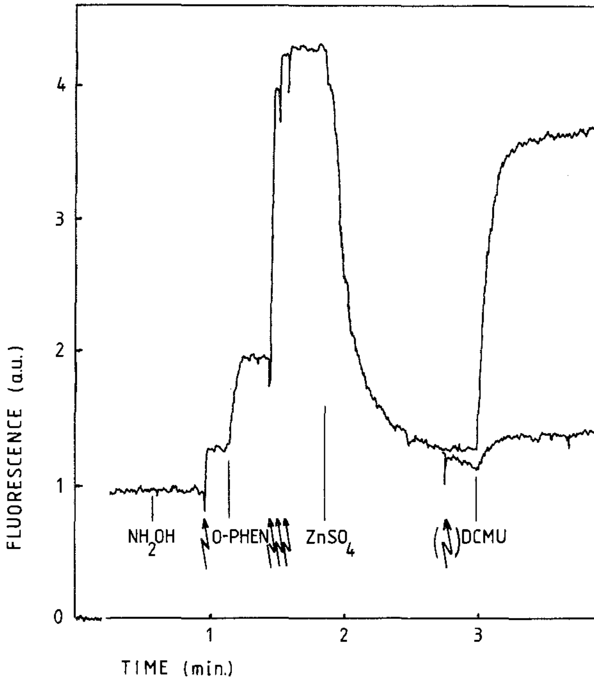


FIG. 4. Changes of the chlorophyll fluorescence yield in dark-adapted spinach chloroplasts, measured with very weak green modulated light. After addition of hydroxylamine (1 m*M*), one saturating flash (arrow), and addition of *o*-phenanthroline (*o*-PHEN, 2 m*M*) the fluorescence yield corresponds to that shown in Fig. 3, after one flash. Centers that were still open were then closed by three flashes, and the *o*-phenanthroline removed by the addition of 5m*M* $ZnSO_4$. After the fluorescence decrease (due to the reaction $Q_A^-Q_B \rightarrow Q_AQ_B^-$) the DCMU-induced rise was large (upper trace), but not if a saturating flash was applied before DCMU addition (lower trace). The fluorescence units correspond to Φ/Φ_0.

effect is induced "from above," starting from nearly 100% Q_B^-. As mentioned in Thielen and van Gorkom (1981b), this situation can be obtained by preillumination in the presence of hydroxylamine and *o*-phenanthroline, which acts like DCMU, to reduce all Q_A, followed by the removal of *o*-phenanthroline with excess Zn^{2+} (Bennoun and Li, 1973). Then the DCMU-induced fluorescence rise is very large indeed and it can be prevented almost completely by a saturating flash just before DCMU addition. Measured in this way, the amplitude of the effect is more impressive, because most PSII centers are involved and the nonlinearity mentioned above now enhances the signal.

Nevertheless, it is clear that the two-electron gate does not operate in all PSII centers. It was not detectable in PSIIβ (Thielen and van

Gorkom, 1981b) and under some conditions, notably at low pH (pH < 7), it did not seem to work in all PSIIα centers either (Lavergne and Etienne, 1980). Probably the "non-B-type" centers, as Lavergne and Etienne called the centers without a two-electron gate, are PSII centers in which X is operating instead of Q_A (see Section V). Joliot and Joliot (1981a) have reported a reduction of Cyt *b*-563 by X^-, but otherwise the path of electron transport at the reducing side of non-B-type PSII centers is unknown. Whatever its path, this electron transport may well be coupled very directly to ATP synthesis; Schreiber (1984) found that the addition of ATP after preillumination induced a rapid Chl fluorescence yield increase of the non-B-type PSII, presumably due to reversed electron transport, which did not require a transmembrane pH gradient as an intermediate.

In the measurements of Figs. 3 and 4, complications by charge accumulation at the oxidizing (electron donor) side of the RC were avoided by addition of a one-electron donor. Without such a donor, part of the Q_A^- induced by DCMU addition would be reoxidized rapidly by back reaction with the oxidants stored in the oxygen-evolving complex and would superimpose a period four oscillation on the observed fluorescence changes (Wollman, 1978). On the other hand, this phenomenon can be used to study the correlation of the period two oscillations at the reducing (electron acceptor) side with the period four oscillation at the oxidizing side. Such fluorescence measurements (van Gorkom *et al.*, 1982) allowed us, for instance, to exclude the theoretical possibility of a rapid exchange of oxygen-evolving enzymes between PSII RCs pointed out by Lavorel (1976).

The presence of Q_B^- decreases the affinity of the PSII complex for DCMU (Laasch *et al.*, 1983). At low DCMU concentrations ($\leq 10\ \mu M$), the presence of Q_B^- may be accompanied by a very slow phase (much slower than $Q_{A\beta}$ reduction) in fluorescence induction curves (Joliot and Joliot, 1983). Quantitative analysis of the redox reactions and binding equilibria involved in electron transport from Q_A^- to the PQ pool in a particular experiment, especially when a herbicide is used as well, would already be rather involved if no further complications arose. Actually, protonation equilibria must be taken into account. PSIIβ (and part of PSIIα, depending on the conditions) participates in herbicide binding and perhaps in PQ binding, but does not exhibit binary oscillations (Thielen and van Gorkom, 1981b), and the relevant concentrations of "free" PQ, plastoquinol, and herbicide in the thylakoid membrane are rather uncertain. An apparently simple question such as: "What is the equilibrium constant of the reaction $Q_A^-Q_B \rightleftharpoons Q_AQ_B^-$?" is actually very hard to answer (see Robinson and Crofts, 1983, and Crofts *et al.*, 1984, for an interesting attempt).

In the presence of ferricyanide the fluorescence induction area in the presence of DCMU is enhanced nearly twofold (Ikegami and Katoh, 1973). It appears that in this case an electron acceptor with a midpoint potential of about 400 mV is able to oxidize Q_A^- very rapidly in a DCMU-insensitive reaction (Velthuys and Kok, 1978). This species, known as C400 or A_H, turns out to be the non-heme iron situated close to Q_A and Q_B (Petrouleas and Diner, 1986).

Our knowledge of electron transport beyond Q_A is still very incomplete. It may be expected that this area of PSII research will continue to inspire scientists to design ingenious fluorescence experiments and carry out complicated quantitative analyses for some time to come. This region of photosynthetic electron transport, where the advantages of fluorescence yield measurements are most obvious, is the target not only of most herbicides, but also of endogenous inhibitors such as formate and acetate; these inhibitors bind in competition with (the noninhibiting) bicarbonate, strongly suggesting a physiological control mechanism (Vermaas and Govindjee, 1981; Snel and van Rensen, 1984).

VII. Excitation Sinks

Not all changes in fluorescence quenching are caused by changes in the photochemical activity of PSII. The PQ pool in its oxidized form has been mentioned as an example of an electron transport intermediate, which quenches the Chl fluorescence (Vernotte *et al.*, 1979). It dissipates excitation energy, no doubt at the expense of the quantum efficiency of photosynthesis. Addition of quinones is known to decrease the fluorescence of PSII (Amesz and Fork, 1967), but it would be surprising if evolution had failed to produce some protection against energy dissipation via the endogenous quinones. In fact, it seems that Chl and PQ are somehow kept apart: addition of detergents greatly enhances fluorescence quenching by the PQ pool and the reduction of the pool is then accompanied by an absorption band shift of Chl *a*, suggesting a close association of PQ with Chl (van Gorkom *et al.*, 1974). This is also observed in the oxygen-evolving PSII preparations isolated with Triton X-100 (e.g., Berthold *et al.*, 1981), which are now widely used. In isolated chloroplasts, in the absence of detergents, the quenching by the PQ pool is somewhat variable. We have observed values ranging from virtually zero in spinach chloroplasts to up to 30% of the variable fluorescence in tobacco chloroplasts (Thielen and van Gorkom, 1981b).

Other quenching intermediates are normally very short-lived. They have no influence on the quantum efficiency of PSII, because at times much shorter than the turnover time of PSII (0.2–1 ms; Bouges-Boc-

quet, 1973) a subsequent photoreaction would be wasted anyway. Mauzerall (1972) was the first one to show (Fig. 5) that the Chl fluorescence yield increases with complicated kinetics in the nanosecond and microsecond range rather than simultaneously with the reduction of Q_A, which occurs in less than 1 ns. The rise phase of a few microseconds is not related to electron transport, but reflects the decay of carotenoid triplets, which are formed in the antenna by transfer from the Chl triplet state (Duysens *et al.*, 1975). The more rapid fluorescence rise is now attributed to the reduction of P^+. Butler (1972) proposed that P^+ is an efficient fluorescence quencher, and this has been amply demonstrated under conditions where the lifetime of P^+ was greatly increased, e.g., at cryogenic temperatures (Butler *et al.*, 1973; Den Haan *et al.*, 1973; Murata *et al.*, 1973; Mathis and Vermeglio, 1974) and at pH 4 (van Gorkom *et al.*, 1976; Pulles *et al.*, 1976; Haveman and Mathis, 1976). Reduction of P^+ is now known to occur normally with half-times of about 20 and 200 ns, depending on the redox state of the oxygen-evolving complex (Sonneveld *et al.*, 1979; Brettel *et al.*, 1984). A half-time of P^+ reduction around 35 μs has often been reported (Zankel, 1973; Joliot, 1975; Gläser *et al.*, 1976; Joliot and Joliot, 1977; Eckert and Renger, 1980) and may be characteristic of centers in which *X* is the electron acceptor (Meiburg *et al.*, 1984). A half-time of 100–200 μs is observed when $P^+Q_A^-$ decays by back reaction only (van Gorkom and Donze, 1973; Haveman and Mathis, 1976). It is noteworthy that all these

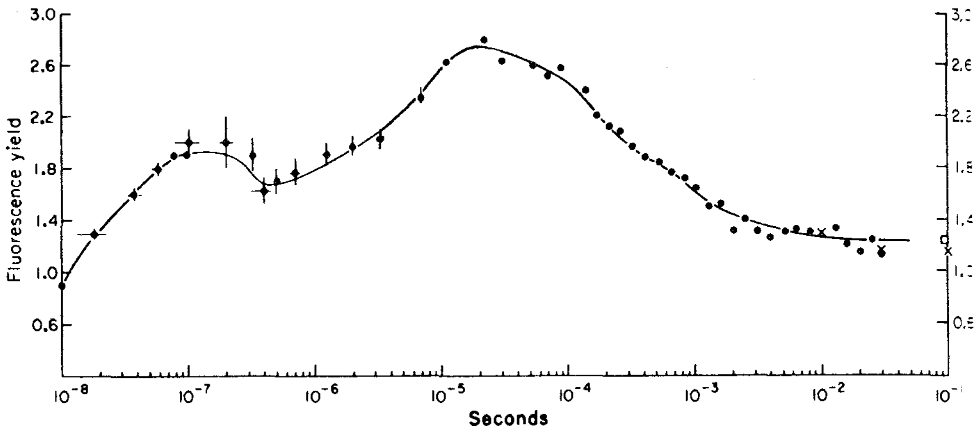


FIG. 5. Chlorophyll fluorescence yield changes in dark-adapted cells of the green alga *Chlorella* after a saturating laser flash. The rise near 20 ns is ascribed to P^+ reduction, that in the microsecond range to the disappearance of carotenoid triplets formed by the flash. The subsequent decrease is due to reoxidation of Q_A^-. (From Mauzerall, 1972.)

reduction phases except the last one—which does not affect the fluorescence yield—were observed first in fluorescence and confirmed later by absorbance measurements.

An even shorter-lived quencher is I^-, the reduced pheophytin which is thought to act as an intermediate in the reduction of Q_A. The state P $I^-Q_A^-$, accumulated by strong illumination at low potential, was found to quench fluorescence nearly as effectively as the "open" RC, as shown in Fig. 6 (Klimov *et al.*, 1977). The quenching by P^+ and I^- (and presumably by any chlorophyllous species of modified electronic configuration—singlet excited, triplet, oxidized, or reduced—in close contact with the antenna) may be explained on the basis of their absorption spectra, which show considerable overlap with the Chl fluorescence spectrum and extend well into the near-infrared (Mathis, 1981; van Gorkom and Thielen, 1982). Excitation transfer to these molecules is probably irreversible.

The quenching by I^- was recently used by Meiburg *et al.* (1983) to measure the membrane potential required to reverse the equilibrium $I^-Q_A \rightleftharpoons I\ Q_A^-$. It was found that these two components differ less than 350 mV in midpoint potential and span the full width of the dielectric barrier formed by the thylakoid membrane.

Other fluorescence quenchers may appear under nonphysiological conditions. Although not mentioned in the literature, it is now clear that

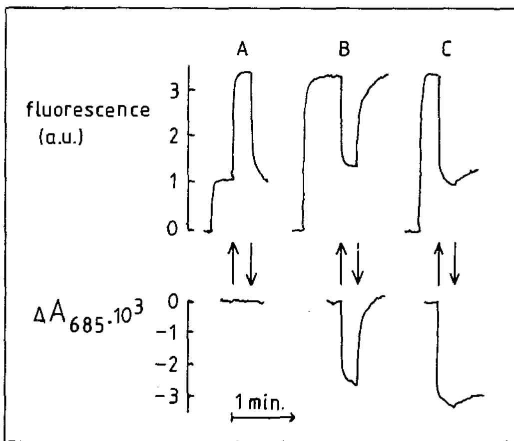


FIG. 6. Chlorophyll fluorescence yield changes in PSII particles measured with modulated excitation: (A) without additions, (B) at an ambient redoxpotential of −200 mV, (C) at −400 mV. Upward and downward arrows indicate actinic light on and off, respectively. The concomitant absorbance changes at 685 nm were spectrally identified as a photoreduction of pheophytin *a*. (From Klimov *et al.*, 1977.)

an oxidized carotenoid near the RC quenches fluorescence efficiently. This species appears on illumination at low temperature if Cyt *b*-559 is in the oxidized state (Schenck *et al.*, 1982) and at physiological temperatures in the presence of some chemicals like tetraphenylboron (Velthuys, 1981a). We have observed a pronounced fluorescence quenching in the latter case (J. P. Dekker and H. J. van Gorkom, unpublished); at low temperature the quenching by an oxidized secondary donor has been known for some time (Visser and Rijgersberg, 1975).

VIII. Conclusion

The study of changes in the fluorescence yield of Chl *a* has contributed enormously to our present knowledge of photosynthetic electron transport. Although more direct methods such as the measurement of absorbance or EPR were usually required to obtain definite answers, the questions to be answered very often originated in fluorescence studies. In all probability the measurement of fluorescence changes will continue to play this role in the discovery and first characterization of as yet undetected properties of the RC and of photosynthetic electron transport.

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11

Delayed Fluorescence: Current Concepts and Status

PAUL ANDREW JURSINIC
Northern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
Peoria, Illinois 61604

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ABBREVIATIONS AND SYMBOLS

A_1	Primary photosystem I acceptor
A_2	Secondary photosystem I acceptor
BChl	Bacteriochlorophyll
Car	Carotenoid
Car^T	Carotenoid triplet
Chl	Chlorophyll
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea, diuron
I	Pheophytin, the primary charge acceptor of the reaction center
I^-	Reduced form of I
J	Rate of generation of the chlorophyll *a* singlet state
LHC II	Light-harvesting antenna chlorophyll *a/b*–protein complex of photosystem II
P	Reaction center chlorophyll or bacteriochlorophyll
P^+	Oxidized form of P
P*	Excited singlet state of P
P^F	Radical pair state (P^+I^-) of photosynthetic bacteria
PQ	Plastoquinone
P430	Photosystem I acceptor (iron–sulfur protein)
P680	Reaction center chlorophyll of plant photosystem II

P700	Reaction center of plant photosystem I
P960	Reaction center of purple photosynthetic bacteria *R. viridis*
PSI	Photosystem I
PSII	Photosystem II
ϕ_f	Quantum yield of chlorophyll *a* fluorescence
ϕ_l	Quantum yield of delayed fluorescence
ϕ_o	Quantum yield of chlorophyll *a* fluorescence when reaction centers are open
Q_A	Primary quinone acceptor of PSII and purple bacteria
Q_A^-	Reduced form of Q_A
Q_B	Secondary quinone acceptor in its uncharged (quinone) form
Q_B^-	Q_B in its singly reduced (semiquinone) form
Q_B^{2-}	Q_B in its doubly reduced (quinol) form
RC	Reaction center
Rps.	*Rhodopseudomonas*
τ_f	Fluorescence lifetime with competing photochemical process operating
τ_0	Natural lifetime of fluorescence with no competing photochemical processes
Z	First electron donor to $P680^+$

ABSTRACT

Delayed fluorescence, which is also known as luminescence, delayed light emission, and delayed luminescence, is the glow of photosynthetic material after it is illuminated. Although delayed fluorescence reflects an insignificant loss of the total energy stored by photosynthesis, it is a sensitive intrinsic probe of photosynthesis. This chapter is a review of theories, methods, and advances made in the field of delayed fluorescence in the past few years. Earlier reviews are cited for those who desire a comprehensive point of view. Special emphasis is placed on the radical pair hypothesis and how its application bridges the phenomena of delayed fluorescence and Chl *a* fluorescence. The usefulness of delayed fluorescence as an analytical tool in photosynthesis research is shown by a discussion of recent applications and advances.

I. Introduction

An intriguing phenomenon that occurs in plant material is its glow after being illuminated. This postillumination emission of light has been referred to in the literature by a number of terms: delayed fluorescence, delayed light emission, delayed luminescence, and luminescence. Here the term delayed fluorescence will be used. The original discovery of delayed fluorescence in plant material was made by Strehler and Arnold (1951) when they tried to use the firefly luminescence technique to detect light-induced ATP formation in the green alga *Chlorella* (see Arnold, Chapter 2, this volume). If a suspension of algae that had been illuminated produced ATP, then the addition of firefly extract (luciferin) should produce an emission of light. However, it was discovered that light was emitted even when the luciferin was left out. The light emission

was, in fact, from chlorophyll *a* (Chl *a*) and was generated by fundamental photosynthetic reactions, not by the ATP–luciferin reaction. Delayed fluorescence was found to be a ubiquitous phenomenon, being observed in leaves (Strehler and Arnold, 1951), chloroplasts (Strehler, 1951), and photosynthetic bacteria (Arnold and Thompson, 1956).

Since that time, the investigation of delayed fluorescence from plant material has proved its fundamental relation to photosynthetic reactions. Although delayed fluorescence reflects an insignificant loss of the total energy stored by photosynthesis, it is a sensitive indicator of the many reactions that compose photosynthesis. This sensitivity makes delayed fluorescence an extremely complex phenomenon that is influenced by many variables. This can be very frustrating; however, with awareness and control of the variables, delayed fluorescence becomes an important intrinsic probe. If the variables are not controlled or are unknown, which has been the case in many publications, delayed fluorescence measurements lead to confusing and contradictory conclusions. Delayed fluorescence is a tool that must be used with care. At this time, prudence dictates that it be used in conjunction with other analytical measurements to obtain a complete picture of an experimental system. The interpretation of delayed fluorescence alone is less likely to lead to unequivocal conclusions than are most other techniques when they are applied individually.

This chapter will be devoted to what the author feels have been major advances in the field of delayed fluorescence over the past few years. The reader should consult the fine reviews that exist in the earlier literature to gain a comprehensive point of view. Crofts *et al.* (1971) and Fleischman and Mayne (1973) emphasized the modulation effects of membrane potential and pH gradient; Mar and Roy (1974) stressed kinetic modeling; Lavorel (1975) reviewed data, methods, and theory; Malkin (1977a) presented kinetic analysis, various theoretical mechanisms, and modulation effects; Malkin (1977b) gave a very readable general review; Amesz and van Gorkom (1978) presented a general review of the recombination hypothesis and its experimental support; Fleischman (1978) reviewed the literature characterizing delayed fluorescence and chemiluminescence in photosynthetic bacteria; Govindjee and Jursinic (1979) reviewed the relationships between the reactions of photosynthesis and Chl *a* fluorescence and delayed fluorescence.

The experimental methods for measuring delayed fluorescence will not be discussed here. The interested reader is directed to earlier reviews (Lavorel, 1975; Govindjee and Jursinic, 1979) and Lavorel *et al.*, Chapter 4, this volume.

II. Distinction of Delayed Fluorescence from Other Light Emission

A. Some General Characteristics of Delayed Fluorescence

Radiant energy striking a plant is absorbed by two pigment systems (Govindjee and Govindjee, 1975) consisting of various spectral forms of Chl *a* and accessory pigments. Virtually all of the absorbed energy is used for photochemistry (see van Gorkom, Chapter 10, and Norris and van Brakel, Chapter 3, this volume), which accounts for the nearly perfect efficiency of photosynthetic reactions in plants (Sun and Sauer, 1971) and in photosynthetic bacteria (Wraight and Clayton, 1974; Cho *et al.*, 1984). However, about 2–3% of the energy is reemitted from the pigment systems as fluorescence (Latimer *et al.*, 1956) and 0.03% as delayed fluorescence (Jursinic and Govindjee, 1982).

In oxygen-evolving organisms, delayed fluorescence is found to originate predominantly but not exclusively from photosystem II (PSII). This is supported by the following lines of evidence. In algal mutants that lack PSII, delayed fluorescence is either very weak or absent; the action spectra for PSII activity and delayed fluorescence are essentially identical; and delayed fluorescence has a 60- to 90-fold greater intensity in PSII- than in photosystem I (PSI)-enriched particles (see citations in Amesz and van Gorkom, 1978; Govindjee and Jursinic, 1979). Fluorescence of Chl *a* also originates from both photosystems. This is particularly clear from the results of the fluorescence emission spectra at 77°K, which have been reviewed by Lavorel and Etienne (1977).

A general energy level diagram for a pigment system is shown in Fig. 1 to assist in understanding how delayed fluorescence compares to other emission processes. The emission spectra of delayed fluorescence and fluorescence are essentially the same in plants (Arnold and Davidson, 1954; Arnold and Thompson, 1956; Lavorel, 1969; Sonneveld *et al.*, 1980) as well as in photosynthetic bacteria (Arnold and Thompson, 1956; Clayton, 1965; Zankel, 1969; Carithers and Parson, 1975). These spectra indicate that both fluorescence and delayed fluorescence are from the lowest excited singlet state of Chl *a* or bacteriochlorophyll *a* (BChl *a*). This is quite different from phosphorescence (Krasnovsky *et al.*, 1975), which originates from an excited triplet state and is shifted to the red end of the spectrum (lower energy) compared to the excited singlet state (see Fig. 1). Based on emission spectra, it seems that the pigment that is excited can be in the reaction center (RC) as well as in the light-harvesting system. This is most clearly shown in the blue-green alga *Anacystis nidulans*, where emission comes from RC Chl and from phyco-

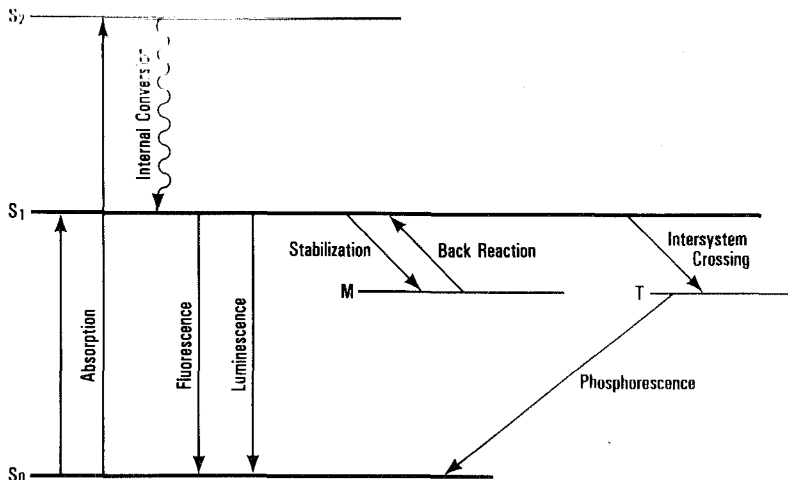


FIG. 1. Energy level (Joblanski) diagram of a pigment system in photosynthetic material. S_0, S_1, and S_2 are the electronic ground state, first excited singlet state, and second excited singlet state of the pigment system; *M* represents an undefined metastable state of the photochemical system; and *T* is the triplet state. The energy levels of the *M* and *T* states are not necessarily equal.

bilins by uphill energy transfer (Sonneveld *et al.*, 1980). The phycobilins are accessory pigments of the light-harvesting complex of *Anacystis* (see Fork and Mohanty, Chapter 16, this volume).

The origin of delayed fluorescence emission from the antenna pigment bed in plants has been demonstrated by observing polarization of delayed fluorescence. When thylakoids are put into a strong magnetic field, they orient so that their membranes are perpendicular to the direction of the field (Breton *et al.*, 1973). Under these conditions Chl *a* fluorescence is polarized, indicating an orientation of the long-wavelength transition moment, Q_y, parallel to the membrane (Becker *et al.*, 1973; Geacintov *et al.*, 1974). Delayed fluorescence in the 10–100 ms range, following illumination by continuous light, is polarized in magnetically oriented thylakoids (Farkas *et al.*, 1981b). The angle of delayed fluorescence polarization is parallel to the membrane and the Q_y transition moment, which is consistent with delayed fluorescence exciton migration from the RC to the antenna pigment bed. Delayed fluorescence emission then occurs when the excited antenna pigments decay to their ground states.

The difference between fluorescence and delayed fluorescence is in the origin of the excited singlet state of the emitting pigment molecule.

As shown in Fig. 1 for fluorescence, absorption of light leads to the population of many levels of excited singlet states. By internal conversion, these high-level excited states decay to the lowest excited singlet state, from which emission (fluorescence) occurs. The emission of fluorescence ceases within 1–2 ns when the excitation illumination, which populates the excited singlet state, is stopped. This emission has also been called *prompt* fluorescence. In photosynthetic material, most of the absorbed energy is stabilized in an undefined metastable state labeled *M* in Fig. 1, which has a lower energy than the excited singlet state. This is an equilibrium reaction highly favored in the direction of stabilization. A small probability of repopulation of the excited singlet state (back reaction) exists. This gives rise to delayed fluorescence, the generation of the excited singlet state from a lower-energy metastable state. The emission of delayed fluorescence will continue as long as the metastable state is populated. This can be up to hours after excitation illumination is stopped.

Two characteristics distinguish fluorescence and delayed fluorescence. Since delayed fluorescence originates from a metastable state, it is affected by many variables (temperature, membrane potential, external magnetic fields) to which fluorescence often, but not always, is immune. This will be dealt with in Sections III,B,1 and III,B,4. The other major difference is the lifetime of the emission. The lifetime of Chl *a* fluorescence is, in most cases, less than 1.5 ns (see reviews by Govindjee and Jursinic, 1979, and Moya *et al.*, Chapter 7, this volume). Delayed fluorescence lifetimes are much longer and quite diverse: 2–4 ns (Shuvalov and Klimov, 1976; Godik and Borisov, 1979, 1980), 100–200 ns (Sonneveld *et al.*, 1980), and many different components in the microsecond, millisecond, and second ranges (consult any of the earlier reviews cited in the Introduction).

B. *Theories of Delayed Fluorescence*

1. TRIPLET THEORY

On receiving excitation energy, the PSII reaction center produces triplet states by intersystem crossing (Stacy *et al.*, 1971) (see Fig. 1). These triplet excitations may decay by radiationless transitions, or two triplets may undergo fusion and produce an excited singlet state and a ground state. These excited singlet states then decay to the ground state with the emission of delayed fluorescence. The production of triplet states (see Hoff, Chapter 9, this volume) in plants has been observed only when normal photochemistry has been blocked by light saturation or inactiva-

tion. When RCs are closed by light pulses of high intensity, Chl and carotenoid (Car) triplets are formed and have been detected by their absorption change (Mathis, 1977; Breton *et al.*, 1979) and Chl *a* fluorescence quenching (Mauzerall, 1976). Also, the presence of triplets has been shown by the existence of phosphorescence (Krasnovsky *et al.*, 1975, 1977) (see Fig. 2) and by microwave-induced changes in Chl *a* fluorescence at 2°K (Hoff *et al.*, 1977a). It has been suggested that the thermoluminescence glow peak, labeled Z, arises from metastable triplet states when photochemistry is saturated (see, e.g., Sane *et al.*, 1977). Details of thermoluminescence are presented by Sane and Rutherford (Chapter 12, this volume).

There is no doubt that triplet states can be generated in photosynthetic systems. However, it has not been demonstrated that triplet–triplet fusion gives rise to delayed fluorescence in plants under nonsaturating light conditions. Section III,A,1 discusses how the triplet state of the RC radical pair $[P680^{+}\ I^{-}]^{T}$ may explain components of delayed fluorescence decay in the microsecond range.

2. CHARGE RECOMBINATION THEORY

This is the most widely accepted theory of delayed fluorescence. Essentially, it states that photosynthesis is a photoreaction that leads to

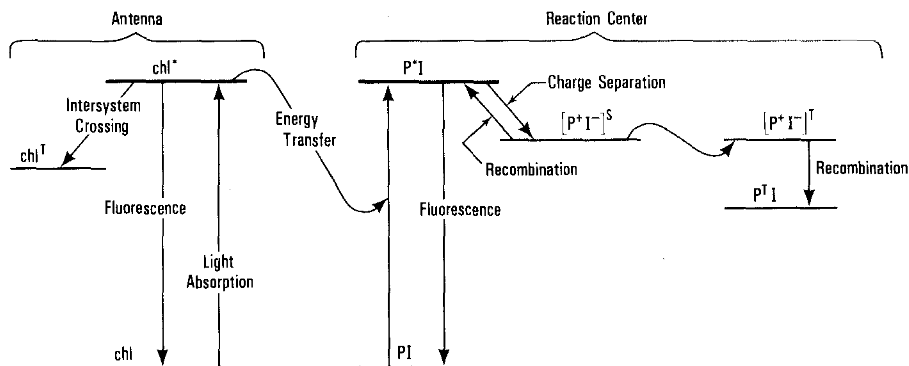
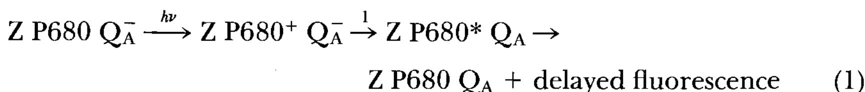


FIG. 2. Scheme for light absorption and charge separation in photosynthetic material that includes a radical pair $P^{+}I^{-}$. As discussed in the text, this arrangement is applicable to both photosynthetic bacteria and plants. The symbols are defined as follows: Chl, antenna chlorophyll; Chl* antenna chlorophyll in the excited singlet state; P, reaction center pigment Chl *a* or BChl *a*; I, primary electron acceptor pheophytin or bacteriopheophytin in the reaction center; P*, reaction center chlorophyll or bacteriochlorophyll in the excited singlet state; P^{T}, the triplet state; $[P^{+}I^{-}]^{S}$, the radical pair in the singet state; and $[P^{+}I^{-}]^{T}$ the radical pair in the triplet state. In photosynthetic bacteria, antenna Chl is replaced by BChl.

oxidized and reduced products, and delayed fluorescence is due to recombination of these charged products. For PSII, this can be written as:



$$Z\ P680\ Q_A^- \xrightarrow{h\nu} Z\ P680^+\ Q_A^- \xrightarrow{1} Z\ P680^*\ Q_A \rightarrow$$
$$Z\ P680\ Q_A + \text{delayed fluorescence} \qquad (1)$$

where $P680^+$ and $P680^*$ are the RC Chls of PSII in the oxidized and excited singlet states, respectively; Q_A is the primary quinone electron acceptor; Z is the first electron donor to the RC; and reaction 1 is charge recombination. This theory was originally described by Arthur and Strehler (1957) and Strehler and Lynch (1957) and was presented in the above framework by Lavorel (1969), Mohanty *et al.* (1971), and van Gorkom and Donze (1973). Besides these original papers, details of the theory and supporting evidence have been thoroughly described in the reviews cited at the beginning of this chapter. The charge recombination theory has recently been altered to accommodate the role of pheophytin or bacteriopheophytin in the primary photochemistry of RCs. The primary photoreaction is now believed to be generation of the radical pair $P680^+I^-$, where I^- is reduced pheophytin. This theory is discussed in the next section.

3. RADICAL PAIR HYPOTHESIS

This theory is a refinement of the charge recombination theory and was first used to describe the charge separation reaction that occurs in RCs of photosynthetic bacteria (Parson and Ke, 1982; Hoff, Chapter 9, this volume). It has been found to be applicable to PSI and II RCs of plants. This theory also gives an understanding of temperature and magnetic field effects on delayed fluorescence.

The RC of photosynthetic bacteria contains a photochemically reactive BChl complex (P), a bacteriopheophytin which is the electron acceptor (I), and two quinones (Q_A and Q_B) (Parson and Cogdell, 1975; Okamura *et al.,* 1982; Norris and van Brakel, Chapter 3, this volume). The antenna pigments absorb light and transfer energy to P, which gives rise to the excited singlet state (P*). Within 5 ps charge separation occurs, and a radical pair state (P^+I^-, also known as P^F) is formed (Parson and Cogdell, 1975; Blankenship and Parson, 1979). Under conditions of active photosynthesis, the electron is transferred from I^- to Q_A with a halftime of 120–240 ps (see, e.g., Rockley *et al.,* 1975). The electron on Q_A^- is then passed on to Q_B in about 100 μs (Blankenship and Parson, 1979).

When bacterial photosynthesis is inactivated by chemical reduction of Q_A, the lifetime of the P^+I^- state is 6–10 ns (Cogdell *et al.,* 1975; Parson

and Cogdell, 1975). The P^+I^- state can decay to the ground state as a singlet state or it can proceed by way of a metastable triplet state $[P^+I^-]^T$ (see Fig. 2). The spins on P^+ and I^- oscillate between the singlet $[P^+I^-]^S$ and triplet $[P^+I^-]^T$ states. When an external magnetic field is applied, the triplet state splits into three energy sublevels, T_1, T_0, and T_{-1}, which correspond to the different spin quantum numbers. Only the T_0 level is close to the singlet energy level, so singlet-to-triplet exchange is reduced in a magnetic field. This greatly changes the manner in which $[P^+I^-]^S$ decays to ground state. It is this effect of magnetic field on reactions that distinguishes the radical pair mechanism from other types of chemical reactions (Muss *et al.*, 1977). Magnetic field effects are not observed in most chemical reactions because the splitting of energy levels caused by an external magnetic field is insignificant compared to thermal fluctuations.

The $[P^+I^-]^S$ state can also recombine to give the excited singlet state P*I, which can decay to the ground state with emission of light (delayed fluorescence) (Shuvalov and Klimov, 1976; Godik and Borisov, 1979, 1980). This emission is considered to be delayed fluorescence and not prompt fluorescence for the following reasons:

(1) In active RCs of bacteria with Q_A oxidized, fluorescence occurs with a 60–200 ps lifetime (Borisov and Godik, 1973; Paschenko *et al.*, 1977; Godik and Borisov, 1979). When Q_A is reduced, the lifetime of the emission increases 15- to 40-fold to 2.5–3 ns (Godik and Borisov, 1979). On the other hand, the measured quantum yield increases at most four-fold. In the case of emission by a single fluorophore, the quantum yield and the lifetime change in parallel according to the following relationship: $\phi_f = \tau_f/\tau_0$ where ϕ_f is the quantum yield of fluorescence, τ_f is the fluorescence lifetime, and τ_0 is the natural lifetime (Guilbault, 1973). The breakdown of this relationship for emission from RCs of bacteria can be explained by the occurrence of fluorescence and delayed fluorescence simultaneously.

(2) The $[P^+I^-]^S$ state is about 0.04–0.08 eV lower in energy than the P*I state, which is indicated by a decrease in delayed fluorescence as the temperature is decreased (Shuvalov and Klimov, 1976; van Grondelle *et al.*, 1978; Godik and Broisov, 1979; Godik *et al.*, 1982). This temperature dependence (thermal activation energy) is expected for delayed fluorescence but not for fluorescence.

(3) An external magnetic field decreases the RC triplet yield and induces an emission increase (Blankenship *et al.*, 1977; Hoff *et al.*, 1977b; Rademaker *et al.*, 1979). This is in agreement with the radical pair hypothesis, since the magnetic field favors the singlet over the triplet decay

route for P^+I^-. This magnetic field effect is a quality of delayed fluorescence, not fluorescence, since the first singlet excited state (from which fluorescence originates) is not changed by a magnetic field.

The data discussed up to this point support the radical pair model of RC photochemistry. However, in one detailed study that used RCs from *Rhodopseudomonas sphaeroides* and *Rhodospirillum rubrum,* deviations from the theory were found (Schenck *et al.*, 1982). The decay rates of $[P^+I^-]^S$, the quantum yield of $[P^+I^-]^T$, and the dependence on light intensity of the decay kinetics of delayed fluorescence all varied with temperature, magnetic field, and isotope replacement. Known decay paths of $[P^+I^-]^S$ did not adequately explain these relationships. Nevertheless, at present the radical pair description is the most comprehensive model available for the RC (see Norris and van Brakel, Chapter 3, this volume).

This discussion so far has given evidence supporting the radical pair hypothesis of photoreactions and delayed fluorescence that occur in the RC of bacteria. This hypothesis also seems applicable in plant photosynthesis.

In plant preparations (algae, chloroplasts, thylakoids, and PSII particles), the emission yield increases three- to fourfold on photoreduction of the quinone acceptor Q_A, with a similar increase in emission lifetime from 0.4 to 1.7 ns (Briantais *et al.*, 1972; Moya *et al.*, 1977). In these cases the $\phi_f = \tau_f/\tau_0$ relationship holds. When Q_A is chemically reduced with dithionite the emission lifetime increases from 0.4 to 4.3 ns, whereas the quantum yield of emission increases about 2.5-fold (Shuvalov *et al.*, 1980). Clearly, the $\phi_f = \tau_f/\tau_0$ relationship does not hold in this case. Shuvalov *et al.* (1980) also measured an absorption change with a 4-ns decay time that could be attributed to the disappearance of $P680^+I^-$. They concluded that the 4-ns emission was actually delayed fluorescence generated when $P680^+I^-$ recombined.

The reason for the discrepancy in the data when Q_A is reduced photochemically or chemically may be the trivial one of different sample materials. However, the following explanation that involves delayed fluorescence is also possible. The Chl *a* fluorescence yield (ϕ_f) is a function of the many decay routes of the singlet excited state:

$$\phi_f = k_f/(k_f + \kappa_p + k_d + k_t) \qquad (2)$$

where the various first-order rate constants are for fluorescence (k_f), trapping at a photochemical center (k_p), nonradiative deactivation (k_d), and end transfer to another pigment (k_t) (Lavorel and Etienne, 1977). It has generally been assumed that as all Q_A becomes reduced photochemistry ceases, k_p becomes zero, and the fluorescence yield attains a maxi-

mum value. However, in terms of the radical pair hypothesis, this is not the case. With Q_A fully reduced, reaction 3 in Fig. 3 is halted. Reaction 2 in Fig. 3 becomes enhanced in the reverse direction, and the singlet excited state of Chl *a* is now generated as delayed fluorescence. Reactions 1 and 2 of Fig. 3 continue in the forward direction; that is, photochemistry continues. For centers in the P680 I Q_A or P680 I Q_A^- state, charge separation still takes place, so k_p is unchanged, as is ϕ_f. The increase in quantum yield of fluorescence is, in this picture, a greatly enhanced delayed fluorescence emission (Klimov and Krasnovsky, 1981). For a different view, see van Gorkom, Chapter 10, this volume. In fact, when photochemistry is inhibited by chemical reduction of Q_A and photochemical reduction of I, the fluorescence yield does not increase but decreases to approximately its level when Q_A is oxidized (Klimov *et al.*, 1977). The apparent increase in fluorescence lifetime when Q_A is reduced is due to this delayed fluorescence, which has a 6–10 ns lifetime, not being differentiated from actual Chl *a* fluorescence. The reported validity of the $\phi_f = \tau_f/\tau_0$ relationship as Q_A was photochemically reduced (Briantais *et al.*, 1972; Moya *et al.*, 1977) must have been coincidental.

In PSII particles that had Q_A reduced with dithionite, the light emis-

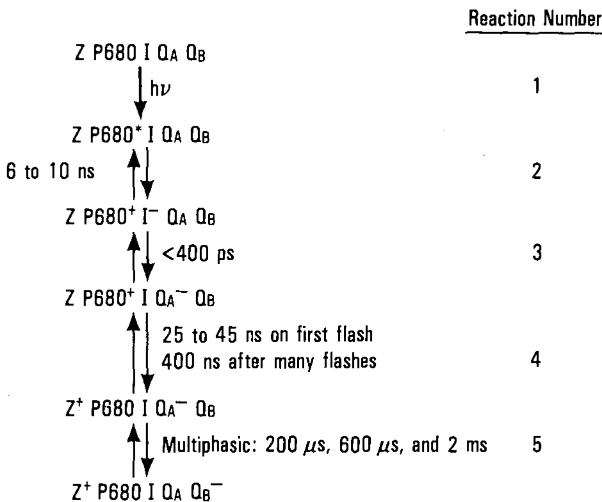


FIG. 3. Schematic illustration of charge transfer in PSII of plants. Z is the first electron donor to the reaction center Chl P680, I is the pheophytin, Q_A is the primary quinone electron acceptor, and Q_B is the secondary quinone electron acceptor. References for the lifetimes of the various reactions are: reactions 2 and 3 (Klimov and Krasnovsky, 1981), reaction 4 (van Best and Mathis, 1978; Sonneveld *et al.*, 1979; Brettel and Witt, 1983; Eckert *et al.*, 1984), and reaction 5 (Mauzerall, 1972; Stiehl and Witt, 1969).

sion was observed to decrease on lowering on the temperature from 0 to −100°C, which gave an activation energy of 0.04–0.08 eV (Klimov *et al.*, 1978). This immediately suggests that the emission is delayed fluorescence because Chl fluorescence does not exhibit an activation energy. This emission was believed to originate from recombination of the radical pair $P680^+I^-$, since photoreduction of I^- completely inhibited the emission (Klimov *et al.*, 1978).

The radical pair origin of delayed fluorescence in higher plants and algae was also confirmed by modulation of its amplitude by a magnetic field. In spinach chloroplasts and the green alga *Chlorella*, the application of a magnetic field caused an increase in delayed fluorescence (Rademaker *et al.*, 1979). This is expected in the radical pair hypothesis because the triplet decay mode $[P680^+I^-]^S \rightarrow [P680^+I^-]^T$, which is nonluminescent, becomes less favorable in a magnetic field.

Electric field effects on Chl *a* fluorescence of thylakoids have also given support to the radical pair hypothesis. Intense electric fields can be generated in thylakoid membranes by application of external electric gradients. For this to occur the thylakoids are suspended in hypotonic medium, where they form spheres or blebs (Arnold and Azzi, 1977; see Section III,B,1.). In dark-adapted blebs the applied electric field causes the Chl *a* fluorescence to increase (Meiburg *et al.*, 1983; van Gorkom *et al.*, 1984). This has been interpreted as a blockage of reaction 3 in Fig. 3 by the electric field. If the artificial electron donor tetraphenylboron is present, then illumination of blebs causes Q_A to be reduced without the oxygen-evolving system being oxidized, and the Chl *a* fluorescence yield is high. Application of an electric field under these conditions causes a decrease in Chl *a* fluorescence yield. The interpretation is that the electric field causes the electron on Q_A^- to transfer back to I, which gives an RC in the form of P680 I^- Q_A. This form of the RC is closed to photochemistry. $P680^+I^-$ cannot be generated, so the recombination and its 2–4 ns delayed fluorescence (variable fluorescence) do not occur. This is seen as an inhibition of Chl *a* fluorescence with application of the electric field.

III. Delayed Fluorescence in Higher Plants and Algae

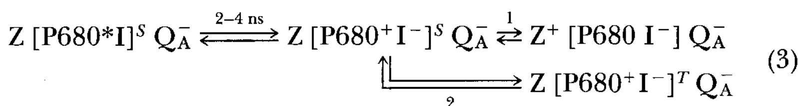
A. *A Bridge between Nanosecond and Microsecond Range Decay of Delayed Fluorescence*

According to the radical pair hypothesis, delayed fluorescence originates from the recombination of $P680^+I^-$ and occurs with a 2–4 ns

lifetime. However, delayed fluorescence has decay kinetics in the range of hundreds of ns and beyond (see any of the earlier reviews cited in Section I). The answer to this is, in principle, quite simple: the $P680^+I^-$ charge pair is in equilibrium with many other states. All of these states have their own lower decay rates and physical characteristics. These equilibria are highly unfavorable to the $P680^+I^-$ state, so these slower-decaying delayed fluorescence components are much lower in quantum yield. The fundamental 2–4 ns decay (recombination rate) has been measured (Klimov *et al.*, 1978) only when equilibrium with these other states has been eliminated by proper chemical treatment of the sample. The study of delayed fluorescence has now turned to the identification and characterization of these equilibrium states.

1. WHEN THE PRIMARY QUINONE ACCEPTOR (Q_A) IS REDUCED

In chloroplasts and algae treated with dithionite, delayed fluorescence decay components with 100–200 ns and 0.7–1 μs lifetimes have been observed (van Best and Duysens, 1977; Sonneveld *et al.*, 1980). The 0.7–1 μs component is enhanced if Q_A is reduced (van Best and Duysens, 1977) and if the measurement is made at room temperature. (For the position of Q_A in PSII, see Fig. 1 in Duysens, Chapter 1, this volume.) At low temperature, in samples treated with hydroxylamine (which affects the electron donor side of PSII), and at low pH this component is eliminated (Sonneveld *et al.*, 1980). The 100–200 ns component is greatly enhanced at 77°K. Also, on application of an external magnetic field, this component has an increased lifetime and amplitude (Sonneveld *et al.*, 1980). It is likely that the equilibrium states these components reflect can be represented as follows:



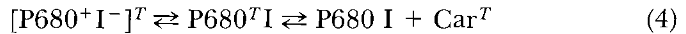
$$\mathrm{Z\,[P680^{*}I]^{S}\,Q_A^-} \xrightleftharpoons{2\text{–}4\text{ ns}} \mathrm{Z\,[P680^{+}I^{-}]^{S}\,Q_A^-} \overset{1}{\rightleftharpoons} \mathrm{Z^{+}\,[P680\;I^{-}]\,Q_A^-}; \quad \mathrm{Z\,[P680^{+}I^{-}]^{S}\,Q_A^-} \overset{2}{\rightleftharpoons} \mathrm{Z\,[P680^{+}I^{-}]^{T}\,Q_A^-} \qquad (3)$$

where the symbols are as previously defined, reaction 1 is the reduction of $P680^+$ by the donor Z, and reaction 2 is a spin realignment from the singlet to the triplet state.

The 0.7–1 μs component is associated with reaction 1 in Eq. (3). The Z $P680^+ \rightarrow Z^+P680$ reaction takes place with a lifetime of 20–45 ns on the first flash (van Best and Mathis, 1978), approximately 400 ns after many flashes (Sonneveld *et al.*, 1979), 25 μs after hydroxylamine treatment (den Haan *et al.*, 1976), and 200 μs at low pH (Haveman and Mathis, 1976). A more thorough discussion of the kinetics after many flashes is given in Section III,B,9. After many flashes the kinetic agreement between delayed fluorescence decay and $P680^+$ reduction is good, and the

elimination of the 0.7–1 μs component by hydroxylamine or low pH is explained. Under these conditions of inhibition, the 0.7–1 μs component of delayed fluorescence decay should be replaced by 25 and 200 μs components. To my knowledge, these components have not been looked for under the condition of chemical reduction of Q_A.

The 100–200 ns component has been attributed to reaction 2 in Eq. (3). The triplet character of this equilibrium state is indicated by its magnetic field dependence (Sonneveld *et al.,* 1980). It is interesting to note that generation of Car^T (carotenoid triplets) is dependent on the redox state of the PSII RC. When Q_A is chemically reduced, production of Car^T is enhanced; but when P680 I^- Q_A^- is generated photochemically, it is inhibited (Klimov *et al.,* 1980). It is possible that reaction 2 starts a series of equilibria in the triplet manifold as follows:



$$[P680^+ I^-]^T \rightleftarrows P680^T I \rightleftarrows P680\ I + Car^T \quad (4)$$

where all the symbols are as before. The Car^T is known to decay with a lifetime of 3–7 μs (Breton *et al.,* 1979; Mathis *et al.,* 1979).

Reaction centers can be put in the P680 I Q_A^- state, which is closed to *normal* photochemistry. Illumination of PSII, with DCMU present, causes P680 I Q_A^- to form with the reoxidation of Q_A^- having a 1-s half-time (Bennoun, 1970). When the oxygen-evolving system has been inhibited with hydroxylamine, decay of Q_A^- when DCMU is present has a >20-min half-time (Joliot and Joliot, 1981). Both these methods for generating Q_A^- were used by Jursinic and Govindjee (1982). They found that excitation when Q_A^- was present still gave delayed fluorescence with components with 5–10 μs and 35–40 μs half-time. It was suggested (Jursinic and Govindjee, 1982) that charge separation occurred between P680 and an acceptor other than Q_A, perhaps I. This charge pair then recombined to give the μs-range delayed fluorescence. The occurrence of μs-range delayed fluorescence after excitation with Q_A^- was present had also been reported by Lavorel (1973) and recently by Meiburg *et al.* (1984). Using the technique of double-flash excitation of samples with DCMU present, Meiburg *et al.* (1984) demonstrated that delayed fluorescence when Q_A was reduced did show a recovery time. By altering the time between flashes it was found that delayed fluorescence after the second flash recovered to its maximum amplitude with a 35-μs half-time. Clearly, this had no relation to the 1-s half-time of reoxidation of Q_A^-, but instead was most likely related to another intermediate X_a^- or I^- reoxidation, as proposed by Eckert and Renger (1980). [See van Gorkom, Chapter 10, this volume, for a discussion of X_a (≡*X* or Q_2).]

It is possible that equilibrium between the triplet states described earlier underlies the μs-range delayed fluorescence decay that has been

reported under conditions when the RC was in a state (P680 I Q_A^-) closed to normal photosynthesis (Lavorel, 1973; Jursinic and Govindjee, 1982). This may be a case where triplet states do lead to delayed fluorescence, however, by repopulating the radical pair state $[P680^+I^-]^S$ through an equilibrium that involves spin realignment.

2. WHEN THE PRIMARY QUINONE ACCEPTOR (Q_A) IS OXIDIZED

When Q_A is not chemically reduced, the reaction scheme in Fig. 3 takes place. The electron on I moves to Q_A in less than 400 ps (Klimov and Krasnovsky, 1981), and it is this charge transfer that results in a major stabilization of the charge separation against the 2–4 ns $P680^+I^-$ recombination. All of the reaction steps in Fig. 3 are equilibria whose reverse reactions lead to regeneration of $P680^+I^-$, the ultimate source of delayed fluorescence. All of these equilibrium states have different kinetics, and this gives rise to the large number of decay components of delayed fluorescence in the microsecond and longer range (see earlier reviews cited in Section I). The condition of Q_A being oxidized or reduced prior to illumination results in formation of different equilibrium states and different decay components of delayed fluorescence. The identification of the equilibrium state a particular component is associated with is still not complete. The large number of decay components make proper experimental controls extremely important so that correct conclusions can be drawn from delayed fluorescence data.

B. New Applications and Advances in the Understanding of Delayed Fluorescence

1. EFFECTS OF MEMBRANE POTENTIAL ON DELAYED FLUORESCENCE

As previously explained, delayed fluorescence in plants originates from the repopulation of excited singlet states of Chl *a* from stored energy. The large-scale loss of this energy is avoided by charge migration and stabilization into states that have a high activation energy for recombination of the charge (Arnold and Azzi, 1968). Shifts in the thylakoid membrane pH gradient or electric potential can greatly stimulate delayed fluorescence by decreasing these activation energy barriers. The literature in this area has been discussed in earlier reviews (Lavorel, 1975; Malkin, 1977a; Govindjee and Jursinic, 1979).

A number of new developments have occurred in the area of modulation of delayed fluorescence by electric fields across thylakoid mem-

branes. The stimulation of delayed fluorescence is best understood in the charge recombination theory. Charge separation is known to be vectorial across the thylakoid membrane (Witt, 1979; Junge and Jackson, 1982). An electric field aligned with the dipole axis of the separated charge can shift the potential energy of the system and alter the probability or rate of charge recombination. The electric field can be generated as a diffusion potential through a salt jump (Kraan *et al.*, 1970), as a photochemically generated potential (Witt, 1979; Junge and Jackson, 1982), and as an externally applied electric field (Arnold and Azzi, 1971). All of these techniques have been used to stimulate delayed fluorescence.

Delayed fluorescence in the millisecond range, following illumination with continuous light or multiple flashes (the phosphoroscope technique), shows enhancement by an electric field. This was not the case for ms delayed fluorescence of thylakoids after single-flash excitation (Jursinic *et al.*, 1978). Jursinic *et al.* concluded that a proton gradient or low internal pH of the thylakoid was needed for the electric field enhancement of ms-range delayed fluorescence.

If broken chloroplasts are suspended in a hypotonic medium, they will expand into spherical objects known as blebs (Arnold and Azzi, 1977). Millisecond-range delayed fluorescence in blebs, following continuous illumination (Arnold and Azzi, 1977) or a single flash (Ellenson and Sauer, 1976), can be enhanced 1000-fold by an externally applied electric field. This electric field enhancement after a single flash is in apparent disagreement with the work of Jursinic *et al.* (1978). A possible explanation may be the different preparations used: thylakoids and blebs. Perhaps blebs have a low internal pH or proton gradient under dark-adapted conditions and thylakoids do not. In support of this possibility is the fact that the field enhancement of delayed fluorescence was abolished when the membrane integrity of blebs was destroyed by sonication, treatment with the nonionic detergent Triton X-100, or heating (Ellenson and Sauer, 1976).

The electric field induced within the membrane shows a strong angular dependence with respect to the direction of the external field (Ellenson and Sauer, 1976). This selectively stimulates specific regions of the bleb membrane. Since Chl *a*, from which delayed fluorescence originates, has a particular orientation in the membrane (Breton and Vermeglio, 1982), a high degree of polarization is expected from the electric field-enhanced delayed fluorescence. A significant electroselection of the emission polarization has been found (Farkas et al., 1980, 1981a; de Grooth and van Gorkom, 1981). These results support the vectorial charge separation of PSII across the thylakoid membrane and the orien-

tation of the long-wavelength transition moment, Q_y, of Chl *a* parallel to the plane of the membrane.

The electric field stimulation of delayed fluorescence has usually been explained as an increase in the rate of the back reaction. Another possibility is that the electric field increases the probability that recombination will lead to the emission of a photon. This possibility was investigated by de Grooth and van Gorkom (1981), who found that an electric field pulse of a few ms duration caused a bust of delayed fluorescence as well as a drop in Chl *a* fluorescence that corresponded to a drop in Q_A^- concentration. The integrated delayed fluorescence and extent of change of the Q_A^- concentration remained the same with different durations of electric field pulses. Thus, the proportionality between recombination and photon emission was not altered by the electric field.

The external electric field stimulation of delayed fluorescence rises rapidly (R phase), within about 50 μs in chloroplasts suspended in distilled water, and subsequently decays in less than 1 ms to a slow phase (S phase) (Ellenson and Sauer, 1976). These phases probably originate from different precursors and membrane locations and may provide a technique for studying different locations on the thylakoid membrane. New information has now become available about these phases. At low pH the phases are kinetically very distinct; however, at pH 9 they are no longer separated (Symons *et al.,* 1984a). When chloroplasts are suspended in hypotonic medium, blebs form over a 1-h period. During the first few minutes of hypotonic treatment only the S phase is observed, and with longer times the R phase develops (Symons *et al.,* 1984b). Another difference between the phases is that the R phase is polarized perpendicular to the applied field, whereas the S phase is not polarized (Farkas *et al.,* 1980).

The magnitude and kinetics of the electric field stimulation of delayed fluorescence are strongly dependent on the electric conductivity of the bleb membrane (Farkas *et al.,* 1981a). This property has been used to study ionophores that increase membrane conductivity (Farkas *et al.,* 1982). Information was obtained about the kinetic differences between ionophores that act as ion or proton carriers and those that form channels for ions and protons and about their ionic selectivity. This may be an important technique for better understanding membrane and ionophore interactions.

Delayed fluorescence in the microsecond range was found not to be affected by membrane fields (Jursinic *et al.,* 1978). It was hypothesized that the recombining charges do not span the entire thylakoid membrane, and therefore they have a reduced field effect. It was concluded that the primary charge separation spanned less than 0.5 nm, whereas

the secondary charges that give rise to millisecond-range delayed fluorescence were approximately 1.1 nm apart (Jursinic *et al.*, 1978). Measurements by Venediktov *et al.*(1980) of ms-delayed fluorescence enhanced by a diffusion potential also showed the separation to be approximately 1.2 nm. However, a separation distance of 2.5 nm was reported by Ortoidze *et al.* (1979) based on external electric field stimulation of millisecond delayed fluorescence from dried films of chloroplasts. The latter value is questionable because there is a poor fit between the experimental and theoretical curves for stimulation of delayed fluorescence versus field strength, on which it is based.

The absence of electric field stimulation of microsecond-range delayed fluorescence (Jursinic *et al.*, 1978) has recently been confirmed (Meiburg *et al.*, 1984) for an external electric field applied to blebs. However, this lack of stimulation was observed only if the field was applied during the excitation flash. If the electric field was applied as a pulse after the excitation flash, stimulation of delayed fluorescence was observed. Thus, Meiburg *et al.* suggested that delayed fluorescence in the μs range resulted from two different mechanisms: the field-sensitive delayed fluorescence originates from recombination of $P680^+$ Q_A^- and the field-insensitive delayed fluorescence from recombination of $P680^+$ X_a^-. [X_a is a PSII acceptor that is observed to function when Q_A is reduced (Eckert and Renger, 1980; see Section III,A,1).] The $P680^+$ Q_A^- charge pair would span the entire thylakoid membrane and so be exposed to the total electric field, while the $P680^+$ X_a^- charge pair would not. This is, in principle, the same as the argument already employed by Jursinic *et al.* (1978). Meiburg *et al.* (1984) suggest that μs-range delayed fluorescence originates predominantly from recombination of the $P680^+$ X_a^- charge pair, which is insensitive to applied electric fields.

As discussed earlier, chromatophores of photosynthetic bacteria have delayed fluorescence with a 4–6 ns lifetime, arising from recombination of P^+ and I^-. This electron transfer is believed to be vectorial with some component across the chromatophore membrane. This hypothesis concerning the primary charge separation in photosynthetic bacteria is upheld. A transmembrane electric potential generated by hydrolysis of ATP (Borisov *et al.*, 1980) or a light-induced membrane potential (Kotova *et al.*, 1981) caused an increase in intensity and a shortening of the lifetime of delayed fluorescence. This was a rather direct demonstration of an increased recombination rate caused by an electric field.

2. DEPENDENCE ON EXCITATION INTENSITY

Results related to the dependence of the amplitude of delayed fluorescence on excitation intensity fall into two categories: (1) dependence on

the square of the intensity (I^2) at low light levels and linear dependence at high light levels (Jones, 1967; Lavorel, 1971; Stacy *et al.*, 1971), and (2) linear dependence at low light levels with saturation at levels of light that saturate PSII photochemistry (Ruby, 1971; Zankel, 1971; Jursinic and Govindjee, 1977a; Wong *et al.*, 1978). As pointed out by McCauley and Ruby (1981), the I^2 dependence was observed when delayed fluorescence was measured with the phosphoroscope technique, whereas the linear dependence occurred when microsecond and submicrosecond excitation flashes were used. McCauley and Ruby found that delayed fluorescence originated from two types of RCs; one type had a small antenna and delayed fluorescence that was constant with flash number, while the other type had a large antenna and significant delayed fluorescence only after two or more flashes, that is, after multiple hits. These findings provide an explanation for the different light intensity dependences found with the phosphoroscope and single-flash methods. In the phosphoroscope measurement, the excitation flashes are of sufficient duration that multiple hits can occur. Therefore at low light intensities, the RCs with large antennas are preferentially excited, and the I^2 dependence of multiple hits is observed. At higher intensities, the centers with large antennas become saturated and the centers with small antennas become important, exhibiting their linear dependence on excitation intensity. When delayed fluorescence is measured with single flashes of microsecond or shorter duration that do not give significant multiple hits, delayed fluorescence comes from the centers with small antennas (McCauley and Ruby, 1981), which are associated with linear dependence.

3. A CONTINUUM OF KINETIC STATES

Decay of delayed fluorescence in the seconds range after continuous illumination is known to take two forms: $L(t) = L(0)/(1 + kt)$ or $L(t) = L(0)/(1 + kt)^2$ where $L(t)$ and $L(0)$ are the delayed fluorescence at any time and at time zero after the excitation light is terminated, and k is a decay constant (Mar and Roy, 1974; Lavorel, 1975; Ellenson and Sauer, 1976). The first equation is specific for a reaction between reactants of the same concentration, a biequimolecular reaction. In the seconds time range, the reactants are expected to be the S states of the oxygen-evolving complex (see Wydrzynski, 1982) and the reduced quinone molecules. Heating and Tris washing were used to change the concentration of reactants in the oxygen complex and so disturb the equimolecular arrangement. The biequimolecular form of the kinetic decay was unaltered (Lavorel and Dennery, 1982). Clearly, there was another reason for this type of decay kinetics besides equal concentrations of reactants.

A mathematical analysis was completed (Lavorel and Dennery, 1982) that predicted the biequimolecular type of decay without requiring reactants of the same concentration. Lavorel and Dennery had to assume that there were many equilibrium states with different decay constants and that these states became exponentially distributed in the seconds time range. These equilibrium states were not identified, and what controlled their distribution was not determined. This work has emphasized the danger in using decay kinetics for predicting reaction mechanisms. Also, the existence of a continuum of equilibrium (kinetic) states is consistent with the large number of charge carriers and reaction pathways occurring in photosynthesis.

4. EFFECTS OF Cl^- DEPLETION

It has long been recognized that Cl^- anions are required for maximal rates of electron transport in isolated chloroplasts (Izawa *et al.*, 1969, 1983; Govindjee *et al.*, 1983). Cl^- is known to function on the electron donor side of PSII, perhaps near the manganese of the oxygen-evolving complex of PSII (see Fig. 1 in Duysens, Chapter 1, this volume). Delayed fluorescence has been successfully used to provide a better understanding of the reversible inhibition caused by Cl^- depletion.

The slow components (in the seconds range and longer) of delayed fluorescence decay are associated with the recombination of S_2 and S_3 states of the oxygen evolution system (Barbieri *et al.*, 1970; Joliot *et al.*, 1971; Lavorel, 1975) with Q_A^- and Q_B^- (also see Sane and Rutherford, Chapter 12, this volume). In Cl^--depleted samples, this slow component of delayed fluorescence decay is inhibited after a single flash (Muallem and Laine-Boszormenyi, 1981; Theg *et al.*, 1984) or continuous illumination (Theg *et al.*, 1984). These results are consistent with the idea that one effect of Cl^- depletion is to increase the stabilization of the S_2 and S_3 states of the oxygen evolution system. These states are stabilized against recombination and the delayed fluorescence associated with it.

Samples depleted in Cl^- also have an altered microsecond-range delayed fluorescence. The delayed fluorescence intensity 150 μs after a flash is known to oscillate with a period of four in phase with oxygen flash yields (Zankel, 1971) (see Fig. 4). When a dark-adapted sample is illuminated with light flashes, charged states (S states*) of the oxygen-evolving system are generated (see a review by Wydrzynski, 1982). After the third flash the most highly charged state, S_4, is formed and leads to

* The S states of the oxygen-evolving complex (S_0, S_1, S_2, S_3, and S_4) should not be confused with the ground state ($\mathbf{S}_0$) and excited states ($\mathbf{S}_1$ and $\mathbf{S}_2$) of Chl or BChl, discussed earlier in this chapter.—editors.

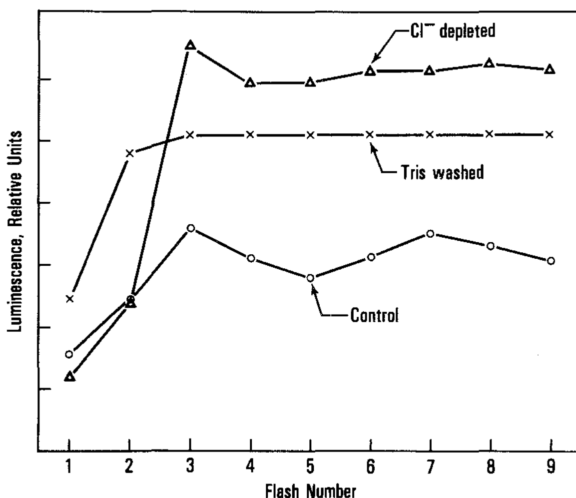


FIG. 4. Effects of Tris washing and Cl^- depletion on delayed fluorescence (labeled as luminescence) 150 μs after a flash as a function of flash number. (○) Control thylakoids; (△) Cl^- depleted; (x) Tris-washed (0.8 *M*, pH 8.0). These data were collected in the author's laboratory, using methods similar to those described by Jursinic and Stemler (1982). Tris washing was carried out as described by Jursinic and Govindjee (1977a), and Cl^- depletion as described by Theg *et al.* (1984). All samples were dark-adapted for more than 10 min and flashes were given at 2 Hz.

the evolution of oxygen. These S states are in equilibrium with the RC as follows: $S_{n+1} \cdots$ P680 $Q_A^- \rightleftharpoons S_n \cdots$ P680$^+$ Q_A^-. The greater the S state charge, the farther to the right this equilibrium resides. This results in higher concentrations of P680$^+$ Q_A^- and the delayed fluorescence generated by its recombination. When the oxygen-evolving system is destroyed, for example by Tris washing, this component of delayed fluorescence is enhanced after two flashes (Jursinic and Govindjee, 1977b; Bouges-Bocquet, 1980; Boussac and Etienne, 1982) (see Fig. 4). This reflects an accelerated recombination reaction between P680$^+$ and Q_A^- when electron donors that reduce P680$^+$ have been depleted (Govindjee and Jursinic, 1979). In this way, μs-range delayed fluorescence acts as a counter for the number of charges available on the electron donor side of PSII. In Cl^--depleted thylakoids, delayed fluorescence is normal after the first and second flashes but is greatly enhanced after the third or more flashes (see Fig. 4). Hence two electrons were available between the Cl^- depletion block and P680 (Theg *et al.*, 1984). From these delayed fluorescence data, it was concluded that Cl^- depletion stops the oxygen evolution system from exceeding the S_2 state (also see Itoh *et al.*, 1984).

One electron comes from the S_1 to S_2 advance, and the other from Z. However, if another intermediate exists between the oxygen evolution system and P680 (see, e.g., Kambara and Govindjee, 1985), then both electrons would come from this region.

5. BICARBONATE DEPLETION (FORMATE PRETREATMENT)

Monovalent anions such as formate, acetate, and particularly bicarbonate can control the rate of electron flow during the Hill reaction (Warburg and Krippahl, 1960; Good, 1963; Stemler and Govindjee, 1973; Stemler and Jursinic, 1983). It is believed that a low pH, bicarbonate is displaced by other anions, and this leads to a drastic decrease in the rate of electron flow from water to various electron acceptors (see reviews by Govindjee and van Rensen, 1978; Vermaas and Govindjee, 1981a,b, 1982; Stemler, 1982). For example, pretreatment of thylakoids with formate results in membranes that have formate bound to them (Stemler and Murphy, 1983) in competition with bicarbonate. Under this condition the quinone reactions $Q_A^- Q_B \rightarrow Q_A Q_B^-$ and $Q_A^- Q_B^{2-} PQ \rightarrow Q_A Q_B^- PQ^{2-}$ are greatly slowed, and the Hill reaction is inhibited (see review by Stemler, 1982; Vermaas and Govindjee, 1982). When formate is not bound or bicarbonate is added to displace formate, the rates of the quinone reactions become high, and the Hill reaction is enhanced (see, e.g., Siggel *et al.,* 1977; Stemler and Murphy, 1983; Stemler and Jursinic, 1983; Snel and van Rensen, 1984).

While these general characteristics of formate- and bicarbonate-treated samples have been determined by a variety of measuring techniques, delayed fluorescence has been applied and has corroborated some of these conclusions. The $Q_A^- Q_B \rightarrow Q_A Q_B^-$ reaction is a transition between states that one expects to be involved in delayed fluorescence. Any blockage of this stabilization reaction would be expected to enhance delayed fluorescence. In fact, complete blockage of this reaction with diuron was found (Jursinic and Stemler, 1982) to enhance delayed fluorescence in the range 150 μs to 4 s. Also, when thylakoids were pretreated with formate and depleted of bicarbonate the delayed fluorescence was enhanced, but to a smaller extent. This strengthened the argument that under these conditions the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ reaction was slowed (as shown by other techniques: Jursinic *et al.,* 1976; Siggel *et al.,* 1977; Farineau and Mathis, 1983).

6. EMISSION YIELDS OF FLUORESCENCE AND DELAYED FLUORESCENCE

The possible ways in which the delayed fluorescence yield ϕ_l may depend on the fluorescence yield ϕ_f have been discussed by Lavorel

(1968, 1975). The relationship between fluorescence intensity (number of photons emitted) F and absorbed light intensity I_a is $F = \phi_f I_a$. Since fluorescence and delayed fluorescence both reflect the presence of singlet excitons in the pigments associated with the RCs of PSII, Lavorel, by analogy, suggested the following relationship for delayed fluorescence: $L = \phi_l J$ where L and ϕ_l are the delayed fluorescence intensity and quantum yield and J is the rate of generation of the Chl *a* singlet state. Essentially there are two extreme cases that differ in the mobility assumed for the excitons generated for delayed fluorescence. If the excitons are unable to migrate over the pigment bed, the emission will occur from pigments connected to an open RC where charge recombination has just occurred. In this case $\phi_l = \phi_o$, where ϕ_o is the fluorescence yield for an open RC. In the other case, when excitons are able to migrate over the entire pigment bed of PSII, ϕ_l will be proportional to ϕ_f.

These relationships have been tested by many different research groups, but one difficulty has remained unsolved: how to change ϕ_f without simultaneously changing J. It was demonstrated by Clayton (1969) and Mar *et al.* (1975) that delayed fluorescence in the millisecond range was modulated in the same way as ϕ_f. These data are particularly difficult to interpret because proton gradients and membrane potentials, which alter J, were not controlled. Wraight (1972) used the phosphoroscope method to look at delayed fluorescence 1 ms after illumination. His samples were uncoupled to eliminate any complications from proton gradients and membrane potentials. During the induction phase Q_A becomes reduced. This causes an increase in fluorescence attributed to an increase in ϕ_f. Also, an increase in delayed fluorescence is expected that is attributed to enhanced recombination, larger J, as one of the recombination substrates (Q_A^-) builds up. If $L = \phi_l J$ is correct and $\phi_l = \phi_f$, one expects the dependence of delayed fluorescence on ϕ_f to be greater than first order. In fact, first-order dependence was observed, and it was concluded that ϕ_l was invariant (not related to ϕ_f). The delayed fluorescence changes were related only to the reduction level of Q_A and enhanced recombination.

Barber *et al.* (1977), Hipkins (1978), and Malkin and Barber (1978) have found that the emission yield of delayed fluorescence components in the range of hundreds of milliseconds follows ϕ_f. However, for components in the 1–10 ms range, ϕ_l follows the fluorescence yield of open traps, ϕ_o. In these experiments proton gradients and membrane potentials were eliminated with ionophores, Q_A was kept in a reduced state, and ϕ_f was modulated by alterations in the divalent salt content of the reaction medium. On the other hand, delayed fluorescence in the 0.1–3.8 ms range decreased as fluorescence increased in uncoupled chloroplasts and PSII particles at 20 and −50°C (Itoh and Murata, 1973; Itoh,

1980). Delayed fluorescence in the 6–60 μs range followed ϕ_f and was attributed to changes in the radiationless deexcitation of the Chl singlet excited state (Wong *et al.,* 1978). Thus, the correlation between ϕ_f and ϕ_l depends on the part of the delayed fluorescence decay that is being observed. This makes questionable a simple analysis of the dependence of delayed fluorescence on the Chl *a* fluorescence yield. The various reactions that underlie delayed fluorescence at different times after illumination are important and must be considered.

From the point of view of the radical pair hypothesis (Section II,B,3), a relationship quite different from the one just presented is expected between delayed fluorescence and fluorescence yield. The radical pair hypothesis maintains the ϕ_f is the same in P680 I Q_A and P680 I Q_A^- centers. In this model the increase in the measured fluorescence signal (variable fluorescence) when Q_A is reduced is actually delayed fluorescence with a 2–4 ns lifetime. The presence of the delayed fluorescence exciton on a P680 I Q_A center or its migration to a neighboring P680 I Q_A^- center is unimportant because both centers have the same fluorescence yield. The increase in fluorescence yield are, in fact, delayed fluorescence, not fluorescence; i.e., ϕ_f is really ϕ_l.

The literature cited earlier in this section does demonstrate a correlation between millisecond-range delayed fluorescence and variable fluorescence yield. If the latter is actually delayed fluorescence, the correlation is not surprising. The variable fluorescence (delayed fluorescence of 2–4 ns lifetime), of course, is modulated by many changes at the RC (Q_A^- concentration, concentration of divalent salts, pH, membrane potential, temperature, *S* states of the oxygen-evolving systems, etc.) that alter the great number of equilibria which underlie delayed fluorescence. It is precisely for this reason that 1–10 ms delayed fluorescence might be correlated with variable fluorescence (delayed fluorescence with a 2–4 ns lifetime) in a different way than 200-ms and longer delayed fluorescence.

7. PHOSPHORYLATION OF THYLAKOID PROTEINS

Bennett (1977, 1979, 1980) has shown that the light-harvesting antenna chlorophyll *a*/*b*-protein complex of PSII (LHC II) is reversibly phosphorylated. It has been suggested that this phosphorylation of LHC II regulates the distribution of absorbed excitation energy between PSI and II *in vivo* (Allen *et al.,* 1981; Kyle *et al.,* 1982; Haworth *et al.,* 1982; Canaani *et al.,* 1984; also see chapters by Briantais *et al.,* Chapter 18, Fork and Mohanty, Chapter 16, and Govindjee and Satoh, Chapter 17, this volume).

Possible effects of protein phosphorylation on the primary photochemistry of PSII were investigated by measuring delayed fluorescence (Jursinic and Kyle, 1983). The delayed fluorescence was measured in the range 12 μs to 1 ms after a single flash; three decay components, with half-times of decay of 4–6 μs, 35–45 μs, and 280–300 μs, were observed. These decay rates were unchanged by protein phosphorylation. However, in samples with phosphorylated proteins, the amplitudes were enhanced twofold for the two fastest components and by 40% for the 300-μs component. This change in amplitude but not in kinetics was interpreted as meaning that the stabilization reactions that took place in less than 1 ms (see Fig. 3) were not altered by protein phosphorylation. From Chl *a* fluorescence measurements (Jursinic and Kyle, 1983), it was concluded that the Q_B^-/Q_B ratio in dark-adapted material was higher in samples with phosphorylated proteins. It was proposed that Q_B^- was more stable due to a change in the accessibility of an endogenous reductant to Q_B, or to an increase in dissipative cycling of charge around PSII.

8. DELAYED FLUORESCENCE IN THE SECONDS RANGE

In dark-adapted plant material, absorption of light results in the separation of charge at the RC. The positive charges are ultimately located on the *S* states of the oxygen-evolving system (Kok *et al.*, 1970; Wydrzynski, 1982). These charged states deactivate with half-times of tens of seconds (Forbush *et al.*, 1971; Joliot *et al.*, 1971; see Sane and Rutherford, Chapter 12, this volume).

Bennoun (1970) compared Chl *a* fluorescence and delayed fluorescence in the seconds range in the presence of DCMU. In samples whose oxygen systems were destroyed by treatment with hydroxylamine, there was no Q_A^- reoxidation or millisecond-range delayed fluorescence. In samples with only DCMU present, Q_A^- was oxidized with a 1.5-s half-time, and delayed fluorescence in the seconds range was observed. It was hypothesized that recombination of Q_A^- with the S_2 state generated this delayed fluorescence. Decay of the S_3 state, recombination of S_3 and Q_A^-, was also associated with a weak delay fluorescence in the seconds range (Joliot *et al.*, 1971).

Injection of DCMU into a sample that has been preilluminated will cause the $Q_A^- \, Q_B \rightleftharpoons Q_A \, Q_B^-$ equilibrium to be shifted entirely to the Q_A^- Q_B state (Velthuys and Amesz, 1974; Wollman, 1978). Flash-illuminated samples into which DCMU has been injected have greatly enhanced delayed fluorescence with decay times of 1 and 2 s (Lavergne and Etienne, 1980). This delayed fluorescence has been assigned to S_2 Q_A^-

and S_3 Q_A^- recombination, where the Q_A^- concentration is induced by the injection of DCMU.

Delayed fluorescence in the tens of seconds range has been studied in thylakoids by Rutherford and Inoue (1984). (Also see Lavergne and Etienne, 1981; Lavergne, 1982.) Based on the flash number dependence of the amplitude and the inhibition by DCMU and Tris washing, this component of delayed fluorescence has been identified with S_2 Q_B^- and S_3 Q_B^- recombination. A similar component has also been reported in leaf disks from spinach (Rutherford *et al.,* 1984). It is interesting to note that reoxidation of Q_B^- has been shown to occur with a 22-s half-time (Lavergne and Etienne, 1981; Robinson and Crofts, 1983), in good agreement with this delayed fluorescence, which has a half-time of decay of tens of seconds (Rutherford and Inoue, 1984; Rutherford *et al.,* 1984).

Avron and Schreiber (1979) reported that delayed fluorescence in the seconds range can be enhanced by addition of ATP to chloroplasts. To observe this effect, the chloroplasts had to be preilluminated for approximately 3 min to light-activate a latent ATPase activity (Bakker-Grunwald, 1977; Avron and Schreiber, 1977). When ATP was introduced it was hydrolyzed, and a proton gradient was generated across the thylakoid membrane, which is known to increase recombination of charge and delayed fluorescence (see any of the earlier reviews cited in Section I).

Related to the work just discussed is the finding that seconds-range delayed fluorescence in the green alga *Chlorella* was inhibited when tri-*n*-butyltin (TNBT) was added to the sample (Joliot and Joliot, 1980). TNBT is a potent inhibitor of ATPase. It was hypothesized that in the dark a proton gradient was maintained in the algal cell chloroplast by ATPase activity, with ATP provided by the mitochondria. As suggested by Avron and Schreiber (1979) for isolated chloroplasts, this proton gradient in chloroplasts of *Chlorella* would enhance recombination of charges and delayed fluorescence.

9. PHOTOSYSTEM II HETEROGENEITY

As discussed in Section III,B,2, to understand the dependence of delayed fluorescence on excitation intensity, the heterogeneity in PSII must be considered. A more detailed discussion of this heterogeneity will now be given (also see van Gorkom, Chapter 10, in this volume).

The rise in the Chl *a* fluorescence transient with DCMU present is not a simple exponential but is sigmoidal with fast and slow kinetics (Melis and Homann, 1976, 1978; Melis and Duysens, 1979; Melis and Schrei-

ber, 1979). The interpretation is that two types of PSII (α and β) exist, and that they differ in the configuration of their antenna pigment. The β-type PSIIs have small numbers of antenna pigments (associated with their RCs) that are isolated from one another, and these account for the slow kinetics of the Chl *a* fluorescence rise. The α-type PSIIs have aggregates of antenna pigments that are three- to fourfold larger than those in the β-type PSIIs and are connected to one another. In these connected PSIIs, energy from a closed RC can be transferred to an open neighboring RC. These α-type PSIIs account for the fast kinetics of the Chl *a* fluorescence rise. Heterogeneity in PSII antenna size has also been inferred from electron microscopy (Armond *et al.*, 1977), the flash intensity dependence of the oxygen evolution pattern (Jursinic, 1979), and the light-saturation behavior of oxygen flash yields in the steady state (Ley and Mauzerall, 1982).

Heterogeneity in the quinones of PSII also occurs (see a review by Vermaas and Govindjee, 1981b; also see van Gorkom, Chapter 10, in this volume). The $Q_A^- Q_B \rightarrow Q_A Q_B^-$ reaction can be driven in the reverse direction by rapid injection of DCMU or dithionite, which is monitored by an enhancement in Chl *a* fluorescence yield (Velthuys and Amesz, 1974; Wollman, 1978). However, less than 50% of the total variable fluorescence is sensitive to the DCMU injection, and this has been interpreted as meaning that PSIIs exist that do not have Q_B (Wollman, 1978; Lavergne and Etienne, 1980; Lavergne, 1982). Thus, there is heterogeneity in PSII based on the electron acceptors that are present: there are B-type (Q_B present) and non-B-type (Q_B not present) PSIIs.

The Chl *a* fluorescence transient with DCMU present has been recorded after various preillumination flashes (Thielen and van Gorkom, 1981). The transient was separated into fast (α) and slow (β) components; it was found that the α component oscillated with a periodicity of two, being large after odd-numbered flashes. The β component did not oscillate. it was suggested that the α component was the same as a B-type center and that the β component was the same as a non-B-type center.

The recombination of charges that occurs at RCs in these different types of PSIIs has unique properties. The B-type centers do not recombine when the oxygen-evolving system is in the S_0 or S_1 state. Thus, recombination in these centers has a flash number (or S-state) dependence (Lavergne, 1982). The non-B-type centers decay with no dependence on flash number. The delayed fluorescence associated with the B-type center is found to be highly stimulated by pH jumps and to be dependent on flash number, being very low on the first flash and oscillating with a period of four (Lavergne and Etienne, 1981; Lavergne, 1982). Delayed fluorescence associated with non-B-type PSIIs is not stimulated

by pH jumps and does not change with flash number. These different PSIIs are very likely the same as those found by McCauley and Ruby (1981), discussed in Section III,B,2. The B-type (α) PSII is the same as the PSII with a large antenna that gave delayed fluorescence after multiple hits and was sensitive to hydroxylamine treatment. The non-B-type (β) PSII is the same as the PSII with a small antenna that had the same delayed fluorescence on all flashes.

Another type of heterogeneity in PSII centers of thylakoids has been hypothesized by Conjeaud *et al.* (1979). These authors studied the kinetics of the Z $P680^+ \rightarrow Z^+$ P680 reaction in control and Tris-washed thylakoids by following the absorption change at 820 nm due to $P680^+$ reduction. In control thylakoids, the Z $P680^+ \rightarrow Z^+$ P680 reaction was found to be multiphasic, with components of 25–45 ns (van Best and Mathis, 1978; Eckert *et al.*, 1984), 200–250 ns (Brettel and Witt, 1983; Eckert *et al.*, 1984), 2–10 μs (Renger *et al.*, 1978; Conjeaud *et al.*, 1979; Brettel and Witt, 1983; Eckert *et al.*, 1984), 20–35 μs (Glaser *et al.*, 1974; Conjeaud *et al.*, 1979; Brettel and Witt, 1983; Eckert *et al.*, 1984), and 200–400 μs (Glaser *et al.*, 1974; Renger *et al.*, 1978; Brettel and Witt, 1983; Eckert *et al.*, 1984). Delayed fluorescence components have been observed in thylakoids that correlate with all but the 20–35 ns component (Govindjee and Jursinic, 1979; Sonneveld *et al.*, 1980).

In Tris-washed thylakoids, the ability to evolve oxygen is destroyed (Yamashita and Butler, 1969); and here the Z $P680^+ \rightarrow Z^+$ P680 reaction has a pH-dependent rate between 2 and 45 μs (Conjeaud *et al.*, 1979; Conjeaud and Mathis, 1980). It was suggested by Conjeaud *et al.* (1979) that the 6- and 22-μs components of this reaction, observed in control thylakoids, came from centers with inhibited oxygen evolution ability. A heterogeneity in the oxygen-evolving ability of thylakoids may be created by the procedure used to isolate thylakoids.

A 6–10 μs decay component occurs in delayed fluorescence and had been attributed to the Z $P680^+ \rightarrow Z^+$ P680 reaction (Lavorel, 1973; Jursinic and Govindjee, 1977b; Govindjee and Jursinic, 1979). Interestingly, this component does not occur in intact cells of *Chlorella* (van Best and Duysens, 1977). It had been suggested (Jursinic and Govindjee, 1977b) that this was a significant difference between intact cells and thylakoids. Buttner and Babcock (1984) observed delayed fluorescence components in both Tris-washed and control thylakoids of 5 and 50 μs at pH 8, and of 18 and 90 μs at pH 4.5. They suggested that the occurrence of these pH-dependent components in control thylakoids was due to heterogeneity in the activity of PSIIs. Thylakoid preparation inhibited a portion of the PSII (similar to Tris washing), causing the Z $P680^+ \rightarrow Z^+$ P680 reaction to have pH-dependent kinetics in the microsecond rather than the nanosecond range.

C. Delayed Fluorescence from Photosystem I

1. PHOTOSYSTEM I PARTICLES

Up to this point in the chapter, the discussion has dealt mostly with delayed fluorescence from PSII of plants. This section will present data that support the emission of delayed fluorescence from PSI.

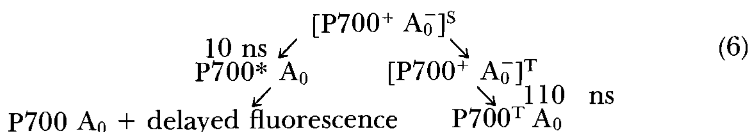
The reaction complex of PSI may be written as follows (cf. Parson and Ke, 1982; see Fig. 1 in Duysens, Chapter 1, this volume): P700 A_0 A_1 F_x $F_{A,B}$, where P700 is the RC Chl *a* (Kok, 1961), A_0 is a Chl *a* monomer (Fenton *et al.,* 1979; Heathcote *et al.,* 1979; Shuvalov *et al.,* 1979a; Baltimore and Malkin, 1980), A_1 may be a bound plastoquinone (see discussions in Rutherford and Heathcote, 1985), and F_x and $F_{A,B}$ are iron–sulfur proteins (Ke, 1973; Bolton, 1977; Shuvalov *et al.,* 1979a,b).

On arrival of an exciton at the PSI RC, charge separation† takes place, producing $P700^+$ A_0^- A_1 F_x $F_{A,B}$ within a few ps, which then stabilizes to $P700^+$ A_0 A_1 F_x $F_{A,B}^-$. In PSI particles, Shuvalov (1976) observed delayed fluorescence after flashes of 2-μs duration that had an emission maximum at 710 nm, and activation energy of 0.65 eV, and a decay time of 20 ms. This delayed fluorescence is believed to be due to recombination of $P700^+$ $P430^-$, where P430 is either F_x or $F_{A,B}$, or both. This assignment was supported by the elimination of delayed fluorescence if P700 was chemically oxidized or P430 was chemically reduced prior to illumination. Other evidences for its PSI origin are: an emission spectrum identical to PSI fluorescence, the same light saturation characteristics as the photooxidation of P700, and the fact that these particles have only PSI activity.

In PSI particles with iron–sulfur proteins, and A_1 reduced, flash excitation is believed to give rise to charge separation as follows:

$$\mathrm{P700\ A_0\ A_1^-\ F_x^-} \xrightarrow{h\nu} \mathrm{P700^*\ A_0\ A_1^-\ F_x^-} \rightarrow \mathrm{P700^+\ A_0^-\ A_1^-\ F_x^-} \qquad (5)$$

Since magnetic field effects are observed, it is believed that $P700^+$ A_0^- is a radical pair. This radical pair can decay as follows:



$$\begin{array}{ccc} & [\mathrm{P700^+\ A_0^-}]^S & \\ 10\ \mathrm{ns} \swarrow & & \searrow \\ \mathrm{P700^*\ A_0} & & [\mathrm{P700^+\ A_0^-}]^T \\ \swarrow & & \searrow\ 110\ \mathrm{ns} \\ \mathrm{P700\ A_0} + \text{delayed fluorescence} & & \mathrm{P700^T\ A_0} \end{array} \qquad (6)$$

† The reader should be warned that in the early literature, the symbol A_1 was used for A_0.—editors.

where $[P700^+ A_0^-]^S$ is the radical pair in the singlet state and $[P700^+ A_0^-]^T$ in the triplet state. The 10-ns recombination of $[P700^+ A_0^-]^S$ to give $P700^* A_0$ was observed by Shuvalov *et al.* (1979b), using the $P700^+$/P700 absorption change at 694 nm. The lengthening of the lifetime of the delayed fluorescence decay from 110 to 130 ns by a magnetic field is explained by assuming that the decay of the radical pair is largely determined by singlet-to-triplet exchange and triplet recombination (Sonneveld *et al.*, 1981). This explanation is similar to that proposed for RCs of photosynthetic bacteria and PSII (see Section II,B,3).

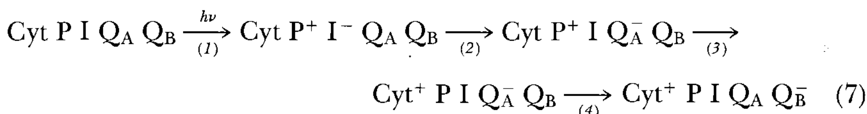
2. BUNDLE-SHEATH CHLOROPLASTS

Use of bundle-sheath chloroplasts is another technique for detecting PSI delayed fluorescence. Bundle-sheath chloroplasts of the so-called C_4 plants have been reported to be completedly devoid of PSII activity (Woo *et al.*, 1970), which simplifies the measurement of PSI delayed fluorescence. Measurements of millisecond-range delayed fluorescence by the phosphoroscope method were made in bundle-sheath and mesophyll protoplasts (Gregory *et al.*, 1979). There was no observable delayed fluorescence from the bundle-sheath preparation. Ross *et al.* (1982) also attempted to measure ms-range delayed fluorescence from bundle-sheath protoplasts. In this work, a tunable dye laser was used for excitation between 695 to 720 nm. This allowed excitation specific for the PSI antenna system, and thus the method avoided measuring delayed fluorescence from contaminated PSII protoplasts. No ms-range delayed fluorescence was observed from PSI under these conditions.

IV. Delayed Fluorescence in Photosynthetic Bacteria

A. Decay after One Flash

Delayed fluorescence has been observed in whole photosynthetic bacteria (Fleischman, 1978) and has been studied in detail in membrane fragments of *Rhodopseudomonas* (*Rps.*) *viridis* (Fleischman, 1974; Carithers, and Parson, 1975). In the work by Carithers and Parson (1975), delayed fluorescence was observed in the millisecond range following excitation with 10-μs flashes. The normal reaction sequence is as follows (see Norris and van Brakel, Chapter 3, this volume):



$$\text{Cyt P I } Q_A Q_B \xrightarrow[(1)]{h\nu} \text{Cyt } P^+ I^- Q_A Q_B \xrightarrow[(2)]{} \text{Cyt } P^+ I Q_A^- Q_B \xrightarrow[(3)]{} \text{Cyt}^+ \text{ P I } Q_A^- Q_B \xrightarrow[(4)]{} \text{Cyt}^+ \text{ P I } Q_A Q_B^- \quad (7)$$

where Cyt is the cytochrome *c*-558 (C558), P is the reaction center BChl. I is bacteriopheophytin, Q_A is the first quinone electron acceptor, and Q_B is the second quinone electron acceptor. Reactions (*3*) and (*4*) are charge stabilization reactions that can be inhibited by chemical oxidation of C558 prior to illumination and addition of *o*-phenanthroline, respectively. Under these conditions delayed fluorescence is greatly enhanced and has a half-time of decay of 600 μs. The decay of P^+, followed by its absorbance change at 850 nm (the peak is at 960 nm), has an identical half-time. This is a classic example of delayed fluorescence generated by charge recombination ($P^+ Q_A^-$); the charged species decay with the same kinetics as delayed fluorescence, since all further charge stabilization reactions have been eliminated.

The main decay route for $P^+ Q_A^-$ is not delayed fluorescence. The rate constant for charge decay is approximately 1000 s^{-1}, whereas a comparison of delayed fluorescence and fluorescence intensities indicates that energy is being returned to the bulk BChl with a rate constant of 3.7 s^{-1}. Also, temperature is observed to affect only the delayed fluorescence amplitude, and not the decay kinetics. It has been hypothesized (Carithers and Parson, 1975) that decay of $P^+ Q_A^-$ is predominantly by a nonradiative process, believed to be tunneling of an electron from Q_A^- to P^+.

B. Decay after Multiple Flashes

Delayed fluorescence has also been measured, following 0.5-μs laser flashes, in RC preparations of *Rps. sphaeroides* that contained Q_A or Q_A and Q_B (Arata and Parson, 1981). In centers with only Q_A the delayed fluorescence has a lifetime of 100 ms, corresponding to the decay of P^+ Q_A^-, which is followed by an absorption change. Centers that have Q_A and Q_B have much lower delayed fluorescence and a decay lifetime of about 1 s, corresponding to the decay of $P^+ Q_B^-$. When *o*-phenanthroline, which blocks the $Q_A^- Q_B \rightarrow Q_A^- Q_B^-$ reaction, is present in these centers, the delayed fluorescence behaves as in RCs that have only Q_A.

Flesichman (1974) also measured delayed fluorescence in chromatophores of *Rps. viridis* in the millisecond range, following multiple excitations, with the phosphoroscope method. Temperatures below 0°C were used to stop the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ reaction. The delayed fluorescence observed decayed with a 7-ms half-time, as did the $P^+ Q_A^-$ state, which was followed by ESR absorption spectroscopy. The $P^+ Q_A^-$ recombination was temperature-insensitive, whereas the delayed fluorescence had a 0.2-eV activation energy. It was concluded that $P^+ Q_A^-$ recombination proceeded by parallel radiative and nonradiative routes. The delayed

fluorescence decay observed in these measurements in *Rps. viridis,* which is 10 times slower than that measured by Carithers and Parson (1975), is probably due to differences in sample preparation, solution redox potential, and buildup of unidentified high-energy states during the multiple illuminations of the phosphoroscope method.

Using the phosphoroscope method, Arata *et al.* (1977) measured delayed fluorescence in chromatophores of *Chromatium vinosum* in the millisecond time range. They found components with 8 ms and 100–200 ms half-times, in good agreement with the phosphoroscope measurements of Fleischman (1974) in *Rps. viridis.* When *o*-phenanthroline was included, the delayed fluorescence decay and the P^+ decay, followed by absorption change, were in good agreement. Reagents that dissipated proton gradients and membrane potential were found to decrease the delayed fluorescence intensity. Under these conditions of multiple excitation, the modulation of delayed fluorescence by factors other than the $P^+ Q_A^-$ concentration become obvious.

Measurements of delayed fluorescence after single flashes have also been made in chromatophores of *Rps. sphaeroides,* and these decayed with a half-time of 120 μs, much faster than the decay of P^+ or Q_A^- (Carithers and Parson, 1976). When multiple flashes were given with a period less than 15 s, the delayed fluorescence intensity was enhanced 11- to 18-fold, but the decay kinetics remained the same with a 120-μs half-time. This enhancement occurred only under conditions that favored generation of $P Q_A^-$. The membrane potential was not involved, since the ionophore gramicidin only partially inhibited delayed fluorescence. It was hypothesized that the reduction of $P^+ Q_A^-$ leads to storage of free energy, which is available for delayed fluorescence. The mechanism involved in this sharing of free energy was not defined.

Recombination can also occur between P^+ and Q_B^-. Chromatophores of *Rps. viridis* were illuminated with a series of 40-μs flashes of subsaturating intensity (Fleischman, 1984). Reduction of P^+ was followed by absorption changes, and these had two phases with half-times of 0.38 and 27.6 s at 20°C and pH 7. A delayed fluorescence component with the 0.38-s absorbance component and had the same pH dependence. This component was due to $P^+ Q_B^-$ with Q_B^- stabilized by protonation.

The 27.6-s component was from charge stabilized on other components (quinones) past Q_B. An acid–base jump could induce delayed fluorescence in the seconds range in preilluminated chromatophores of *Rps. viridis* (Fleischman *et al.,* 1984). The time between the cessation of illumination and the pH jump was important. The amplitude of the stimulated delayed fluorescence decreased with a half-time of 20–30 s, which is quite similar to the 27.6-s component of $P^+ Q_B^-$ recombination

(Fleischman, 1984). It seems likely that the acid–base shift causes the generation of Q_B^- by backflow of electrons from quinones other than Q_A or Q_B.

V. Concluding Remarks

In this chapter we have discussed delayed fluorescence in plants and photosynthetic bacteria with an emphasis on developments during the past few years. A connection between what has been called variable Chl *a* fluorescence and delayed fluorescence has now been strongly supported by the radical pair hypothesis in PSII. The experimental evidence at this time seems very convincing and additional experimentation in the next few years will be interesting. The usefulness of delayed fluorescence in photosynthesis research is dependent on the correct identification of various decay components with particular reactions. This is a fertile ground for continued work, since gaps in our knowledge are very wide indeed. The heterogeneity in PSII has now been recognized; delayed fluorescence measurements will possibly assist us in our understanding of this heterogeneity.

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12

Thermoluminescence from Photosynthetic Membranes

PRAFULLACHANDRA VISHNU SANE
National Botanical Research Institute
Rana Pratap Marg
Lucknow 226001, Uttar Pradesh, India

ALFRED WILLIAM RUTHERFORD
Service de Biophysique
Départment de Biologie
Centre d'Études Nucléaires de Saclay
91191 Gif-sur-Yvette, France

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ABBREVIATIONS AND SYMBOLS

ADRY	Acceleration of deactivation reactions in the water-splitting enzyme Y
ANT2P	2-(3-Chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene
CCCP	Carbonyl cyanide-*m*-chlorophenylhydrazone
DBMIB	2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCCD	Dicyclohexylcarbodiimide
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea; diuron
D_z	An unknown donor in PSII
EPR	Electron paramagnetic resonance
kD	Kilodalton
P680	Primary electron donor (chlorophyll) in PSII
P700	Primary electron donor (chlorophyll) in PSI
P960	Primary electron donor (bacteriochlorophyll) in *Rhodopseudomonas viridis*
Pheo	Pheophytin
PQ	Plastoquinone
PSII	Photosystem II
Q_A	Primary plastoquinone electron acceptor in PSII
Q_B	Secondary plastoquinone electron acceptor in PSII
RC	Reaction center
S_n (n = 0, 1, 2, 3, 4)	Charge accumulation states of photosystem II O_2-evolving enzyme where n is the number of positive charges accumulated
Tris	Trishydroxyaminomethane
TL	Thermoluminescence
Z	Electron donor to $P680^+$ which gives rise to signal II-very fast

ABSTRACT

The reactions responsible for some of the thermoluminescence peaks in plants have recently been identified, and it has become clear that thermoluminescence is due to the same reactions responsible for delayed fluorescence. This work and the possible origins of the other thermoluminescence peaks are discussed. The advances in the understanding of thermoluminescence have led to a much broader use of thermoluminescence as a probe of electron transfer reactions in photosystem II. In particular, it can be used to investigate the function of the O_2-evolving enzyme. Charge storage, S state oscillations, deactivation of the S states, the involvement of protons, the relative stability of the S states, and S state formation in the nonfunctional enzyme have all been studied by using thermoluminescence. In addition, the electron acceptor side of photosystem II has been investigated by this technique. The effects of herbicides and resistance to herbicides, the effect of thermophilic adaptation, and the redox and protonation state of the electron acceptor Q_B have all been monitored. All of these diverse and sometimes unique measurements, taken together with the fact that they can be done as easily on leaves as on isolated membranes, make thermoluminescence a technique that is becoming a much more frequently used probe in photosynthesis research.

I. Introduction

Pulses of light are emitted from preilluminated photosynthetic membranes when they are warmed from low temperatures in the dark. This

phenomenon is called thermoluminescence (TL) and its study can provide useful information concerning photosynthetic electron transport (for an earlier review, see Inoué and Shibata, 1982; also see Inoué, 1983). The light emitted as thermoluminescence results from the reversal of light-driven charge separation; i.e., it is due to recombination of a positive and a negative charge on the primary reactants. This recombination occurs after a series of back reactions in which a pair of charges return to the primary reactants via a series of secondary electron acceptors and donors. Although in plant systems two photosystems are present, most (if not all) of the TL seems to arise from photosystem II (PSII).

II. The Early Work and a Historical Perspective

The first reports of TL in photosynthetic material were made by Arnold and Sherwood (1957) and by Tollin and Calvin (1957). In both reports dried chloroplasts were used, and it seems likely that the TL observed reflected severely damaged systems. However, Arnold and Sherwood (1957) stated that TL was also present in leaves and in algae. (See Arnold, Chapter 2, this volume.) Later, Arnold (1966) showed the presence of TL in cell suspensions of the green alga *Chlorella.* Although a low-temperature (−160°C) band was reported for the first time, the resolution in this work was not good enough to show more than one band at higher temperatures.

The work of Arnold and Azzi (1968), Rubin and Venediktov (1969), and Shuvalov and Litvin (1969) represented a significant step forward in this area of research. In these reports the first well-resolved TL curves from photosynthetic material were presented. Rubin and Venediktov (1969) resolved four peaks between −50 and 50°C in samples illuminated during cooling and only one major band in samples illuminated at −50°C. Under the latter conditions it was found that the presence of DCMU* (which blocks electron flow from Q_A^- to Q_B; see Fig. 1) shifted the band from 25 to 10°C. The interpretations of these phenomena given in this work remain valid today and are effectively the same as those used recently to identify the origin of the charge pairs involved in the formation of each peak (see Section V). Arnold and Azzi (1968) and Shuvalov and Litvin (1969) generated the TL curves by illumination at liquid nitrogen temperature (77°K) and so obtained slightly different results from Rubin and Venediktov (1969). Both groups characterized the low-temperature Z band and both found that one of the high-tem-

* Called diuron; see list of abbreviations and symbols.

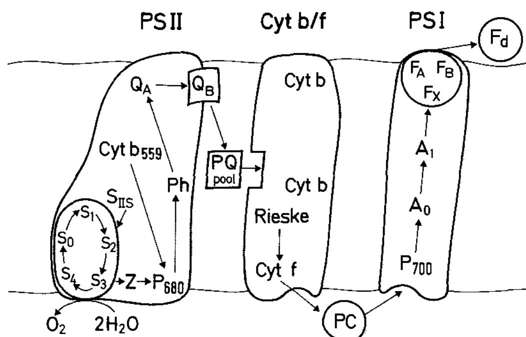

FIG. 1. **Photosynthetic electron transport in plants. The scheme shows three membrane-bound protein complexes: (1) the PSII and the O_2-evolving complex; (2) the cytochrome (Cyt) *b/f* complex; and (3) the PSI complex. The components of PSII are defined in the list of abbreviations and symbols. The cytochrome components in the Cyt *b/f* complex are self-evident; Rieske stands for Rieske iron–sulfur center. In PSI, A_0 and A_1 are early electron acceptors, and F_X, F_A, and F_B are iron–sulfur centers which act as secondary electron acceptors; PC is plastocyanin and Fd is ferredoxin. (Compare with Fig. 1 in Duysens, Chapter 1, this volume.)**

perature bands was insensitive to (or enhanced by) diuron while the other bands were diminished. Arnold and Azzi (1968) reported that the peaks in the region −40 to 50°C were absent in PSII-less mutants and present in PSI-less mutants, clearly demonstrating their origin in PSII. Shuvalov and Litvin (1969) correlated the peaks with phases of delayed light emission (also called delayed fluorescence; see Jursinic, Chapter 11, this volume), and this remains valid at least at higher temperatures and has been "rediscovered" recently (see Section VI). This group also calculated the activation energies for the state responsible for each peak.

Arnold and Azzi (1971) also subsequently calculated the activation energies for the states giving rise to each peak and showed that certain bands could be charged by one or two flashes, suggesting an association with charge storage involved in O_2 evolution. At about the same time, Fleischman (1971) reported TL in purple bacteria, which correlated with charge recombination in the reaction center (RC) measured directly by other techniques.

In these few early papers can be found the elements of nearly all the subsequent work on the subject. These elements include the attribution of TL to recombination of primary reactants, the involvement of reverse electron transfer from secondary donors and acceptors in PSII, the origin of most of the plant TL in PSII, the correlation of delayed light with TL, and the uses of different illumination regimes to produce different

redox states. In subsequent work these and other groups produced a great deal of data relevant to these points. Other techniques have provided great improvements in our understanding of photosynthetic electron transfer, and correlations have been made that have allowed the specific charge storage states involved in TL to be identified (see Section V). As a result of the identification of the TL peaks, the technique can now be used as a powerful probe of photosynthetic electron transfer in its own right. It has already provided results which have been important in PSII research (see Section VIII).

III. Methods Used for Measuring Thermoluminescence

Thermoluminescence is measured with instruments that vary in design but are rather simple in principle. A sample (chloroplasts, part of a leaf, algae, etc.) which has been preilluminated is warmed, usually from 77°K to about 70°C in the dark. A photomultiplier measures any light emitted by chlorophyll *a* or bacteriochlorophyll in the sample during the warming. The warming rate is controlled and the light emitted is recorded as a function of temperature. The temperature is measured by a thermocouple, which is usually in direct contact with the sample.

Variations in the nature of the preillumination have been used. In some work, dark-adapted samples are illuminated at room temperature and then during cooling of the sample down to 77°K. Sometimes continuous illumination has been given at a fixed temperature between room temperature and 77°K. There are also a number of reports in which saturating flash excitation has been used, usually at temperatures high enough that stable charge separation occurs with a high quantum yield (−20°C). Experiments have been done in which flashes are given at a higher temperature followed by continuous illumination at low temperatures. In experiments where excitation is given at temperatures higher than 77°K, the sample is usually rapidly cooled in the dark to trap the photoinduced states before TL is recorded.

IV. Nomenclature

Unfortunately, the nomenclature used by different groups is not identical. The equivalence of peaks in the different nomenclatures has been one of the major controversies and sources of confusion in TL research. The problem arises for a number of reasons, including the following: (1) the temperature maximum of a peak is dependent on the heating

rate, and different heating rates are used by different groups; (2) in some kinds of experiments several peaks overlap; (3) differences in the intactness of the material can result in significant photochemical differences which are manifest as differences in the TL curve; and (4) most important, some experiments are carried out with continuous illumination during cooling or at fixed temperatures, while others use saturating flashes.

Basically there are two systems of nomenclature, an alphabetic system and a numerical system. Table I represents a tentative assignment of the equivalence of peaks in the different nomenclatures. This table is not 100% accurate, since there are examples in the literature in which re-

TABLE I
Thermoluminescence in Plants

Peak	Approximate emission temperature (°C)	Origin[a]	Approximate phase of delayed luminescence at room temperature[b]	Comments
Z	−160	?		Emission max. 740 nm; excitation max. blue light
Z_v	−70 (variable)	$D_z^+Q_A^-$ (?)	200–500 μs	Oscillates with preflash number; maxima coincide with S_1
I (A)	−20	$Z^+Q_B^-$ (?) and/or $Z^+Q_A^-$ (?)	Hundreds of milliseconds 4 ms	Oscillates with preflash number; maxima coincide with S_3
II (D)	−0	$S_2Q_A^-$ and $S_3Q_A^-$	1.5 s	Oscillates with flash number when diuron is added after excitation; maxima coincide with S_2 and S_3
III	10	$S_{2/3}Q_B^{2-}$ (?)	30 s	Formed in leaves or in the presence of DCCD
IV (B)	25	$S_2Q_B^-$ and $S_3Q_B^-$	30 s	Oscillates with flash number; maxima coincide with S_2 and S_3
V (C)	50	Signal II-slow Q_A^- (?)	Minutes	Oscillates with flash number when diuron is added after excitation; maxima coincide with S_0 and S_1

[a] For a discussion of the origin of the TL, see Section V.
[b] For a discussion of the relationship between TL and delayed light emission, see Section VI.

searchers have used the same nomenclature but have misassigned the peaks.

V. Characterization and Identification of the Origins of the Thermoluminescence Peaks

Until recently, most of the research with TL has had as its central theme the identification of the reactions responsible for the TL peaks. Much of this work has been done with samples frozen under illumination or continuously illuminated at temperatures where the photochemistry has not been well defined by other techniques. Since PSII has multiple donors and acceptors, each with different temperature dependence, these experimental conditions give rise to TL which is inherently difficult to interpret. Despite this, much data has been published and some conclusions have been drawn about the origins of the TL. The TL measurements in which flash illumination is used have proved more interpretable, since discrete states are formed which have already been well characterized by other techniques (flash-induced fluorescence, absorption, electron paramagnetic resonance, O_2 polarography measurements). A current scheme of photosynthetic electron transfer in plants is shown in Fig. 1 (cf. Fig. 1 in Duysens, Chapter 1, this volume). For recent reviews of photochemistry in PSII and PSI, see van Gorkom (1985) and Rutherford and Heathcote (1985), respectively. Figure 2 shows an idealized TL curve where all the bands are present (in practice, no experimental data have been reported where all of the bands are well resolved

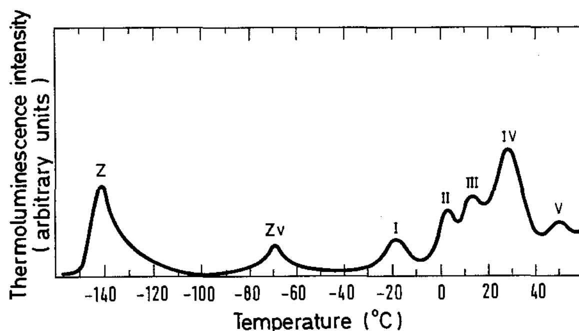

FIG. 2. An idealized thermoluminescence (TL) curve from plant photosynthetic membranes. The curve shows all the TL peaks well resolved (this situation is actually not found in practice). The designation of the peaks, the conditions for their formation, and their origins are discussed in Section V (also see Table I).

in the same sample). In this section the evidence from continuous and flash excitation studies on the origin of each TL peak will be discussed.

A. The Z Peak

In plant material illuminated at 77°K a TL band appeared at −160°C (Arnold and Azzi, 1968; Shuvalov and Litvin, 1969). The peak was preferentially excited by blue light (Arnold and Azzi, 1968; Shuvalov and Litvin, 1969) but could also be charged with red light (Shuvalov and Litvin, 1969; Sane *et al.*, 1974) and gamma rays (Sane *et al.*, 1974). The peak was also present in boiled material and in isolated chlorophyll and its emission maximum was at 740 nm (Shuvalov and Litvin, 1969; Sane *et al.*, 1974). On the basis of these observations, most workers have assumed that this band is not related to photosynthetic electron transfer. Although it was suggested that the Z band may be phosphorescence from the decay of chlorophyll triplets (Sane *et al.*, 1974), this is doubtful since chlorophyll phosphorescence occurs at longer wavelengths. (See Hoff, Chapter 9, this volume, for a discussion on triplets.) Shuvalov (1976) reported that chlorophyll emission at 740 nm may be due to triplet–triplet fusion. Even if this is the type of emission seen as the Z peak, there is still no information on the nature of the energy storage state stably photoinduced at 77°K which is the precursor of this luminescence. It is of interest that the charging of the Z band corresponds to a quenching of fluorescence measured at low temperatures (Kyle *et al.*, 1983).

B. The Z_v Peak

In a study of the effect of illumination temperature on TL, Ichikawa *et al.* (1975) found a peak which had a maximum 10 to 30°C higher than the illumination temperature between −55 and −196°C. This band was designated Z_v (where v stands for variable), and it was suggested that it may be partially due to the lower-temperature Z peak and the higher-temperature peak I (for A band) (Ichikawa *et al.*, 1975; Desai *et al.*, 1977). Desai *et al.* (1977) showed that the Z band could largely account for the Z_v peak at temperatures lower than −100°C. It is less clear, however, that peak I can account for the Z_v band at temperatures above −100°C. Indeed, in several reports there are clearly two different bands between −70 and −10°C (see Ichikawa *et al.*, 1975; Inoué *et al.*, 1977; Inoué and Shibata, 1978a,b, 1979). Thus there is little doubt that the Z_v band exists as a band in its own right, at least in the temperature range

−100 to −20°C. A survey of the literature indicates that the Z_v peak is not observed in data obtained by illumination during freezing. The Z_v peak was present in isolated PSII particles and absent in PSI particles (Ichikawa *et al.*, 1975).

This peak is present when the O_2-evolving system is inhibited by Tris (Ichikawa *et al.*, 1975; Inoué *et al.*, 1977) or by NH_2OH (Vass *et al.*, 1984). For a recent review on the O_2-evolving system, see Govindjee *et al.* (1985a). It is also present in chloroplasts and leaves from plants grown under conditions where the O_2-evolving enzyme is not activated, i.e., in intermittent light-grown plants (Ichikawa *et al.*, 1975; Inoué *et al.*, 1976a), dark-grown spruce leaves (Inoué *et al.*, 1976b,c), and manganese-depleted algae (Inoué, 1976). The peak's sensitivity to diuron (Ichikawa *et al.*, 1975) may be at least partly due to an effect of ethanol (Vass *et al.*, 1984).

It was shown recently that a series of flashes given at 2°C prior to continuous illumination at −80°C results in variations in the intensity of the Z_v band (Vass *et al.*, 1984). The intensity of the band oscillated with flash number, showing maxima after 0, 4, and 8 flashes. It was concluded that the peak resulted from recombination of an electron located prior to the DCMU block (probably Q_A^-) with a positive charge on an unknown donor, D_z, which is in some way associated with the *S* states (see Fig. 1). Cytochrome *b*-559 was ruled out as the donor because of its relatively low potential and its high stability (Vass *et al.*, 1984).

The results of Vass *et al.* (1984) have been repeated and extended recently (Demeter *et al.*, 1985b,c). For unknown reasons trypsin digestion greatly enhanced the Z_v band while the higher temperature peaks were abolished. It was also found that despite being largely insensitive to DCMU, the amplitude of the band seemed to be modulated by the redox state of Q_B under some conditions. Demeter *et al.* (1985b) calculated that the luminescence equivalent to the Z_v peak would decay in 200–500 μs at ambient temperature.

C. Peak I (the A Band)

A peak between −40 and −10°C was formed when samples were cooled under illumination or continuous illumination was given at a fixed temperature between −10°C and 77°K (see, e.g., Rubin and Venediktov, 1969; Shuvalov and Litvin, 1969; Lurie and Bertsch, 1974a; Desai *et al.*, 1975). Inoué *et al.* (1976a) showed that this band was charged by illumination at −20°C more than by illumination at −65°C.

There is a great deal of evidence indicating that this band arises in PSII: it is charged by red and not far-red light (Shuvalov and Litvin,

1969; Lurie and Bertsch, 1974a; Desai *et al.*, 1975), it is present in PSII particles and not in PSI particles (Lurie and Bertsch, 1974a; Ichikawa *et al.*, 1975; Sane *et al.*, 1977), and it is present in mutants lacking PSI and absent in mutants lacking PSII (Arnold and Azzi, 1968; Lurie and Bertsch, 1974a). This peak is lost if DCMU is present (see, e.g., Arnold and Azzi, 1968; Rubin and Venedictov, 1969; Shuvalov and Litvin, 1969). However, only a single turnover is expected in the presence of DCMU, and since that turnover yields a more stable charge pair (peak II, the D band) the DCMU effect on peak I does not provide information on the location of the charge pair responsible for this peak.

Other treatments which block electron transfer between PSII and PSI (PQ extraction, $HgCl_2$, DCCD) had little effect on peak I (Sane *et al.*, 1983b), indicating that the electron involved in the formation of this peak is located on an acceptor closer to the reaction center than the PQ pool.

Some information exists on the location of the positive charge involved in peak I. Treatments that inhibit the O_2-evolving enzyme, i.e., treatment with Tris (Inoué *et al.*, 1977), Triton X-100 (Ichikawa *et al.*, 1975), and NH_2OH (Demeter *et al.*, 1979), and tetranitromethane addition (Sane *et al.*, 1983a), all had little effect on this peak. In contrast, peak I was lost when plant material was grown under conditions where the O_2 enzyme did not develop (i.e., intermittent light-grown plants, dark-grown spruce, and manganese-depleted algae) and it appeared as the O_2-evolving enzyme was photoactivated (Inoué, 1976; Inoué *et al.*, 1976a,b,c). It was also shown that if a series of flashes was given at room temperature prior to continuous illumination at −65°C, the intensity of peak I oscillated with flash number (Inoué and Shibata, 1978a,b). Maxima were observed after the second and sixth flashes. At first it was suggested that the positive charge involved in peak formation was on an unknown side path component that could donate a charge when the S_3 state was present (Inoué and Shibata, 1978a,b). Later, however, Inoué and Shibata (1979) and Inoué (1981) suggested that the S_4 state may be involved.

Peak I in Tris-washed chloroplasts could be partially charged by a single flash given at −15°C, and it was tentatively attributed to $Z^+Q_B^-$ recombination (Rutherford and Inoué, 1984a). The attribution of this band to $Z^+Q_B^-$ does not necessarily contradict its assignment to S_4, since Z^+ in the presence of S_3 may constitute S_4. In untreated chloroplasts there are no flash data pertaining to the location of the negative charge involved in peak I formation, although the inverse relationship between the intensity of peak I and peak IV reported by Laüffer *et al.* (1978) may point toward the involvement of Q_B^-.

More recently a detailed study of peak I has been reported (Demeter *et al.,* 1985b,c). When DCMU was added after preillumination with 2 flashes, the amplitude of the peak generated by illumination at −80°C was enhanced. This indicates that Q_A^- is involved in the recombination reaction that gives rise to this peak. Under some conditions the amplitude of the peak seemed to be modulated by the redox state of Q_B^-, but the mechanism of this effect is not understood.

The calculated kinetics of the corresponding luminescence at room temperature was 4 ms and it was suggested that Z^+ was the source of the positive charge involved in the luminescent reaction (Demeter, 1985b).

D. Peak II (the D or Q Band)

When whole algae or leaves are frozen under illumination, a peak is usually present between 0 and −12°C. This band is intensified when diuron is present (Rubin and Venediktov, 1969; Desai *et al.,* 1975). In isolated chloroplasts, freezing under illumination generates this peak only if diuron is present (Ichikawa *et al.,* 1975; Lurie and Bertsch, 1974a; Sane *et al.,* 1977; Demeter *et al.,* 1979).

Peak II is not excited by far-red light, indicating that it arises from PSII (Desai *et al.,* 1975). Lurie and Bertsch (1974a), Sane *et al.* (1977) showed that the PSII particles contained peak II while in PSI particles it was absent or diminished.

The intensification of peak II by the addition of diuron is accompanied by the loss of peaks I and IV and a diminution in peak III (Rubin and Venediktov, 1969; Lurie and Bertsch, 1974a; Ichikawa *et al.,* 1975; Demeter *et al.,* 1979). Desai *et al.* (1975) showed that peak II was accompanied by a decrease in fluorescence yield, indicating that Q_A^- oxidation was associated with the emission of this luminescence (see Fig. 3). From this fluorescence work and the effect of diuron it seemed likely that Q_A^- is the source of the electron for peak II formation (Desai *et al.,* 1975). The possible involvement of the *S* states in peak II formation was pointed out by Sane *et al.* (1977) on the basis of its sensitivity to heat treatment.

Rutherford *et al.* (1982) showed that a single flash given at −15°C to diuron-treated chloroplast gave rise to a peak II (Fig. 4). This was attributed to $S_2Q_A^-$ recombination. When diuron was added to chloroplast after two flashes a peak II was also formed, and this was attributed to $S_3Q_A^-$ recombination (Demeter, 1982; Demeter *et al.,* 1982; see also Rutherford *et al.,* 1982). In this way oscillations of peak II with flash number were demonstrated (Demeter, 1982; Demeter *et al.,* 1982).

Sane *et al.* (1983b) showed that the diuron peak II was lost when the O_2-evolving enzyme was inhibited by Tris washing or tetranitromethane

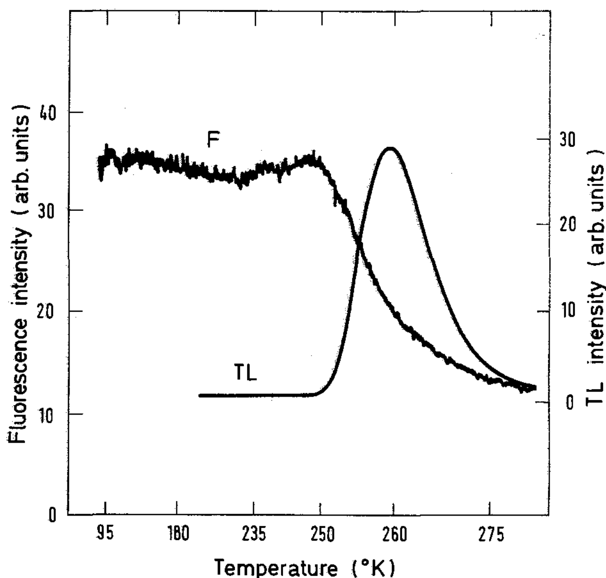

FIG. 3. Relationship between TL peak II and the chlorophyll *a* fluorescence (F) yield in spinach leaf. The leaf had been frozen to 77°K under intense white light. (From Desai *et al.*, 1975.)

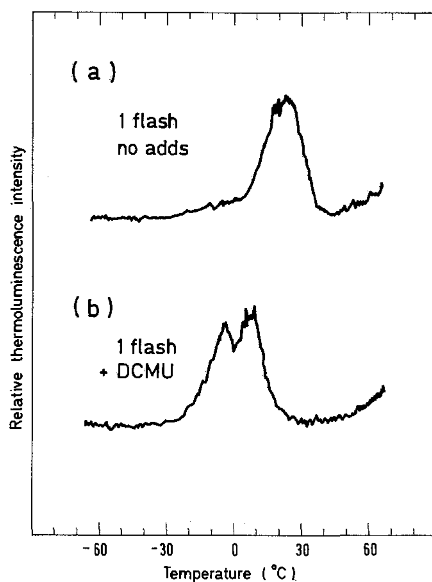

FIG. 4. Thermoluminescence by a single flash at −15°C in spinach chloroplasts: (a) no additions; peak maximum is at 25°C (peak IV); (b) +DCMU (diuron); peak maximum is at 0°C (peak II). (From Rutherford *et al.*, 1982.)

addition. The ADRY reagents CCCP (Sane *et al.*, 1983a) and ANT2P (Renger and Inoué, 1983) were shown to diminish the intensity of peak II, in agreement with its assignment to $S_{2/3}Q_A^-$.

E. Peak III

Peak III, as described by Desai *et al.* (1975) and Sane *et al.* (1977, 1983a,b), is a TL peak at 10°C in samples frozen under illumination. Although peak III is clearly observed in intact algal cells and leaf disks, it is not normally observed in untreated chloroplasts (Sane *et al.*, 1977, 1983a).

This peak may have been designated B_1 in some reports, but in most work with chloroplasts and/or flash illumination the B_1 peak is more likely to be equivalent to peak IV and peak III is not observed.

The presence of diuron decreases peak III, but the effect is less marked than for peak I or IV (Sane *et al.*, 1977, 1983a,b). This effect could indicate that the electron involved in formation of this peak is from an intermediate after the diuron block or that more than one turnover is required for peak III formation.

Sane *et al.* (1983b) showed that when DCCD was added to chloroplasts peak III became prominent. It has been suggested that DCCD blocks electron transfer into the plastoquinone pool (Sane *et al.*, 1979); if this is accepted, then the source of the electron for peak III formation could be Q_B^{2-}.

When peak III was induced in chloroplast by the presence of DCCD it was found that treatments which destroyed the O_2-evolving enzyme completely eliminated peak III (Sane *et al.*, 1983a).

From the available data it is not possible to definitely assign the charge pair responsible for peak III formation; however, reasonable candidates would be $S_2Q_B^{2-}$ or $S_3Q_B^{2-}$ recombination. This recombination probably occurs only when Q_B^{2-} transfer to the plastoquinone pool is blocked by DCCD in chloroplasts. It is not clear then why this peak should be present in the absence of DCCD in intact leaves and algae. Flash experiments in the presence of DCCD may help to determine the origin of this peak.

F. Peak IV (the B Band or B_1 and B_2 Bands)

1. CONTINUOUS ILLUMINATION STUDIES

Peak IV is a prominent peak at temperatures around 30°C in chloroplasts, leaves, and algal cells and is eliminated by diuron (see e.g.,

Arnold and Azzi, 1968; Rubin and Venediktov, 1969; Lurie and Bertsch, 1974a; Desai *et al.*, 1975; Ichikawa *et al.*, 1975) and by DBMIB (which blocks oxidation of the PQ_{pool} and also electron transfer from Q_A^- to Q_B; see Fig. 1) (Demeter *et al.*, 1979). Extraction of the PQ pool diminishes this peak and it is reconstituted by adding back the extracted plastoquinone (Sane *et al.*, 1983b). The peak was also lost when DCCD was present and was diminished in the presence of $HgCl_2$, an inhibitor of plastocyanin, PC (Fig. 1; Sane *et al.*, 1983b). From these results Sane *et al.* (1983b) suggested that peak IV was inhibited whenever electron flow between the two pigment systems was blocked and that electron flow beyond PC was necessary for the appearance of this peak. It was suggested that this electron flow to PSI might be necessary if some kind of energized state of the membrane played a role in generating the peak (Sane *et al.*, 1983b). However, evidence from previous work with uncouplers of photophosphorylation do not support such a suggestion (Demeter *et al.*, 1979). The presence of this peak in mutants lacking PSI (Arnold and Azzi, 1968; Lurie and Bertsch, 1974a) and in isolated PSII particles (Lurie and Bertsch, 1974a) also weighs against the requirement for PSI.

The absence of peak IV in material where the O_2-evolving system is not developed (Inoué, 1976; Inoué *et al.*, 1976a,b) or inhibited (Inoué *et al.*, 1977; Rozsa and Demeter, 1982; Sane *et al.*, 1983a) suggests that the S states are involved in the appearance of this peak. The reduction of peak IV intensity in the presence of ADRY reagents may support the involvement of the higher S states in the formation of this peak (Ichikawa *et al.*, 1975; Demeter *et al.*, 1979; Sane *et al.*, 1983a). Which S state may be involved has not been determined in continuous light studies.

2. THE FLASH DATA

The peak induced by flash excitation in untreated chloroplasts is at around 30°C. After a series of flashes given at room temperature, the intensity of this band was found to oscillate with a period of four (Inoué and Shibata, 1978a,b). The maxima occurred after the 2nd, 6th, and 10th flashes. From Kok's S state model (Kok *et al.*, 1970) it was concluded that this peak is related to the S_3 state. The weaker TL band observed at the same temperature after the first flash was attributed to S_2. It was subsequently shown that the peak at about 25°C (peak IV) induced by a single flash was shifted to 0°C (peak II) if diuron was present (see Fig. 4) (Rutherford *et al.*, 1982).

It was suggested that the flash-induced peak, in the absence of diuron, is due to recombination of a charge on S_2 or S_3 with an electron on an

intermediate beyond the diuron inhibitory site (Rutherford *et al.*, 1982). Involvement of PQH_2 from the pool was ruled out since its oxidation (by excitation of PSI with far-red light in the presence of methyl viologen as an electron acceptor) did not alter peak IV. It was therefore proposed that Q_B^- was the source of the electron involved in formation of this peak. Indeed Q_B^- was the most likely candidate since this is the state in which electrons are stably stored after a flash before leaving PSII upon a second flash, according to the two-electron gating mechanism (Bouges Bocquet, 1973; Velthuys and Amesz, 1974).

Since Q_B^- was known to be stable in the dark in approximately 30% of the centers (Fowler, 1977; Wollman, 1978), it was evident that Q_B^- could be involved in recombination after every flash. It was also predicted that the relative TL amplitude on the first and second flashes would depend on the ratio of Q_B to Q_B^- present in the dark. This ratio would also determine the oscillation pattern. A number of experiments were carried out to test this:

1. Dark adaptation resulted in decreased Q_B^- in the dark and consequently in a higher TL amplitude after the first flash and a change in the flash pattern from one with maxima on 2 and 6 flashes to one with maxima on flashes 1 and 5 (see Fig. 5) (Rutherford *et al.*, 1982). Taken to an extreme, after 6 h of dark adaptation the TL remaining showed a period of two oscillations with maxima on odd-numbered flashes (Demeter and Vass, 1984).
2. Preillumination with strong light resulted in an increase in the concentration of Q_B^- stable in the dark, and consequently the oscillation pattern showed maxima on flashes 2 and 6 (Rutherford *et al.*, 1982).
3. Low-temperature illumination given before or after flash excitation resulted in the introduction of a single electron into the electron acceptor complex of PSII at the expense of cytochrome *b*-559. This inverted the Q_B/Q_B^- ratio without changing the *S* states. Hence, in dark-adapted chloroplasts which showed an oscillation with maxima on flashes 1 and 5, low-temperature illumination given after flash excitation changed the pattern to one with maxima on flashes 2 and 6 (see Fig. 5) (Rutherford *et al.*, 1982).
4. Chemical oxidation of Q_B^- to Q_B by washing with potassium ferricyanide increased the thermoluminescence on the first flash relative to that on the second, as predicted (Rutherford *et al.*, 1984a).

All of these results strongly support the conclusion that the flash-induced peak IV arises from $S_2Q_B^-$ and $S_3Q_B^-$ recombination. By using this conclusion together with the *S* state model of O_2 evolution (Kok *et al.*, 1970) and the two-electron gate theory for Q_B^- formation (Bouges

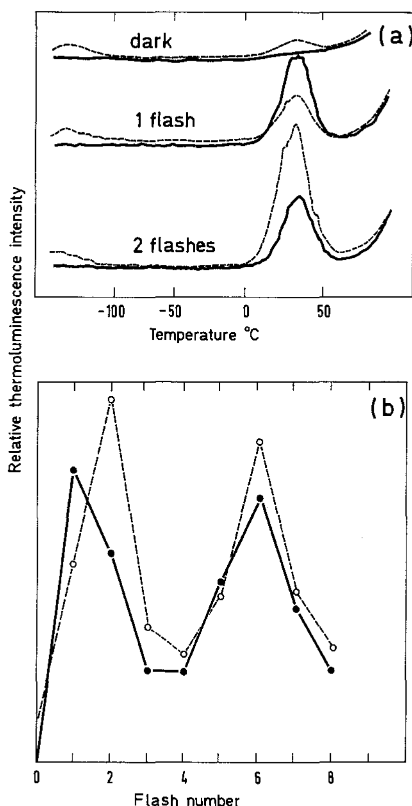

FIG. 5. Thermoluminescence (peak IV) recorded after a series of flashes in dark-adapted spinach chloroplasts: (a) TL curves; (b) plot of peak IV insensity versus flash number. Flashes were given at 20°C and samples were rapidly frozen in the dark. Dashed lines are from samples which were illuminated at 77°K before TL was recorded. Solid lines are from samples that did not receive this extra illumination. (From Rutherford *et al.*, 1982.)

Bocquet, 1973; Velthuys and Amesz, 1974), the TL flash pattern was simulated for the first time (Rutherford *et al.*, 1982). Using the same criteria, Demeter and Vass (1984) also simulated the oscillation pattern for peak IV.

G. Peak V (the C Band)

There are several reports of a TL band at approximately 50°C (see e.g., Rubin and Venediktov, 1969; Desai *et al.*, 1975; Ichikawa *et al.*, 1975). This band was preferentially charged in leaves when they were

illuminated at 77°K (Desai *et al.*, 1975; Ichikawa *et al.*, 1975). The peak has been shown to be insensitive to (Rubin and Venediktov, 1969) or enhanced by diuron (Desai *et al.*, 1975; Ichikawa *et al.*, 1975). A relationship between this band and PSI photochemistry has been proposed, since in leaves this band is charged by 740-nm light (Desai *et al.*, 1975, 1983). In addition, the band was shown to be present in Tris-washed chloroplasts (Inoué *et al.*, 1977) and when the O_2-evolving enzyme was not developed (Inoué *et al.*, 1976a,b,c). Sane *et al.* (1977) also reported that PSI-enriched subchloroplasts were relatively enriched in this band.

In contrast to the idea that peak V results from PSI, recent evidence indicates unequivocally that PSII is involved. Demeter *et al.* (1984) reported that a peak at 50°C oscillated with flash number in chloroplasts where diuron was added after flash excitation. The oscillation pattern showed maxima associated with the S_0 and S_1 states. The fact that diuron induces this band indicates that Q_A^- is involved in its formation. The location of the positive charge is more difficult to determine. The involvement of cytochrome *b*-559$^+$ was ruled out on the grounds that it does not compete with the *S* states as a donor at physiological temperatures. Demeter *et al.* (1984) also discounted the involvement of signal II-slow in the formation of this peak. This argument was based on a comparison of EPR data in the literature on the stability of signal II-slow and effects of inhibitors on this signal. It was concluded that the band arose either from recombination of $S_0Q_A^-$ and $S_1Q_A^-$ or from recombination of an unidentified donor and Q_A^-. However, it is unlikely that S_0 and S_1 would be thermodynamically equivalent, and if they were able to supply positive charges to a recombination reaction, the reactions for S_0 and S_1 would not occur at the same temperature. EPR experiments in which signal II-slow and Q_A^- were monitored directly indicated that recombination of the charges on these components could be responsible for luminescence at 50°C (A. W. Rutherford, unpublished).

Further data indicate that peak V is not due to recombination in PSI. First, work by Sane *et al.* (1983b) showed that $HgCl_2$, which inhibits between plastocyanin and P700, had little effect on this peak, indicating that the charge storage state would have to be P700$^+$. It is highly unlikely that P700$^+$ would be sufficiently long-lived, under the conditions of the experiment, to play such a role. Second, peak V is present in PSII particles (Berthold *et al.*, 1981) that are effectively uncontaminated by PSI (A. W. Rutherford, unpublished).

There are two ways to reconcile the observation that peak V is charged by 740-nm light. First, it can be assumed that two different bands emit at this temperature. Although this is a rather unsatisfactory answer, it is certainly possible. Alternatively, it is possible that peak V occurs by re-

combination in PSII (possibly recombination of signal II-slow and Q_A^-) but can be enhanced by PSI turnover in some way (e.g., by PQ_{pool} oxidation or electrochemical gradient formation).

H. Origins of Thermoluminescence in Plants: An Overview

Table I shows the conclusions on the origins of each of the TL peaks. It is clear that a number of the assignments are tentative. The assignments that are clear (peak II as $S_{2/3}Q_A^-$ and peak IV as $S_{2/3}Q_B^-$) have been obtained by comparisons with the literature data on the well-characterized physiological charge storage system in PSII. The photochemistry of PSII at higher and at lower temperatures is less well characterized, and thus the conclusions concerning the origins of the charges involved in the other TL bands will remain tentative until complementary experiments are carried out by other techniques. The involvement of Z^+ in peak I, for example, might be tested by EPR work in this temperature region. The D_z donor suggested to be involved in the Z_v band may correspond to the new $g = 4$ donor recently discovered by EPR (Casey and Sauer, 1984; Zimmermann and Rutherford, 1984).

The relationship between the TL peaks and certain charge pairs in PSII is shown schematically in Fig. 6.

I. Thermoluminescence in Photosynthetic Bacteria

Few studies of TL in bacteria have been published. Fleischman (1971) reported that *Rhodopseudomonas viridis* showed a single TL peak under anaerobic conditions but two peaks under aerobic conditions. Comparison of the TL data with direct optical and EPR measurements of P960, the primary bacteriochlorophyll donor, and the reaction center-bound cyctochromes (Fleischman and Cooke, 1971) led to the suggestion that the luminescence was due to a positive charge trapped on P960 recombining with an electron on a secondary acceptor (Fleischman, 1971). Further work on the slow delayed light emitted under similar conditions, or the delayed light induced by increasing the pH, led to the conclusion that the luminescence was due to $P960^+Q_B^-$ recombination (Fleischman *et al.*, 1984). It is likely that this $P960^+Q_B^-$ recombination, which gives rise to delayed fluorescence on a time scale of seconds, is also responsible for one of the TL peaks (D. Fleischman, personal communication). (See Jursinic, Chapter 11, this volume, for further discussions on delayed fluorescence or delayed light emission.)

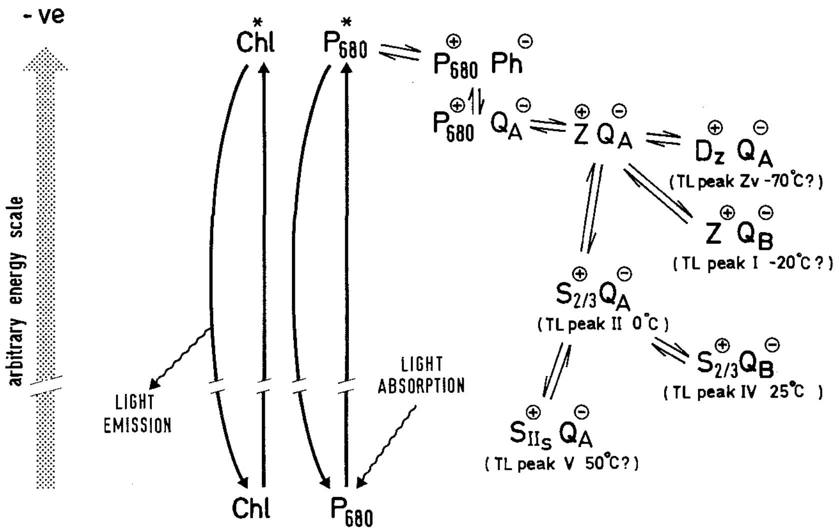

FIG. 6. A scheme of electron transport in PSII showing the formation of a series of charge pairs and their relationship (in some cases, possible relationship) to the TL bands. (See list of abbreviations and symbols.) The charge pairs are placed on an energy scale relative to each other, although the energy difference between each pair is arbitrary. Forward electron transfer results in the formation of a series of charge pairs, each more stable than the preceding one. A relationship between the stability of the charge pair and the temperature of the TL peak is evident.

Govindjee *et al.* (1977) also reported TL from photosynthetic bacteria. However, this was not due to charge recombination. It was shown to arise from magnesium protoporphyrin IX, a precursor of bacteriochlorophyll, which accumulates in the organism.

VI. Relationship between Thermoluminescence and Delayed Fluorescence

The common origin of TL and delayed fluorescence has been discussed since the first reports of the phenomena. In particular, Shuvalov and Litvin (1969) correlated certain TL bands with phases of delayed fluorescence. Although this was questioned by Lurie and Berstch (1974b), recent work has verified their assignment of the TL peak in the presence of diuron to a phase of delayed fluorescence occurring in 1.5 s (Rutherford *et al.*, 1982; Rutherford and Inoué, 1984a; Rane and Sane, 1985).

Experiments designed specifically to demonstrate the common origin of TL and delayed fluorescence were reported by Desai *et al.* (1982). (For a discussion of delayed fluorescence, see Jursinic, Chapter 11, this volume.) The interconversion of TL and delayed fluorescence was demonstrated (Fig. 7). The identification of the major TL bands as being due to $S_{2/3}Q_A^-$ (peak II) and $S_{2/3}Q_B^-$ (peak IV) recombination allowed a more specific correlation to be made. The $S_{2/3}Q_A^-$ peak was correlated with the 1.5-s phase, which had been well characterized earlier (Lavergne and Etienne, 1980), and an $S_{2/3}Q_B^-$ phase was predicted to be present which decayed on a time scale of tens of seconds (Rutherford *et al.*, 1982). Such a phase of delayed fluorescence decaying in 30 s and oscillating with the predicted pattern after a series of flashes was subsequently discovered (Rutherford and Inoué, 1984a) (see Fig. 8). In the same work the flash-induced TL band at −7°C in Tris-washed chloroplasts, tentatively attrib-

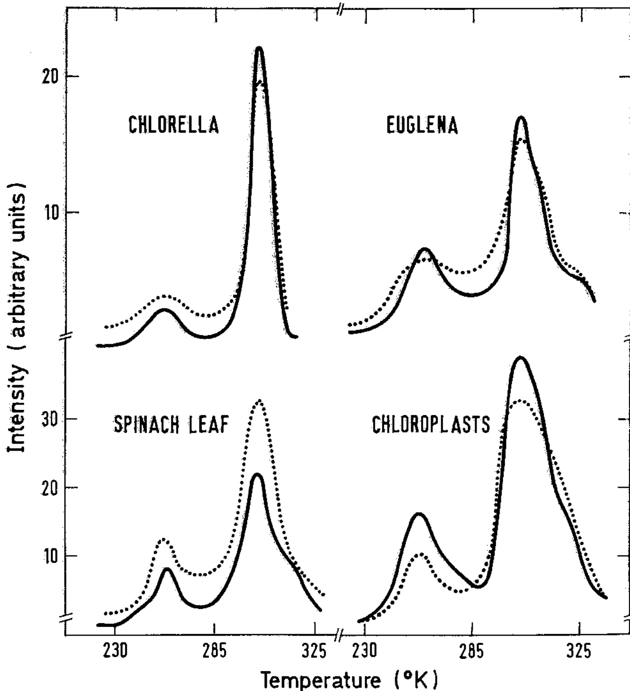

FIG. 7. Comparison of TL curves (——) with delayed light emission (·····) observed in different photosynthetic materials. The delayed light emission pattern was obtained by plotting the intensity of delayed light emission observed 2.5 s after excitation at different temperatures. (From Desai *et al.*, 1982.)

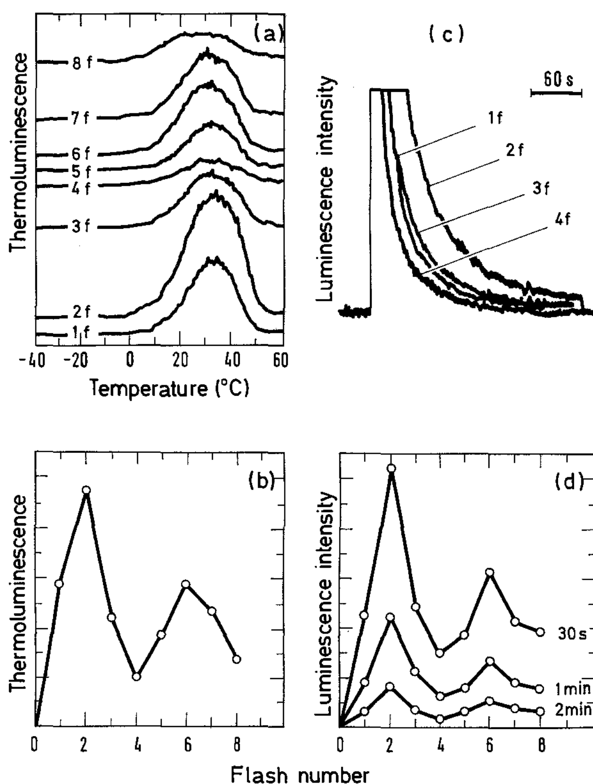

FIG. 8. Relationship between flash-induced TL (peak IV) and delayed fluorescence (luminescence) in spinach leaves: (a) TL recorded after a series of flashes (f); (b) TL intensity (peak IV) plotted as a function of flash number; (c) delayed fluorescence recorded after a series of flashes; (d) delayed fluorescence intensity at 30 s, 1 min, and 2 min after flash excitation plotted as a function of flash number. All flashes were given at room temperature. (From Rutherford *et al.*, 1984d.)

uted to $Z^+Q_B^-$ recombination, was correlated with a phase of delayed fluorescence decaying on a time scale of hundreds of milliseconds.

Similar experiments have been done by Rane and Sane (1985), who also attempted to correlate the different components of delayed fluorescence with the TL peaks by using continuous light excitation. The inhibitors diuron and DCCD were used to generate specific TL peaks and results similar to those cited above were obtained; in addition, peak III, tentatively attributed to $S_{2/3}Q_B^{2-}$ recombination (see Section V), corresponded to a delayed fluorescence phase decaying in 35 s (Rane and Sane, 1985).

Since the TL peaks are well separated from each other, their origins may, at least in some cases, be determined more easily than those of the overlapping kinetic phases of delayed fluorescence. For this reason it seems likely that future research aimed at establishing the charge pairs responsible for the unidentified TL peaks (see Section V) will be useful in understanding the origins of the equivalent phases of delayed fluorescence.

VII. Physical Parameters Obtained from Thermoluminescence

In early work (Arnold and Azzi, 1968, 1971; Shuvalov and Litvin, 1969) activation energies were calculated for a number of TL peaks, using the unmodified Randall–Wilkins theory (Randall and Wilkins, 1945). This theory was developed from work with inorganic crystals and assumes a single-step migration of trapped electrons to more stable locations (positive charges), emitting light in the process. The calculated activation energies based on measurements of the initial rise of the individual peaks (Shuvalov and Litvin, 1969; Tatake *et al.*, 1981) and those based on the temperature of the peak maximum and peak half-heights (Arnold and Azzi, 1968; Lurie and Bertch, 1974b; Tatake *et al.*, 1981) were contradictory. Experimental error certainly contributed to the large discrepancies between the results obtained by different groups. Since most of the data were measured on TL curves generated by continuous illumination while freezing—conditions which produce a range of different states and associated TL peaks—it is not suprising that difficulties were encountered in trying to obtain data from overlapping peaks. Another serious problem with most of these calculations was that the activation energy was obtained by using an arbitrarily decided frequency factor. By a number of different methods, Tatake *et al.* (1981) calculated the activation energies for the glow peaks without an assumed frequency factor. The frequency factor itself and the lifetime of the states responsible for each glow peak were calculated by using the values for the activation energies. It was found that the frequency factors were unreasonably large, and it was concluded that simple application of Randall–Wilkins theory was not appropriate for the major TL peaks from plants.

In a contemporary study, Vass *et al.* (1981) attempted to resolve the problem of overlapping glow peaks by using a computer-assisted curve resolving technique. Using data from the whole of each of seven re-

solved peaks, they calculated the activation energy, the frequency factor, the free energy of activation, and the lifetime of the states responsible for the TL by slightly modified version of the Randall–Wilkins theory. It was noted that the activation energies and the lifetimes of the states at the temperature maxima of the peaks were greater than would be expected if the TL band reflected simply recombination of the primary charge separation step, and it was suggested that the TL peak also represented reversal of the charge translocation and stabilization steps (Vass *et al.*, 1980, 1981; Demeter *et al.*, 1981). DeVault *et al.* (1981, 1983) also postulated the involvement of reversal of the charge translocation steps to account for the unusual physical propeties of the TL curves in plants. They proposed that the light-emitting charge recombination could occur only when charges, stabilized on relatively distant components, returned to the primary reactants via a series of temperature-dependent equilibrium reactions. It was demonstrated that such a mechanism could explain the abnormally large apparent activation energies and apparent frequency factors (DeVault *et al.*, 1983). The involvement of reversal of secondary electron transfer steps in TL had been clear for several years (see e.g., Rubin and Venediktov, 1969) and was the basis for most of the experiments on the origin of the TL peaks, but was not taken into account in previous work where Randall–Wilkins theory was applied to plant TL.

Further modifications of the understanding of the physical side of TL came from Vass and Demeter (1984), who took into account nonradiative depletion of the traps. These authors also presented data taken at different heating rates, from which activation energies for nonradiative depletion were calculated.

A relationship between the apparent free energy of activation and the midpoint potentials of the charge transfer states was discussed briefly by Vass *et al.* (1981). After the identification of the flash-induced TL bands at 0 and 25°C as arising from $S_{2/3}Q_A^-$ and $S_{2/3}Q_B^-$, respectively, it was pointed out that the differences in the activation energies for these two bands should include a contribution from the free energy difference between the redox midpoint potentials of the Q_A/Q_A^- and Q_B/Q_B^- couples (Rutherford *et al.*, 1982; *cf.* DeVault *et al.*, 1983). Based on the identification of these bands, and using computer-assisted curve fitting to the flash-induced TL, Demeter and Vass (1984) calculated the redox span between these two redox couples from the apparent free energies of activation. The value of 70 mV between the E_m of Q_A/Q_A^- and Q_B/Q_B^- is reasonable in comparison with the value obtained from kinetic data (reviewed in Crofts and Wraight, 1983). However, the reliability of the

values for the apparent free energy of activation calculated from the abnormal frequency factors and activation energies remains to be demonstrated. The conclusion from TL work that $S_2Q_B^-$ and $S_3Q_B^-$ are identically stable (at normal pH) indicated that S_2 and S_3 have similar midpoint potentials (Rutherford *et al.*, 1982; Demeter and Vass, 1984). This is in agreement with the conclusions drawn earlier from kinetic considerations (Bouges Bocquet, 1980).

It has been demonstrated that one of the factors important in the formation of millisecond delayed fluorescence is the presence of electrochemical potential gradients across the photosynthetic membrane (see e.g., Crofts *et al.*, 1971; Jursinic, Chapter 11, this volume). Such gradients could also influence the position and amplitude of TL bands under some circumstances. For the work done with small numbers of flashes and dark-adapted broken chloroplasts, it is very unlikely that such gradients exist. Indeed, some work with gramicidin under these circumstances showed no significant effects on TL (G. Renger and A. W. Rutherford, unpublished data). Similarly, continuous illumination of broken chloroplasts almost certainly results in blockage of electron transfer (due to the absence of a PSI electron acceptor system) before significant gradients can build up (see e.g., Rutherford and Inoué, 1984a). Thus, under these circumstances too it does not seem likely that electrochemical gradients play any role in TL (see Vass *et al.*, 1981).* In contrast, in intact algae or leaves or in chloroplasts with an intact or exogenous acceptor system (e.g., methyl viologen), such gradients could modify TL to a significant extent. Such effects would be especially marked when continuous illumination at room temperature is given. The effects of ionophores on TL in such systems should help to determine the extent of any putative effects of electrochemical gradients and might also provide an explanation for the discrepancies in phenomenology and interpretation of TL observed with different biological materials and different illumination regimes.

VIII. Thermoluminescence as a Probe of PSII Photochemistry

Since the origins of some of the TL peaks have been clearly determined, it has been possible to use the peaks as probes of the photochemistry of PSII. In this section some applications of thermoluminescence in photosynthesis research are described.

* For studies on the effects of electric field on thermoluminescence, see Knox and Garab (1982) and Knox *et al.* (1984).—editors.

A. Temperature Dependence of the Water Oxidation Reactions

Having established the relationship between flash-induced peak IV and the S_2 and S_3 states of the O_2-evolving enzyme, Inoué and Shibata (1978a,b) studied the temperature dependence of S state formation. Flash excitation was given between 17 and −35°C, and from the changes in the oscillation pattern of peak IV intensity the following conclusions were drawn: (1) at −10°C water already bound to the "oxygen-evolving enzyme" can be oxidized, but a second cycle of water oxidation does not take place, indicating that binding of water to the enzyme is inhibited at this temperature. (2) At −20°C, the S_3-to-S_4 step is apparently blocked. (3) At −35°C, the S_2-to-S_3 state is blocked. (4) At −65°C the S_1-to-S_2 step still occurs. Although some of these results may be related to the temperature dependence of electron transfer on the acceptor side of PSII, they have proved useful in subsequent research and until recently they were the only data available on the temperature dependence of the O_2-evolving enzyme.

B. Effects of Herbicides

The effects of certain electron transport inhibitors were observed in some of the earliest reports of TL in plants (see Section V). Attempts have been made to classify herbicides by their effects on the TL peaks (Droppa *et al.,* 1981; Vass and Demeter, 1982). It was found that the emission temperature of the herbicide-induced peak II (now known to be due to $S_2Q_A^-$ recombination) varied depending on the type of herbicide used, and it was suggested that these differences (or the differences in the physical parameters calculated from the peak) might reflect differential herbicide effects on the stability of Q_A^-. The validity of this classification is doubtful since multiple turnover effects due to the excitation by continuous illumination and the ADRY effects of phenolic herbicides on the electron donor side of PSII (Rutherford *et al.,* 1984b; Mathis and Rutherford, 1984) were not taken into account. Nevertheless, it was clearly demonstrated that TL could be used to do herbicide displacement experiments to investigate the proximity of binding sites of different herbicides (Droppa *et al.,* 1981; Demeter *et al.,* 1982).

Demeter *et al.* (1985a) recently applied TL to the study of herbicide resistance in plants. It was shown that the emission temperature of the $S_2Q_A^-$ peak (II) was unchanged in atrazine-resistant mutants but that the $S_{2/3}Q_B$ peak was shifted to a lower temperature (from 30 to 15°C). This effect was interpreted as being due to a lowering of the midpoint redox

potential (E_m) of the Q_B/Q_B^- redox couple by approximately 30 mV in resistant chloroplasts.

Herezeg *et al.* (1979) investigated the effect of pyridazinone herbicides on TL. These herbicides greatly diminished the peaks appearing between 0 and 40°C. It was concluded that they acted on the O_2-evolving enzyme.

C. Deactivation of the S States

The recombination of positive charges on the S states with an electron from Q_B^- was demonstrated experimentally for the first time by using TL (Rutherford *et al.,* 1982). This agreed with earlier suggestions that Q_B^- could be the source of deactivating electrons for S_2 (Bouges Bocquet, 1975; Diner, 1977), and subsequent work has provided further support for the importance of this reaction as a deactivation pathway (Robinson and Crofts, 1983; Demeter and Vass, 1984; Rutherford and Inoué, 1984a; Rutherford *et al.,* 1984a,c,d; Vermaas *et al.,* 1984). The suggestion that TL on flash 2 results from analogous $S_3Q_B^-$ recombinations is more controversial (Rutherford *et al.,* 1982). The almost identical temperature maxima of the $S_2Q_B^-$ and $S_3Q_B^-$ TL peaks indicate that these states have similar stabilities and hence similar decay rates. This agrees well with the observation that S_2 and S_3 deactivation rates have similar half-times (30 s) in chloroplasts (see e.g., Joliot and Kok, 1975). In contrast, the work of Diner (1977) indicated that acceptor side electrons were not involved in S_3 deactivation; however, the $S_3Q_B^-$ state was probably present in only a small proportion of the centers under the conditions of these experiments. It was pointed out (Rutherford and Inoué, 1984a; Rutherford *et al.,* 1984c,d) that the TL data could not distinguish betwen two possible pathways for TL formation from the $S_3Q_B^-$ state: first, $S_3Q_B^-$ recombination luminescence, $S_3Q_B^- \xrightarrow{\nearrow TL} S_2Q_B \xrightarrow[e]{\nearrow} S_1Q_B$; and second, nonrecombination deactivation of S_3 followed by recombination of $S_2Q_B^-$, $S_3Q_B^- \xrightarrow[e]{\nearrow} S_2Q_B^- \xrightarrow{\nearrow TL} S_1Q_B$. Nevertheless, the fact that the $S_2Q_B^-$ TL is shifted to a higher temperature by decreased pH (due to protonation of Q_B^-) while $S_3Q_B^-$ is unaffected (perhaps due to S_3 deprotonation canceling out Q_B^- protonation) indicates that true $S_3Q_B^-$ recombination is being observed (Rutherford *et al.,* 1984a,c).

Measurements of the stability of peak IV were used by Inoué and Shibata (1978a,b) and by Laüffer and Inoué (1980) as a probe of the lifetime of S_2 and S_3. It was pointed out in subsequent work that these measurements reflect only those centers in which Q_B^- is present (Rutherford *et al.,* 1984c,d). However, by giving a period of illumination at 77°K the centers which deactivated in the state S_2Q_B could be detected. It was

found that deactivation of S_2 is more rapid in centers where Q_B^- is present than in centers where Q_B is present (Rutherford *et al.*, 1984c,d).

By performing deactivation experiments at low pH (conditions where the $S_2Q_B^-$ and $S_3Q_B^-$ bands have different emission temperatures) it was possible to show that S_3 deactivated via S_2 (Rutherford and Inoué, 1984b). This agreed with earlier conclusions from measurements of O_2 evolution (see, e.g., Joliot and Kok, 1975).

The involvement of Q_B^- in recombination reactions with S_2 and S_3 provided an explanation for the existence of stable Q_B^- in dark-adapted chloroplasts. It was assumed that Q_B^- was stable whenever it was present with S_0 or S_1 but that it would recombine whenever it was with S_2 or S_3. This led to the prediction that Q_B^- is present in 25% of the centers in a dark-adapted sample (Rutherford *et al.*, 1982; see also Velthuys, 1980). By using TL it was shown that dark-stable Q_B^- was present in up to 50% of the centers when broken chloroplasts were dark-adapted after a period of strong illumination (Rutherford *et al.*, 1982). It was suggested that this could be due to $S_2Q_B^{2-}$ recombination to form $S_1Q_B^-$, which could occur in the presence of a fully reduced PQ pool (Rutherford and Inoué, 1984; Rutherford *et al.*, 1984a,c). As a result of the involvement of Q_B^- in deactivations of S_2 and S_3, one or two preflashes followed by dark adaptation not only synchronizes the S states in S_1 but also lowers the concentration of Q_B^- which is stable in the dark. This effect was observed by using TL (Rutherford *et al.*, 1984a).

D. Function of ADRY Reagents

By measuring the $S_2Q_A^-$ (peak II) and $S_{2/3}Q_B^-$ (peak IV) TL bands with flash excitation in the presence of the ADRY reagent ANT2P, Renger and Inoué (1983) studied the function of this reagent. It was shown that ANT2P works as a mobile species deactivating S_2 and S_3 (i.e., eliminating peak IV) at substoichiometric concentrations. At subzero temperatures, the ANT2P was able to deactivate S_2 (i.e., eliminate peak II) only in centers in which the chemical was bound.

E. Effect of Bicarbonate Depletion

Bicarbonate depletion affects electron transport between Q_A and the pool (for a review, see Vermaas and Govindjee, 1982). Thermoluminescence has been used to investigate this effect. Sane *et al.* (1984) showed that bicarbonate-depleted chloroplasts exhibited TL like that observed in the presence of diuron in samples frozen under illumination. In a study with flash excitation, Govindjee *et al.* (1984) found that the $S_{2/3}Q_B^-$

recombination peak (IV) was shifted to higher temperatures and oscillations of the peak intensity with flash number were inhibited in bicarbonate-depleted chloroplasts. These effects were interpreted as being due to inhibition of electron transfer in the acceptor quinone complex, possibly due to a conformational change that may slow down electron transfer from Q_B^{2-} to the PQ pool.

F. Electron Transport in Thermophilic Species

Govindjee *et al.* (1985b) reported flash-induced peaks in the thermophilic alga *Synechococcus vulcanus.* Interestingly, it was found that the $S_{2/3}Q_B^-$ recombination band (peak IV) had an emission maximum at 55°C instead of 30°C in normal chloroplasts. The stability of the peak was predictably greater, and this was also reflected in the slow deactivation times of the S states, which were measured with conventional O_2 electrode techniques.

G. Thermoluminescence as a Probe of the S States When O_2 Evolution Is Inhibited

By using isolated PSII membranes, flash-induced TL was recorded in samples in which the 24-kilodalton (kD) polypeptide was depleted by salt washing. Under these conditions O_2 evolution was inhibited by 60% and yet the $S_{2/3}Q_B^-$ TL band (peak IV) was unaffected in terms of its amplitude. However, oscillations of the TL intensity with flash number no longer occurred, the peak was shifted to a slightly lower temperature. Reconstitution of the 24-kD polypeptide regenerated O_2 evolution and the normal peak position. This work (A. W. Rutherford, M. Yuasa, and Y. Inoué, unpublished) is an indication that S state advancement can take place in the absence of the 24-kD polypeptide.

These observations were extended by Ono and Inoué (1985) who showed that the $S_{23}Q_B^-$ band was still present even when the 33-kD polypeptide was removed. The inhibition of oscillations observed in these studies, although originally attributed to a direct effect of the polypeptide depletion on S_3 advancement, might in fact be due to a chloride depletion lesion (see review by Zimmermann and Rutherford, 1985 and the discussion by Ono *et al.,* 1986).

H. Electron Transport in PSII Measured in Leaves

Most of the measurements of PSII electron transfer listed above can be done as easily in leaves as in isolated membranes. Already TL has

provided measurements of charge accumulation on the S states and Q_B, deactivation of S_2, the redox state of Q_B in the dark, and the effects of herbicides in leaves (Rutherford *et al.*, 1984c,d). These kinds of measurements may prove useful in investigations of more physiological feedback processes which may influence electron transfer in living systems. Also, electron transfer in systems where membrane isolation is difficult (i.e., pine leaves or perhaps lichens) can be studied with TL (see Inoué *et al.*, 1976b,c).

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III
Bioluminescence

III

Bioluminescence

13

Bioluminescence in Bacteria and Dinoflagellates

JOHN WOODLAND HASTINGS
Department of Cellular and Developmental Biology
The Biological Laboratories
Harvard University
Cambridge, Massachusetts 02138

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ABBREVIATIONS

A	Acceptor of electrons
BL	Bioluminescence
CL	Chemiluminescence
cAMP	Cyclic adenosine monophosphate
Chl, Chl*	Chlorophyll and its electronically excited singlet
D	Donor of electrons
FMN (or F) and $FMNH_2$	Flavin mononucleotide and its reduced form, respectively
$h\nu$	Photon
kb	Kilobase
kD	Kilodalton
K_m	Michaelis–Menten constant
LBP	Luciferin binding protein
M_r	Molecular weight
mRNA	Messenger ribonucleic acid

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q	Quanta
NADP	Nicotinamide-adenine dinucleotide phosphate

ABSTRACT

Light emission in living organisms may be due either to prior absorption of light (photoluminescence) or to an exergonic chemical reaction (chemi- or bioluminescence). In photosynthesis the chemical species formed in the primary step following light absorption are similar to those postulated to be the penultimate states in many chemi- and bioluminescent reactions. Mechanistically, bioluminescence may be viewed as the reverse of photosynthesis. Bioluminescence occurs in organisms of many different phyla, including the bacteria and dinoflagellates described here. Most of the enzymes and substrates (luciferases and luciferins) are unrelated, indicative of evolutionarily different origins. In the bacteria light emission is continuous and occurs as the result of a reaction which shunts the respiratory pathway: a mixed-function oxidation of reduced flavin and long-chain aliphatic aldehyde by molecular oxygen. In dinoflagellates light emission is distinctly different. The substrate (luciferin) is an open-chain tetrapyrrole which bears a similarity to chlorophyll, and its oxidation results in luminescence. This occurs primarily in the form of flashes emitted from subcellular organelles. In the living cell control of the reaction and of the light flash is postulated to involve pH changes.

I. Introduction

A. *Energization of Electronically Excited States in Living Organisms*

There are two types of light emission in living organisms: *photoluminescence,* which is dependent on the prior absorption of light, and *chemiluminescence* (CL) or *bioluminescence* (BL), in which the energy from an exergonic chemical reaction is transformed to light energy.

Photoluminescence is the principal concern of this volume; it includes two distinctly different types of emission. The first includes fluorescence and phosphorescence, namely the reemission of light directly from electronically excited states (singlet and triplet) populated by the absorption of light. The second, which is well known to occur in photosynthetic systems, is the so-called delayed light emission or delayed fluorescence (see Lavorel, 1975). Energy from excited chlorophyll (Chl*) is utilized to form long-lived intermediates (oxidized and reduced) with lifetimes ranging from nanoseconds (ns) to hours (Jursinic, Chapter 11, this volume). These intermediates may then be annihilated to repopulate singlet excited states of Chl and thus give rise to the delayed light. Although this emission is tied to the prior absorption of light, its codiscoverer, Strehler, argued (Arthur and Strehler, 1957; Strehler and Lynch, 1957), and correctly we believe, that it should be considered a CL.

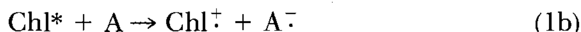
In this chapter we shall specifically discuss bioluminescence (Herring, 1978). BL is a CL, but it requires an enzyme (a luciferase) and does not involve prior absorption of light. The energy from a chemical reaction is channeled to result in the formation of electronically excited singlet states whose emission in the blue-green (approximately 500 nm) corresponds to at least 50 kcal/einstein (210 kJ/einstein).

B. Bioluminescence: Mechanistic Similarity to Photosynthesis

It is not generally appreciated that the resemblance between the fundamental photochemical steps in photosynthesis and BL may be more than superficial. Mechanistically, BL may be viewed as the reverse of photosynthesis: the primary chemical states formed in photosynthesis are comparable to what may be penultimate states in BL.

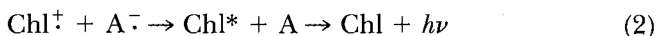
The absorption of a photon by Chl populates an excited state whose energy is subsequently captured chemically in the form of a primary oxidant and a primary reductant (Clayton, 1980; Parson and Ke, 1982). With A as the electron acceptor, these steps can be represented as:

$$\mathrm{Chl} + h\nu \rightarrow \mathrm{Chl}^* \qquad (1a)$$



$$\mathrm{Chl}^* + \mathrm{A} \rightarrow \mathrm{Chl}^{+\cdot} + \mathrm{A}^{-\cdot} \qquad (1b)$$

For the most part these intermediate species give rise to a stable oxidant and reductant. However, some may recombine and, as mentioned above, result in the reemission of light, "delayed" light in the form of emission from the singlet excited state of Chl.



$$\mathrm{Chl}^{+\cdot} + \mathrm{A}^{-\cdot} \rightarrow \mathrm{Chl}^* + \mathrm{A} \rightarrow \mathrm{Chl} + h\nu \qquad (2)$$

In BL, part of the energy made available in a luciferase-catalyzed substrate oxidation (always by molecular oxygen) is conserved in the form of an electronically excited state. Intermediate states may be very similar to those responsible for delayed light in photosynthetic systems. One proposed mechanism of CL and BL, which has received a great deal of attention, emphasizes this point (Faulkner, 1978; Schuster and Schmidt, 1982; Wilson, 1985), namely the chemically induced electron exchange mechanism (Koo *et al.*, 1978). According to this hypothesis, the several steps just prior to excited state formation in CL and BL [Eq. (3)] involve first the transfer of an electron from a donor (D) to an acceptor (A). The acceptor, now $\mathrm{A}^{-\cdot}$, which in the model contains a weak oxygen–oxygen bond, cleaves spontaneously to form products C and $\mathrm{B}^{-\cdot}$; the latter should be a stronger reductant than $\mathrm{A}^{-\cdot}$, so the electron is trans-

ferred back to $D^{\dot{+}}$ with energy release and formation of D*. All in all, these steps result in the formation of oxidant and reductant species similar to those postulated in photosynthetic systems, namely radical pairs, which are then annihilated by electron transfer with the concomitant formation of an excited state.

$$\begin{array}{l} A + D \rightarrow A^{\dot{-}} + D^{\dot{+}} \\ \qquad\quad \downarrow \\ \qquad C + B^{\dot{-}} \\ \qquad\quad B \qquad D^* \rightarrow D + h\nu \end{array} \tag{3}$$

BL and photosynthesis may thus share mechanisms which produce species capable of giving light emission via the transfer of a single electron.

C. Different Luciferins and Luciferases: Different Biochemical Mechanisms and Evolutionary Origins

Bioluminescence is not restricted to bacteria and algae; it also occurs in many other organisms in many different phyla, ranging from jellyfish and brittle stars to worms, fireflies, molluscs, and fish, to mention just a few (Harvey, 1952; Herring, 1978). Although detailed biochemical information concerning the structures of the luciferins (substrates) and luciferases (enzymes) is available for only some seven groups of organisms, the systems are very different (Table I). It has thus been estimated that among the present-day bioluminescent systems, the ability to emit light may have originated many different times during the course of evolution (Hastings, 1983).

Light emission in bacteria occurs continuously as the result of a reaction which shunts the respiratory pathway (Grogan, 1984; Hastings *et al.*, 1985); electrons at the oxidation level of reduced flavin are diverted to O_2 in a mixed-function oxidation specific for reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde (RCHO).

$$FMNH_2 + O_2 + RCHO \xrightarrow[\text{luciferase}]{\text{bacterial}} FMN + RCOOH + H_2O + h\nu \tag{4}$$

Bacterial luciferases are heterodimeric (α, β) proteins of molecular weight (M_r) about 80,000 with a single active center per dimer. Bound to luciferase, both the intermediate luciferase flavin peroxide and the product hydroxyflavin, postulated to be the emitter in the pure system, have been spectrally characterized.

Dinoflagellate BL is distinctively different in many respects: originating in a large eukaryotic cell, the light emission occurs primarily in the form of flashes (lasting about 100 ms) emitted from subcellular organ-

TABLE I
Biochemistry of Seven Luminous Systems

Luminous organisms (genera)	Luciferins, other factors	Luciferases, M_r	Emission λ_{max} (nm)
Bacteria (*Photobacterium; Vibrio*)	Reduced flavin and long-chain aldehyde	80,000	495–500
Dinoflagellates (*Gonyaulax; Pyrocystis*)	H^+, tetrapyrrole (bile pigment)	420,000	475
Coelenterates (*Aequorea; Renilla*)	Ca^{2+}, coelenterazine (Imidazopyrazine nucleus)	21,000	460–490
Annelids (*Diplocardia*)	H_2O_2, *N*-isovaleryl-3-aminopropanal	300,000	500
Molluscs (*Latia*)	Enol fomate form of an aldehyde, or an aromatic or terpene aldehyde	170,000	500
Crustacea (*Vargula* = *Cypridina*)	Imidazopyrazine nucleus	68,000	465
Insects (*Photinus; Photuris*)	ATP, Mg^{2+} (benzo)thiazole nucleus	100,000	560

elles (Hastings, 1978; Sweeney, 1979; Widder and Case, 1982a,b; Johnson *et al.*, 1985). The reaction involves the oxidation by molecular O_2 of a single substrate, the chromophore responsible for the fluorescence of the organelles seen in some species (Eckert, 1966a; Johnson *et al.*, 1985). Luciferin from *Pyrocystis lunula* bears a chemical similarity to Chl (Dunlap *et al.*, 1981) and may be sequestered at pH > 7 by a luciferin binding protein (LBP). Dinoflagellate luciferase, the monomer of which is a large (140 kD) single-chain polypeptide, is also localized at the organelles, as judged by immunocytochemical studies (Nicolas *et al.*, 1985). As discussed below, the biochemical control of the reaction and of the light flash appears to involve pH changes.

II. Bacterial Bioluminescence

A. Discovery: Relationship to Respiration

Prior to the advent of electric lights and refrigeration, it was noted that fish or flesh of animals might emit light, and even today the occasional refrigerator with a defective light may allow this BL to come to the attention of a midnight-snack seeker. The history of attempts to account for such luminescence is fascinating, and early observers sometimes invoked the supernatural (see Harvey, 1957). During the latter half of the nineteenth century, the bacterial origin of the light was established and

the diversity of bacterial species and habitats was recorded. Both marine and freshwater species were recognized, as well as the existence of luminous vibrios, including vibrios isolated from patients with Asiatic cholera (Harvey, 1952).

A relationship between the pathways of respiration and BL in bacteria (Fig. 1) had been correctly inferred long before the isolation of the luciferase (Strehler, 1953) and the demonstration of requirements for $FMNH_2$ (McElroy *et al.,* 1953) and aldehyde (Strehler and Cormier, 1954). With starved cells, the addition of glucose results in a prompt increase in both light intensity and respiration, and the addition of cyanide reduces O_2 consumption but does not necessarily inhibit and may actually stimulate light emission (Harvey, 1952). High ambient [O_2] causes a decrease in the intensity of BL in growing cultures without reducing the levels of luciferase synthesized or its *in vitro* activity (Ulitzur *et al.,* 1981), this decrease being attributed to the fact that less reduced substrate is available to luciferase in the cell. Because of its unusually high affinity for O_2, the luminescent system has been used for O_2 measurement in special applications (Chance and Ohnishi, 1978). More recently, an O_2 electrode which incorporates luminous bacteria as the detector has been perfected and employed (Lloyd *et al.,* 1981).

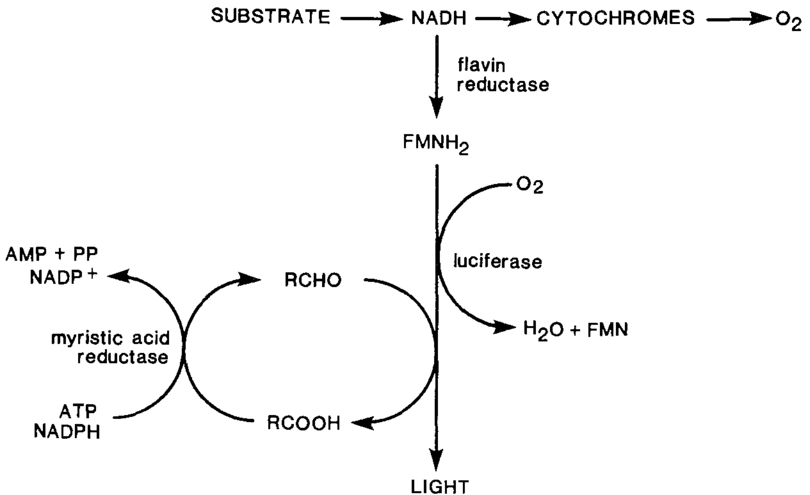


FIG. 1. The bacterial luciferase reaction shown in relation to the cellular electron transport pathway, from which it derives its reducing power for the supply of its substrate $FMNH_2$. The mixed-function oxidation of $FMNH_2$ and long-chain aldehyde (RCHO) results in the production of light, acid (RCOOH), and FMN. The recycling of acid catalyzed by myristic acid reductase to form aldehyde is also shown.

Although luminescence in bacterial cultures is continuous and quite steady, this might not be so at the level of the individual cell. Indeed, there have been reports of discrete flashes and/or an oscillation (8 cps) in the light output in single cells (Berzhanskaya *et al.,* 1973, 1975). However, these reports were not confirmed (Haas, 1980).

B. Biochemistry: Flavin Intermediates

1. LUCIFERASE

Luciferases of all bacterial species are homologous heterodimeric (α–β) proteins; they catalyze the bioluminescent mixed-function oxidation of $FMNH_2$ and a long-chain aldehyde by molecular O_2 [see Eq. (4) above]. The two subunits are themselves homologous (Baldwin *et al.,* 1979a), but there is only a single active center per dimer, and isolated subunits, either in solution or immobilized, exhibit no luciferase activity individually (Watanabe *et al.,* 1982).

Structurally, bacterial luciferases appear to be simple: no metals, prosthetic groups, or non-amino acid residues appear to be involved. A possible exception is *Photobacterium leiognathi* luciferase, reported to be a glycoprotein (Balakrishnan and Langerman, 1977); this has not been confirmed (Ziegler and Baldwin, 1981), but the possibility should probably not be completely excluded. In *Vibrio harveyi* the complete amino acid sequence of the α subunit has been deduced from the cloned genes (Cohn *et al.,* 1985). The luciferases from different bacterial species differ slightly in M_r; in some but not all cases hybrid molecules with subunits from different species may be active (Meighen and Bartlett, 1980; Ruby and Hastings, 1980). Many structural mutants of luciferase have been isolated and characterized (Cline and Hastings, 1971, 1972). Kinetic (catalytic turnover time) mutants are associated with the α subunit, while thermal instability occurs equally frequently with lesions in α and β subunits. However, while the active center appears to be associated more with the α subunit, there is evidence that the β subunit is also involved (Cline, 1973; Meighen and Bartlett, 1980). The aldehyde binding site appears to be located at the interface between the subunits (Tu and Henkin, 1983). Two inactive mutant luciferases, one with a defective α and the other with a defective β subunit, have been shown to complement to form wild-type luciferase (Anderson *et al.,* 1980).

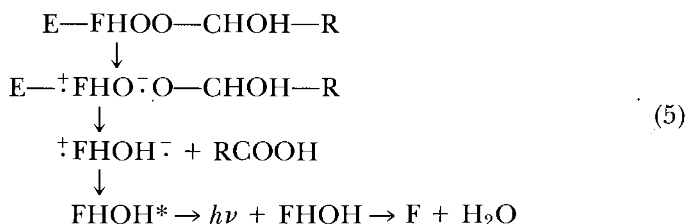
Bacterial luciferase activity is sensitive to proteolysis by trypsin or chymotrypsin (Baldwin *et al.,* 1979b). This is accompanied by hydrolysis of one or a small number of peptide bonds within a "protease labile" region of the α subunit, a region believed to be part of (or related to) the active

center (Dougherty *et al.*, 1982). It appears to be located approximately 100 residues from the carboxy terminus of the α subunit and to span about 20 residues, with five or six trypsin- and two chymotrypsin-sensitive sites. Both FMN and orthophosphate protect the region from proteolysis (Holzman and Baldwin, 1980a,b). Proteolysis of the α subunit results in the generation of a large (28 to 30 kD) species plus many smaller fragments and a loss of enzymatic activity. However, all these fragments remain associated under nondenaturing conditions. The β subunit remains altogether unaffected.

There is a highly reactive thiol at the active center whose alkylation completely inactivates luciferase (Ziegler and Baldwin, 1981). Inactivation studies with long-chain *N*-alkylmaleimides had suggested that the active center must have a hydrophobic character. Studies with spin-labeled maleimides indicated that the cysteine lies in a hydrophobic cleft at least 17 Å in length (Merritt and Baldwin, 1980). More recently, the thiol has been shown to be at the aldehyde binding site (Fried and Tu, 1984).

2. REACTION PATHWAY; INTERMEDIATES

The reaction steps and intermediates postulated for the reaction of bacterial luciferase with $FMNH_2$, O_2, and aldehyde are shown in Fig. 2. An interesting feature of the reaction is its inherent slowness: at 20°C the time required for a single catalytic cycle is about 20 s. This is attributable mostly to the lifetime of the intermediate 4a-peroxydihydro-FMN; isolated and purified at a low temperature, it has been shown to have absorption peaking at 372 nm and fluorescence emission centered at about 490 nm (Hastings *et al.*, 1973; Balny and Hastings, 1975). Its reaction with long-chain aldehyde is postulated to form an intermediate flavin peroxyhemiacetal (FHOO—CHOH—R), which then [Eq. (5)] reacts via an intermolecular electron exchange mechanism to form a long-chain acid (RCOOH) and populate the singlet excited state of 4a-hydroxy-FMN (FHOH*). After emission, this should break down promptly to FMN and H_2O (Kurfürst *et al.*, 1984); all intermediates are luciferase-bound.



$$\begin{array}{c} \text{E—FHOO—CHOH—R} \\ \downarrow \\ \text{E—}^{\dot{+}}\text{FHO}^{\dot{-}}\text{O—CHOH—R} \\ \downarrow \\ ^{\dot{+}}\text{FHOH}^{\dot{-}} + \text{RCOOH} \\ \downarrow \\ \text{FHOH}^* \rightarrow h\nu + \text{FHOH} \rightarrow \text{F} + \text{H}_2\text{O} \end{array} \quad (5)$$

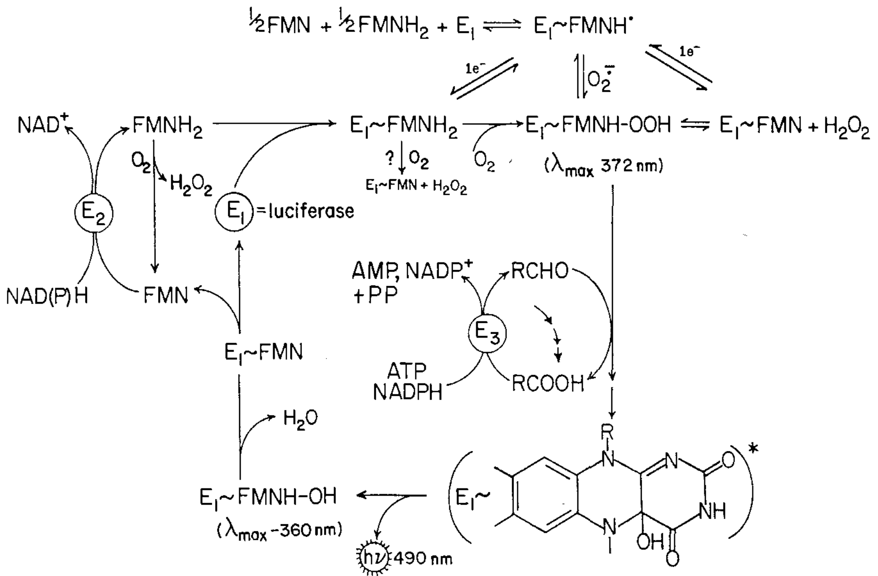


FIG. 2. Details of the steps and intermediates proposed for the luciferase reaction. The reaction leading to light emission starts with the formation of $FMNH_2$ from FMN and NAD(P)H; this is catalyzed by FMN reductase (E_2). Luciferase (E_1)-bound $FMNH_2$ reacts with molecular oxygen to form the intermediate flavin 4a-peroxide, which then reacts with a long-chain aldehyde to form an excited species (designated as the flavin 4a-hydroxide). The fatty acid that is formed is then released and is reconverted to aldehyde by a third enzyme (E_3, myristic acid reductase). The ground state hydroxyflavin loses water to form FMN, which can recycle. Formation of the blue radical, along with its reaction with superoxide ion, is shown at the top.

The oxygen-containing intermediate is stabilized by aldehyde analogs, long-chain compounds such as alcohols which inhibit the reaction and bind at the aldehyde site (Hastings *et al.*, 1966a). At 0°C with added dodecanol the isolated luciferase–peroxyflavin intermediate has a lifetime of many hours instead of many minutes (Tu, 1979). Nakamura (1982) found that the binding affinity for long-chain aliphatic fatty acids increases with chain length between 10 and 24 carbons. Luciferase–peroxyflavins have also been prepared with several different flavin derivatives (Tu, 1982).

Under certain conditions the luciferase neutral radical semiquinone, having absorption in the red (500–700 nm), may be formed (Kurfürst *et al.*, 1982). This enzyme-bound radical is inactive for light emission either with or without aldehyde, but it can nevertheless react with the superox-

ide radical to give BL (Kurfürst *et al.*, 1983), probably via a pathway involving the flavin peroxide (Fig. 2).

3. EMITTERS; COLOR OF BIOLUMINESCENCE

In a high quantum yield CL or BL reaction, a reaction product or intermediate occurs in a singlet electronically excited state, which, being in the ground state subsequent to its emission, should be identifiable by its fluorescence (Hastings and Tu, 1981). Oxidized FMN, a product in the bacterial reaction, has fluorescence centered at about 525 nm, very different from BL *in vitro* ($\lambda_{max} \sim 490$ nm). When reduced flavin analogs were used in the reaction in place of $FMNH_2$, the color of the BL was altered (Fig. 3), but again it did not correspond to the fluorescence emission of the analogs used (Mitchell and Hastings, 1969). These observations could be explained if the emitter is an unstable intermediate flavin species and thus difficult to detect. Indeed, simultaneous kinetic measurements of absorbance and BL revealed that the decay of BL occurred more rapidly than the appearance of FMN, indicative of such a transient intermediate species subsequent to light emission (Kurfürst *et al.*, 1984). Its absorption ($\lambda_{max} \sim 360$ nm) and fluorescence emission ($\lambda_{max} \sim 490$ nm) are consistent with the postulate that it is the luciferase-bound flavin 4a-hydroxide (Fig. 2).

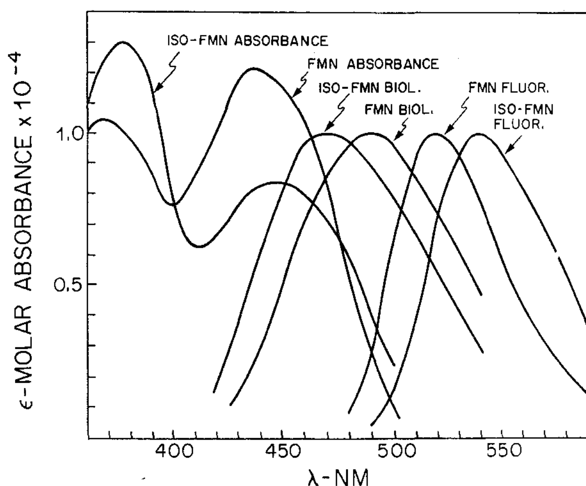
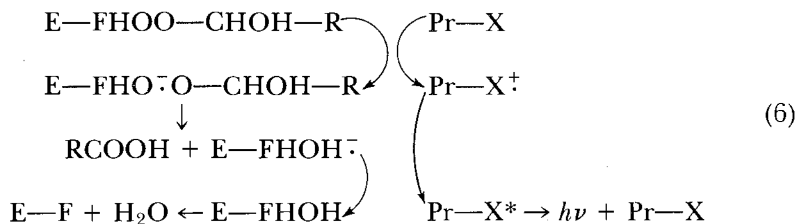


FIG. 3. Absorption (left; absorbance) and fluorescent emission spectra (right; fluor) for FMN and iso-FMN, along with bioluminescence emission spectra (center; biol.) for luciferase reactions initiated with $FMNH_2$ and iso-$FMNH_2$. Abscissa: wavelengths (λ) in nanometers (nm). The fluorescence and bioluminescence intensity values are normalized.

In some species and strains of luminous bacteria the color of the light emission *in vivo* matches that *in vitro*. But in other cases the spectra are different, even though the same luciferase reaction and the same flavin intermediates appear to be involved. The two documented examples are in *Photobacterium phosphoreum* (Gast and Lee, 1978), where blue light ($\lambda_{max} \sim 475$ nm) is emitted, and a strain of *Vibrio fischeri*, which emits yellow light ($\lambda_{max} \sim 535$ nm; Ruby and Nealson, 1977).

The blue emission is postulated to involve a chromophore identified as 6,7-dimethyl-8-ribityllumazine (Koka and Lee, 1979), bound (1 : 1) to a specific "blue fluorescent" protein having an M_r of about 20,000 (Small *et al.*, 1980). The striking yellow emission in *V. fischeri* involves an apparently analogous "yellow fluorescent" protein (Leisman and Nealson, 1982) in which the chromophore is FMN (J. W. Hastings, S. Ghisla, G. Leisman, and K. Nealson, unpublished). Given the formation of the luciferase 4a-peroxyflavin as an intermediate, how might the singlet excited state of a chromophore on an accessory protein be populated? One possibility is Förster-type energy transfer (Knox, 1975; van Grondelle and Amesz, Chapter 8, this volume). This seems reasonable in the case of the yellow emission, but would not be very efficient for the blue-emitting species (Ward, 1981). As diagrammed in Eq. (6), we propose instead that the accessory protein-bound chromophore (Pr-X, where X may be either lumazine or FMN) acts as an alternative electron donor, transferring an electron to the luciferase flavin peroxyhemiacetal, and becoming excited on electron transfer back to the chromophore. Thus, starting with the same flavin peroxyhemiacetal, the excited state of an accessory protein-bound chromophore is formed directly, along with long-chain acid and hydroxyflavin (FHOH), without the intermediacy of any other excited (or "energized") state.



C. Physiology: Control of Luminescence

Bacterial BL is emitted continuously by virtue of a reaction which occurs as a shunt of the electron transport pathway. Under different

conditions, light emission may differ in intensity; in nature this is probably due in many cases to differences in the cellular content of luciferase, and thus to its biosynthesis, rather than to changes in the rate of the bioluminescent reaction itself. In fact, the conditions which stimulate or repress luciferase synthesis have provided important insights into the function of light emission in bacteria.

1. AUTOINDUCTION

Control of luciferase synthesis at the transcriptional level is mediated by a substance called autoinducer, produced constitutively and secreted by the cells themselves. Enzymes involved in aldehyde synthesis are similarly controlled (Ulitzur and Hastings, 1979a; Miyamoto *et al.*, 1985). Autoinducer in *V. fischeri* (Fig. 4) is postulated to interact with a product of the *luxL* operon to trigger the transcription of luciferase mRNA (Engebrecht and Silverman, 1984). At low cell densities, as described by Nealson *et al.* (1970, 1972) and by Barak and Ulitzur (1981) on solid medium, growth and division occur exponentially but there is no luciferase synthesis. At a higher cell density luciferase synthesis is then initiated, and occurs at a rate far greater than growth. Thus, a large difference in the specific luciferase content of the cells occurs over the course of growth under these conditions (Fig. 5).

Autoinduction and thus luciferase synthesis would be expected to occur when cell densities are high, as in light organs, in the gut, or in parasitic infections (Nealson and Hastings, 1979). On the other hand, under conditions where autoinducer cannot accumulate, luminous bacteria would not produce luciferase during growth and should thus be nonluminous. This prediction has been confirmed (Ulitzur and Hastings, 1979b; Rosson and Nealson, 1981). Although autoinducer exhibits species specificity, many species of nonluminous vibrios (which produce little or no luciferase) do excrete a substance that causes autoinduction in *V. harveyi* (Greenberg *et al.*, 1979). The structure of *V. harveyi* autoinducer is not known, but that of *V. fischeri* was determined by Eberhard *et al.* (1981; Fig. 4); synthetic autoinducer exhibits full activity. There are some strains of *P. leiognathi* and *P. phosphoreum* that synthesize luciferase

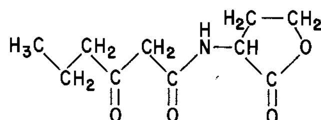


FIG. 4. **Structure of autoinducer of *Vibrio fischeri*. (From Eberhard *et al.*, 1981.)**

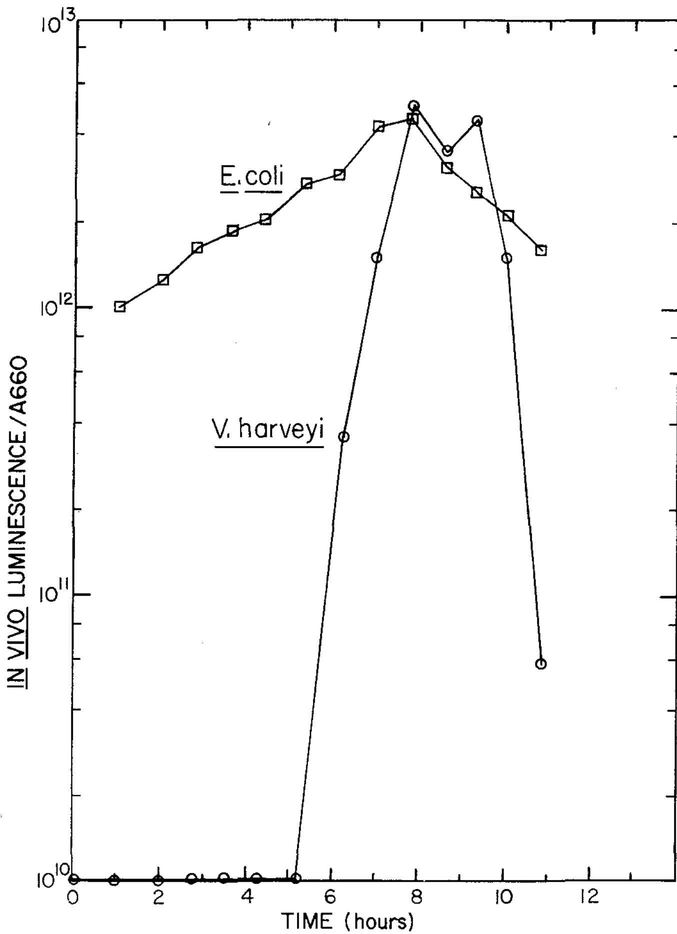


FIG. 5. Changes in the amount of bioluminescence activity per cell as a function of time during growth in a complex medium. Wild-type *Vibrio harveyi* (circles) is compared with *Escherichia coli* (squares) carrying the luciferase structural gene cloned on a plasmid (pWH102). The ordinate values reflect cellular amounts of luciferase; the large changes with time in *V. harveyi* are attributed to autoinduction. (From Gupta *et al.*, 1986.)

in the absence of autoinducer (Katznelson and Ulitzur, 1977; Rosson and Nealson, 1981).

2. GENETIC ORGANIZATION AND CONTROL

The structural genes for the luciferase from *V. harveyi* have been cloned and shown to be expressed in *E. coli* on 4–5-kb DNA fragments

(Belas *et al.*, 1982; Gupta *et al.*, 1983, 1985, 1986; Baldwin *et al.*, 1984). Cohn *et al.* (1983) showed that the *luxA* and *luxB* genes, coding for the luciferase α and β subunits, respectively, are linked, separated by a noncoding region about 80 bases upstream from the start of the structural gene for *luxB*. Homology between the α and β subunits was demonstrated (Cohn *et al.*, 1985), confirming the results of Baldwin *et al.* (1979b).

With these clones of *V. harveyi*, expression of the luciferase gene is constitutive and not subject to autoinduction (Fig. 5; see Section II,C,1). Moreover, the addition of long-chain aldehyde is required for the expression of BL in *Escherichia coli in vivo.* Even a larger 12-kb fragment failed to express autoinduction or aldehyde synthesis functions in *E. coli* (Gupta *et al.*, 1983); the same was true for clones spanning 18 kb in the regions adjoining *luxA* and *luxB* genes (Miyamoto *et al.*, 1985).

By contrast, Engebrecht *et al.* (1983) cloned a 16-kb fragment from *V. fischeri* which provided *E. coli* with the ability to express BL *in vivo,* exhibiting autoinduction and not requiring exogenous aldehyde for light emission. Two operons, *luxL* and *luxR,* with seven *lux* genes, were defined for the complete Lux phenotype, and functions were assigned to these genes (Engebrecht and Silverman, 1984).

3. ARGININE AS A COINDUCER

Cells growing in a minimal medium produce much less luciferase (~1%), and also less luminescence, irrespective of autoinducer. Added arginine stimulates luciferase synthesis specifically and dramatically, but only in the presence of autoinducer. Mutants that produce higher amounts of luciferase when grown on minimal medium were isolated by Waters and Hastings (1977). Analysis of these mutants (Makemson and Hastings, 1979) indicated that the stimulatory effect of exogenous arginine is distinct from that of autoinducer and cAMP (see below) and involves transcription; the mediator may be arginine or argininyl-tRNA.

4. CATABOLITE REPRESSION: GLUCOSE AND cAMP

The synthesis of the luminescent system of *V. harveyi* is subject to catabolite repression; glucose represses luciferase synthesis and cAMP reverses this repression (Nealson *et al.*, 1972). As in other catabolite-sensitive systems, the control overrides inducer. This repression and stimulation suggests that luciferase should be viewed as an enzyme that has some important function(s) under some nutritional conditions while being repressed under others. Catabolite repression by *N*-acetylglucosamine produced from the breakdown of chitin by chitinase produced

extracellularly by luminous bacteria in the gut tract may occur (Nealson and Hastings, 1979; Ruby and Morin, 1979; Baguet *et al.*, 1983). Mutants resistant to catabolite repression have been isolated as "bright on glucose" phenotype.

5. IRON

Bioluminescence and the synthesis of luciferase are decreased by added iron in *V. harveyi* growing in a minimal medium (Makemson and Hastings, 1982). Such cultures emit less light and possess less luciferase per cell than those grown under conditions of growth-limiting iron concentrations; it was suggested that this might have significance in relation to a postulated function of luciferase, namely as a terminal carrier of electrons to O_2. Also, the effects of iron may be functionally significant in relation to symbiosis (Toranzo *et al.*, 1983); iron limitation by the host might limit bacterial growth while maximizing the BL. The repression by iron is not significantly reversed by cAMP, but addition of glucose in the presence of iron causes further repression, which is partially reversible by cAMP. Haygood and Nealson (1984) reported a similar repression by iron in *V. fischeri*.

6. OXYGEN

Molecular O_2 is an absolute requirement in the bioluminescent reaction, but the K_m for the reaction of luciferase-bound $FMNH_2$ with O_2 may be very low, down to 15 n*M* (Lloyd *et al.*, 1985). But O_2 also has another independent role, acting to control the synthesis of luciferase and other components of the luminous system (Nealson and Hastings, 1977). In strains of some species (*P. phosphoreum* and *V. fischeri*) O_2 exerts a differential effect on the synthesis of luciferase compared to other cell components: in low O_2 growth is stopped or limited while luciferase synthesis continues, resulting in cells with very high cellular levels of luciferase, much higher than those of cells grown in air. This appears relevant to the physiology of light organ symbiosis: by limiting O_2 in the light organ, the host can limit the growth of bacteria in the organ while assuring that those that do grow are packed with luciferase, thus specialized optimally for light emission.

The low K_m values for O_2 mean that luciferase could provide an alternative terminal carrier for electrons to O_2, thus allowing aerobic metabolism (albeit at a low rate) under microaerophilic conditions where the cytochrome pathway is limited. Under such conditions luciferase might support aerobic growth on nonfermentable substrates (Makemson and Hastings, 1984, 1986).

D. Ecology: Functions of Bacterial Bioluminescence

1. HABITATS

Luminous bacteria are best known as members of the marine microbial community, where at least six species are recognized (Baumann *et al.,* 1980, 1983; Table II); they are found in considerable numbers (1–10% of viable colonies on some media) in seawater samples. Although these may be described as "free living," they probably do not grow much in seawater, which is not a good growth medium; their occurrence there is presumably related to dispersal from saprophytic, commensal, parasitic, or symbiotic modes of growth (Nealson and Hastings, 1979; Hastings and Nealson, 1981).

Freshwater species are known from the isolation of luminous *Vibrio* and other species from rivers and lakes in Europe and Japan (Harvey, 1952), but they have not been studied extensively. There are also species (*Xenorhabdus* sp.) which are symbiotic with soil nematodes; they collaborate in the parasitization of insects—resulting in luminous (but dead) caterpillars (Poinar *et al.,* 1980). These bacteria are unusually large, bril-

TABLE II
Associations of Luminous Bacteria

Mode	Habitat or host	Species
Free living	Seawater	All marine species: *Photobacterium phosphoreum, Photobacterium leiognathi, Photobacterium logei, Vibrio harveyi, Vibrio fischeri, Vibrio splendida, Vibrio orientalis, Altermonas hanedai*
	Freshwater, soil	*Vibrio cholerae, Xenorhabdus luminescens* (?)
Saprophytic	Dead marine animals, fish, wounds, meat	All marine species (as above), *Xenorhabdus luminescens* (?)
Commensal	Outer surfaces and digestive tracts of marine fish and invertebrates	All marine species (as above)
Parasitic	Marine crustacea	*Vibrio harveyi, Vibrio fischeri, Photobacterium phosphoreum*
	Terrestrial and freshwater	*Vibrio cholerae*
	Animals	*Xenorhabdus luminescens*
Symbiotic/parasitic	Nematode/caterpillar	*Xenorhabdus luminescens*
Light organ symbionts	Teleost fish and squid	*Vibrio fischeri, Photobacterium leiognathi, Photobacterium phosphoreum*

liantly pigmented, and produce an antibiotic that inhibits the growth of other bacteria (Paul *et al.*, 1981). This prevents the dead animals from putrefying, thereby promoting their ingestion and dispersal by predators.

2. ASSOCIATIONS: FUNCTIONS OF LUMINESCENCE

The most exotic associations recognized for luminous bacteria involve specialized light organs in which a pure culture of luminous bacteria is maintained as in a chemostat at high cell density (virtually packed cells and at high light intensity (Dunlap, 1984). Exactly how this is achieved—the initial infection, exclusion of contaminants, nutrient supply, control of metabolism, and biosynthesis—is not well understood. In this symbiotic association the bacteria receive a niche and nutrients while the fish is supplied with light, which it can use in many different ways. Light emission may serve offensive (predation), defensive (escape from predation), and intraspecies communication functions (Morin *et al.*, 1975; McFall-Ngai and Dunlap, 1983; Morin, 1983).

The function of light emission by bacteria in parasitic, commensal, or saprophytic modes is less obvious. Positive selection for luminescence in these cases could be related to its being seen and acted on by other species. By ingesting the luminous material, for example, other organisms could cause the dispersion and propagation of the bacteria (Hastings and Nealson, 1977; Morin, 1983; Andrews *et al.*, 1984). Luminous bacteria growing on a substrate, whether it is a parasitized crustacean, the surface of a dead fish, or a fecal pellet, could produce sufficient light to attract other organisms to feed on the material, thus enhancing the propagation of the bacteria.

III. Dinoflagellate Bioluminescence

A. *"Phosphorescence," Red Tides, and the Functions of Luminescence*

The sparkling "phosphorescence" seen at night when seawater is disturbed is due in many instances to the BL of unicellular dinoflagellates. For example, the BL of the famous phosphorescent bays (Puerto Rico, Jamaica) is due to a single predominant species, *Pyrodinium bahamense*, high populations of which persist throughout the year (Seliger *et al.*, 1970, 1971). Many (but not all) dinoflagellates are photosynthetic, and not all dinoflagellates are bioluminescent: no freshwater species are bioluminescent and only a fraction of the marine species are capable of

emitting light (Sweeney, 1979). Conspecific luminous and nonluminous varieties of *Gonyaulax excavata* (Swift *et al.*, 1973; Schmidt *et al.*, 1978) and *Noctiluca miliaris* (Eckert and Findlay, 1962) have been reported.

The so-called red tides are usually caused by a single predominant dinoflagellate species (Sweeney, 1975). Some species are responsible for the production of a neurotoxin (e.g., saxitoxin); some are bioluminescent and may give rise to a spectacular display of light during the night.

The function of BL in dinoflagellates has attracted interest. Burkenroad (1943) speculated that dinoflagellate luminescence, stimulated by predators, might alert the predators of those predators and thus indirectly protect the dinoflagellates. Instead, the effect might be a direct one (Morin, 1983); for example, flashing caused by the mechanical stimulation from an approaching predator might startle or otherwise divert the predator. This idea was supported by the experiments of Esaias and Curl (1972), who found that predation rates on luminous *Gonyaulax polyedra* by copepods were less at the phase of peak luminescence. More recent experiments have also supported this idea (White, 1979; Buskey *et al.*, 1983).

B. Biochemistry: Soluble and Particulate Fractions

1. LUCIFERIN, LUCIFERASE, AND THE LUCIFERIN BINDING PROTEIN

A linear tetrapyrrole structure has been proposed for dinoflagellate luciferin (Dunlap *et al.*, 1981; Fig. 6), but neither intermediates nor products in the luminescent reaction have been isolated or identified. The luciferin, which is subject to rapid nonenzymatic autoxidation in air, has absorption maxima at 245 and 390 nm and fluorescence peaking at 474 nm (Njus, 1975; Dunlap and Hastings, 1981a). Paradoxically, the fluorescence emission spectrum of the luciferin before its light-emitting reaction corresponds to that of the BL, yet the oxidation of luciferin must *precede* the formation of the excited state.

Soluble extracts of *G. polyedra* in buffer contain luciferase in three different M_r classes of about 400,000, 135,000, and 35,000 (Krieger *et al.*, 1974). The 135-kD species is the native monomer, while the 35-kD species is a proteolytic fragment thereof. Activity does not require any non-covalently bound metal, prosthetic group, or other cofactor. Extracts also contain a luciferin binding protein (LBP; M_r about 120,000), which sequesters the luciferin at an alkaline pH and releases it under acidic conditions (Fogel and Hastings, 1971; Sulzman *et al.*, 1978). It may thereby play a role in the control of luminescence and the triggering of flashing.

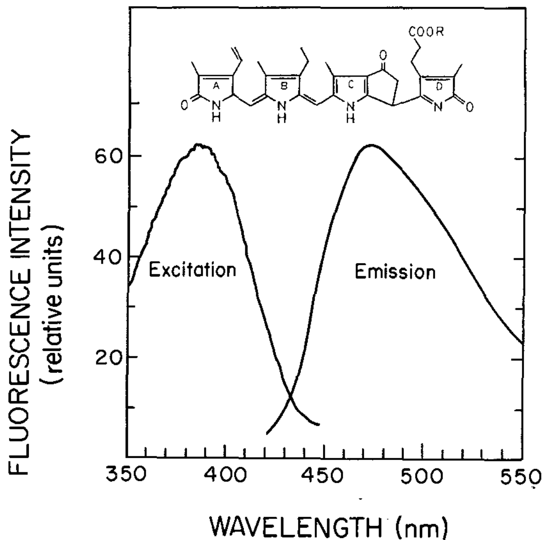


FIG. 6. Corrected excitation and emission spectra of partially purified *Dissodinium* luciferin, determined with a Farrand MK-1 spectrofluorometer (from Njus, 1975) along with its possible partial structure (inset; Dunlap *et al.*, 1981).

But the particulate fraction in *G. polyedra* extracts also has activity: particles (termed *scintillons*, or light-emitting units) purified from extracts made at pH 8 emit a flash similar to that of the living cell when the pH is rapidly lowered from 8 to 5.7 (DeSa *et al.*, 1963; Hastings, 1978). The soluble and particulate fractions apparently utilize the same luciferin, luciferin-binding protein, and luciferase.

2. SOLUBLE FRACTION

The characteristic BL of *Gonyaulax* and other dinoflagellates occurs as flashes coming from subcellular microsources or organelles; the soluble system may therefore derive from their breakage during extraction. Whatever may be its origin, the pH dependence of the soluble fraction is of interest in relation to the *in vivo* control of the reaction. Extracts made at pH 8 do not emit light during extraction, and in the particle-free supernatant the luciferase is found in its higher-M_r forms (135,000 and/ or 400,000). By then simply lowering the pH from 8 to 6.5 the light-emitting reaction will occur, as a consequence of two pH-dependent processes: (1) activation of luciferase, which is inactive at pH 8 but active at pH 6.5, and (2) release of luciferin from its binding protein at the acidic pH (Fogel and Hastings, 1971; Sulzman *et al.*, 1978). In such extracts, the activation is thus a steep function of pH; if the assay is

performed instead with purified luciferase, free luciferin, and no binding protein, the curve is not so steep.

In extracts made at an acid pH the proteolysis of the luciferase is catalyzed by an endogenous protease, yielding the active lower-M_r fragment (35,000). A striking fact is that the 35-kD luciferase no longer exhibits the sharp cutoff with pH; it has good activity at pH 8. Since the LBP binds the substrate at pH 8, LBP acts as an inhibitor of the 35-kD luciferase reaction at this pH.

Kinetically, the reaction of purified 35-kD luciferase is related to enzyme and substrate concentrations in the classical way. In its reaction with free luciferin at pH 8 and room temperature, the light intensity (reaction velocity) rises to a maximum within 0.5 s and decays exponentially, the apparent first-order rate constant being dependent on enzyme concentration. The maximum initial intensity and the total light are proportional to the luciferin concentration, but the first-order rate constant for the decay of luminescence is unaffected.

Although there are some differences, the luciferins and luciferases of different species appear to be similar and to cross-react (Hastings and Bode, 1961; Hamman and Seliger, 1972; Lecuyer *et al.,* 1979). In a comparative study of four dinoflagellate species (*G. polyedra, Gonyaulax tamarensis, Dissodinium lunula,* and *Pyrocystis noctiluca*), it was found that the luciferins and luciferases cross-react in all combinations (Schmitter *et al.,* 1976). Luciferases from extracts made at pH 8 all possess high M_r values (200,000–400,000) with similar pH–activity profiles. The active single chain of luciferase was 135 kD in the first two species and 60 kD in the latter two. Extracts made at pH 6 yielded luciferases with good activity at pH 8 but of lower M_r; the breakdown was attributed to endogenous proteases. An LBP was extracted from the two *Gonyaulax* species, but none was detected in the soluble fraction from the two others.

3. THE PARTICULATE SCINTILLON FRACTION

The active light-emitting particles, which contain luciferase, luciferin, and LBP (Fogel and Hastings, 1972; Fuller *et al.,* 1972; Henry and Hastings, 1974), are referred to as scintillons (flashing units), but the original report that they are functionally associated with guanine crystals (DeSa *et al.,* 1963) was retracted (Fogel *et al.,* 1972). The identity of scintillons has not been established with certainty, but based on observations described below, they are now believed to correspond to the fluorescent subcellular organelles from which bioluminescence emanates *in vivo,* and which are released on cell lysis (Figs. 7 and 8; see also Section III,C below). These may correspond to the dense bodies seen in the

FIG. 7. Endogenous fluorescent organelles in *Gonyaulax polyedra*. (A and C) Intact cell with test (cell wall) removed, viewed by bright field illumination (left) and fluorescence (right) focusing at upper cell surface. Note fragments of test at left and upper right in (A). (B and D) Lysed cell, also by bright field (left) and fluorescence (right), showing exuded particles. Note that test remains in place. Zeiss IM-35 inverted epifluorescence microscope with 40× Neofluor objective. Filters for fluorescence excitation, 395–420 nm; emission, 450–560 nm (Zeiss set 487718). (From Johnson *et al.*, 1985.)

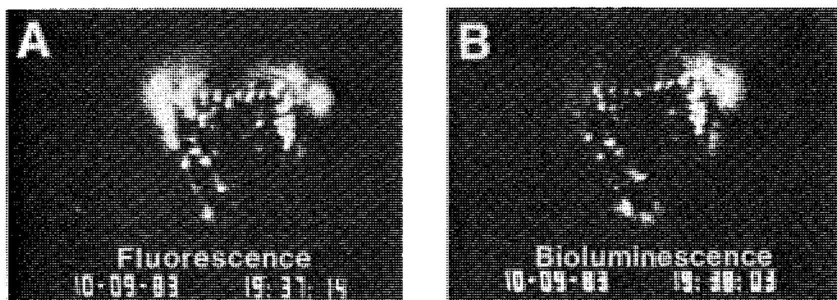
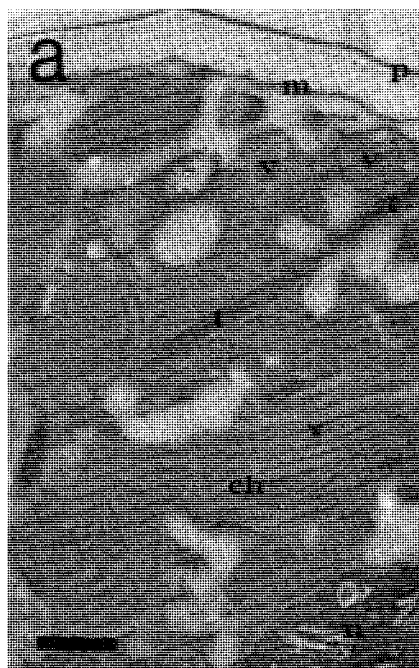


FIG. 8. Colocalization of endogenous fluorescence with bioluminescence: (A) fluorescence of particles before stimulation; (B) bioluminescence at peak of flash, stimulated by Ca^{2+} addition (no fluorescence excitation). Images intensified with a Zeiss/Venus TV-3 video camera, 40× Neofluor objective photographed from video monitor with Tri X film, developed with Acufine. (From Johnson *et al.*, 1985.)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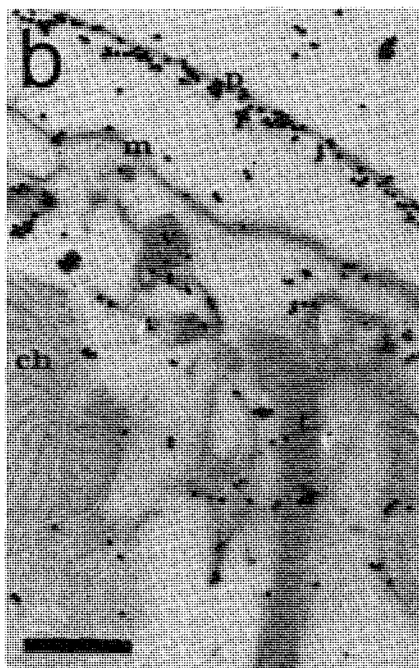
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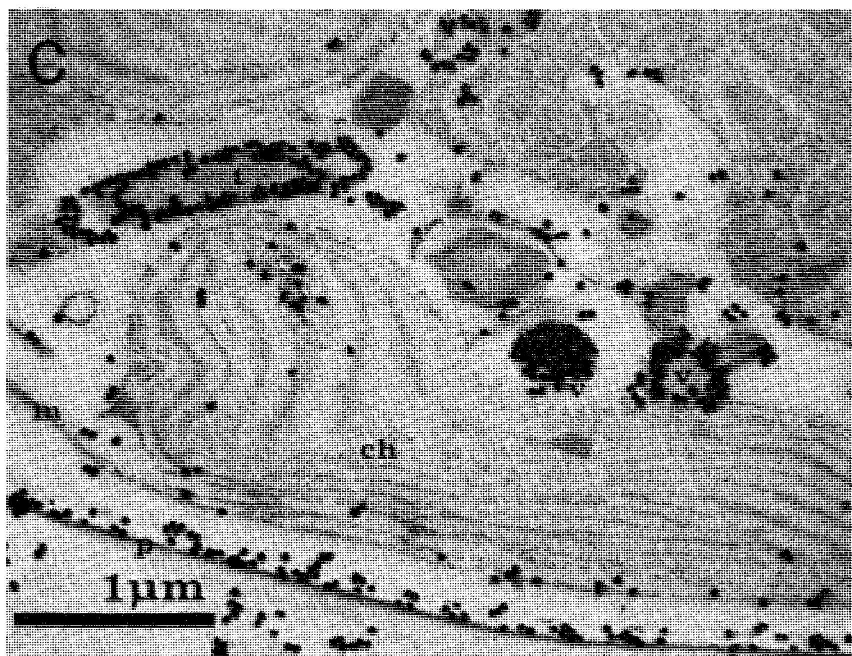
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electron microscope and found to be labeled with antibody raised against luciferase (Fig. 9).

As with the soluble elements, the key to the activity of scintillons is pH. If scintillons at pH 8 are rapidly shifted to pH 5.7, a flash occurs which is kinetically similar to the flash of the living cell (Hastings *et al.*, 1966b). Using this assay, *G. polyedra* scintillons have been purified by sucrose isodensity gradient centrifugation; the activity sediments with a peak density of 1.23 g ml^{-1}. From velocity sedimentation, an M_r of about 10^9 was estimated, corresponding to a particle of about 0.5 μm diameter. The number of fluorescent particles in different fractions after sucrose density gradient centrifugation is proportional to the scintillon activity (Johnson *et al.*, 1985). Both the flash kinetics and the photon yield of scintillons are independent of concentration over a wide range, indicating that the functional molecular components are not significantly dissociated on dilution and that no other factors are required for luminescence.

Although living cells flash repeatedly on repeated stimulation, scintillons *in vitro* emit only a single flash following a pH jump. However, discharged scintillons may be "recharged" *in vitro* by readjusting to pH 8 and incubating with luciferin (Fogel and Hastings, 1972; Fuller *et al.*, 1972); this also restores the fluorescence of the particles, as seen microscopically (Johnson *et al.*, 1985). The addition of luciferin to freshly isolated scintillons prior to assay may also stimulate their activity. This is referred to as "supercharging." As with recharging, the luciferin is bound by the particle; this is presumably due to a particle-associated LBP.

Although scintillon activity has been found in all dinoflagellate species examined, there appear to be some differences, and the amount of activity may be low in some species. In fact, more than one organelle or subcellular compartment may be responsible for scintillon activity, and they may differ in different species. This is suggested first by the fact that in *G. polyedra* and *G. tamarensis* the major part of the activity bands at a density that peaks between 1.20 and 1.23 g ml^{-1}, while in *D. lunula* and *P. noctiluca* the values are centered around 1.15 to 1.16 g ml^{-1} (Schmitter *et al.*, 1976). Especially intriguing in this connection are the facts that

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FIG. 9. Thin sections of glutaraldehyde-fixed and embedded *Gonyaulax polyedra* cells incubated overnight with polyclonal antibodies: (a) gold-labeled goat anti-rabbit antibody; (b) normal rabbit serum followed by gold-labeled goat anti-rabbit antibody; (c) rabbit antiluciferase antibody followed by gold-labeled goat anti-rabbit antibody. Two organelles are specifically labeled: dense vesicles (v) and trichocysts (t); m, Plasma membrane; ch, chloroplast; p, pellicle; n, nucleus. Scale bars, 1 μm. (After Nicolas *et al.*, 1985.)

in extracts of *G. polyedra* both of the above density classes of scintillons occur (Schmitter, 1973) and that antibodies raised against the purified luciferase label two different organelles in *G. polyedra* (Fig. 9).

C. Cell Biology: Subcellular Sources and Scintillons

1. SUBCELLULAR SOURCES AND SCINTILLONS

Bioluminescence in dinoflagellates occurs primarily in the form of flashes (~0.1 s), which emanate from subcellular "microsources," variously estimated to be about 0.5 to 1.5 μm in diameter (Sweeney, 1980). Several possibilities have been suggested for their ultrastructural identity in different species.

a. *Noctiluca*. *Noctiluca miliaris* is a large cell (about 0.5 mm). Below its pellicle there is a very thin layer of cytoplasm (0.11 $\pm$ 0.07 μm; Nawata and Sibaoka, 1979) enclosing the acidic (pH 3.5) vacuole (Nawata and Sibaoka, 1976), which occupies most of the cellular volume. Light microscope examination revealed apparently synchronous flashes from a myriad of microscopic sources within the peripheral cytoplasmic compartment. Eckert (1966a,b) has recorded BL flashes photometrically from individual microsources. The microflashes resemble whole-cell flashes but are somewhat shorter. This can be explained by the 5- to 10-ms asynchrony of microsource triggering that results from the conduction time of the triggering action potential.

Individual "microflashes" were shown to originate from strongly phase-retarding inclusions which were also highly fluorescent. Many such phase-retarding inclusions were seen, but only about 5% were bioluminescent, with an invariant correlation with fluorescence. The possibility that lipid vesicles are identical with the phase-retarding bodies was suggested (Sweeney, 1978).

b. *Gonyaulax*. Observations similar to those of Eckert (1966a), namely the colocalization of BL and fluorescence to subcellular particles, have recently been reported for *G. polyedra* (Fig. 8; Johnson *et al.*, 1985). The fluorescence was attributed to luciferin. The observations were made by image-intensified video microscopy, so that many sources could be viewed and recorded simultaneously. The *Gonyaulax* cell is structurally very different from and much smaller (~40 μm in diameter) than the *Noctiluca* cell. It possesses a thick cellulose theca, lacks a large central vacuole, and, with chloroplasts and other inclusions, presents a more opaque image, so that the fluorescent sources (~0.5 μm) cannot be as

easily visualized by light or phase contrast microscopy (Fig. 7). As judged by fluorescence microscopy, they are located cortically during both day and night—this in contrast to the chloroplasts, which exhibit day–night changes (Schmitter, 1973; Herman and Sweeney, 1975; Rensing *et al.*, 1980). In thin sections incubated with rabbit polyclonal antibody directed against *G. polyedra* luciferase (Dunlap and Hastings, 1981b), followed by treatment with a goat anti-rabbit antibody labeled with colloidal gold, there was specific labeling of two organelles: dense vesicles and the compartment between the trichocyst capsule and shaft (Fig. 9; Nicolas *et al.*, 1985).

The bioluminescent particles described by Johnson *et al.* (1985) appear to correspond to the dense vesicles, as judged by their shape and size. Trichocysts are apparently not visualized by fluorescence, but it is possible that they contain luciferase but not luciferin.

c. *Pyrocystis*. Widder and Case (1981, 1982a,b) studied the distribution and activity of subcellular bioluminescent sources in *P. fusiformis*. Unlike *Noctiluca* and *Gonyaulax*, the light sources did not exhibit strong fluorescence, although weak fluorescence was detected by image intensification. But, as in *Noctiluca*, there may be a large number of apparently similar organelles, only some of which are associated with BL and fluorescence.

During the night phase BL occurs in response to mechanical stimulation from microsources that are dispersed throughout the cytoplasm. During the day BL does not occur in response to mechanical stimulation, but it can be evoked by the addition of a weak acid. In this case it emanates from an orange spherical object in the central region next to the nucleus (Sweeney, 1981). As seen in the electron microscope, this body is comprised of many short rounded rods, approaching spheres, about 0.25–0.5 μm. By night these rods disperse throughout the cytoplasm, and it has therefore been proposed that they are the structural counterparts of microsources in bioluminescence (Sweeney, 1982). However, they have not been shown to correspond to the mechanically stimulable microsources, and they were not labeled by antiluciferase antibodies (Nicolas, M. T., Sweeney, B. M., and Hastings, J. W., unpublished).

Widder and Case (1982b) confirmed the occurrence of a "perinuclear" glow in *P. fusiformis*, occurring in response to "acid stimulation" in day-phase cells at a time when the mechanically stimulable microsources have disappeared from the periphery of the cell. In cells kept in constant darkness, however, these mechanically stimulable microsources remain dispersed in the cytoplasm during the first hours of the day phase, but the perinuclear glow nevertheless develops normally, and the circadian

rhythm of luminescence persists. This suggested to the authors that the acid-stimulable glow adjacent to the nucleus may originate from structures other than the mechanically stimulable microsources, possibly ones involved in the synthesis of bioluminescent substrates prior to packaging in microsources.

D. Physiology: Control of Luminescence

1. THE FLASH TRIGGERING ACTION POTENTIAL: EXCITATION–EMISSION COUPLING

The bioelectric control and triggering of bioluminescent flashing in *Noctiluca* was elucidated by Eckert (1965, 1966a). At room temperatures a typical flash reaches maximum amplitude in 10 to 20 ms and decays 50% in about the same time. Such flashes occur with a latency of 2 to 3 ms in response to a characteristic all-or-none conducted action potential, the polarity of which is apparently opposite to that of metazoan action potentials. This is attributed to morphological factors; if the vacuole is designated as "external," both the stimulus and response have orthodox polarities (Eckert and Sibaoka, 1968).

The action potential may thus occur in *Noctiluca* across the vacuolar membrane with the cytoplasmic potential (negative with respect to the vacuole at rest) overshooting zero and becoming transiently electropositive. The action potential can be initiated by electrical stimulation, but it is also evoked, as in nature, by mechanical stimulation. Since the action potential is conducted over the cell, triggering of individual microsources more remote from the stimulus site occurs later; thus flashes occur in a wave with local triggering as the action potential passes. Based on the locations and the latency difference of the light coming from two different emitting sites, a conduction velocity of about 60 cm s^{-1} was estimated (Eckert, 1966a). As a consequence of channel openings (increased membrane conductance) in the vacuolar membrane there is positive current flow from the vacuole to the cytoplasm.

Although flashes are all-or-none, they exhibit facilitation and also summation. Changes in the intensity of the flashes exhibited by the whole cell ("macroflash") occur because of gradations in intensity of microflashes rather than as a result of changes in the number of responsive organelles (Eckert and Reynolds, 1967).

To account for the flash, we postulate a model involving a voltage-gated H^+ channel in a relevant membrane, triggered by the propagated action potential; the pH change results in the release of luciferin from LBP and its reaction with pH-activated luciferase (Hastings, 1978). A

flash results from the concerted reaction of many luciferases operating, in effect, in synchrony. If excess luciferin is liberated from LBP during the transient drop in pH, it can be recaptured and available for subsequent flashes.

The study of *N. miliaris* by Nawata and Sibaoka (1979) supported this model. They were able to alter the pH and ionic composition of the large acidic vacuole and to demonstrate a relationship between the pH of the vacuole and the amplitude of the flash-triggering potential. They calculated that the pH of the entire cytoplasmic compartment (assumed to have a pH of about 7 at rest) would be transiently lowered to about 4.5. They concluded that the vacuolar membrane during activity behaves like an H^+ electrode and that the flash-triggering potential is generated by a transient increase in H^+ permeability that allows protons to carry current through it from the vacuole into the cytoplasm.

2. MECHANICAL STIMULATION AND SPONTANEOUS BIOLUMINESCENCE

The generation of an action potential and flashing by mechanical stimulation has been attributed to membrane deformation (Eckert, 1965) or to shear forces (Christianson and Sweeney, 1972; Hamman and Seliger, 1972); in species possessing flagella, the attachment sites could play a role in translating shear forces into membrane deformation. Calcium is postulated to act as the triggering cation in nature because its presence in the external medium at concentrations of about 10 mM appears to be required for mechanical stimulability during the dark phase (Hamman and Seliger, 1972, 1982). Substances which bind calcium and block membrane calcium channels also interfere with mechanically stimulated flashing.

The understanding of mechanical stimulation has been hampered by the difficulty in varying and/or quantitatively measuring stimulus intensity (Sweeney and Hastings, 1957; Biggley *et al.*, 1969; Reynolds *et al.*, 1969; Widder and Case, 1981). Christianson and Sweeney (1972) found that the threshold stimulus for cells in the middle of the day phase is greater than that for cells in the middle of the night phase.

Since some of the species are motile, flagellar motions and/or collisional events might give rise to spontaneous flashing, especially during the nighttime. Flashes that are apparently spontaneous do occur (Sweeney and Hastings, 1958; Biggley *et al.*, 1969), and while such flashing from a dense culture seems frequent, it is not when calculated on a per cell basis: on average, each cell spontaneously emits one or two flashes per day (Hastings and Krasnow, 1981).

Krasnow *et al.* (1981) attempted to identify the origin of spontaneous flashes; they are not the result of cell-to-cell or cell-to-wall collisions. No correlation (for any reason) was found between the occurrence of one flash and a subsequent one—i.e., bursts, trains, etc.

In *G. polyedra* (and some other species as well; see Sweeney, 1979) a spontaneous glow occurs in undisturbed cells, but is is very dim ($\sim 10^{-4}$ q/s per cell at its maximum) and, as described below, exhibits a circadian rhythm (Sweeney and Hastings, 1958). Krasnow *et al.* (1981) investigated whether flashing and glow are interdependent in any way but found no positive indications. The spontaneous glow may be due to the activity of a proteolytic fragment of luciferase (see Section III,D,6).

3. CHEMICAL STIMULATION

A variety of chemical agents evoke bioluminescent flashing and glow. Some of these are presumed to act by initiating the BL-triggering action potential, while others may penetrate and bypass the membrane, acting more directly at the level of the subcellular light-emitting sites. With the demonstration that bioluminescent particles (scintillons) can be isolated *in vitro* by extraction at pH 8 and then triggered *in vitro* by lowering the pH to 5.7, the direct chemical stimulation of *in vivo* luminescence has been interpreted as an alteration of the local pH at these sites, by H^+ from without or within (Hastings, 1978; Nawata and Sibaoka, 1979; Hamman and Seliger, 1982). The control of intracellular pH is known to have an important role in a variety of physiological processes (Busa and Nuccitelli, 1984).

The specificity of H^+ for the triggering of the bioluminescent system in all dinoflagellates is further indicated by the observation that *in vivo* emission is stimulated in all species by weak acids at pH 5. Hamman and Seliger (1982) consider that all other cations which stimulate *in vivo* BL (NH_4^+, La^{3+}, K^+, and Ca^{2+} at concentrations ranging from 10^{-2} to 10^{-4} *M* are the most effective) act to depolarize the cell membrane and to result ultimately in an increase of H^+ ions at BL sites.

4. PHOTOINHIBITION OF MECHANICALLY STIMULATED BIOLUMINESCENCE

Sweeney *et al.* (1959) reported the action spectrum for photoinhibition of BL in *G. polyedra,* with maxima at 450 and 700 nm. Light at 600 nm was ineffective. As discussed by Hamman *et al.* (1981b), different mechanisms (or combinations thereof) may be involved in this inhibition. These include a reduction in the actual BL capacity, a decrease in the sensitivity or responsivity of the mechanoreceptor mechanism, a block-

age in the propagation of the action potential, and an inhibition or inactivation of the coupling between the action potential and the luminescent sites. Bode *et al.* (1963) showed that in *G. polyedra* photoinhibition did not involve the first of these. During the night, yields of extractable luciferin were increased following exposure to light; this was attributed to a photoinduced decrease in the susceptibility of the luminescent sites to the mechanical stimulation to which the cells are subjected during harvesting. Consistent with this, Hamman and Seliger (1982) showed that the luminescent response of cells to chemical stimulation, which bypasses the mechanoreceptor, was not decreased by light treatment as was mechanically stimulated luminescence.

Different results have been obtained in different studies, and there may also be some species differences. Esaias *et al.* (1973) reported the action spectrum for photoinhibition of mechanically stimulated luminescence in *Gonyaulax catenella, G. acatenella,* and *G. tamarensis* to be almost the inverse of the result of Sweeney *et al.* (1959), with a single maximum at ~560 nm and no action in the blue (<450 nm) or red (>650 nm). Hamman *et al.* (1981b) suggested that the same may be true for *G. polyedra.* Hamman *et al.* (1981a) found blue light with a maximum at 436 nm to be most effective for photoinhibition in *D. lunula;* the effect dropped off sharply between 450 and 500 nm. This was attributed to a nonphotoactive shielding pigment in the region of BL emission. Similar sensitivities were determined for *Pyrodinium bahamense* and three species of *Pyrocystis;* no photoinhibition by light in the yellow and red regions (500–650 nm) was found.

5. PHOTOSTIMULATION OF BIOLUMINESCENCE

In three dinoflagellate species, excitation by red light from a laser or a xenon flash lamp results in the emission of luminescence that matches BL spectrally (Hickman and Lynch, 1981; Sweeney *et al.,* 1983). The experimental conditions were too different in these two studies to compare them in detail from a mechanistic viewpoint. Hickman and Lynch (1981) exposed *P. lunula* to brief (0.1 μs) high-energy (2 joule) laser pulses at 585 nm and recorded bioluminescent flashes; these appeared similar kinetically to mechanically stimulated flashes. The intriguing results of Sweeney *et al.* (1983), mostly with *G. polyedra,* suggest that Chl is the photoreceptor and that photosystem II of photosynthesis is involved. It was established that neither the evolution of O_2 nor the production (or movement) of H^+, both of which figure in the biochemistry of both photosynthesis and dinoflagellate BL, are involved in the light-induced emission. Sweeney *et al.* (1983) also reported that the light-

stimulated BL was favored by anaerobic conditions; whether or not luminescence occurred under strict anaerobic conditions was not established.

6. CIRCADIAN RHYTHMS

A circadian rhythm of BL occurs in some, possibly many, dinoflagellates; properties of this rhythmicity are known in large part from studies in *G. polyedra* (Hastings, 1959; Sweeney, 1983). Bioluminescent flashing occurs on stimulation and this response is far greater during the night than during the day. Cultures maintained under conditions of constant temperature and constant dim light continue to exhibit rhythmicity for weeks but, lacking the synchronizing cues of light–dark circles, the rhythm exhibits a "circadian" period, i.e., one which differs somewhat from 24 h. In addition, the amplitude of the rhythm damps with time, probably due to desynchronization between individual cells (Njus *et al.*, 1981).

Daily changes in the cellular concentrations of both luciferase and luciferin can account for the bioluminescence rhythm. Indeed, it was recently shown that luciferase must be synthesized *de novo* and destroyed each day, a finding of importance in relation to the cellular circadian mechanism (Dunlap and Hastings, 1981b; Johnson *et al.*, 1984). The peak phases of luciferase concentration and luminescent flashing are coincident, but the peak of the spontaneous glow of luminescence occurs many hours later (Krasnow *et al.*, 1980). Although this may seem paradoxical, it may be noted that the glow is very weak and that the phase corresponds to the time at which luciferase would be undergoing proteolysis. The transient occurrence of the 35-kb proteolytic fragment, whose activity is not so subject to regulation by pH, might be responsible for the glow.

Photosynthesis also exhibits a pronounced circadian rhythm (Hastings *et al.*, 1961); changes in photosystem II may account for this rhythm (Samuelsson *et al.*, 1983). Concomitantly, fluorescence is about twice as high during the day than during the night phase (Govindjee *et al.*, 1979).

IV. Concluding Remarks

This chapter has put forward a new thesis, namely that the fundamental steps in bioluminescence may have similarities to photochemical steps in photosynthesis and, indeed, that bioluminescence may be viewed as the reverse of photosynthesis. In the primary photochemical step in photosynthesis, chlorophyll acts as an electron donor; delayed light may

be viewed as luminescence coming from chlorophyll excited by its recombination with an electron derived from one of the many different species that had served as initial electron acceptors. Thus, delayed light may have many kinetic components but always exhibits chlorophyll fluorescence. In bioluminescence a peroxide formed by reaction with molecular oxygen participates in chemically induced electron exchange; the peroxide acts as the electron acceptor in the initial electron transfer, but the donor may differ. Thus, in the final electron transfer back to the donor the emission corresponds to the fluorescence of that particular species. These postulates and perspectives may help clarify our understanding of the fundamental photochemistry in luminescence and the mechanisms involved in the interconversion of chemical and radiant energy.

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IV

Light Emission from Rhodopsins

14

Light Emission from Bacteriorhodopsin and Rhodopsin

RAJNI GOVINDJEE
THOMAS EBREY
Department of Physiology and Biophysics
University of Illinois at Urbana–Champaign
Urbana, Illinois 61801

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ABBREVIATIONS AND SYMBOLS

bR Bacteriorhodopsin
ϕ_f Fluorescence yield
τ_f Lifetime of fluorescence

ABSTRACT

The energy-transducing membrane of *Halobacterium halobium*, the purple membrane, contains a single chromoprotein, bacteriorhodopsin (bR). As in photosynthetic systems, light absorption by bR produces a proton gradient across the plasma membrane, which in turn leads to ATP production. The chromophore structure and the primary photochemistry of bR are similar to those of the visual pigment rhodopsin. Several investigators have studied the fluorescence properties of bR and rhodopsin in an attempt to obtain information about the primary photochemistry of these pigments.

The fluorescence yield of bR is temperature-dependent, whereas the photochemistry is temperature-independent from 5 to 300°K. The quantum yield of fluorescence is very low, 2.5×10^{-4}, and the lifetime is ≤2 ps at room temperature. The fluorescence yield increases 10- to 15-fold in going from room temperature to 77°K. The fluorescence yield of rhodop-

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sin is temperature-independent from 5 to 300°K and its yield is even lower than that of bacteriorhodopsin. The bathorhodopsin formation time shows both a temperature dependence and a deuterium effect. In a homolog of rhodopsin in which the cis-to-trans photoisomerization is hindered, the fluorescence yield is higher than in rhodopsin itself.

I. Introduction

Tracing the pathway of the utilization of light energy in photosynthesis has been very profitably pursued by studying the characteristics of that part of the absorbed light which is not used for photochemical processes but rather is reemitted as fluorescence. The success of these studies, as shown in other chapters in this book, encouraged many people interested in light energy transduction in bacteriorhodopsin (bR) and rhodopsin to investigate fluorescence from these pigments. It is probable that many features of the fluorescence from these two pigments are similar, for both contain retinal as their chromophore and their primary photochemistry is a cis–trans isomerization about a double bond of the retinal chromophore (Rosenfeld *et al.*, 1977; Honig *et al.*, 1979). This chapter concentrates on bR because rhodopsin has a very low quantum yield of fluorescence and very few studies of this pigment are available; nevertheless, the rhodopsin results are of some relevance in helping to understand bacteriorhodopsin's fluorescence and so they will be discussed briefly.

Bacteriorhodopsin is a chromoprotein found in the purple membrane fraction of the plasma membrane of *Halobacterium halobium.* It consists of 248 amino acids whose sequence has been determined (Khorana *et al.*, 1979; Ovchinnikov *et al.*, 1979). The peptide chain is folded into seven α-helical segments that span the thickness of the membrane (Henderson and Unwin, 1975; Engelman *et al.*, 1980), and these segments are connected by short hydrophilic loops. The N-terminal region consists of four or five amino acid residues projecting beyond the surface of the membrane on the outside, whereas the C terminus consists of approximately 20–25 amino acids projecting beyond the membrane surface on the cytoplasmic side. The retinal chromophore of bR is attached to lysine 216 of the protein via a protonated Schiff base.

Bacteriorhodopsin exists in two forms: (1) light-adapted bR, which has an absorption maximum at 568 nm, and (2) dark-adapted bR, with a maximal absorbance at 558 nm. The chromophore of light-adapted bR is almost 100% all-*trans*-retinal, whereas dark-adapted bR has 50% 13-*cis*- and 50% all-*trans*-retinal. Because light absorption by the all-*trans* form of bR leads to proton pumping, almost all experiments have been

done with the light-adapted form of bR. Unless otherwise indicated, we will discuss only experiments on light-adapted bR.

Light absorbed by bR initiates a photochemical cycle during which the pigment goes through a series of spectroscopically distinct intermediates, K, L, M, N, and O, before returning to its original state (Fig. 1; also see a review by Stoeckenius *et al.,* 1979). This photocycle is coupled to the transport of approximately two protons across the cell membrane (Ort and Parson, 1979; Bogomolni *et al.,* 1980; Govindjee *et al.,* 1980; Renard and Delmelle, 1980). The step bR → K is photochemical; the rest of the later steps are dark reactions. The intermediate K is stable at 77°K and irradiation with long-wavelength light converts K back to bR; the quantum efficiency of the K → bR backreaction is 0.7 and is temperature-independent (Hurley and Ebrey, 1978). The quantum yield of the photocycle is 0.3 and is also independent of temperature (Becher and

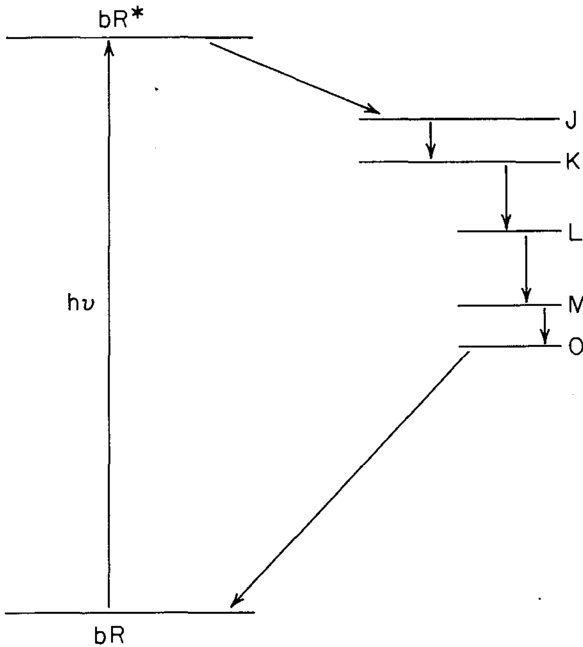

FIG. 1. Photocycle of bacteriorhodopsin (bR). The first photoproduct stable at 77°K is called K (absorption λ_{max}, 610 nm). Intermediate J (λ_{max}, 625 nm) is a ground state species which is unstable at all temperatures. K decays in the dark through branched pathways, to intermediates L (λ_{max}, 550 nm), M (λ_{max}, 412 nm), and O (λ_{max}, 640 nm), before returning to the ground state. The quantum efficiency of K (and M) formation is about 0.3.

Ebrey, 1977; Goldschmidt *et al.*, 1977; Hurley and Ebrey, 1978; Iwasa *et al.*, 1980).

A nonphysiological but quite interesting form of bR is formed when the pH of a sample is lowered to approximately 3; it is called the acid bR or the acid blue bR form, because of its blue color in contrast to the purple color of bR (Oesterhelt and Stoeckenius, 1971; Moore *et al.*, 1978; Mowrey *et al.*, 1979). The absorbance maximum of acid bR is at 605 nm. Bacteriorhodopsin is a quite unusual protein in that it is stable at this and even lower pH values, possibly because it contains no histidine. Acid bR is probably formed from bR by the displacement of normally tightly bound divalent cations by the high concentration of protons (Chang *et al.*, 1985). Deionization of the purple membrane by a variety of means leads to the formation of a species spectrally identical to acid blue bR (Kimura *et al.*, 1984; Chang *et al.*, 1985).

The fluorescence from bR is quite weak at room temperature but is enhanced considerably as the temperature is lowered to 77°K. The fluorescence emission has a large Stokes shift and there is some disagreement about the exact shape of its spectrum, its yield, and its lifetime at both room and low temperatures. We will discuss each of these features in turn, ending with a discussion of the pathways of light energy in bacteriorhodopsin and the relationship of rhodopsin to bacteriorhodopsin. Some of this material has been reviewed briefly by Ebrey (1982) and by Lewis and Perreault (1982), who provided useful tables summarizing much of the data so far.

II. Fluorescence Emission and Excitation Spectra of Bacteriorhodopsin

A. *Bacteriorhodopsin Fluorescence*

1. ROOM TEMPERATURE FLUORESCENCE

The fluorescence emission from bR at room temperature covers a rather broad range of wavelengths, and the emission maximum has been reported to range from 660 to 790 nm with various preparations from several different laboratories (see Table II in Lewis and Perreault, 1982). Sineshchekov and Litvin (1976, 1977) reported that the emission maximum is at 660 nm, Lewis *et al.* (1976) at 791 nm, Spoonhower (1976) at 730 nm, Alfano *et al.* (1976) and Govindjee *et al.* (1978) at 700–710 nm, and Kouyama *et al.* (1985) at 750 nm. Kriebel *et al.* (1979) found that the emission maximum at a low exciting light intensity (50 $\mu W/cm^2$)

was at 714 nm, but shifted to 735 nm at high-intensity (~3 W/cm^2) laser excitation. They attributed the 735-nm emission to the formation of pseudo-bR (discussed in Sections II,C,2 and V). J. P. Spoonhower (cited in Lewis and Perrault, 1982) did not observe this shift when he varied the exciting light intensity. The reason for the variability in the emission maximum reported above is not clear. It is possible that the different samples are not equivalent and do indeed have different emission maxima. Important variables might be the state of proteolysis of the sample, pH, type of cations bound to the sample, and state of hydration. However, none of these has so far been explored in depth. It seems reasonable, however, to take 740 ± 20 nm as the emission maximum of bR fluorescence at room temperature, this being the value found by most laboratories under most conditions (see Fig. 2). [Figure 2a shows data for acid bR (to be discussed later), and Fig. 2b shows data for bR at pH 7.0.] At pH 7.0 the excitation maximum is around 570 nm and the bR fluorescence shows a very large Stokes shift (Fig. 2b). Furthermore, there is no

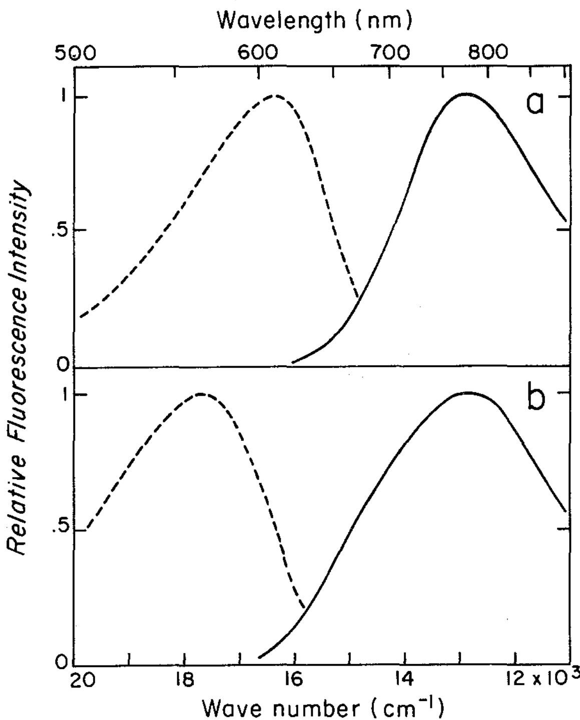

FIG. 2. Corrected fluorescence emission (solid line) and excitation (dashed line) spectra of light-adapted bR at room temperature: (a) pH 2.6; (b) pH 7. (From Kouyama *et al.*, 1985.)

mirror-image relationship between the fluorescence and absorption spectra.

2. LOW-TEMPERATURE FLUORESCENCE

The emission spectrum of bR at low temperatures has been measured in several different laboratories. As the temperature of bR is lowered, one or more of the photocycle intermediates can accumulate to a significant amount, and thus the fluorescence observed must be carefully studied so that it can be assigned to either bR or one of the intermediates present. The fluorescence from the intermediates will be discussed later. The emission spectrum of bR has been clearly observed only at temperatures below about 100°K, where the sole photocycle intermediate present is the primary photoproduct K (except possibly a species proposed by T. Gillbro and co-workers, pseudo-bR; see below). This is possible because it is generally agreed that at 77°K the fluorescence from bR is much greater than the fluorescence from K and thus the latter can be ignored. However, the resulting emission spectra, obtained in different laboratories, are not all identical (see Table I in Lewis and Perreault, 1982), although all agree that the broad unstructured fluorescence emission of bR at room temperature is replaced by a more structured emission spectrum at 77°K. Lewis *et al.* (1976) reported peaks at 678, 733, and 791 nm. Sineshchekov and Litvin (1976, 1977) reported emission maxima at 665 and 720–730 nm in bR suspensions; at 665, 720–730, and 780–790 nm in bR films at neutral pH (the relative intensity of the 665-nm emission being higher in suspensions and that of the 780-nm emission being higher in bR films); and at 720–730 and 780–790 nm in freeze-dried bR samples. Alfano *et al.* (1976) and Govindjee *et al.* (1978) reported emission maxima at 680, 710, and 735–740 nm; and Shapiro *et al.* (1978) at 670, 720, and 790 nm. Gillbro *et al.* (1977) also observed light emission at 77°K, but they suggested that it originated from a photoproduct, pseudo-bR (discussed in Sections II,C,2 and V). Sineshchekov *et al.* (1981, 1984) also reported complex emission from bR irradiated for long times at 77°K and suggested that two fluorescent photoproducts, iso-bR and pseudo-bR, were required to explain their results. The low-temperature emission spectrum is probably more reliable than the room temperature one because of the higher yield of fluorescence. Again, it is conceivable that environmental conditions, noted in Section II,A,1, may affect either the position or intensity of these bands, although at present there is no evidence for this. We tentatively accept the low-temperature emission spectrum of bR as that shown in Fig. 3. [Fig-

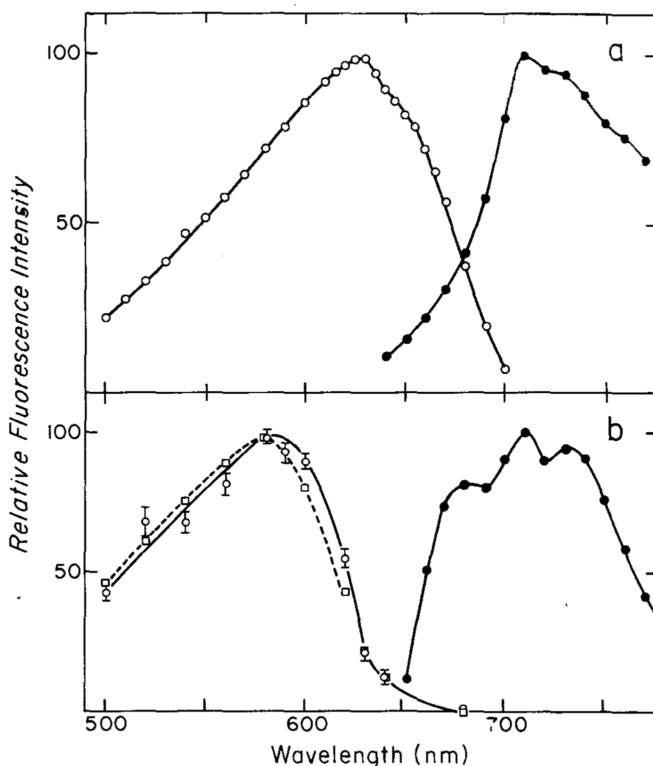

FIG. 3. Corrected fluorescence emission (filled circles) and excitation (open circles) spectra of light-adapted bR at 77°K: (a) pH 3; (b) pH 7. The dashed line in (b) is the percent absorption of the sample. (From Govindjee *et al.*, 1978, and unpublished data of the authors, 1984.)

ure 3a shows data for acid bR (to be discussed later) and Fig. 3b shows data for bR at pH 7.0.]

The excitation spectrum shows a maximum around 585 nm (Govindjee *et al.*, 1978) (Fig. 3b). However, Sineshchekov and Litvin (1977) observed a peak at 585 nm with a shoulder around 510–520 nm in bR suspensions, and a peak around 620–630 nm and a shoulder around 575–580 nm in bR films at pH 7.0.

B. Acid Bacteriorhodopsin Fluorescence

Bacteriorhodopsin is relatively stable over a wide pH range. Lowering the pH to around 3 causes bR to turn blue and the absorbance maximum

shifts from 568 to approximately 600 nm for the acid blue species. The quantum yield of fluorescence of acid bR (pH 2.7), at both 77°K and room temperature, is several times higher than that of bR at neutral pH. According to Kouyama *et al.* (1985), the emission maximum of acid bR at room temperature is similar to that of bR at neutral pH (see Fig. 2a). However, the maximum of the excitation spectrum is red-shifted to around 610 nm. Sineshchekov and Litvin (1977) observed an emission maximum at 720 nm for acid bR at both room temperature and 77°K. The excitation spectrum showed a peak around 630 nm at 77°K. Spectra obtained by the present authors at 77°K are shown in Fig. 3a; the emission spectrum has peaks around 710 and 730 nm and the excitation spectrum has a broad peak around 630 nm.

C. Fluorescence of the Photointermediates

1. PHOTOINTERMEDIATE K

Several lines of evidence indicate that the primary photointermediate, K, is nonfluorescent. Govindjee *et al.* (1978) argued that most or all the fluorescence emission at 77°K is from bR because (1) the excitation spectrum for fluorescence emission at 720 nm at 77°K matches very closely the percent absorption spectrum of bR with a maximum around 585 nm; (2) different steady-state mixtures of bR and K can be produced at 77°K on irradiation by light of different wavelengths, but the fluorescence emission spectra of these mixtures are identical for these different excitation wavelengths; and (3) there is a time-dependent decrease in the fluorescence intensity of a sample in the bR state when a strong measuring light is turned on. The latter argument supports the nonfluorescent character of K because the size of the final decrease in fluorescence intensity is different for different exciting wavelengths and is approximately equal to the amount of K produced in the steady state by each wavelength.

2. PSEUDO-bR

As discussed above, the primary photoproduct of bR, the red-shifted intermediate K, does not appear to have detectable fluorescence at 77°K. On the basis of the effect of preillumination on the fluorescence yield, Gillbro *et al.* (1977) and Kriebel *et al.* (1979) proposed that bR was transformed not only to K but also in parallel to a second primary photoproduct, pseudo-bR, having a very similar absorption spectrum to bR but a much higher fluorescence yield. Gillbro *et al.* (1977) observed that pre-

illumination of the sample at 77°K with 514.5-nm light (1 W/cm^2) caused a 3.5-fold increase in the fluorescence intensity and a blue shift in the emission peaks from 678 and 725 nm to 670 and 720 nm. Also, the intensity of the 670-nm emission band increased much more than that of the 720-nm band. Their excitation spectrum for emission between 670 and 740 nm showed a maximum at 597 nm and a shoulder at 540 nm. These authors reported that long illuminations could increase the fluorescence yield over 50-fold. This is a startling and provocative finding; Sineshchekov *et al.* (1981) reported similar results. Sineshchekov *et al.* (1984) concluded that another species, iso-bR, is also required to explain the fluorescence seen at 77°K after strong irradiation. However, in our experiments we never saw such a large effect of preillumination, although we probably did not use as intense light as these authors. It is unlikely that pseudo-bR was formed so rapidly in our low-temperature measurements that we could not follow its appearance, because we could follow the appearance of K, which is formed with a quite high quantum efficiency, 0.3. Likewise, Lewis and Perrault (1982) reported no increase in yield or change in emission spectrum with increasing exciting light intensity. Nevertheless, it may be that variations in sample preparation or sample handling or a higher exciting light intensity gave rise to these differences in results. The absorption data of Gillbro and Sundström (1983) related to the behavior of their hypothesized photoproduct will be discussed in Section V,A.

3. PHOTOINTERMEDIATES M AND O

Besides the K intermediate, the M and O intermediates have also been studied. Gillbro and Kriebel (1977) reported fluorescence emission from bR samples that were cooled to 77°K under strong illumination; they called these "bleached" samples because their color had changed from purple to yellow. Such samples contain a mixture of photocycle intermediates. The emission spectrum for excitation at 640 nm had a main band at 705 nm with a shoulder at 740 nm. The 740-nm emission is the same as that observed by other research groups for bR samples at 77°K and, as discussed above, has an excitation maximum around 585 nm. However, the excitation spectrum for emission at 705 nm in bleached samples was found to be considerably red-shifted compared to the excitation spectrum of an unbleached sample. The excitation difference spectrum for bleached and unbleached samples showed a maximum around 635 nm. The absorption spectrum of the bleached sample also showed a shoulder around 630 nm. Thus, it seems that the 705-nm emission originates from a species absorbing around 635 nm. The only species absorbing in

this wavelength region are the K and O photointermediates, and K as an emitting species was ruled out above. Thus, the 705-nm fluorescence was attributed to the photointermediate O.

Gillbro and Kriebel (1977) also observed a structured fluorescence spectrum with peaks at 500, 540, and 580 nm from the bleached samples with 454.5-nm excitation. The excitation spectrum for fluorescence emission at 530 nm showed peaks at 375, 390, 419, and 444 nm. This fluorescence emission is attributed to the M intermediate. Sineshchekov *et al.* (1981) also described the fluorescence from bR samples cooled to −60°C under illumination. They reported the formation of the photointermediate M (which they called P419), which when irradiated with 436-nm light produces another intermediate, which they called P585. In contrast to fluorescence from M, observed by Gillbro and Kriebel, Sineshchekov *et al.* (1981) found that P419 (equivalent to M) has little or no fluorescence. However, P585 is highly fluorescent and converts back to P419 with 546-nm light; it does not appear to be equivalent to bR. The emission maximum of P585 is at 740 nm and its quantum yield is 10^{-4} at −62°C. Sineshchekov *et al.* proposed that P585 is not the pseudo-bR reported by Gillbro *et al.* (1977), because the excitation maximum of P585 is at 578 nm whereas that of pseudo-bR is at 597 nm. However, the latter value may be artifactually red-shifted because it was not corrected for differences in the amount of bR at each irradiating wavelength since the exciting light changes the concentration of bR. Thus, the long-wavelength species reported by these different groups may be equivalent.

III. Quantum Yield of Fluorescence of Bacteriorhodopsin

A. Room Temperature Fluorescence Yield

Early estimates of the yield (ϕ_f) of bR fluorescence at room temperature were $1.2–2.4 \times 10^{-4}$ (Spoonhower, 1976, quoted in Lewis and Perrault, 1982); 2.4×10^{-5} (Alfano *et al.*, 1976); 10^{-4} (Sineshchekov and Litvin, 1976, 1977); and more recently $2.5–2.7 \times 10^{-4}$ for light-adapted bR and $0.7–1.2 \times 10^{-4}$ for 13-*cis*-bR (Kouyama *et al.*, 1985), and 10^{-4} for light-adapted bR (Polland *et al.*, 1986). In view of the concurrence of most estimates, we tentatively accept a value of 2.5×10^{-4} as the ϕ_f for bR at room temperature.

B. Temperature Dependence of Fluorescence Yield

Many researchers have noted that the ϕ_f of bR increases when the sample is cooled from room temperature to 77°K. It was initially as-

sumed that the species fluorescing at room temperature was the same as the species fluorescing at low temperature, but more recently this assumption has been questioned (to be discussed later). The temperature dependence of the fluorescence of bR samples has been reported by Sineshchekov and Litvin (1977) and Shapiro *et al.* (1978). Shapiro *et al.* (1978) found that the ϕ_f of bR remains constant and low between 300 and 150°K, below which it increases sharply. The three peaks (670, 720, and 790 nm) observed in the emission spectrum at 77°K are affected differently as the temperature is raised. Above 150°K the 670-nm peak disappears and the 720-nm peak shifts approximately 5 nm toward longer wavelengths, whereas the 790-nm peak remains relatively unaffected. Sineshchekov and Litvin (1977) found two breaks at 173 and 253°K in a plot of log ϕ_f of bR versus $1/T$. The calculated activation energies were 2.7×10^2 cal/mole in the 77–173°K range, 10^3 cal/mole in the 173–253°K range, and 4×10^3 cal/mole in the 253–293°K range. The authors concluded that these regions roughly correspond to the transformations of bR and its photointermediates. The differences between these results and those of Shapiro *et al.* may be due to the presence or the absence of these photointermediates.

Other workers have noted the increase in fluorescence yield to varying degrees in going from room temperature to 77°K. The ratio of the ϕ_f at 77 and 300°K was reported to be 15–20 by Alfano *et al.* (1976); 10 by

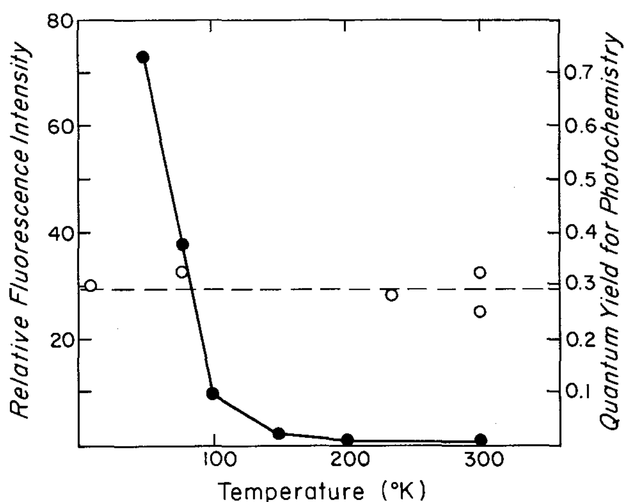

FIG. 4. Temperature dependence of fluorescence intensity (filled circles) and quantum yield of photochemistry (open circles). Fluorescence data are from Shapiro *et al.* (1978); quantum yield of photochemistry data are from Goldschmidt *et al.* (1977), Becher and Ebrey (1977), Hurley and Ebrey (1978), Govindjee *et al.* (1980), and Iwasa *et al.* (1980).

Sineshchekov and Litvin (1977); 10–39 without or with preillumination with 514-nm light by Gillbro *et al.* (1977) and Kriebel *et al.* (1979); and 38 by Shapiro *et al.* (1978). It is possible that some of the variability observed by different investigators was due to the formation of pseudo-bR under some circumstances.

The temperature dependence of the fluorescence as a qualitative phenomenon is quite important because the photochemical transformation of bR to K, the only other process originating from the excited state of bR which has been studied, is temperature-independent (Fig. 4).

IV. Lifetime of the Fluorescence of Bacteriorhodopsin

The lifetime (τ_f) of the fluorescence of bR has been measured at both low and room temperatures. Hirsch *et al.* (1976), using an up-conversion gate technique and multiple-pulse excitation of 10 ps duration, measured the τ_f at room temperature as 15 ± 3 ps. At 90°K, Alfano *et al.* (1976) determined the τ_f as 40 ± 5 ps by using a Kerr gate technique and also multiple pulse-train excitation; at room temperature, the measurement was limited by resolution, ≤8 ps. Shapiro *et al.* (1978), using single-pulse excitation, found the lifetime to be 60 ± 15 ps at 77°K; measurements at room temperature were limited by resolution, ≤20 ps. Mahr and Sagan (1981) reported a value of 20 ps at room temperature. Sharkov *et al.* (1983), using single subpicosecond pulse excitation, found the τ_f to be less than 2 ps at room temperature. If one takes the temperature dependence of the yield, one can calculate the expected room temperature (RT) τ_f from the measured τ_f at 77 or 90°K, assuming that one is always looking at the same state when the temperature is changed. Using the relationship $\tau_f(\mathrm{RT}) = \tau_f(77°\mathrm{K})\ \phi_f(77°\mathrm{K})/\phi_f(\mathrm{RT})$, the estimated values of $\tau_f(\mathrm{RT})$ are approximately 3 ps (Alfano *et al.*, 1976) and 1.5 ps (Shapiro *et al.*, 1978). These are consistent with the measured value of less than 2 ps (Sharkov *et al.*, 1983).

V. Origin of the Fluorescence of Bacteriorhodopsin

A. Primary Photochemistry of Bacteriorhodopsin

In order to understand the models for the origin of the fluorescence of bR it is necessary to make a brief excursion into what is known about the primary photochemistry of bR. This topic is reviewed in more detail

by Honig *et al.* (1979), Ottolenghi (1980), and Birge (1981) as well as by Ebrey (1982). The absorption maximum of K, the primary photoproduct at all temperatures down to 10°K, is red-shifted about 60 nm from that of the bR, as determined by low-temperature spectroscopy of stable mixtures containing the photoproducts. Picosecond absorption spectroscopy seems to suggest that the photoproduct K, which is stable at 77°K but which can also be detected at room temperature, is formed through an intermediate which is unstable at all temperatures. It was proposed (Honig *et al.*, 1979) that this unstable intermediate, called S (Applebury *et al.*, 1978), K′ (Ebrey, 1982), or J (Dinur *et al.*, 1981), is the first ground state product of the photochemistry of bR (Fig. 1). This proposal is based on (1) absorption measurements showing that at room temperature the ground state of bR is repopulated by the time J is formed and thus the excited state must already have depopulated, and (2) the very close similarity between the absorption spectra of J and K, which suggests that they represent slightly different forms of the same species (Applebury *et al.*, 1978). Since K is a ground state species, J must also be a ground state species. Ippen *et al.* (1978) determined the formation half-life of J as 1 ± 0.5 ps. They did not detect the decay of J to K because their measurements were at the isosbestic point of this transition. Recently, Polland *et al.* (1985) determined the formation time of J as 0.7 ps and studied the J-to-K transition in detail. Thus, the 0.7-ps lifetime for the precursor of J, presumably the excited state of bR, bR* (Fig. 1), appears to be consistent with the room temperature τ_f of less than 2 ps; this coincidence may be misleadingly fortuitous (see Section V,B).

Gillbro and Sundström (1983) suggested that the J-to-K transition is due to the decay of their hypothesized species, pseudo-bR, to its photoproduct. They proposed that this transient is seen only if laser intensities are so high that two photoprocesses take place, bR to pseudo-bR (through bR*) and pseudo-bR to its photoproduct (through pseudo-bR*). Their measured J-to-K (pseudo-bR* to the photoproduct ground state) lifetimes at low temperatures were significantly longer than those reported by Applebury *et al.* (1978). Unfortunately, they did not vary the light intensity in order to provide stronger evidence that they were observing a transition which required two photons for its creation.

B. Models of Fluorescence

The absorption of light by bR leads to two processes: the formation of the stable photoproduct K and the emission of fluorescence. Govindjee *et al.* (1978) gave three reasons why the low-temperature fluorescence

from bR must come from a different excited state than that leading to the K photoproduct (Fig. 5). Their first argument was based on the hypothesis that bR and K share a common minimum in their excited state. If bR and K had a common minimum they should fluoresce equally, but in fact bR is much more fluorescent than K. Second, since the photochemistry of bR is temperature-independent while the fluorescence is quite temperature-dependent (Fig. 4), the low-temperature fluorescence must originate from a different state than the low-temperature photochemistry. Finally, the ϕ_f at 77°K (~60 ps) is much longer than the lifetime to form K (~10 ps), requiring two different excited state lifetimes and hence two different excited states. Thus, after excitation, the energy has distributed to two different states or regions of the same state (see the next paragraph). Only a small fraction of the absorbed photons would have to go to the fluorescing state; most would go to the photochemical state. The temperature dependence of the fluorescing state is achieved by postulating an energy barrier.

Thus, a simple model for bR fluorescence is that after excitation, the molecule is initially in its vertically excited state, from which (1) it has the highest probability of fluorescence because the energy gap to the ground

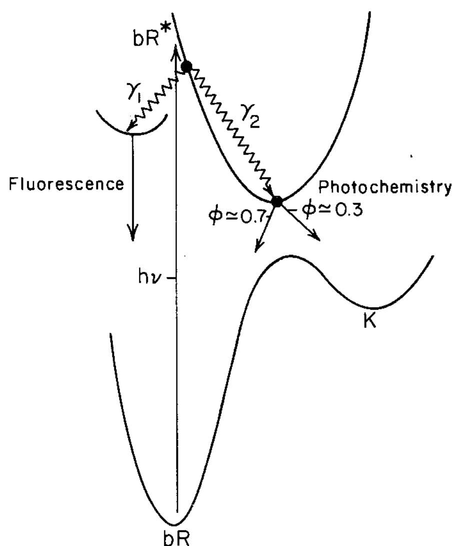

FIG. 5. **Hypothetical energy level diagram for bR and its primary photoproduct K, showing that the fluorescence originates from a state close to 0° while the photochemistry originates from a different state, close to 90°. The population of the two different excited states with quantum efficiencies of γ_1 and γ_2 is shown by the wavy arrows.**

state is largest, but (2) it very quickly (in subpicosecond times) moves along the excited state potential surface to an energy minimum. As soon as it gets away from the vertically excited state the probability of fluorescence decreases greatly. Another consequence of this large movement in the excited state is that the usual relationship between intrinsic lifetime (τ_0), quantum yield (ϕ_f), and radiative lifetime (τ) is no longer valid (Doukas *et al.*, 1984). This suggests that the coincidence between the τ_f for bR calculated from $\tau_f = \phi_f\tau_0$ and the τ_f measured at room temperature may be accidental.

Kouyama *et al.* (1985) propose quite a different model for bR fluorescence based on the similarity between the room temperature emission spectra and different excitation spectra for bR at neutral pH and for acid bR. They hypothesize that normally a proton is transferred in the excited state from one protein group to another in going from bR to its primary photoproduct. Most of the fluorescence from bR at room temperature is postulated to be from the excited state after the proton transfer has taken place on a subpicosecond time scale. Since the emitting species would have a different structure (and possibly absorption) than bR, the lack of a mirror-image relationship between bR absorption and fluorescence at neutral pH could be explained. In acid bR, since the acceptor group on the protein is already protonated in the ground state, there could be no excited state proton transfer. The absorption and emission would be from the same species and thus a smaller Stokes shift would result. This proposed proton transfer is quite different from that proposed by Peters *et al.* (1977) and Applebury *et al.* (1978), which was suggested to occur in picosecond rather than subpicosecond times. Kouyama *et al.* (1985) propose that at 77°K the proton transfer is blocked so the fluorescence is emitted by bR before the proton transfer has taken place. The temperature dependence of fluorescence would then be due to the temperature dependence of the proton transfer reaction. Thus, there are two species that contribute to the fluorescence, depending on pH and temperature. Although this hypothesis is appealing in explaining the large Stokes shift of bR fluorescence, the lack of any evidence for subpicosecond proton transfer makes it difficult to evaluate.

A third type of model for bR fluorescence is that of Gillbro and coworkers (Gillbro and Kriebel, 1977; Gillbro and Sundström, 1983) and a similar model of Sineshchekov *et al.* (1981, 1984). They also suggest the existence of two participating states, but these are the excited states of two different ground states. As mentioned earlier, they propose that most of the bR fluorescence comes from a species formed photochemically from bR and termed by them pseudo-bR (Sections II,C,2 and V).

Presumably the temperature dependence of fluorescence is due to the temperature dependence of the photoconversion process to pseudo-bR. Some of the experimental results of Gillbro and co-workers have been corroborated in general by Sineshchekov *et al.* (1981), but these workers suggest that a second photoproduct, iso-bR, is also required to explain the fluorescence behavior of bR at 77°K after strong irradiation. Thus, it may be that under some circumstances very strong illumination can lead to the formation of a highly fluorescent species. However, our failure and that of the others to see the enormous fluorescence enhancements observed by these two research groups suggests that it can be seen only under special environmental conditions or under much more intense illumination.

VI. Fluorescence from Rhodopsin

Fluorescence from both bovine and squid rhodopsin has been reported by Alfano, Callender, and co-workers (Doukas *et al.*, 1981, 1984, 1985) and from frog and bovine rhodopsin by (Sineshchekov *et al.* (1983) and Sineshchekov and Litvin (1985). Doukas *et al.* (1984) found that at room temperature squid rhodopsin has a broad emission band with a maximum at approximately 620 nm, while bovine rhodopsin fluorescence emission is shifted about 40 nm to shorter wavelengths. Sineshchekov *et al.* (1983) reported a well-resolved emission maximum at 580 nm for frog rhodopsin. The ϕ_f is quite low for both bovine and squid pigments, $1.2 \pm 0.5 \times 10^{-5}$ (Doukas *et al.*, 1984). The measured τ_f is less than 15 ps at room temperature (Doukas *et al.*, 1984); the τ_f calculated from the ϕ_f should be about 0.1 ps. Doukas *et al.* (1985) found that the ϕ_f is independent of temperature from 5 to 40°K, as well as independent of whether the rhodopsin is in H_2O or D_2O. Since the first step in the bleaching of rhodopsin that is kinetically resolvable with absorption measurements on a picosecond time scale, the batho′-to-batho transition (see Peters *et al.*, 1977; Honig *et al.*, 1979), is dependent on temperature and deuteration, Doukas *et al.* inferred that the primary photochemical event must precede the deuterium- and temperature-dependent step. For the visual pigment rhodopsin, Doukas *et al.* (1984) proposed that the fluorescence and photochemistry come from different regions of the excited state. They noted that in a molecule like rhodopsin (or bacteriorhodopsin for that matter), which undergoes photochemistry along an excited state surface similar to the one shown in Fig. 5, the fluorescence is much more likely to take place close to 0°, i.e., close to the vertical excited state, than at 90°, the minimum of the excited state

where the photochemical transformation takes place. This is because the rate of fluorescence, according to the Einstein relationship, goes as the cube of the energy difference between the ground and excited states, so that as one gets away from 0° the energy difference, and thus also the probability of radiative deexcitation, diminishes. The phenomenon that different regions of the excited state surface are the origins of fluorescence and photochemistry will be seen only when a molecule moves a great deal on its excited state surface, as happens during photoisomerization. Doukas *et al.* (1985) showed that a homolog of rhodopsin in which the cis–trans photoisomerization is hindered (Buchert *et al.*, 1983) has a much higher ϕ_f than rhodopsin itself.

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V

Special Features of Different Organisms: Relationship of Fluorescence to Biochemistry and Physiology

15

Fluorescence Properties of Photosynthetic Bacteria

JAN AMESZ
HENK VASMEL
Department of Biophysics
Huygens Laboratory of the State University
2300 RA Leiden, The Netherlands

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ABBREVIATIONS AND SYMBOLS

BChl	Bacteriochlorophyll
BPheo	Bacteriopheophytin
B800–850, B875	Light-harvesting pigment–protein complexes containing BChl absorbing at 800 and 850 and at 875 nm, respectively
I	Primary electron acceptor
P	Primary electron donor
Q_A	First stable electron acceptor (quinone)
RC	Reaction center

ABSTRACT

This chapter provides a review of the fluorescence properties of photosynthetic bacteria. Section II is devoted to the properties of light-harvesting systems and energy transfer between the various pigment molecules that are contained in these systems. In purple bacteria the pigments are part of pigment–protein complexes that are embedded in the intracytoplasmic membrane, and at least in some species a fairly detailed and quantitative picture begins to emerge of the structure of these complexes and of the organization of energy transfer. In green bacteria the antenna system is more complicated and a quantitative picture is still lacking. In these bacteria most of the antenna is accounted for by the chlorosomes, which are located outside the membrane, and the efficiencies of the energy transfer pathways between the various pigments of the antenna system are more uncertain.

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Other characteristics of fluorescence are presented in Section III. A discussion is given of the yield of fluorescence as a probe of the efficiency of trapping of excitation energy by the reaction center. Similar but often more specific information comes from the application of phase and flash fluorometry to determine the lifetimes of the excited state of bacteriochlorophyll. Finally, measurements of fluorescence polarization of membranes and isolated pigment–protein complexes of purple bacteria are discussed. In recent years these techniques have yielded information about the orientation of the photosynthetic pigments and, together with other data, about the spatial organization of pigment–protein complexes.

I. Introduction

The photosynthetic bacteria are a group of photosynthetic prokaryotes that are characterized by the presence of bacteriochlorophyll (BChl) and by a photosynthetic mechanism that is distinguished from that of higher plants and algae by the presence of only one photosystem. They do not evolve oxygen in the light. For both traditional and practical reasons the term photosynthetic bacteria is not meant to include the cyanobacteria (blue-green algae), which do evolve oxygen, have a pigment constitution similar to that of red algae (see Fork and Mohanty, Chapter 16, this volume), and have a photosynthetic mechanism similar to that of eukaryotic algae and higher plants.

The order of the photosynthetic bacteria (Rhodospirillales) is divided into two suborders: the purple bacteria (Rhodospirillineae) and the green bacteria (Chlorobiineae), each of which is divided into two families. For the Rhodospirillineae these are the Rhodospirillaceae and the Chromatiaceae, formerly known as Athiorhodaceae and Thiorhodaceae, respectively. The Chlorobiineae are divided into the families Chlorobiaceae and Chloroflexaceae, the green and brown sulfur bacteria and the filamentous green bacteria, respectively (see review by Trüper and Pfennig, 1978 and Imhoff *et al.*, 1984 for recent modifications of the taxonomy of the photosynthetic bacteria; also see Fig. 1 in Govindjee and Satoh, Chapter 17, this volume).

With the exception of a few species of purple bacteria such as *Rhodopseudomonas (Rps.) viridis* (Eimhjellen *et al.*, 1963) and of the recently discovered species *Heliobacterium chlorum* (Gest and Favinger, 1983; Brockmann and Lipinski, 1983), which contain BChl *b* and BChl *g*, respectively, all photosynthetic bacteria contain BChl *a* (Fig. 1). In purple bacteria this pigment is located in the intracytoplasmic membrane, which forms invaginations that are continuous with the cytoplasmic membrane (see Kaplan and Arntzen, 1982). The green bacteria possess, in addition to BChl *a*, large quantities of BChl *c*, *d*, or *e*. These pigments

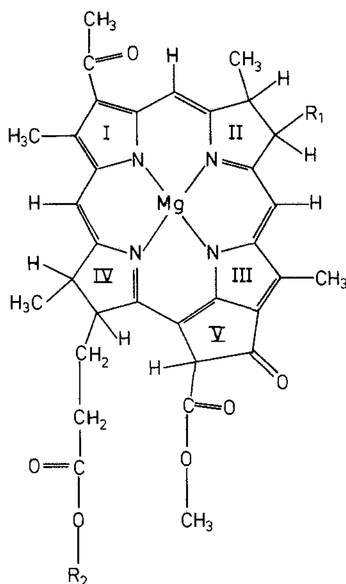

FIG. 1. Structures of bacteriochlorophylls *a* and *b*. The substituents R_1 and R_2 for BChl *a* are —H and either phytyl or geranyl–geranyl (Katz *et al.*, 1972), respectively. For BChl *b* they are =CH—CH_3 (with omission of the adjacent hydrogen on ring II) and either phytyl (in *Rhodopseudomonas viridis;* Scheer *et al.*, 1974) or 2,10-phytadienyl (in *Ectothiorhodospira halochloris;* Steiner *et al.*, 1981). (Also see Fig. 1 in Seely and Connolly, Chapter 5, this volume for a comparison with structures of Chl *a*, Chl *b*, etc.)

have an extra double bond in ring II of the tetrapyrrole system (Fig. 2; also see Fig. 1 in Seely and Connolly, Chapter 5, this volume) and are thus more closely related to chlorophyll than to BChl *a* or *b*. They are located in the chlorosomes, oblong bodies that are attached to the cytoplasmic membrane, which itself contains BChl *a* (Remsen, 1978; Amesz and Knaff, 1986).

In organic solution BChl *a* shows a strong absorption band (the Q_y band) in the near-infrared region near 770 nm (Table I), a weaker band (the Q_x band) in the visible region near 590 nm, and strong bands (B_x and B_y) in the near-ultraviolet. The Q_y and Q_x bands are the most important ones for studies of energy transfer and fluorescence. The Q_y band of BChl *b* is located near 795 nm, those of BChl *c*, *d*, and *e* near 650–660 nm. Excitation of the Q_x or *B* bands is followed by rapid radiationless conversion to the lowest singlet state (the Q_y band), from which fluorescence emission occurs.

The different pigmentation of purple and green bacteria is reflected in their absorption spectra. BChl *a*-containing purple bacteria show ab-

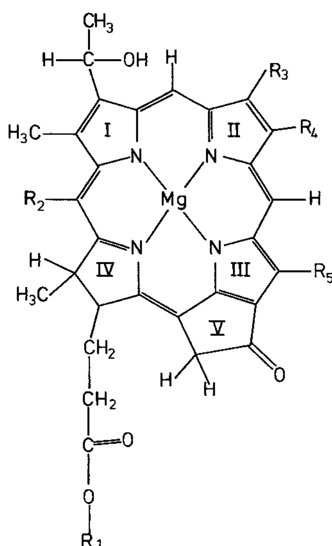

FIG. 2. Structures of bacteriochlorophylls *c*, *d*, and *e*. R_1: farnesyl, but several minor esterifying alcohols have been reported (Caple *et al.*, 1978; Smith *et al.* 1983a; Braumann *et al.*, 1986). The BChl *c* isolated from *Chloroflexus aurantiacus* is esterified mainly with stearyl (Risch *et al.*, 1979). R_2–R_5 are various substituents: each bacteriochlorophyll exists as a number of homologs (Purdie and Holt, 1965; Holt *et al.*, 1966; Gloe *et al.*, 1975; Smith *et al.*, 1980, 1982).

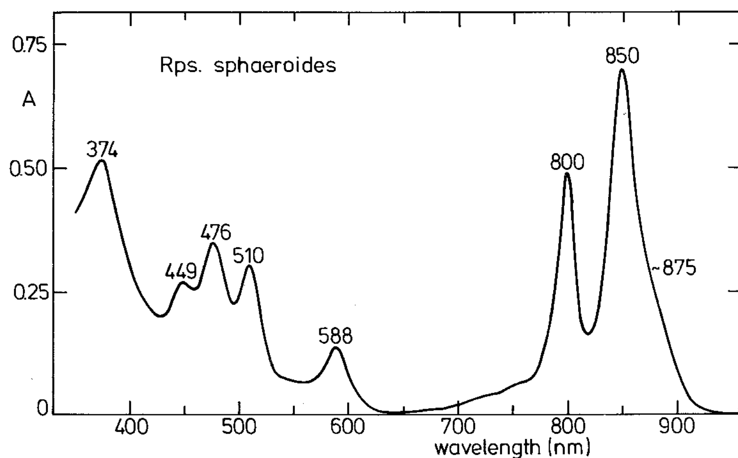

FIG. 3. Absorption spectrum of whole cells of *Rhodopseudomonas sphaeroides*. The bands at 449, 476, and 510 nm are due to spheroidene, the major carotenoid in this species; the others belong to BChl *a*. *A*, absorbance.

TABLE I
Absorption and Fluorescence Bands of Bacteriochlorophyll

Bacterio-chlorophyll	*In vitro*[a] Q_y absorption band (nm)	*In vivo* Q_y absorption band (nm): Representative species	Antenna	Primary electron donor	Fluorescence maximum[b] (antenna) (nm)
BChl *a*	770[c]	*Rps. sphaeroides*	800–880	870	895[d]
		P. aestuarii	795–835	830–840	817[e]
BChl *b*	794[f]	*Rps. viridis*	830–1020	985	1040[g]
BChl *c*	660[h]	*P. aestuarii*	745	[i]	774[e]
		Cfl. aurantiacus	740	[i]	753[j]
BChl *d*	654[h]	*Chl. vibrioforme* f.sp. *thiosulfatophilum*	727	[i]	—[k]
BChl *e*	647[h]	*Chl. phaeovibrioides*	725	[i]	—
BChl *g*	763[l,m]	*Heliobacterium chlorum*	788[n]	798[o]	813[p]

[a] Dissolved in acetone.
[b] Only the strongest fluorescence emission bands are given, as measured at room temperature.
[c] Sauer *et al.* (1966).
[d] Goedheer (1972).
[e] Swarthoff *et al.* (1982).
[f] Eimhjellen *et al.* (1963).
[g] Olson and Clayton (1966).
[h] Gloe *et al.* (1975).
[i] BChl *c, d,* and *e* do not function as the primary electron donor.
[j] H. J. M. Kramer and T. Swarthoff, unpublished observations.
[k] Not determined.
[l] Brockmann and Lipinski (1983).
[m] Dissolved in dioxane.
[n] Gest and Favinger (1983).
[o] Fuller *et al.* (1985).
[p] van Dorssen *et al.* (1985).

sorption bands in the near-infrared region at 800–890 nm due to BChl *a*, and bands in the visible region due to BChl *a* and carotenoids (Fig. 3). The near-infrared absorption bands of BChl *b* are at still longer wavelengths. The absorption spectra of green bacteria are dominated by the absorption bands of BChl *c, d,* or *e* near 750 and 460 nm (Fig. 4). Typical absorption and emission bands of the various BChls are summarized in Table I.

The long-wavelength bands of the BChls *in vivo* thus show considerable red shifts of up to 220 nm (corresponding to 2800 cm^{-1} compared

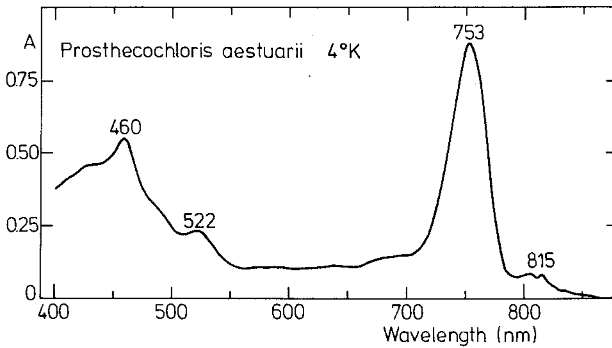

FIG. 4. Absorption spectrum of the green sulfur bacterium *Prosthecochloris aestuarii*, measured at 4°K, showing bands of BChl *c* (460 and 753 nm), BChl *a* (near 815 nm), and carotenoid (522 nm).

to the corresponding bands in organic solution (Table I). The cause of these red shifts is still a matter of dispute (Pearlstein, 1982). Experiments with model systems have shown that similar shifts can be observed on formation of dimeric or oligomeric BChl *a* (Gottstein and Scheer, 1983; Scherz and Parson, 1984) and BChl *c, d,* and *e* (Smith *et al.*, 1983b), and thus would be due to exciton interaction between the transition dipoles of neighboring BChl molecules. Calculations based on this mechanism show a reasonable fit to the observed absorption spectra of several well-defined pigment complexes (Knapp *et al.*, 1985; Vasmel, 1986). However, calculations have shown that similar red shifts can also be brought about by interaction with charged groups that are in close vicinity of the pigments, such as charged amino groups on the proteins to which the BChl molecules are bound (Eccles and Honig, 1983). The formation of charge transfer bands has also been discussed (Maslov *et al.*, 1983). Some bands, especially those at longer wavelengths, show an additional red shift on cooling (Vredenberg and Amesz, 1966; Rijgersberg *et al.*, 1980), the cause of which is not known.

II. Light-Harvesting Systems and Energy Transfer

A. Purple Bacteria

The pigments of purple photosynthetic bacteria are bound to proteins that are contained in the intracytoplasmic membrane. Two types of pigmented membrane proteins may be distinguished: the antenna (or light-harvesting) proteins and the reaction center complex.

The antenna protein complexes account for most of the pigment present in the cells; they contain BChl *a* (or BChl *b* as in *Rps. viridis*) and carotenoids and can be solubilized by detergent extraction and subsequently purified by standard biochemical methods. These pigment–protein complexes have been isolated from a variety of species, including *Rhodospirillum (R.) rubrum, Rps. sphaeroides,* Rps. capsulata,* Rps. acidophila,* and *Chromatium (C.) vinosum.* The number of different antenna complexes that are present in the membrane varies for different species. *Rhodospirillum rubrum* contains only one, B875 (Sauer and Austin, 1978; Cogdell *et al.*, 1982; Picorel *et al.*, 1983), whereas four different complexes have been isolated from *Rps. acidophila* (Cogdell *et al.*, 1983; Angerhofer *et al.*, 1986) and from *C. vinosum* (Hayashi and Morita, 1980).

The light-harvesting complexes that have been most extensively investigated are those of *Rps. sphaeroides* and *Rps. capsulata.* These species contain two different antenna complexes: the B800–850 complex that is responsible for the BChl *a* absorption bands near 800 and 850 nm of intact cells (Fig. 3) and B875 (Clayton and Clayton, 1972; Cogdell and Crofts, 1978; Feick and Drews, 1978; Sauer and Austin, 1978; Cogdell and Thornber, 1979; Broglie *et al.*, 1980). Both complexes contain carotenoid. For B800–850 a molecular ratio of BChl to carotenoid of three (Cogdell and Thornber, 1979) and more recently a ratio of two (Radcliffe *et al.*, 1984) have been reported; for B875 the ratio appears to be close to one (Broglie *et al.*, 1980). The protein moiety of the complexes consists of two different peptides, the α- and β-apoproteins. The isolated B800–850 complex consists of highly ordered aggregates of these subunits (Kramer *et al.*, 1984a); the size of these aggregates depends on the method of preparation. The primary structure of the apoproteins of a number of antenna complexes has been determined (Brunisholz *et al.*, 1981; Cogdell and Valentine, 1983; Gogel *et al.*, 1983; Tadros *et al.*, 1983, 1984; Theiler *et al.*, 1984). They all contain a homologous hydrophobic core of about 20 amino acids, which probably form an α-helical stretch that traverses the membrane. For recent reviews of the properties of light-harvesting complexes, refer to Cogdell and Thornber (1980), Cogdell and Valentine (1983), Thornber *et al.* (1983), and Amesz and Knaff (1986).

Almost all fluorescence of the isolated B800–850 complex is emitted in a band near 865 nm and comes from BChl 850 (Cogdell *et al.*, 1981). The fluorescence of BChl 800 is approximately 50 times weaker (van

* *Rhodopseudomonas sphaeroides* and *Rps. capsulata* have recently been renamed *Rhodobacter (Rh.) sphaeroides* and *Rh. capsulatus,* respectively (Imhoff *et al.*, 1984).

Grondelle *et al.,* 1982), and the action spectrum for BChl 850 fluorescence (see Fig. 5) shows about equal efficiency for light absorbed by BChl 800 and by BChl 850 (Kramer *et al.,* 1984a). These observations indicate a high efficiency of energy transfer from BChl 800 to BChl 850, with an estimated rate of energy transfer of 3×10^{11} s^{-1} at 4°K and a dipole–dipole distance between BChl 800 and BChl 850 of about 20 Å (van Grondelle *et al.,* 1982).

As noted already by Wassink and co-workers (Vermeulen *et al.,* 1937) and by Duysens (1952), intact cells and membrane vesicles (the so-called chromatophores) of purple bacteria have their main emission band in the long-wavelength region around 890–900 nm (1040 nm in *Rps. viridis*; see Olson and Clayton, 1966). Thus most of the emission comes from the long-wavelength absorbing BChl, which is contained in the B875 complex in *Rps. sphaeroides* and in corresponding antenna complexes in other bacteria. This is true at room temperature (Goedheer, 1972; Feick *et al.,* 1980; Rijgersberg *et al.,* 1980) as well as at 77°K and at 4°K (Goedheer, 1972; Mechler and Oelze, 1978; Rijgersberg *et al.,* 1980). Studies by Monger and Parson (1977) suggest that an array of B875 complexes, together with several reaction centers, form domains that are embedded in a "lake" of B800–850 (see Van Grondelle and Amesz,

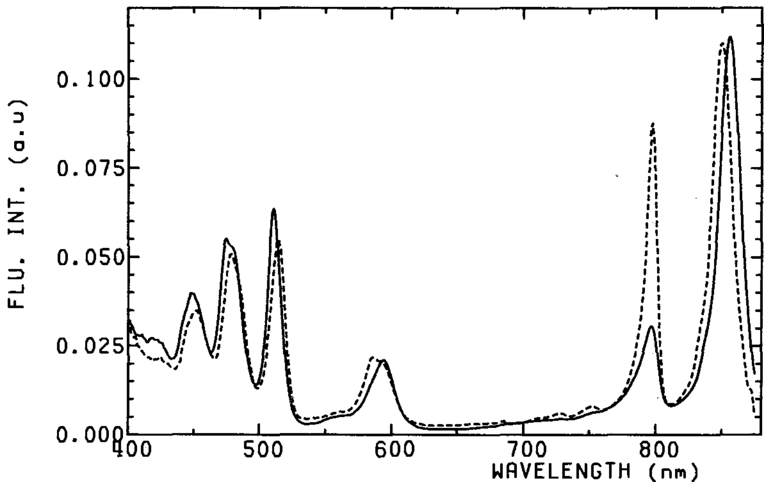

FIG. 5. Excitation spectrum for BChl *a* fluorescence of the B800–850 complex from *Rps. sphaeroides* (dashed line), measured at 4°K. The solid line shows the excitation spectrum of a preparation from which most of the BChl *a* 800 had been removed with lithium dodecyl sulfate (Kramer *et al.,* 1984a). Fluorescence intensity, arbitrary units.

Chapter 8, this volume). The emission bands of BChls absorbing at shorter wavelength are weaker (Clayton, 1966; de Klerk *et al.*, 1969; Thornber, 1970; Goedheer, 1972; Mechler and Oelze, 1978; Feick *et al.*, 1980; Rijgersberg *et al.*, 1980; Sebban and Moya, 1983), indicating that efficient energy transfer occurs from complexes such as B800–850 to the antenna complex that contains the long-wavelength absorbing BChl (see Fig. 6).

High efficiencies of energy transfer from short-wavelength to long-wavelength absorbing BChl, approaching 100%, are also indicated by the fluorescence excitation spectra (Duysens, 1952; Amesz and Vredenberg, 1966; Ebrey, 1971; Wang and Clayton, 1971; Goedheer, 1973). Efficiencies of energy transfer from carotenoid to BChl *a* vary from about 30% in *R. rubrum* and *Rps. palustris* to about 50% in *C. vinosum* (Duysens, 1952; Ebrey, 1971; Goedheer, 1973) and 80–90% in *Rps. sphaeroides* (Goedheer, 1959; Cogdell *et al.*, 1981). A similarly high efficiency was observed in the mutant GIC of *Rps. sphaeroides*, which contains neurosporene instead of spheroidene (Cogdell *et al.*, 1981). This indicates that it is the structure of the antenna complex rather than the chemical nature of the carotenoid that determines the transfer efficiency. Figure 7 shows a scheme of energy transfer pathways for *Rps. sphaeroides*.

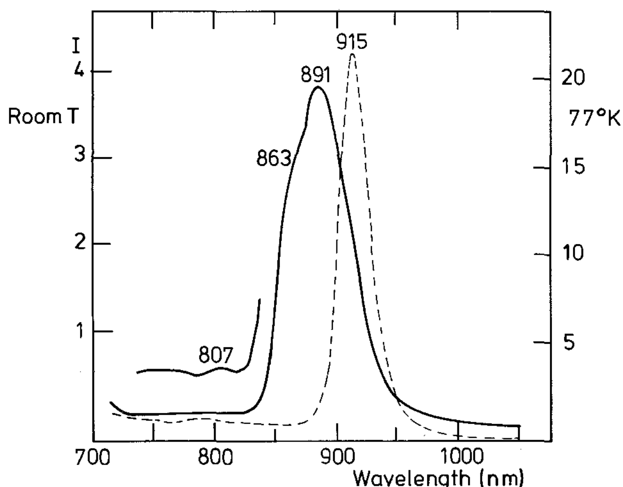

FIG. 6. Fluorescence emission spectra of whole cells of *Rps. capsulata*, measured at room temperature (solid line) and at 77°K (dashed line). The upper curve between 720 and 850 nm was measured at a higher amplification (Feick *et al.*, 1980).

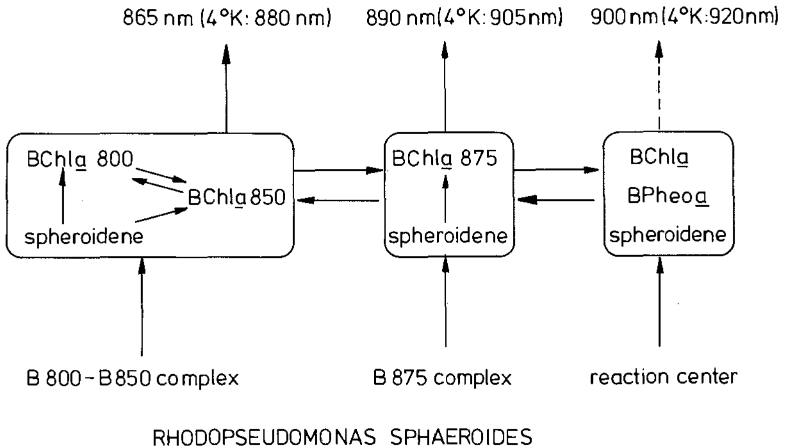

FIG. 7. **Scheme for excitation energy transfer in *Rps. sphaeroides*. Here and in Figs. 9 and 12, dashed arrows refer to emission observed in isolated complexes. For a discussion of the rates of energy transfer, see van Grondelle and Amesz (Chapter 8, this volume).**

B. Green Bacteria

1. CHLOROFLEXACEAE

Whole cells of the gliding green bacterium *Chloroflexus* (*Cfl.*) *aurantiacus* exhibit emission bands of BChl *c* and BChl *a* (Betti *et al.*, 1982). On excitation of BChl *c*, located in the chlorosome, emission bands are detected at 753, 806, and 884 nm (Fig. 8). The first two bands are also observed in isolated chlorosomes (Betti *et al.*, 1982). These chlorosomes contain a small amount of firmly bound BChl *a* absorbing at 792 nm, which has been suggested to function as a base plate protein in a manner analogous to that of the BChl *a* protein in green sulfur bacteria (Staehelin *et al.*, 1978, 1980; see Section II,B,2). The peak at 884 nm is due to BChl *a* emission from long-wavelength absorbing BChl *a* in the B808–866 light-harvesting complex (Vasmel *et al.*, 1986), which is contained in the membrane and was recently isolated by Feick and Fuller (1984). Practically no fluorescence is emitted from the BChl *a* absorbing at 808 nm, a situation similar to that encountered in the B800–850 complexes of *Rps. sphaeroides* and *Rps. capsulata* (see Section II,A). Together with the action spectrum for BChl 866 emission, this indicates that the efficiency of energy transfer from BChl 808 to BChl 866 is close to 100%. Energy transfer from carotenoid to BChl *c* has also been observed in both intact cells (H. J. M. Kramer and T. Swarthoff, unpublished) and isolated

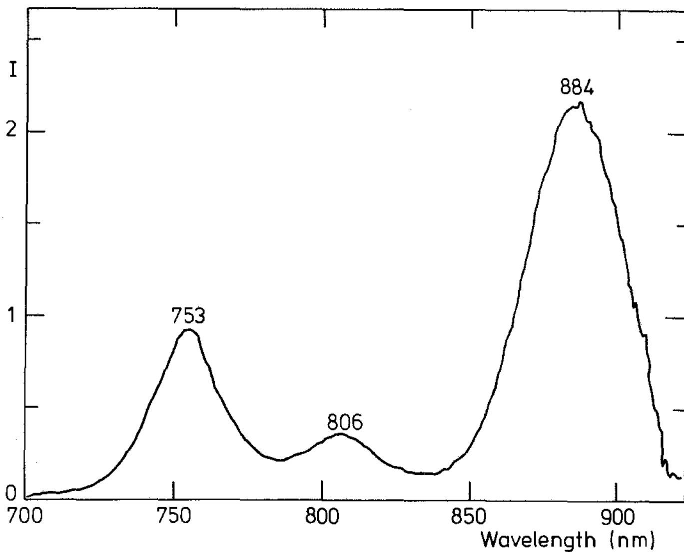

FIG. 8. Fluorescence emission spectrum of whole cells of *Cfl. aurantiacus* on excitation at 470 nm (H. J. M. Kramer and T. Swarthoff, unpublished). Maxima in the uncorrected spectrum reported by Betti *et al.* (1982) were at somewhat shorter wavelengths.

chlorosomes. In the latter case its efficiency is about 65% (van Dorssen *et al*, 1986b). Figure 9 shows a scheme for energy transfer and fluorescence in *Cfl. aurantiacus*.

2. CHLOROBIACEAE

Emission spectra of green sulfur bacteria were measured first by Krasnovskii *et al.* (1962) and subsequently by various other investigators Sybesma and Olson, 1963; Goedheer, 1972; Borisov *et al.*, 1977; Stadnichuk and Litvin, 1977; Swarthoff *et al.*, 1982). The main fluorescence band in these species comes from BChl *c*. For *Prosthecochloris (P.) aestuarii* this band is located at 774 nm at room temperature (Table I) and at 784 nm at 4°K (Swarthoff *et al.*, 1982). In addition, a weaker band is present at 814–816 nm which is due to BChl *a*. On cooling, the latter band is replaced by one at 828–830 nm (see Fig. 10). Minor emission bands at 635 and 675 nm (Krasnovskii *et al.*, 1962) seem to be largely due to "artifacts" formed during aging of the cells (Karapetyan *et al.*, 1980).

Whereas the BChl *c* emission band is due to the chlorosomes (see Section I), the BChl *a* emission bands of intact cells at 814 and 828 nm are presumably due to the so-called light-harvesting BChl *a* protein

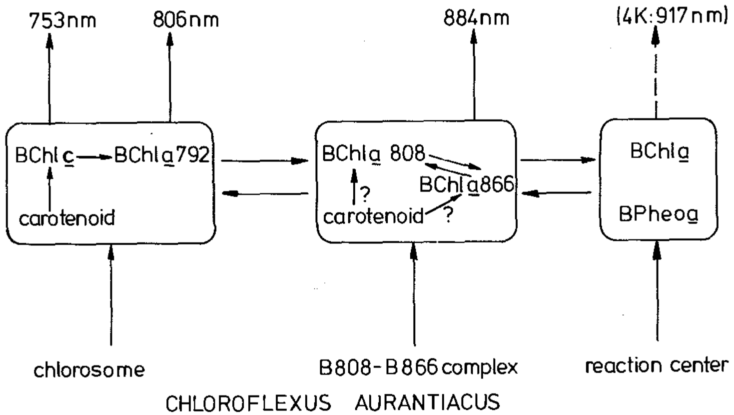

FIG. 9. Scheme for energy transfer in *Cfl. aurantiacus.* Compare with Fig. 7.

complex. X-Ray studies of the crystalline preparation have shown that it consists of three identical subunits, each containing seven BChl *a* molecules, with a total molecular weight of 140,000 (Matthews *et al.*, 1979). The complex does not contain carotenoid. The BChl *a* protein is at-

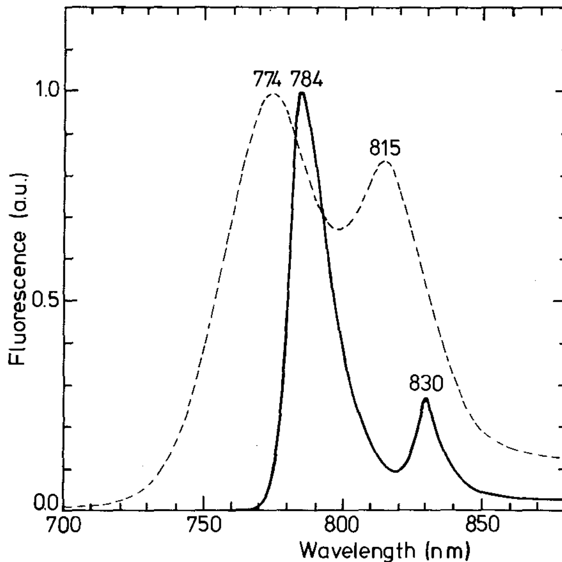

FIG. 10. Emission spectra of whole cells of *P. aestuarii,* measured at room temperature (dashed line) and at 4°K (solid line). The spectra have been normalized at their maxima (Swarthoff *et al.*, 1982).

tached to the cytoplasmic membrane, from which it can be released by the use of chaotropic agents (Olson and Thornber, 1979). Electron micrograph studies suggest that it is arranged in a regular array and forms the base plate to which the chlorosome is attached (Olson, 1980; Staehelin *et al.*, 1980). At room temperature, the main absorption band of the BChl *a* protein is at 809 nm (Olson, 1980). At 4°K the spectrum can be resolved into seven bands, ranging from 793 to 825 nm (Whitten *et al.*, 1980), which are thought to represent the exciton bands of the seven interacting BChl *a* molecules of each subunit (Pearlstein, 1982).

At room temperature the main emission band of the BChl *a* protein is at 817 nm (Sybesma and Olson, 1963; Fowler *et al.*, 1973). On cooling, this band is gradually replaced, as in intact cells, by an emission band at 828 nm, which probably originates from the long-wavelength transition at 825 nm in the absorption spectrum (Fig. 11). As shown by Rijgersberg and co-workers (Rijgersberg, 1980; Swarthoff *et al.*, 1982), the relative intensities of the emission bands at 817 and 828 nm at various temperatures indicate a thermal equilibrium between the energy levels of the two transitions (see van Grondelle and Amesz, Chapter 8, this volume). Such an equilibrium must be established within the lifetime of the excited

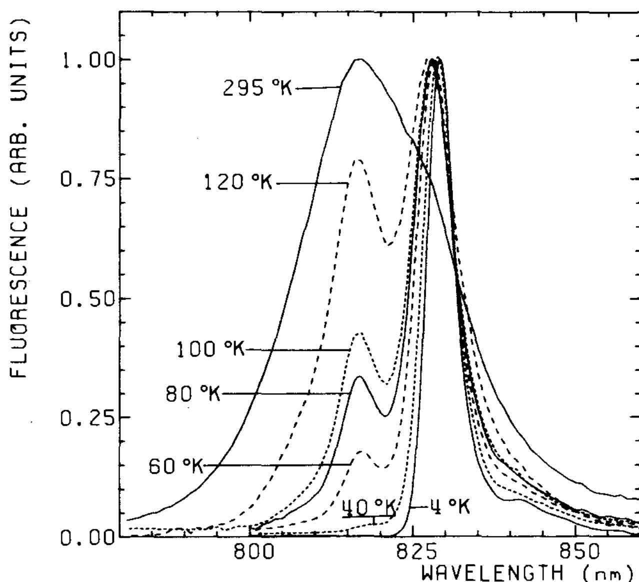

FIG. 11. Fluorescence emission spectra of the BChl *a* antenna protein complex from *P. aestuarii* at different temperatures. The spectra are normalized at their maxima (Swarthoff *et al.*, 1982).

complex, which indicates high rates of energy transfer between the two transitions.

Part of the BChl *a* contained in the membrane is bound to the so-called core complex, which is associated with the reaction center (Vasmel *et al.*, 1983b; Hurt and Hauska, 1984). Quantitative evidence concerning the efficiencies of energy transfer from the chlorosome to BChl *a* and to the reaction center is scarce, since action spectra of fluorescence of intact cells are not available. Such action spectra are more difficult to obtain than those of purple bacteria because of the very weak BChl *a* absorption bands (see Fig. 4), which are, moreover, poorly resolved at room temperature. Accurate measurements of quantum efficiencies of photochemical reactions, which would set a lower limit on the overall efficiency of energy transfer from the chlorosome to the reaction center, are not available either. By comparing the relative efficiencies of cytochrome *c* oxidation for light absorbed at 812 and 774 nm, Olson and Sybesma (1963) arrived at an estimate for the efficiency of energy transfer from BChl *c* to BChl *a* of 60–70%. Within the chlorosome, carotenoid transfers its excitation energy to BChl *c* with an efficiency of 50–60% at 4°K (H. J. M. Kramer and T. Swarthoff, unpublished). Figure 12 shows a scheme of the pathways of energy transfer in green sulfur bacteria.

Although it is usually assumed that energy transfer from the chlorosome to the core complex and to the reaction center occurs via the BChl *a* protein (Staehelin *et al.*, 1978; Olson, 1980), such transfer has not been directly demonstrated, and recent experiments with isolated pigment–

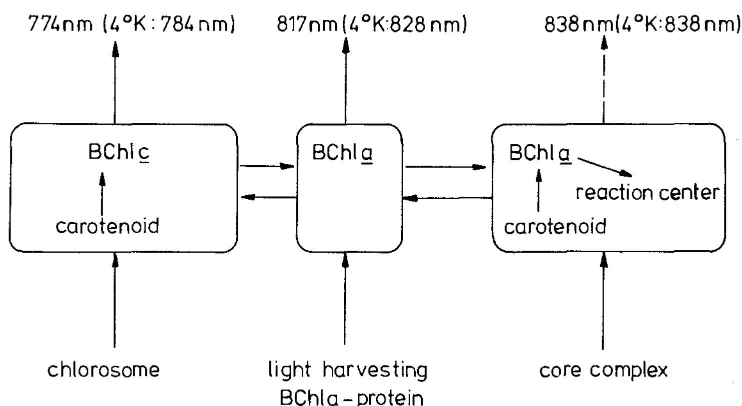

FIG. 12. Scheme for energy transfer in *P. aestuarii*. Compare with Figs. 7 and 9. Recent measurements have shown that the chlorosomes contain small amounts of BChl *a* (not shown in the scheme) (Gerola and Olson, 1986), which probably functions as an intermediary in the energy transfer to the light-harvesting BChl *a* protein (van Dorssen *et al.*, 1986a).

protein complexes indicate that the efficiency of energy transfer from the BChl *a* protein to the core complex, at least in these complexes, is low (Kramer *et al.,* 1982).

By detergent treatment of membrane fragments of *P. aestuarii,* Swarthoff and Amesz (1979) obtained a pigment–protein complex (the PP complex) with a molecular weight of about 600,000. The PP complex contained a functional reaction center and about 75 BChl *a* molecules (Swarthoff and Amesz, 1979). About 20 of these are contained in the core complex and the remaining BChls in subunits of the BChl *a* protein (Vasmel *et al.,* 1983b). In addition to BChl *a*, the complex contains some carotenoid and a BChl *c* derivative absorbing near 670 nm, which is preferentially associated with the core complex (Vasmel *et al.,* 1983b; Braumann *et al.,* 1986).

Two bands are observed in the emission spectrum of the PP complex. The first one, located at 817 nm at room temperature and at 828 nm at 4°K, could be assigned to the BChl *a* protein on the basis of its excitation spectrum (Kramer *et al.,* 1982). The second one is located at 838 nm. This band is very weak in the emission spectra of intact cells (Stadnichuk and Litvin, 1977). It appears to originate from the core complex and is at least partly due to delayed fluorescence originating from a reversal of the primary light reaction (Kramer *et al.,* 1982).

III. Physical Parameters of Fluorescence

A. Fluorescence Yield and Trapping

On arrival of the excitation energy in the reaction center, electron transfer occurs from BChl to an acceptor molecule. The primary electron donor is BChl *a* in all species of green and purple bacteria except for those that contain BChl *b*. In purple bacteria, and probably also in green bacteria, it is in a dimeric form (Okamura *et al.,* 1982; Parson and Ke, 1982; Deisenhofer *et al,* 1984; also see Norris and van Brakel, Chapter 3, this volume). It is usually called P (P870 and P985 for BChl *a* and BChl *b* containing purple bacteria, respectively, P840 for Chlorobiaceae, P865 for Chloroflexaceae). The number indicates the approximate location in nanometers of the long-wavelength bands. The primary charge separation thus may be written as:

$$P^*I \rightarrow P^+I^- \quad (1)$$

where P* denotes the excited state of P and I is the primary acceptor molecule. In purple bacteria and Chloroflexaceae the primary electron acceptor is bacteriopheophytin (BPheo) *a* or *b*, whereas in Chlorobiaceae

it is probably BChl *c* (van Bochove *et al.* 1984; Nuijs *et al.*, 1985; Braumann *et al.*, 1986). The electron is rapidly transferred (in purple bacteria in about 200 ps) to secondary electron acceptors. In purple bacteria and Chloroflexaceae the secondary acceptors are quinones (ubiquinone or menaquinone); in Chlorobiaceae the electron acceptor chain includes iron–sulfur centers (Amesz, 1984; Amesz and Knaff, 1986; Blankenship, 1985).

Experiments with purple bacteria have shown that the state of the reaction center is an important factor in determining the fluorescence yield of the antenna BChl (also see Duysens, Chapter 1, this volume):

1. In the state P I Q_A (where Q_A denotes the first quinone acceptor), excitation energy in the antenna is trapped by the reaction center, and the fluorescence yield is low (at the so-called F_0 level, varying from 1 to 4.5% for different bacterial strains; Wang and Clayton, 1971; Kingma, 1983).

2. When the reaction center is in the state P^+, e.g., due to earlier illumination, the fluorescence yield is high, since energy transfer from the antenna cannot occur (Vredenberg and Duysens, 1963). Nevertheless, there are indications that some quenching of the antenna still occurs when P870 is in the oxidized state (Heathcote and Clayton, 1977; Kingma, 1983; Sebban *et al.*, 1984; see also Section III,B).

3. If the reaction center is in the state P I Q_A^-, the energy will be trapped and the state $P^+I^-Q_A^-$ is formed. However, electron transfer to Q_A cannot take place, and a rapid back reaction occurs (half-time ~ 10 ns; see Parson and Ke, 1982), resulting in reexcitation of P. Now P* may transfer its energy back to the antenna again, resulting in a high yield of fluorescence. At about 100°K this extra fluorescence component is absent. This phenomenon is a special case of delayed fluorescence (see Jursinic, Chapter 11, this volume; van Grondelle *et al.*, 1978; van Bochove *et al.*, 1981; Borisov *et al.*, 1985); because of its short lifetime it is sometimes experimentally difficult to distinguish from "normal" ("prompt") fluorescence.

4. When the reaction center is in the state P I^-, which can in some instances be brought about by illumination under reducing conditions (Parson and Ke, 1982; Amesz and Knaff, 1985), one would expect a high fluorescence yield again, because the primary charge separation cannot occur. However, the available evidence (see Shuvalov and Klimov, 1976; van Grondelle *et al.*, 1978; van Bochove *et al.*, 1981) suggests that the fluorescence yield is in fact close to the F_0 level. The same was observed in photosystem II of plant photosynthesis (Klimov *et al.*, 1978). It has been suggested that I^- in both cases acts as a fluorescence quencher.

As first demonstrated by Clayton (1966), isolated reaction centers of *Rps. sphaeroides* are nonfluorescent or weakly fluorescent in the photochemically active state P I Q_A. An increase in long-wavelength fluorescence at about 900 nm is observed when the reaction centers are brought into the state P I Q_A^- by addition of the strong reductant sodium dithionite (Clayton, 1966; Slooten, 1972; Schenck *et al.*, 1981). This indicates that, when forward electron transport is blocked, the charge separation $P^+I^-Q_A^-$ results in the rapid back reaction to P*I Q_A^-. Then P* returns to its ground state by fluorescent decay. The emission band shifts to 920 nm on cooling to 4°K (den Blanken *et al.*, 1982). However, a remarkable difference between this emission and that from chromatophores (see item 3 above) is the observation that the reaction center fluorescence increases rather than decreases on cooling (Clayton, 1977). When measured in the state P $I^-Q_A^-$, the fluorescence yield appears to be roughly the same as in the state P I Q_A^- (Schenck *et al.*, 1981). For a detailed discussion of these and related experiments, see Parson and Ke (1982) and Woodbury and Parson (1984).

In isolated reaction centers of *Cfl. aurantiacus* in the state P I Q_A^- a long-wavelength fluorescence band is located at 917 nm at 4°K (Vasmel *et al.*, 1983a). Its excitation spectrum, shown in Fig. 13, is practically identical to the low-temperature absorption spectrum of the same preparation, which means that all reaction center pigments transfer their excitation energy with nearly 100% efficiency to the primary electron donor P865.

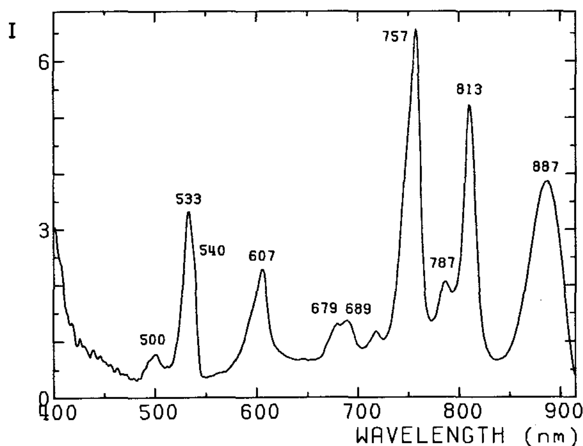

FIG. 13. Excitation spectrum at 4°K for the BChl *a* emission at 917 nm of isolated reaction centers of *Cfl. aurantiacus,* measured in the presence of dithionite (Vasmel *et al.*, 1983a).

B. Fluorescence Lifetimes

Experimentally observed fluorescence lifetimes (τ) of BChl are determined by competing deexcitation pathways for the excited singlet state and are linearly related to the fluorescence yield ϕ_f by the equation:

$$\tau = \phi_f \tau_0 \quad (2)$$

where τ_0 is the intrinsic lifetime, which would be observed if only radiative deexcitation occurred. Fluorescence lifetimes *in vivo* reflect the depopulation of the antenna excited singlet state due to the sum of all nonradiative decay processes, including trapping by reaction centers (see Norris and van Brakel, Chapter 3, this volume). Lifetimes of BChl *a* in several organic solutions have been determined by Connolly *et al.* (1982) and found to vary between 2.3 and 3.6 ns. These numbers and those to be cited below refer to the 1/*e* decay times. By combining these data with the observed fluorescence yields, corresponding intrinsic τ_0 values for BChl *a* are calculated to be 12.0–18.0 ns.

Fluorescence lifetimes can be determined by either picosecond flash spectroscopy or high-frequency phase fluorometry (see Lavorel *et al.*, Chapter 4, and Moya *et al.*, Chapter 7, this volume). A drawback of flash spectroscopy when applied to *in vivo* systems is the fact that the flash energy should be low enough to avoid nonlinear singlet–singlet annihilation effects (see van Grondelle and Amesz, Chapter 8, this volume); for this reason it is also essential to use uniform sample illumination, avoiding "hot spots" in the exciting beam. Taking these precautions, Campillo *et al.* (1977) determined the τ values for the long-wavelength BChl *a* emission by chromatophores from several strains of *Rps. sphaeroides* to be in the range 100–300 ps, with the exception of a mutant (PM-8) lacking a reaction center, which showed a 1.1-ns decay. As expected, the relative ratios of the ϕ_f values corresponded approximately to those of the τ values. The fluorescence yield in strain PM-8 is about 9–10 times greater than in wild-type bacteria (Monger and Parson, 1977), while their τ values are 1.1 ± 0.2 and 100 ± 50 ns, respectively. These differences presumably reflect the effect of trapping of excitation by open reaction centers. Nevertheless, in chromatophores of strain PM-8, the decay time is still significantly shorter than for BChl *a* in organic solution, indicating that considerable quenching of fluorescence occurs in the antenna. Freiberg *et al.* (1984) and Borisov *et al.* (1985) reported a τ of 50–60 ps for chromatophores of *R. rubrum* and *Rps. sphaeroides* when the reaction center was open and of 200 ps when the reaction center was closed (i.e., in the state P^+I) by increasing the intensity of the flash.

Phase fluorometry avoids the problems of high-intensity illumination.

However, with this method the kinetics of deexcitation are not observed, and only an "average" τ is obtained. The existence of more than one decay component for the same fluorescing species can only be established by using more than one frequency of modulation. Moreover, the modulation frequencies used so far were rather low compared to the decay times measured, so that some caution in the interpretation of the shortest τ values reported below may be justified. For recent reviews on the use of phase fluorometry one may consult Gratton *et al.* (1984) and Moya *et al.*, Chapter 7, this volume.

Phase fluorometric determination of fluorescence τ's of purple bacteria was made by Borisov and Godik (1972). For *Rps. sphaeroides* they reported a heterogeneous emission with two decay times of 7 and 800 ps. The short decay time increased to 70 ps on closing the reaction centers. Later experiments (Paschenko *et al.*, 1977; Sebban and Moya, 1983; Sebban *et al.*, 1984) show better agreement with results obtained by flash spectroscopy. Other measurements have allowed spectral resolution of the fluorescent species. The fluorescence decay of chromatophores of *Rps. sphaeroides* could be resolved into two components (Moya *et al.*, Chapter 7, this volume; Sebban and Moya, 1983). A constant emission, attributed to BChl 850, was centered at 865 nm with a decay time of 550 ps. The increase of the total fluorescence and of the average observed decay time on closing of the reaction centers (from F_0 to F_m and from T_0 to T_m, respectively; see Fig. 14) was attributed to a variable component centered at 890 nm (probably emitted by BChl 875). Its decay time would increase from 50 ps in open to 250 ps in closed reaction centers. However, this 50-ps decay is seemingly faster than the 100 ps observed by Campillo *et al.* (1977) for a similar preparation.

The fluorescence decay of chromatophores with closed reaction centers (250 ps) is still fast compared to the decay in the reaction center-less mutants C-71 and PM-8 (650 ps and 1.1 ns, respectively; Campillo *et al.*, 1977; Sebban *et al.*, 1984) and the decay of the isolated B800–850 complex (1.1 ns; Sebban *et al.*, 1984). This again indicates (see previous section) that closed reaction centers still quench the antenna fluorescence, possibly by modification of the quenching properties of a special pool of BChl in the antenna (Razjivin *et al.*, 1982). It should be noted that with open traps the fluorescence lifetimes are also short compared to the measured yields (Wang and Clayton, 1971; Kingma, 1983). This might be explained by recombination fluorescence occurring in open RCs.

The lifetime of fluorescence from isolated reaction centers of *R. rubrum* in the open condition (PI) was recently reported to be 7 ps (Freiberg *et al.*, 1985) and appears to be related to the rate of the pri-

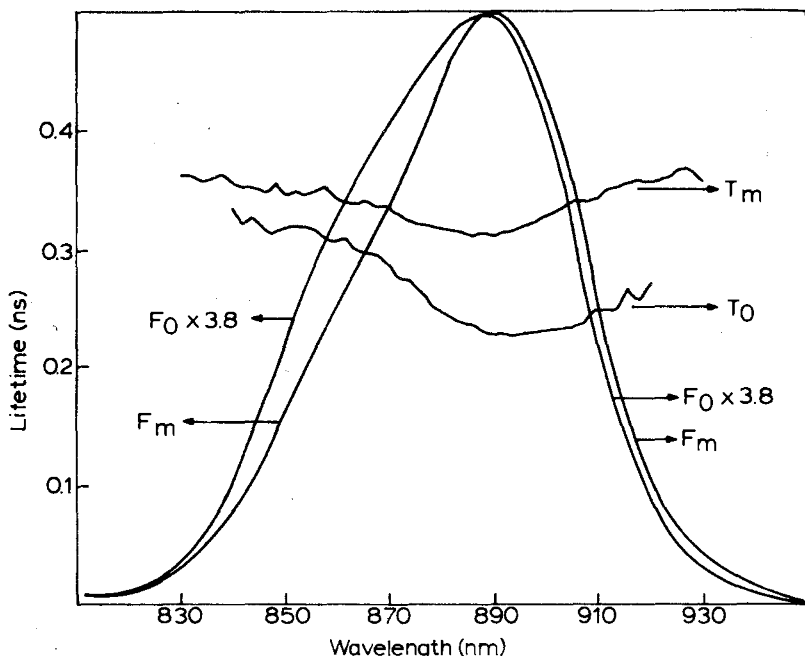

FIG. 14. **Fluorescence emission (*F*) and fluorescence lifetimes (*T*) of chromatophores of *Rps. sphaeroides* as a function of wavelength. F_0 and T_0, open reaction centers; F_m and T_m, closed reaction centers (high light intensity) (Sebban and Moya, 1983).**

mary charge separation. Additional long-lived components are observed when electron transport to Q_A is blocked (Woodbury and Parson, 1984) which are attributed to delayed fluorescence [see (3) above].

Available data on the fluorescence τ's of green bacteria are limited. Applying phase fluorometry to a membrane preparation of *Chlorobium limicola* that still contained fragments of attached chlorosomes, Fetisova and Borisov (1980) deduced that about 90% of the BChl *c* emission (which originates in the chlorosome fragments) decayed with a half-time of 20–50 ps. The remaining fraction had a much longer decay time of 2.7–3.0 ns. If one assumes that the latter fraction came from chlorosome fragments that had been detached from the membrane, comparison of the two τ's would suggest that most of the BChl *c* transferred its energy with an efficiency of nearly 99% to the BChl *a* in the membrane. The τ's of BChl *a* fluorescence itself would amount to 20–60 ps when all traps are open and increase to 200–600 ps after oxidation of the reaction centers (Borisov *et al.*, 1977). Again, a long-lived (2.7-ns) background emission was present, which was responsible for 90% of the fluorescence intensity (open traps) and was attributed to "inactive" BChl *a*. Earlier

measurements with broadband detection on intact cells (Govindjee *et al.*, 1972) gave an average τ of 600 ps, which probably mainly reflects the lifetime of BChl *c*.

Lifetimes of excited states of BChl *a* after a 30-ps laser pulse were determined by Nuijs *et al.* (1985) by absorption difference spectroscopy. In these experiments the absorption in the Q_y band was used to monitor the popualtion and depopulation of the ground state. A lifetime of 280 ps was measured for the isolated BChl *a* protein (see Section II,B,2) of *P. aestuarii*. The same component was present in a membrane preparation with closed RCs, together with a larger 70-ps decay that was attributed to singlet excited BChl *a* in the core complex (see Section II,B,2). The extent of singlet–singlet annihilation appeared to be negligible in these preparations, even with strong exciting pulses. No evidence was found for a significant amount of long-lived excited singlet BChl *a* as reported by Borisov *et al.* (1977).

C. Fluorescence Polarization

Especially during recent years, measurements of polarized fluorescence have proved to be a valuable tool for obtaining information about the structure of the photosynthetic apparatus (Breton and Vermeglio, 1982). Although in principle a large variety of conditions and preparations can be used in studies of fluorescence polarization, it is useful here to distinguish two possible experimental approaches: (a) polarized excitation of (isotropic) samples in which the pigments are randomly distributed, and (b) excitation of oriented (anisotropic) samples.

The second case need be only briefly considered here. It can be used to study the orientation of pigments with respect to the photosynthetic membrane. When the polarization of the emission is measured, the results give information about the orientation of the emission dipoles; with polarized excitation the method yields data about the orientation of the absorption dipoles, which are also obtained by measurements of linear dichroism. These methods have been successfully applied to chloroplasts (see, e.g., Kramer and Amesz, 1982) but as yet they have not been applied to bacterial membranes.

For a random isotropic ensemble of pigment molecules the polarization is given by (Jablonski, 1935):

$$p = (3 \cos^2 \alpha - 1)/(\cos^2 \alpha + 3) \tag{3}$$

where α is the angle between the transition moments involved in absorption and emission, respectively, and p is defined by

$$p = (I_{/\!/} - I_{\perp})/(I_{/\!/} + I_{\perp}) \tag{4}$$

where $I_{//}$ and $I_{\perp}$ are the intensities of the fluorescence polarized parallel and perpendicular to the polarization vector of the excitation light, respectively.

Equation (3) applies only when no reorientation of the molecules occurs during the lifetimes of their excited states. The maximum polarization (p = 0.50) is obtained when the absorption and emission dipoles are parallel, the minimum value (p = −0.33) when they are perpendicular to each other. For BChl *a* this means that the first value should be expected on excitation in the Q_y band, where the emission also occurs, whereas a value of −0.33 should be observed in the Q_x band near 600 nm, if the Q_y and Q_x transitions are perpendicular to each other, as theoretically predicted (Gouterman, 1961). Values approaching these theoretical limits (0.42 and −0.23) have been observed in dilute BChl *a* solutions (Ebrey and Clayton, 1969; Bolt and Sauer, 1981; Breton *et al.*, 1981).

Depolarization of fluorescence may occur by two different mechanisms: (1) rotation during the lifetime of the excited state and (2) energy transfer (Knox, 1968). With BChl *a* dissolved in viscous solvents such as castor oil or cyclohexanol, or bound to membrane proteins, the first effect is very small. The second mechanism, however, is quite important in photosynthetic systems and may be used to advantage to obtain structural information.

Fluorescence polarization spectra of chromatophores and intact cells of various species of purple bacteria showed a fairly strong depolarization of fluorescence at most wavelengths (Ebrey and Clayton, 1969; Goedheer, 1973, 1984; Kramer *et al.*, 1984b). Often, the depolarization was found to be less in the Q_x band than in most of the Q_y band region; in chromatophores of *Rps. sphaeroides* p values of −0.16 (Q_x) and +0.08 (Q_y) at room temperature (Ebrey and Clayton, 1969) and of −0.19 and +0.11 (Kramer *et al.*, 1984b) were measured, and similar results were obtained with *R. rubrum* (Goedheer, 1984; Kramer *et al.*, 1984b). Except at the long-wavelength side of the B875 absorption band at low temperature (Kramer *et al.*, 1984b), the polarization was found to be approximately the same on excitation in the different BChl Q_y bands of *Rps. spaeroides.* This indicates that the transition dipoles of the corresponding BChls have similar orientations, so that energy transfer does not cause a depolarization of fluorescence.

More detailed information has been obtained from studies on isolated pigment–protein complexes from wild and mutant strains of *Rps. sphaeroides* and from *R. rubrum* (Bolt and Sauer, 1981; Breton *et al.*, 1981; Kramer *et al.*, 1984a,b). These results indicated that the BChl *a* Q_y transitions in these complexes are circularly degenerate (i.e., oriented in a

plane without a preferential orientation within the plane). This would explain the relatively low polarization in the Q_y region of the isolated complexes and chromatophores; a significant contribution of structural anisotropy (see Goedheer, 1984, for a discussion) seems less likely, especially for the isolated complexes. For BChl 875 and BChl 850 the Q_x transition dipoles are approximately perpendicular to the plane of the Q_y transitions, but for BChl 800 a different situation applies. For this pigment a *positive* polarization in the Q_x band of approximately +0.07 was observed, indicating that the Q_x transition dipoles lie in approximately the same plane as that formed by the Q_y transitions, all transitions being circularly degenerate. On the basis of these and other results, a working model has been proposed for the structure of B800–850 (Kramer *et al.*, 1984a). This model is discussed in more detail by van Grondelle and Amesz (Chapter 8, this volume).

IV. Conclusions

Studies on photosynthetic bacteria have proved to be essential for an understanding of the mechanism of photosynthesis in general. This certainly applies to studies of the mechanism of the primary and associated electron transfer reactions. The present chapter may serve to illustrate that the same may be said of fluorescence studies.

For purple bacteria a fairly detailed picture is now emerging of the structure of the light-harvesting systems and of the rates and efficiencies of energy transfer between the pigments that constitute these systems. However, there are still many gaps that need to be filled and many aspects that are only poorly understood. One of these concerns the fluorescence properties of isolated reaction centers. In addition, there are many unexplained discrepancies in the reported lifetimes and yields of fluorescence. A large area that has been only superficially explored is that of the fluorescence properties of green bacteria. Nevertheless, advances have been made in all these fields during the past few years, and, with the modern techniques that are available now, there is good reason to believe that many points that are still unclear will be resolved in the near future.

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16

Fluorescence and Other Characteristics of Blue-Green Algae (Cyanobacteria), Red Algae, and Cryptomonads

DAVID CHARLES FORK
Department of Plant Biology
Carnegie Institution of Washington
Stanford, California 94305

PRASANNA MOHANTY
School of Life Sciences
Jawaharlal Nehru University
New Delhi 110067, India

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Light Emission by Plants and Bacteria

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ABBREVIATIONS AND SYMBOLS

A.	*Anacystis*
An.	*Anabaena*
APC	Allophycocyanin
APC-B	Allophycocyanin B, far-red absorbing form of allophycocyanin
B-PE	Bangiophycean phycoerythrin
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
Car	Carotenoid
Chl	Chlorophyll
C-PC	Blue-green algal (or cyanophycean) phycocyanin
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DBMIB	2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DLE	Delayed light emission (delayed fluorescence)
F_λ	Fluorescence emission band at wavelength λ in nanometers (nm)
F_0	Initial fluorescence level seen immediately on illumination
F_M	Maximum fluorescence level attained on illumination
kD	Kilodalton
LHCP	Light-harvesting chlorophyll protein
LiDS–PAGE	Lithium dodecyl sulfate–polyacrylamide gel electrophoresis
M_r	Molecular weight
P.	*Porphyridium*
P700	Reaction center Chl of photosystem I
PBsome	Phycobilisome
PC	Phycocyanin
PCB	Phycocyanobilin
PE	Phycoerythrin
PEC	Phycoerythrocyanin
Porph.	*Porphyra*
PQ	Plastoquinone
PS	Photosystem
PUB	Phycourobilin
R-PC	Rhodophytan phycocyanin
R-PE	Rhodophytan phycoerythrin
SDS	Sodium dodecyl sulfate
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
S.	*Synechococcus*

ABSTRACT

This chapter presents the ways in which blue-green and red algae, as well as cryptomonads and *Prochloron,* have contributed to our understanding of photosynthesis. It is a common characteristic of both the blue-green and red algae that their accessory phycobiliproteins are organized into structures, termed phycobilisomes, that are located on the exterior surface of the thylakoid membrane and serve as light-harvesting units. The four classes of biliproteins (phycocyanin, allophycocyanin, phycoerythrocyanin, and phycoerythrin) that are contained within the phycobilisome are discussed with regard to their distribution, spectroscopic features, and amino acid composition.

Fluorescence emission and excitation spectra at both physiological and low temperatures are described, and the ways in which these studies have been used to elucidate pathways of energy transfer and mechanisms of energy regulation in photosynthesis are mentioned. The use of picosecond fluorescence spectroscopy to study the sequence of energy transfer in the various photosynthetically active pigments is briefly reviewed.
The use of the time course of fluorescence as a diagnostic feature in physiological studies and of delayed fluorescence is briefly discussed. Mention is made of short-term adaptation to changes in light intensity and quality by means of the so-called state I–state II transitions, whereby more balanced excitation of both photosystems of photosynthesis can be achieved. Finally, several specialized features that have allowed the blue-green and red algae to adapt to extremely varied habitats are considered.

I. Introduction

The study of light emission by plants and bacteria continues to provide insights into the basic photoprocesses of photosynthesis as well as into the structural and functional regulation of the photosynthetic apparatus. In this chapter we shall briefly review the use of light emitted by blue-green, red, and cryptomonad algae to gain an understanding not only of the photophysical and photochemical processes of photosynthesis, but also of the variety of cellular, physiological, and adaptative processes of these organisms.

Blue-green algae (cyanobacteria), like the photosynthetic bacteria, are prokaryotes that lack a nucleus and chloroplast and have their photosynthetic membranes located in the cytoplasm. But, like most eukaryotic plants including red algae and cryptomonads, they carry out oxygenic photosynthesis involving two separate photochemical reactions. There are, however, some structural and functional differences between these prokaryotic and the eukaryotic photosynthesizers, which will be described later. The major resemblance between blue-green algae, red algae, and cryptomonads is their possession of photostable phycobilin chromophores which function as accessory light absorbers to harvest solar energy for photosynthesis. These "accessory" pigments extend the photosynthetically active radiation by absorbing the green and yellow wavelengths that are poorly absorbed by chlorophyll (Chl) *a* and transferring them efficiently to Chl *a*, which is the only pigment required for photosynthesis. These accessory pigments give these algae their characteristic colors and aid in their survival in a wide variety of habitats. Both the blue-green and red algae share the common feature of having their accessory colored proteins organized into a unique structure termed the phycobilisome (PBsome).

II. Occurrence and Habitat

A. Blue-Green Algae

The prokaryotic blue-greens can be considered as both algae, since they have an oxygenic type of photosynthesis, and bacteria, since their cellular organization is bacterial in lacking nuclei, mitochondria, a Golgi apparatus, an endoplasmic reticulum, or chloroplasts. The photosynthetic membranes of the cyanophytes are arranged in layers inside the cell and contain all the phycobilins, Chl, and carotenoids (Car) needed for photosynthesis.

The fossil record shows that blue-green algae have existed for about 3 billion years (see, e.g., Hall, 1976). As a result, these organisms are adapted to a wide variety of habitats, from Antarctic lakes to hot springs, salt marshes, and the marine intertidal zone as well as freshwater and terrestrial habitats. Blue-greens constitute the symbiont algae in many lichens. They are also associated with the tissues of plants such as *Azolla*, cycads, and *Anthoceros* (Fritch, 1945). Blue-green algae are ideal experimental material since their filaments or single cells can be readily cultured and used intact or as preparations of photosynthetic membranes, obtained by mechanical or enzymatic techniques.

B. Red Algae

A difficulty encountered in studies of the red algae is the rather limited number of species that are readily available. The majority of red algae live in the sea, but a few genera, especially in the order Bangiales, are freshwater or terrestrial in habitat. Included among these is the pink unicellular *Porphyridium* (*P.*) *cruentum,* which contains a preponderance of phycoerythrin (PE) and grows on soil and damp walls, and the blue-green *P. aerugineum,* which contains phycocyanin (PC) and inhabits fresh water or soil (Fritch, 1945). The great advantage of these species for biophysical research is that they can be readily cultured and are unicellular. The marine red alga *Porphyra* (*Porph.*), a member of the Bangiales although not unicellular, is also an ideal research material since the thallus in some species is composed of only one layer of cells. Since it is physically very resilient, it can be cut to any desired shape for experimental purposes and, if it can be obtained locally, can be maintained in the laboratory for a week or so in seawater. Its long-term culture unfortunately, is difficult to manage (Dawes, 1981).

Most of the red algae belong to the group Florideae, the large majority of these being marine. The thalli of most of these multicellular algae are

usually too thick to be useful for spectroscopic measurements. A few of these genera such as *Delesseria, Halymenia, Phycodrys, Schizymenia,* and *Iridaea* are thin enough to be used for spectroscopic measurements (Fork and Amesz, 1967; Ried and Reinhardt, 1977). Although not suitable for *in vivo* spectroscopic measurements, the filamentous red alga *Griffithsia* can be cultured in the laboratory and used for studies of phycobiliproteins and photosynthetic processes.

C. *Cryptomonads*

The biflagellate cryptomonads comprise a relatively little-known group that contains both pigmented and nonpigmented forms. The few species of cryptomonads that are known are about equally divided between marine and freshwater habitats. No filamentous forms are known.

Like the blue-green and red algae, the cryptomonads contain phycobiliproteins as their major photosynthetically active pigments (Allen *et al.,* 1959; Haxo and Fork, 1959; Ó hEocha and Raftery, 1959; Haxo, 1960). Unlike the blue-green and red algae, the cryptomonads contain Chl *c* in addition to Chl *a* (Haxo and Fork, 1959; Jeffrey, 1976).

D. *Prochloron*

An unusual alga named *Prochloron* was discovered (Lewin, 1977, 1981) inhabiting the tissues of protochordate marine invertebrates in tropical waters (Lewin, 1984; McCourt *et al.,* 1984). Fluorescence emission and excitation spectra of pigments extracted from *Prochloron* show only the presence of Chl *a* and *b*, with the Chl *a*/*b* ratio varying from 2.6 to 12.0. No phycobilins could be detected by fluorescence analysis. This alga appears to be a blue-green on the basis of its cell structure (Whatley, 1977; Moriarty, 1979), metabolism (Fisher and Trench, 1980). partial RNA base sequences (Seewaldt and Stackenbrandt, 1982), and fatty acid and lipid content (Perry *et al.,* 1978; Johns *et al.,* 1981; Murata and Sato, 1983).

The light-harvesting Chl *a*/*b* protein of *Prochloron* differs from the light-harvesting chlorophyll protein (LHCP) of green algae and higher plants by its higher Chl *a*/*b* ratio, its reduced electrophoretic mobility, and the higher molecular weight (M_r) of its main polypeptide. The P700 Chl *a* protein of *Prochloron* is like that of blue-green algae (Takahashi *et al.,* 1982) in having very high M_r proteins obtained with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Larkum and Hiller, 1984).

Prochloron is thus unique in having pigmentation of eukaryotic algae

and higher plants and yet possessing prokaryotic structures typical of the cyanobacteria. If it can be cultured, it will be useful for comparative studies of structure, function, and evolutionary relationships between the blue-green cyanobacteria and other algae.

III. Accessory Pigments: The Biliproteins

There are four classes of biliproteins—PC, allophycocyanin (APC), phycoerythrocyanin (PEC), and phycoerythrin (PE)—that constitute the accessory pigments for blue-green and red algae. These water-soluble, intensely colored, fluorescent biliproteins possess linear tetrapyrrole chromophores that absorb and transfer solar energy to photosynthetic reaction centers.

In some algae the relationship between the amounts of the different phycobiliproteins or the ratio of these pigments to Chl is not constant but varies depending on the color of the light under which the algae have been grown. This adaptation of the pigment ratios can be complementary; i.e., cells grown under blue-green light form more pigment absorbing blue-green light (PE) and appear more red, while algae grown in red light make more red-absorbing pigments (PC) and appear more blue in color (Bogorad, 1975). The obvious ecological significance of chromatic adaptation seems to be that maximum absorption of the available photosynthetically active light can be achieved.

The blue-green algal (or cyanophycean) phycocyanin (C-PC) and APC contain only the blue pigment phycocyanobilin (PCB), while red algal rhodophytan phycocyanin (R-PC) contains both blue PCB and the red phycoerythrobilin pigment. Similarly, PEC possesses both types of tetrapyrrole chromophores (see Gysi and Chapman, 1982).

A third type of bilin chromophore, phycourobilin (PUB), absorbing strongly between 495 and 500 nm and covalently linked to the protein by two thioether linkages, has been proposed to be present in some blue-green algae (Rippka *et al.*, 1974; Bryant *et al.*, 1981; Kursar *et al.*, 1981; also see the review by Glazer, 1981). However, the presence of this chromophore has not been unambiguously proved (Gysi and Chapman, 1982).

The red algae contain three types of PE: R-PE (rhodophytan), B-PE and bPE (both bangiophycean), and R-PC (rhodophytan phycocyanin). APC has been shown to occur in two spectroscopically distinct forms as short-wavelength or long-wavelength fluorescence emitters. The short-wavelength emitting forms of APC (II and III) are found in blue-green and red algae but not in cryptomonads.

The cryptomond biliproteins PE and PC possess α and β subunits (see Gantt, 1979). No APC has been found in the cryptomonads, and it has been proposed that Chl c_2 may be the terminal link in energy transfer between intrathylakoid biliproteins and Chl *a*, but McColl and Berns (1978) showed this to be unlikely since they found only a small Förster spectral overlap integral (Förster, 1948) between PC and Chl c_2 and a large overlap integral between PC and Chl *a* (for a review, see Glazer, 1981). (For a background on energy transfer, see van Grondelle and Amesz, Chapter 8, this volume.) This result suggests that PC transfers energy directly to Chl *a* without going via Chl c_2. Further work is needed to clarify the role of Chl c_2 in the cryptomonads. Analysis of the LHCP of these algae will be useful in determining the role of Chl *c* in cryptomonads.

The amino acid composition of biliproteins is known, and these proteins are rich in acidic as well as aliphatic residues (see Gysi and Chapman, 1982). The complete primary structure of the C-PC and APC of the thermophilic blue-green alga *Mastigocladus laminosus* has been reported (Frank *et al.*, 1978; Sidler *et al.*, 1981). The α and β subunits are homologous, but the α polypeptide is a few amino acid residues shorter than the β.

The absorption characteristics of biliproteins in solution depend on pH, ionic strength, and temperature, and also on the protein concentration. The spectroscopic properties of various biliproteins are summarized by Gysi and Chapman (1982). The biliproteins absorb in the region where there is a gap in the absorption by Chl, in the 400–670-nm range of the solar spectrum: APC at 650–671 nm, PC at 617–620 nm, and PE and PEC at 545–568 nm. PUB shows additional absorption in the 495–498-nm region (McColl, 1982). Glazer and Fang (1973) showed that biliproteins are most stable in acid urea solution, and under this condition the absorption of the bilin chromophore is independent of the nature of the polypeptide chain. The absorption spectrum of the separate α and β subunits can be summed to produce the biliprotein spectrum. Dissociation and reassociation studies with *Synechococcus* 6301 C-PC revealed that the α subunit has one and the β subunit two chromophores (Glazer and Fang, 1973; Glazer *et al.*, 1973). The distribution of chromophores in the subunits of other biliproteins has been obtained from similar studies (see Gantt, 1981; Glazer, 1981).

Biliproteins associate into aggregated forms. Numerous studies on the spectroscopic and thermodynamic parameters of aggregated and disaggregated phycobiliproteins in solution and the effects of solvent, pH, and temperature on pigment–protein and pigment–pigment interactions are available; the reader is referred to a review by Scheer (1981).

IV. The Phycobilisomes

The heteroaggregation of biliproteins into organized supramolecular structures in blue-green and red algae has been termed the phycobilisome (PBsome). Like the chlorosomes of green photosynthetic bacteria, the PBsome constitutes the hydrophilic peripheral light-harvesting pigment–protein complex situated on the stroma side of thylakoid membranes. The cryptomonad biliproteins, as noted earlier, are situated within the intrathylakoid space and do not have PBsome-like structured antennas. The PBsome structures, assemblies, and dynamics have been reviewed by Gantt (1975, 1981), Glazer (1980, 1982, 1984), McColl (1982), and Wehrmeyer (1983). The variations in sizes and shapes of a large number of PBsomes of blue-green algae, red algae, and cyanelles (see Section XII,A) have been compared and reviewed by Gantt (1980). These vary in their M_r from 7 to 15 × 10^6 and contain 300 to 800 tetrapyrrole (bilin) chromophores.

The PBsomes of cyanophytes and rhodophytes provide excellent examples of the organization of a biological structure for efficient function. Gantt's model (1980, 1981) of the PBsome predicted a structural correlation for the flow of absorbed photons from the peripheral region of the PBsome to the interior of the membrane-bound Chl at the reaction center with a sequential transfer of excitation energy in the thermodynamically favorable direction from high to low energy levels. According to this model, the APC core is attached to the thylakoid membrane, and from the APC core rodlike structures project out that contain PC and PE (Fig. 1). When present, PE is located on the peripheral end of the rod structure, which is made up of stacked disks. Glazer *et al.* (1985) found that disk-to-disk transfer was the rate-limiting step for energy flow in the phycobilisome of *Synechocystis* (6701). An upper limit of 8 ps (which was instrument-limited) was found for the transfer time between disks.

Several colorless nonpigmented polypeptides are thought to be associated with the phycobilisome rod structures (Tandeau de Marsac and Cohen-Bazire, 1977; Gantt, 1981). These are called linker polypeptides. The linker elements in rod structures are polypeptides having low M_r's of 30,000 to 70,000. The colorless linker polypeptides associated with the attachment of the disks of the APC core are of higher M_r (70,000). Besides these linker polypeptides, the APC core is suggested to have colored polypeptides that anchor the core to the thylakoid membranes. An unsettled question exists concerning the nature of the terminal emitter of excitation energy to Chl *a* of PSII (and PSI). Initially, APC I

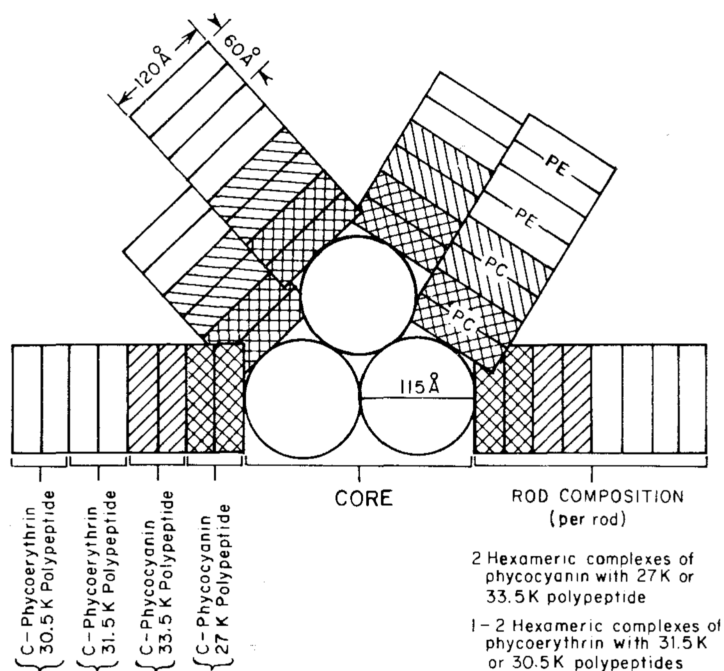


FIG. 1. **Schematic representation of the phycobilisome of *Synechocystis* (6701). The tricylindrical core contains APC and APC-B and associated polypeptides. Two cylinders of the core are proximal to the energy acceptor Chl species in the thylakoid membrane. (From Gingrich *et al.*, 1983; see text for details.)**

Zilinskas *et al.*, 1978) and APC-B (Lundell and Glazer, 1981) were considered to be the final emitters of excitation energy from the PBsome to Chl *a*. With the use of specific protease inhibitors, Rusckowski and Zilinskas (1982) identified a 95-kD colored polypeptide associated with APC I which confers 680-nm fluorescence. These authors also showed evidence that the 95-kD polypeptide remains attached to the thylakoid membrane when the biliproteins are removed by exhaustive washing. Similarly, a 95-kD polypeptide which is colored and fluoresces at 680 nm is viewed as the anchor protein of the red alga *P. cruentum* (Redlinger and Gantt, 1981a,b). Lundell *et al.* (1981) characterized a 75-kD colored polypeptide as the terminal piece in the energy transfer chain in the PBsome of the blue-green alga *Anacystis* (*A.*) *nidulans* (*S. leopoliensis*). MacColl (1982) suggested that the presence or absence of PE may dictate whether a 95-kD or a 75-kD colored anchor polypeptide would be associated with APC to serve as the terminal energy transmitter to Chl *a*.

V. Chlorophyll–Protein Complexes

Thylakoid membranes of blue-green algae are similar to those of higher plants except that they do not contain Chl *b*, do not form grana stacks, and have high contents of saturated or monounsaturated fatty acids in many of their thylakoid lipids.

The use of mild detergent for solubilizing the membranes for the isolation of constituent polypeptides continues to be the method of choice and yields valuable data. Very small PSI particles, similar to those of higher plants, were isolated from *Synechococcus* (*S.*) *cedrorum* (Newman and Sherman, 1978). Stewart and Bendall (1979, 1980, 1981) prepared purified PSII particles with O_2-evolving capacity from the thermophilic alga *Phormidium laminosum,* and England and Evans (1981) prepared O_2-evolving PSII preparations from other thermophilic algae that contained cytochrome (Cyt) *b*-559 as well as phycobilins.

Rusckowski and Zilinskas (1980) resolved four complexes from *Nostoc* sp., two of which had a molecular mass around 110 kD and were composed of three polypeptides. These two Chl proteins showed emission characteristics of PSI with a fluorescence emission at 735 nm at 77°K. The two other complexes, which had 48- and 43-kD polypeptides, fluoresced at 685 nm at 77°K and were assigned to PSII. Nakayama *et al.* (1979) isolated purified PSI and PSII particles and antenna particles from the thermophilic alga *Synechococcus* sp. with digitonin. Katoh and associates (Takahashi and Katoh, 1982; Takahashi *et al.*, 1982; Yamagishi and Katoh, 1983) made further detailed analyses of Chl proteins isolated from PSII and PSI particles from *Synechococcus* sp. Yamagishi and Katoh (1983) showed that a minimum of two Chl proteins were associated with PSII. One was associated with a 40-kD protein, had a fluorescence emission peak at 686 nm at 77°K, and functioned as the antenna, while the other, associated with a 47-kD polypeptide, had a peak at 694 nm and constituted the primary PSII photochemically active Chl. The latter complex is associated with other polypeptides which modify its absorption characteristics.

The PSI of this thermophilic alga was shown to be more heterogenous. Katoh and co-workers isolated five CPI-type (PSI-associated) Chl protein complexes, with Chl/P700 ratios of ~70, that showed emission at 721 nm at 77°K (Takahashi *et al.*, 1982). Öquist *et al.* (1981) showed the importance of the phase transition temperature in the solubilization of Chl proteins. At temperatures below the phase transition of the thylakoid membrane lipids of this thermophilic alga (which is determined by the growth temperature), PSI and its antenna Chl complexes could not be disassociated and solubilized by sodium dodecyl sulfate.

The organization of Chl protein complexes in the thylakoid membranes of *A. nidulans* has been detailed by Guikema and Sherman (1981, 1982, 1983), who identified six different complexes. Hoshina and Fork (1983) obtained aggregates similar in description to those of Guikema and Sherman (1983).

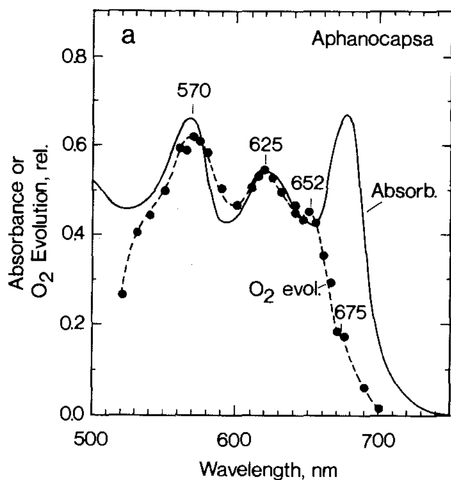
Although a consistent and general picture of the architecture and topography of the Chl proteins of cyanobacterial thylakoids seems to be emerging from studies of the various Chl proteins, it is, nevertheless, still difficult to assign the different forms of Chl *a* or the emission bands seen at 77°K to a particular protein.

Membrane fragments exhibiting PSI and PSII functions have been reported from red algae (Katoh and Gantt, 1979; Stewart and Larkum, 1983). PSII particles containing functionally active PBsomes and having very high O_2 evolution activity have been isolated from *P. cruentum* (Clement-Metral and Gantt, 1983) and are sensitive to DCMU* and hydroxylamine. The absorption spectrum of these PSII PBsome particles minus the PBsome spectrum showed absorption maxima at 675 and 440 nm (Soret band). Excitation of PE (λ = 545 nm) in the PSII PBsome particles produced a fluorescence peak at 688 nm at room temperature. The 696-nm band, which is seen in the unfractionated thylakoid PBsome (Dilworth and Gantt, 1981), is missing in the PSII PBsome preparation.

Redlinger and Gantt (1983) characterized Chl protein complexes separated by SDS–PAGE from *P. cruentum*. The 68-kD PSI complex showed a light-induced P700 change and an emission peak at 77°K at 720 nm with a minor band at 690 nm. Two other bands, one containing a single 52-kD apoprotein and another composed of 52-, 48-, and 40-kD polypeptides, contained no P700 and emitted at 690 nm at 77°K. It is assumed that these two bands belong to PSII and the one containing three polypeptides may have arisen as a result of proteolysis.

The light-harvesting antenna system of cryptomonads is unusual since it contains phycobiliproteins in the intrathylakoid space as well as Chl *c*, which has been proposed to link energy transfer from phycobilins to Chl *a* (Gantt, 1979). Ingram and Hiller (1983), using SDS–PAGE, separated from *Chroomonas* sp. a 100-kD Chl protein complex that associated with PSI, a 42-kD complex resembling that of PSII, and a new 55-kD Chl a/c_2 complex comprising two (24 and 20 kD) peptides. Lichtlé and Duval (1984) used digitonin or lithium dodecyl sulfate–PAGE (LiDS–PAGE) to separate thylakoid Chl protein complexes from *Cryptomonas rufescens*.

* See list of abbreviations and symbols; DCMU blocks electron flow from Q_A^- to Q_B (see Fig. 1 in Duysens, Chapter 1, this volume).—editors.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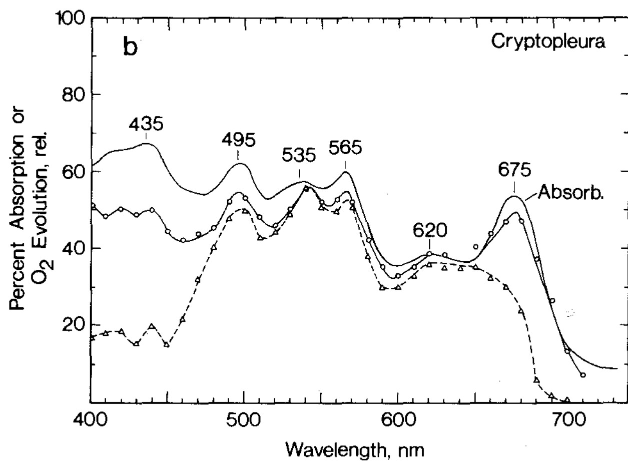
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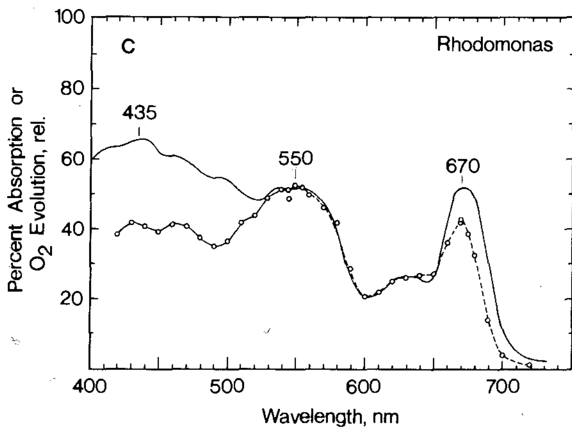
700
Wavelength, nm


c
Rhodomonas
100
80
60
40
20
435
550
670
Percent Absorption or
O_2 Evolution, rel.
400
500
600
700
Wavelength, nm

Three out of four complexes resolved by the latter procedure were identified by spectral emission characteristics as CPI (PSI-associated complexes) and the fourth as a Chl a/c_2 antenna of PSII.

Spectroscopic analyses of Chl protein complexes offer great promise in studies of the primary photochemistry and of the nature and origin of the various luminescence and fluorescence bands seen *in vivo*.

VI. Absorption and Photosynthetic Action Spectra

Because of their unique pigment composition, the red and blue-green algae are useful material for studies of absorption and fluorescence. Since the light absorbed by the phycobilin pigments is spectrally well separated from that absorbed by Chl, it is possible to excite one pigment system predominantly over the other. This has become especially true with the advent of lasers that emit monochromatic blue light, absorbed largely by Chl (442 nm, He–Cd), or green light (515 nm, argon ion) and red light (632.8 nm, He–Ne), absorbed predominantly by PE and PC, respectively.

A. Blue-Green Algae

Early measurements of Emerson and Lewis (1942) showed PC to be an effective sensitizer of photosynthesis in the blue-green alga *Chroococcus*. Absorption spectra and action spectra of photosynthesis for a number of blue-green algae were obtained later by Lemasson *et al.* (1973). Figure 2a shows the absorption spectrum measured at room temperature for *Aphanocapsa* with peaks at 570, 625, and 678 nm. The action spectrum for oxygen evolution has peaks at 570 nm (PE) and 625 nm (PC) and a slight shoulder near 675 nm (probably Chl *a*). An additional shoulder at 652 nm was attributed to APC. Although no absorption band corresponded to this APC action peak at room temperature, an APC absorption peak at 645 nm was seen at 77°K. Both in the red and in the blue region, the efficiency for light absorbed directly by Chl *a* appears to be low, as is also observed with red algae (see below).

FIG. 2. **Absorption spectra and action spectra for oxygen evolution in (a) a blue-green alga (from the data of Lemasson *et al.*, 1973); (b) a red alga (Fork, 1963); and (c) a cryptomonad. (From Haxo and Fork, 1959. Reprinted by permission from Nature 184, 1051–1052, Copyright © 1959 Macmillan Journals Limited.)** The action spectra in (b) were **measured with (circles, solid line) and without (triangles, dashed line) background green light.**

B. Red Algae

Photosynthetic action spectra for a number of red algae were measured by Haxo and Blinks (1950; see also Brody and Emerson, 1959; Haxo, 1960; Fork, 1963), who found high activity for spectral regions of light absorbed by the phycobilins and low activity for light absorbed predominantly by Chl. In agreement with these results, Yocum (1951) and Yocum and Blinks (1954) also found that the quantum efficiencies of red algae were very low at 436 and 675 nm. Duysens (1952) found that the action spectrum for the excitation of Chl fluorescence paralleled the photosynthetic action spectrum for O_2 evolution in having low values in the blue and red spectral regions.

Emerson and co-workers (1956, 1957) continued their systematic study of the "inactivity" of Chl *a* in red light, which was seen more clearly in red and blue-green algae than in the green alga *Chlorella,* where the effect was first noted (Emerson and Lewis, 1943). This pursuit led to the discovery of the Emerson enhancement effect (Emerson *et al.,* 1957; Emerson and Rabinowitch, 1960) and, together with measurements of intracellular redox reactions (Duysens *et al.,* 1961; Kok and Hoch, 1961), led to the idea that photosynthesis requires the cooperation of two light reactions operating in series (for reviews, see Haxo, 1960; Blinks, 1964; Govindjee and Govindjee, 1975).

Figure 2b shows an example of absorption and photosynthetic action spectra measured in the red alga *Cryptopleura crispa* (Fork, 1963). The absorption spectrum has peaks at 435 and 675 nm (Chl); 495, 535, and 565 nm (PE); and 620 nm (PC). The action spectrum measured in the absence of background light follows the absorption spectrum closely in the region of PE and PC absorption but drops away in both the red and blue regions, as found by Haxo and Blinks (1950). The activity of Chl was enhanced in both the red and blue regions when the action spectrum was measured in the presence of background green (546 nm) light absorbed largely by PE. (The difference between these two action spectra is a reflection of the action spectrum for the Emerson enhancement.) This result confirmed that Chl was not inactive in red algae, but that light absorbed by phycobilins is transferred largely to PSII (see Section VIII,A) and that Chl (largely PSI) and phycobilin excitation are both needed for efficient photosynthesis (also see Brody and Emerson, 1959).

C. Cryptomonads

The cryptomonad biliproteins are located on the inner side of the thylakoid membrane and consist of either PC or PE but never both. APC

has, so far, not been detected in the cryptomonads. The cryptomonads also contain Chl *c* and, like the dinoflagellates, have only the Chl c_2 spectral component (Jeffrey, 1969). Absorption spectra measured at 77°K for *Cryptomonas rufescens* show peaks at 568 and 605 nm from phycobilin, shoulders near 630–640 nm from Chl *a* and c_2, and two peaks at 670 and 678 nm attributable to forms of Chl *a* (Lichtlé *et al.*, 1980).

Only few photosynthetic action spectra are available for the cryptomonads. Figure 2c shows for *Rhodomonas lens* that the action spectrum, as with the red algae, follows the absorption spectrum in the green region of PE absorption, but is higher than that of red algae, where Chl *a* absorbs maximally. Similar results were found by Kamiya and Miyachi (1984) for *Cryptomonas* sp.

VII. Fluorescence Emission and Excitation Spectra

A. *Emission Spectra*

Phycobilins transfer their excitation energy to Chl *a* with less than 100% efficiency, and furthermore energy transfer within the PBsome is dependent on environmental conditions. Because of the spectral discrimination between the two photosystems, red and blue-green algae have been used extensively in the elucidation of the pathways of energy transfer and of the mechanisms involved in the regulation of energy distribution in photosynthesis.

1. BLUE-GREEN ALGAE

Figure 3a shows the fluorescence emission spectrum of *Anacystis nidulans* obtained at room temperature from cells excited with light absorbed by both Chl *a* and the phycobilins (Goedheer, 1964; Murata *et al.*, 1966; Murata and Takamiya, 1967). The spectrum shows a main peak at 685 nm (Chl *a*) and a smaller peak near 653 nm (PC + APC). Addition of DCMU to *Anacystis* or *Anabaena* (*An.*) *variabilis* causes the appearance at room temperature of increased fluorescence at 683 and 660 nm and a slight increase at 650 nm (an additional new band around 695 nm is also produced in *Anacystis;* see Papageorgiou and Govindjee, 1967a). These results suggest that energy transfer may also proceed uphill against the thermodynamic gradient from Chl to APC to PC (Ley and Butler, 1977a; Murata, 1977; Wang and Myers, 1977), caused presumably by absorption of thermal energy from nearby molecules (Murata, 1977) (also see Section X).

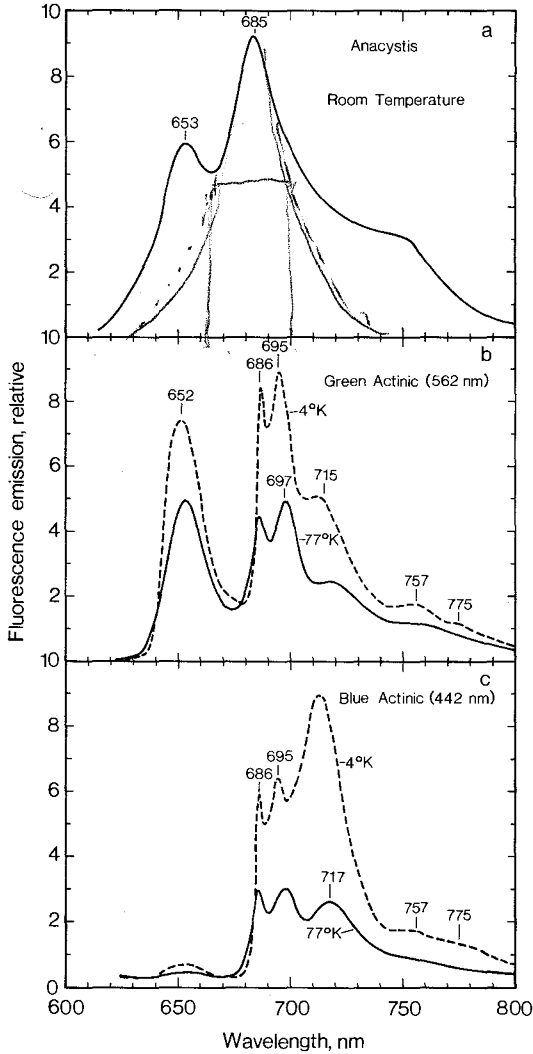


FIG. 3. Fluorescence emission spectra of *Anacystis nidulans* measured (a) at room temperature in light absorbed by both PSI and PSII (replotted from data of Goedheer, 1964). In (b) and (c), the spectra were measured with green (562 nm) and blue (442 nm) actinic light, respectively, at 4 and 77°K (replotted from the data of Rijgersberg and Amesz, 1980).

Cooling produces a strong increase in fluorescence yield of Chl *in vivo* (see, e.g., Murata *et al.*, 1966; Goedheer, 1972) but not of Chl *a* in solution (Goedheer, 1964). As first seen by Brody (1958), the most noticeable effect of low temperature on fluorescence spectra measured *in vivo* is the appearance of enhanced fluorescence at long wavelengths. The peak location of this long-wavelength fluorescence varies from 710 to 740 nm (Murata *et al.*, 1966) depending on the species. In addition, there are two short-wavelength Chl *a* bands (F685 and F695) that are almost universally observed in algae and higher plants (Govindjee, 1963; Murata *et al.*, 1966; Cho and Govindjee, 1970; Butler, 1979; Rijgersberg *et al.*, 1979a,b; Amesz and Rijgersberg, 1981).

Fluorescence emission spectra measured at liquid nitrogen (77°K) or liquid helium (4°K) temperature in *A. nidulans* show the dramatic increase of the fluorescence emission bands compared to what is seen at room temperature (compare Figs. 3b and 3c with 3a; Cho and Govindjee, 1970; Rijgersberg and Amesz, 1980). In *Anacystis* illuminated at 4°K with green actinic light (562 nm), which is absorbed predominantly by phycobilins, there are peaks in the emission spectrum at 686, 695, and ~715 nm (Fig. 3b). Phycobilin excitation, but not Chl excitation (442 nm), produced a strong phycobilin emission band at 652 nm that increased by about 50% on decreasing the temperature from 77 to 4°K (Fig. 3b). Second derivative analysis of this band by Rijgersberg and Amesz (1980) showed it to comprise two bands at 646 (C-PC) and 654 nm (APC). This analysis also revealed a weaker band at 676 nm attributed to the long-wavelength form of APC (APC-B). By using various quinones that quench Chl *a* fluorescence specifically (Amesz and Fork, 1967), Rijgersberg and Amesz (1980) concluded that APC-B contributed only a small fraction to the fluorescence at 685 nm. Moreover, the APC-B fluorescence band has a half-bandwidth of 13–16 nm, whereas the F685 band is considerably narrower, which would also argue against the idea that the F685 band has a significant contribution from APC-B, as suggested by Gantt *et al.* (1977).

The band around 715–717 nm is produced much more strongly by Chl excitation than by phycobilin excitation, suggesting its origin in PSI (Govindjee, 1963; Murata *et al.*, 1966; Cho and Govindjee, 1970; Rijgersberg and Amesz, 1980). Two additional long-wavelength bands can be seen at 757 and 775 nm, both on excitation with 442 and 562 nm, and were attributed by Rijgersberg and Amesz (1980) to vibrational subbands of F695 and F715, respectively. *Anacystis,* however, contains an additional absorption band at 750 nm (Govindjee *et al.*, 1961) which is also fluorescent in this long-wavelength region.

As noted earlier, the F686 and F695 bands are associated with PSII since they are more effectively excited by phycobilins than by Chl *a* (compare Figs. 3b and 3c; also see Rijgersberg *et al.*, 1979a,b; Schreiber *et al.*, 1979).

2. RED ALGAE

Emission spectra measured at room temperature for *P. cruentum* with green actinic light have peaks near 685 nm (Chl *a*) as well as shoulders around 660 and 615 nm (French and Young, 1952; Goedheer, 1964; Krey and Govindjee, 1966; Murata *et al.*, 1966; Singhal *et al.*, 1981; see Fig. 4a). On DCMU treatment or at high light intensities an additional shoulder around 695 nm is also observed (Krey and Govindjee, 1964, 1966). Illumination of *Porph. yezoensis* at room temperature with green light absorbed preferentially by PE produced fluorescence emission peaks at 585 (PE), 655 (PC), and 685 nm (Chl *a*) and shoulders at 638 (PC) and 720 nm (Chl *a*), while excitation of Chl with blue actinic light produced a dominant fluorescence peak at 720 nm (Murata and Takamiya, 1967), and at 732 nm in *Porph. perforata* (Fork *et al.*, 1982). This long-wavelength band apparently originates in PSI (Murata and Takamiya, 1967; Fork *et al.*, 1982) and is not seen in other red or blue-green algae at room temperature (Goedheer, 1972; see Fig. 4a, blue actinic).

On excitation with green light, *P. cruentum* at cryogenic temperatures (Figs. 4b and 4c) has fluorescence maxima near 687, 694, and 712 nm and two long-wavelength bands at 755 and 775 nm (Krey and Govindjee, 1966; Murata *et al.*, 1966; Gantt *et al.*, 1977; Rijgersberg and Amesz, 1980). On excitation with blue light, however, the major emission is at 712–715 nm. Fluorescence bands seen at 646 and 662 nm (Fig. 4b) are attributed to R-PC and APC, respectively (Rijgersberg and Amesz, 1980).

Cyanidium caldarium, a eukaryotic thermophile of uncertain taxonomic position (Allen, 1959; Seckbach *et al.*, 1981), has fluorescence spectra similar to those of red algae. Its fluorescence emission maxima at room temperature are observed near 685 (Chl *a*), 663 (APC), and 655 nm (PC). At 4–77°K the maxima occur at 685–689 and 695 nm, and long-wavelength bands are seen at 712 and 727 nm (Mohanty *et al.*, 1972; Rijgersberg and Amesz, 1980). As with *Porphyridium, Cyanidium* has a long-wavelength band located at 755 nm that is most effectively produced by PSII excitation (see, e.g., Rijgersberg and Amesz, 1980). Bands are also seen at 641, 657, and 664 nm in spectra obtained from cells illuminated with green actinic light. Analysis by Rijgersberg and Amesz

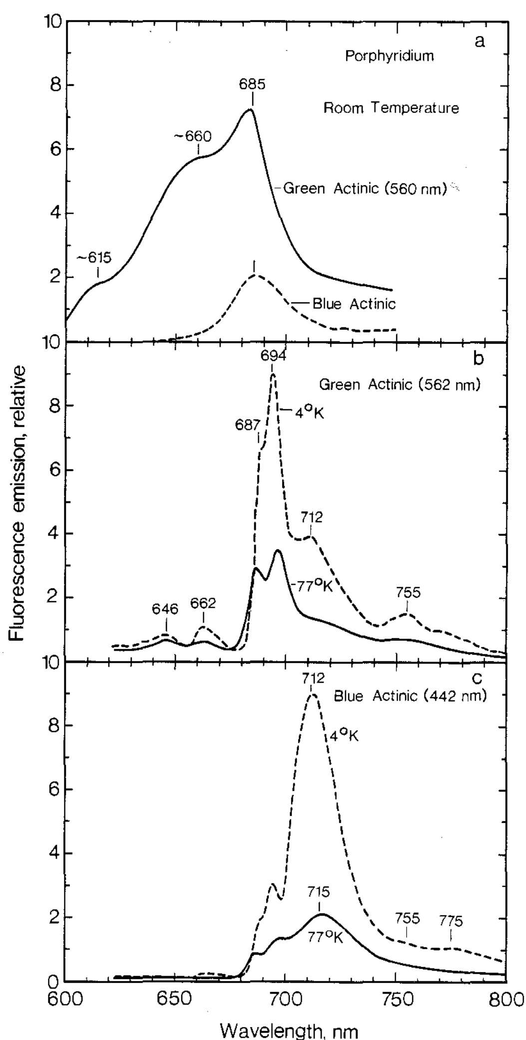


FIG. 4. Fluorescence emission spectra of *Porphyridium cruentum* measured (a) at room temperature with green (560 nm) or blue (442 nm) actinic light (replotted from the data of Singhal *et al.*, 1981). In (b) and (c), the spectra were measured with green (562 nm) and blue (442 nm) actinic light, respectively, at 4 and 77°K (replotted from the data of Rijgersberg and Amesz, 1980).

(1980) revealed a band which probably occurred at 676 nm and which was attributed to APC-B. Excitation of PSI with blue actinic light produced a single prominent band near 727 nm in *Cyanidium.*

3. CRYPTOMONADS

The emission spectrum at 77°K obtained by illuminating *Cryptomonas rufescens* with a broad band of blue-green light that excited both Chl and phycobilins had peaks at 630, 690, and 730 nm as well as a shoulder near 650 nm (Lichtlé *et al.,* 1980). Cells grown at high light intensities had a reduced content of PE in relation to Chl. These samples showed much less fluorescence emission at 630 nm and somewhat less at 690 nm but no change at 730 nm. It was suggested that PE in *cryptomonas* is connected via a phycobiliprotein–Chl antenna to the PSII reaction centers and is independent of a separate Chl antenna that transfers energy to PSII and then to PSI when PSII reaction centers are all closed (Lichtlé *et al.,* 1980).

B. Excitation Spectra

1. BLUE-GREEN ALGAE

As for photosynthetic action spectra (see Section VI,A), room temperature action spectra for fluorescence are dominated by PC activity, while Chl activity is low (Duysens, 1952; Goedheer, 1965; Papageorgiou and Govindjee, 1967b; Govindjee and Mohanty, 1972). In *S. cedrorum,* for example, the action spectrum for F > 705 nm has a peak at 625 nm (Fig. 5), and for *Anacystis* (Goedheer, 1965; Papageorgiou and Govindjee, 1967b) and *Oscillatoria* (Duysens, 1952) the peak is at 630 nm. Chl activity is seen only as a minor peak at 435 nm and a faint shoulder around 670 nm in *S. cedrorum.* The absorbance spectrum of whole cells at room temperature is shown in Fig. 5a for comparison with the fluorescence action spectra.

Action spectra measured at 77°K show that F685 and F695 are more effectively sensitized by phycobilins than by the Chl, while F720 is excited by both phycobilins and Chl (Goedheer, 1965; Murata *et al.,* 1966; Bergeron and Olson, 1967; Cho and Govindjee, 1970). The excitation spectrum for fluorescence at wavelengths longer than 720 nm measured at 77°K in *S. cedrorum* by Goedheer (1965) showed PC peaks at 622 and 631 nm, an APC peak at 650 nm, a shoulder at 672 nm which may be attributable to APC-B, and Chl peaks at 678 and 437 nm (Fig. 5). The 512-nm peak and 470-nm shoulder may represent Car sensitization of Chl fluorescence. Action spectra measured with isolated membranes

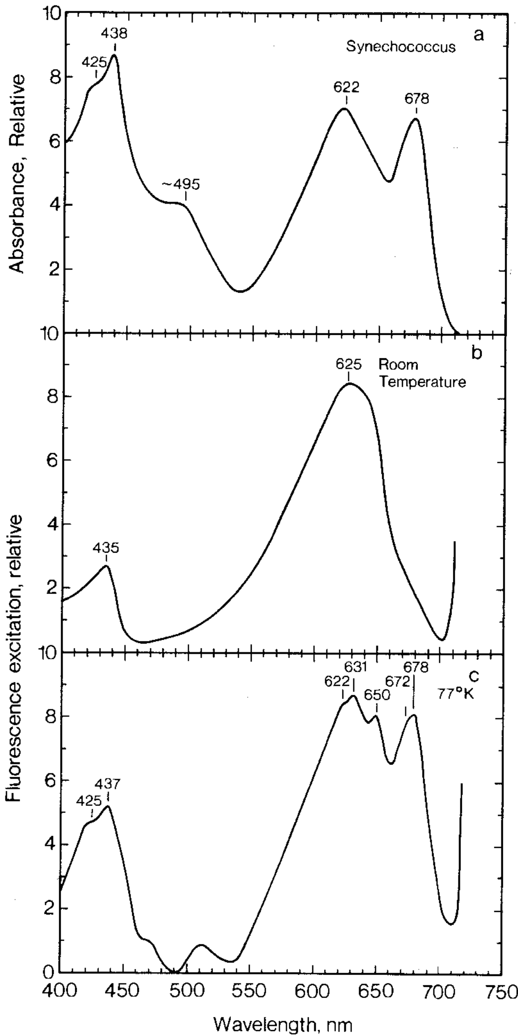


FIG. 5. **Absorption spectrum at room temperature (a) and action spectra for fluorescence at wavelengths >705 nm measured at room temperature (b) and at wavelengths >720 nm measured at 77°K (c) for *Synechococcus cedrorum* (replotted from the data of Goedheer, 1965).**

suggest that energy transfer from β-carotene to Chl occurs in PSI only (Goedheer, 1965, 1969).

Tel-Or and Malkin (1977) determined the relative contribution of different pigments to each of the photosystems in *Phormidium luridum*.

PSI was found to contain about 90% of the Chl *a*, 90% of the Car, and 15% of the PC.

2. RED ALGAE

In the early 1950s it was found that the excitation spectrum from Chl fluorescence mainly follows the absorption of phycobilins (French and

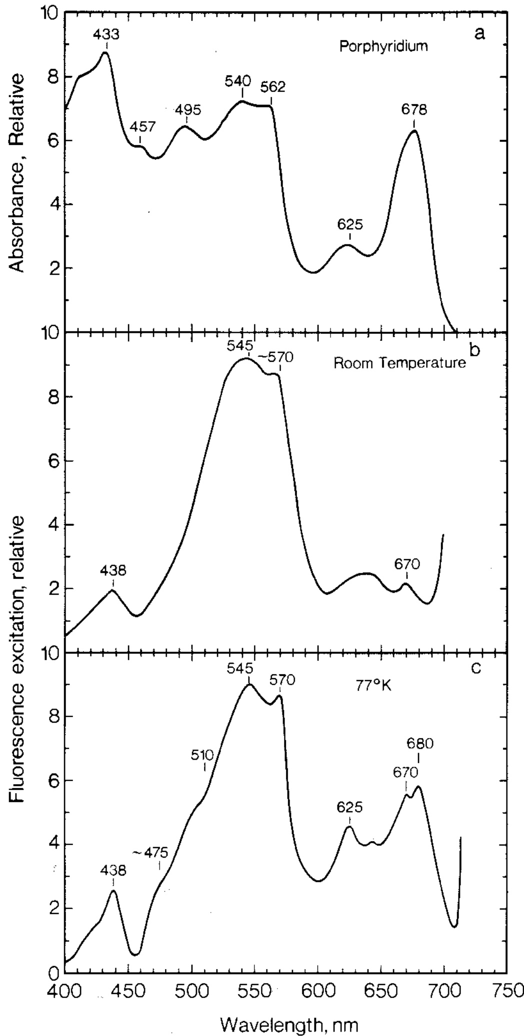


FIG. 6. Absorption spectrum at room temperature (a) and action spectra for fluorescence at wavelengths >705 nm measured at room temperature (b) and at wavelengths >720 nm measured at 77°K (c) for *Porphyridium cruentum* (replotted from the data of Goedheer, 1965).

Young, 1952; Duysens, 1952). At both room temperature and 77°K the action spectra for long-wavelength Chl *a* fluorescence are dominated by PE bands near 545 and 570 nm and a band near 625 nm produced by PC in *P. cruentum* (Figs. 6b and 6c; Goedheer, 1965; cf. Krey and Govindjee, 1966). Bands at 645 and 670 nm in the low-temperature spectrum may represent APC and APC-B, respectively. The much weaker contribution of Chl to the excitation of this fluorescence was reflected by the smaller peaks at 438 and 680 nm. This low-temperature spectrum also reflected a limited capacity of Car to sensitize long-wavelength Chl fluorescence.

3. CRYPTOMONADS

Cryptomonads are unique in possessing Chl *c* in combination with either PE or PC, but not both (Stanier, 1974). Moreover, cryptomonads lack APC (Stanier, 1974) as the linking pigment in energy transfer from phycobilins to Chl *a* (Gantt, 1975). Only a few action spectra for fluorescence of cryptomonads are available. Lichtlé *et al.* (1980) measured excitation spectra of fluorescence at 685 and 730 nm in *Cryptomonas rufescens*. They interpreted their results to mean that energy absorbed by PE is transferred directly to the Chl of PSII reaction centers, an idea in agreement with the intrathylakoid localization of the phycobiliproteins in cryptomonads (Lichtlé, 1978; Gantt *et al.*, 1981).

VIII. Energy Partitioning

A. Energy Transfer

Since the advent of techniques for measuring lifetimes with picosecond resolution, a number of studies have focused on energy transfer *in vivo* between phycobilins and Chl *a* in blue-green and red algae (Porter *et al.*, 1978; Mimuro *et al.*, 1984; Yamazaki *et al.*, 1984).

The first measurements of fluorescence lifetimes of photosynthetically active pigments in intact algae were made by Brody (1960) and Tomita and Rabinowitch (1962), who found transfer times of several hundred picoseconds, which were limited by the experimental techniques then available. The availability of picosecond laser pulses has enabled studies of energy transfer to be made in a much more detailed manner. The sequence of energy transfer was proposed to proceed from PE → PC → APC → Chl *a* by a Förster mechanism (Förster, 1948) involving dipole–dipole interaction (Tomita and Rabinowitch, 1962; Grabowski and Gantt, 1978a,b).

Porter *et al.* (1978) found that excitation of B-PE at 530 nm with a 6-ps pulse in *P. cruentum* led to the production of maximum R-PC fluorescence at 640 nm within 12 ps. Fluorescence of APC at 660 nm reached its

maximum within 24 ps and Chl fluorescence at 684 nm within 50 ps, confirming the energy transfer sequence described above. Brody *et al.* (1981) reached similar conclusions concerning sequential energy transfer in the blue-green alga *A. nidulans* in their studies with a mode-locked Nd^{3+} glass laser and a streak camera. For picosecond excitation, Yamazaki *et al.* (1984) used a synchronously pumped, cavity-dumped dye laser, which generated a pulse train with very high repetition rates (<4 MHz) and lower pulse energies (10^8 photons/cm^2) than the Nd^{3+} glass laser system (10^{14} photons/cm^2). This system allowed measurement of time-resolved fluorescence spectra from intact cells with a resolution of about 25 ps as well as the decay curves associated with individual pigments. Excitation of *P. cruentum* at 540 nm produced fluorescence successively from PE, PC, APC, and Chl *a*. The fluorescence from these components reached its maximum value within 0, 30, 57, and 150 ps, respectively (Fig. 7). For *A. nidulans* excited by 580-nm light, the fluores-

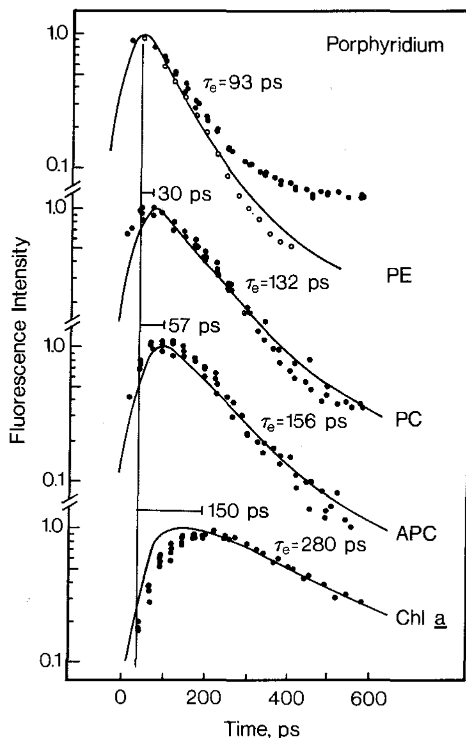


FIG. 7. Fluorescence rise and decay curves for the individual pigments of *Porphyridium cruentum*. The solid line represents the calculated best fit. The open circles in the PE curve plot the fast component of the total decay (data replotted from Yamazaki *et al.*, 1984).

cence of PC, APC, Chl *a*, and APC_{lw} (long-wavelength form of APC) reached its maximum level after 0, 60, 120, and 110 ps, respectively (Fig. 8). These results, and those obtained by Mimuro *et al.* (1984), support the concept of sequential energy transfer among the various phycobilin components of blue-green and red algae with the exception of APC_{lw}, which apparently is not in the main pathway of energy flow to Chl *a*, but rather appears to function as a bypass or a parallel pathway of excitation transfer. Similar conclusions, based on different types of experiments, were reached by Csatorday *et al.* (1978) and by Mohanty *et al.* (1985a).

Karukstis and Sauer (1984) analyzed energy transfer and distribution in *Porph. perforata* by means of time-resolved fluorescence emission spectra, using a single-photon timing system with picosecond resolution. This alga is unusual in having a long-wavelength fluorescence band at room temperature on excitation with blue light absorbed almost exclusively by Chl *a* of PSI (Murata and Takamiya, 1967; Fork *et al.*, 1982) that is analogous to the low-temperature F735 band seen in plants attributable to the antenna Chl associated with PSI (see e.g., Ley and Butler,

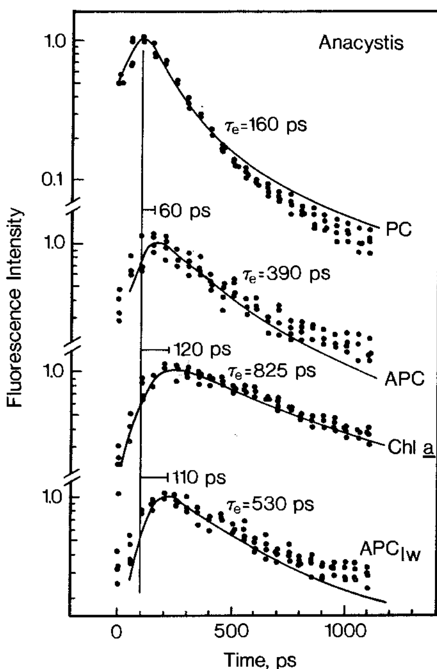


FIG. 8. Fluorescence rise and decay curves for the individual pigments of *Anacystis nidulans*. The solid line represents the calculated best fit (data replotted from Yamazaki *et al.*, 1984).

1976; Butler, 1978). Karukstis and Sauer (1984) found most of the fluorescence in *Porph. perforata* to come from two emission bands at 680 and 740 nm that arose from Chl *a* associated with PSII. The F680 band had a lifetime of 340–380 ps and was proposed to originate in the Chl *a* antenna of PSII, presumably from the excitation lost in transit to the reaction center (Haehnel *et al.,* 1982). A small contribution to the long-wavelength band was thought to come from antenna Chl *a* associated with PSI. This PSI component has a fluorescence lifetime of about 150 ps.

In most red and blue-green algae only a small amount of Chl is associated with PSII; it has been estimated to be ~5% in *P. cruentum* (Ley and Butler, 1977a), ~5–10% in *An. variabilis* (Mimuro and Fujita, 1977), and ~15% in *A. nidulans* (Wang and Myers, 1977). Interestingly, Diner (1979) found that only half of the reaction centers of PSII were coupled to PBsomes. Ley and Butler (1977a) examined fluorescence emission at low temperature in *P. cruentum,* and Butler (1979) developed a model for the pathways of energy flow between the two photosystems. This model has been assumed to apply to red and blue-green algae. As was shown by action spectra measurements, most of the energy absorbed by Chl *a* goes to PSI and most of the energy absorbed by PBsomes goes to Chl *a* associated with PSII, suggesting that PBsomes are linked to small Chl *a* antenna systems. The high efficiency of O_2 evolution in light absorbed by the PBsomes (see Section VI) therefore suggests that efficient energy transfer occurs from Chl *a* associated with PSII to Chl *a* associated with PSI, even when PSII traps are open. According to Ley and Butler, about 50% of the light absorbed by PBsomes is transferred to PSI from PSII when PSII traps are open, and this transfer increases to ~90–95% when traps become closed. Diner and Wollman (1979) compared energy transfer among PSII reaction centers in a wild type and in a mutant of *Cyanidium caldarium* devoid of C-PC with that of the green alga *Chlorella pyrenoidosa.* They observed that although the PSII reaction centers of *Cyanidium* had only about 30 Chl *a* molecules associated with them, compared to 300 in *Chlorella,* the energy transfer between different PSII units in *Cyanidium* is as efficient as that between the much larger PSII units of *Chlorella* (Joliot and Joliot, 1964).

We do not know the various factors that control the flow of energy from PBsomes to PSII. It can be imagined that factors affecting the degree of attachment of PBsomes, such as temperature, light, and ionic constituents, may affect the energy flow (see Section VIII,B).

The quantitative relationship between PSII units and PBsomes is uncertain. On the basis of electron microscopic examination as well as pigment analysis, the ratio of reaction center II units to PBsomes has

been shown to vary between 1 and 4 (Stevens and Myers, 1976; Vierling and Alberte, 1980; Kursar and Alberte, 1983). Analysis of the flash yield of O_2 evolution and fluorescence in *Cyanidium caldarium* indicates that almost half of the PSII units are not attached to PBsomes (Diner, 1979). Ley (1984) made a similar study in *P. cruentum* to estimate the effective cross section for O_2 production measured at 546 nm (PE absorption), and concluded that one PBsome effectively feeds excitation energy to many PSII reaction centers.

B. *Factors Affecting Energy Transfer from Phycobilins to Chlorophyll a*

Since PBsomes contain the major light-harvesting antenna pigments of the blue-green and red algae, the photosynthetic efficiency of these organisms depends on the efficiency of transfer to Chl *a* of the energy absorbed by the phycobilins of the PBsome.

Low-temperature (4–120°K) fluorescence emission spectra of representative blue-green and red algae were found by Rijgersberg and Amesz (1980) to have emission bands in the Chl *a* region near 685, 695 and between 710 and 730 nm, just as in higher plant chloroplasts. The differential increase in the intensity of these bands seen on cooling was assumed to be produced by a decrease in the efficiency of energy transfer between Chl *a* molecules, as assumed for the green alga *Chlorella* (Cho *et al.*, 1966), higher plants (Rijgersberg *et al.*, 1979a), and the purple bacteria (Rijgersberg *et al.*, 1980).

Growth and nutrient conditions affect not only the PC/Chl ratio but also the energy transfer from PC to Chl *a* (Ghosh and Govindjee, 1966; Öquist, 1974; Goedheer, 1976). Ghosh and Govindjee (1966) showed that *A. nidulans* grown in red (PSI) light had decreased efficiency of energy transfer from PC to Chl *a*. However, in cells of *An. azollae* grown in orange (600 nm) light (PSII) an increase of the efficiency of energy transfer from PC to Chl *a* of PSI was observed (Sun, 1983).

It is not certain whether the factors that decrease the PC content of the cell also always increase the efficiency of energy transfer to PSII. It is known, however, that high light and/or high temperature induce high amounts of APC in the cells (Öquist, 1974; Goedheer, 1976) and that the APC content may control energy transfer.

Of special interest in this regard are the studies carried out with photoorganotrophically grown cells of *An. variabilis*. When these cells were cultured in nitrate-free medium they became devoid of phycobilin pigments, but they resynthesized the pigments even in the dark when nitrate was added (Katoh and Ohki, 1975). Dark-regenerated PC does not

transfer energy to Chl *a* of PSII (Ohki and Katoh, 1976), although functional and coupled PBsomes have been isolated from dark heterotropically grown *Tolypothrix tenuis* (Ohki and Gantt, 1983). Light was shown to be required to confer competency to PBsomes to transfer energy to Chl *a* (Katoh and Ohki, 1976). Furthermore, analysis of emission spectra of dark-grown cells of *An. variabilis* suggests that these dark-generated PBsomes funnel excitation energy directly to Chl *a* of PSI (Ohki and Katoh, 1977). Sun *et al.* (1983) suggested that cells of *An. azollae,* grown in an N-free medium in the light, had enhanced energy transfer to Chl *a* of PSI on the basis of analysis of relative emission ratios of PC to Chl *a* and APC to Chl *a*. Subsequent transfer to the dark for several hours decreased the emission of Chl *a* of PSI when the cells were excited with light absorbed by PC. These results seem to indicate that the efficiency of energy transfer of the PBsomes varies as a result of adaptation to changes in environmental conditions. This is an interesting result in view of the general ideas that both red and blue-green algae possess relatively larger numbers of PSI than PSII units (Ley and Butler, 1977a; Mimuro and Fujita, 1977), that most of the energy absorbed by accessory pigments goes to PSII, and that the redox state of PSII regulates energy transfer to PSI (Ley and Butler, 1977b).

IX. Induction of Chlorophyll *a* Fluorescence

When cells, intact chloroplasts, leaves, or leaf segments are illuminated after a period of dark adaptation, they exhibit oscillations in fluorescence yield. The phenomenology of these transient changes in fluorescence yield, called Kautsky transients, has been well documented and is discussed by Briantais *et al.*, Chapter 18, and Govindjee and Satoh, Chapter 17, in this volume, as well as Papageorgiou (1975) and Lavorel and Etienne (1977). In spite of the complexity of these transient changes, fluorescence of variable yield has become a diagnostic feature for *in vivo* electron transport in photosynthesis and is being used increasingly in physiological studies (see Renger and Schreiber, Chapter 19, this volume).

Like higher plants and green algae, blue-green and red algae and possibly cryptomonads show the so-called OIDPSMT transient (for definitions, see Govindjee and Satoh, Chapter 17, this volume). Figure 9a shows a time course of Chl *a* fluorescence measured at 685 nm in *A. nidulans* excited with orange-red light absorbed by PC (Mohanty and Govindjee, 1973a). It may be noted that, unlike the situation for the green alga *Chlorella* (Munday and Govindjee, 1969) and *P. cruentum*

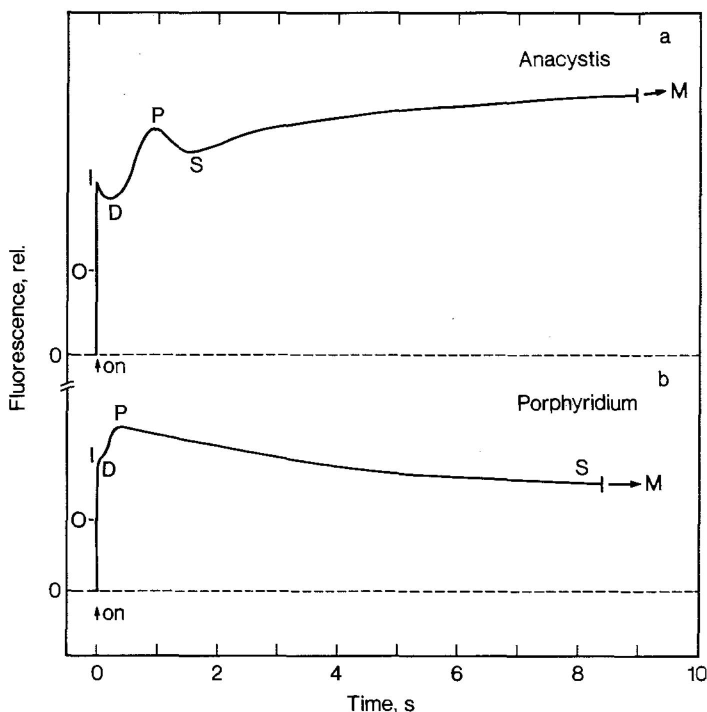


FIG. 9. **Time courses of fluorescence emission at 685 nm for *Anacystis nidulans* (a) and for *Porphyridium cruentum* (b). [(a) adapted from Mohanty and Govindjee, 1973a; (b) from Mohanty *et al.*, 1971a. Reprinted with permission from *Photochem. Photobiol.* 14, 667–682. Copyright 1971, Pergamon Press, Ltd.]**

(Mohanty *et al.*, 1971a; cf. Fig. 9b), the P level in *Anacystis* is not the maximum yield attained during the fluorescence induction period. Rather the large and slow S-M rise is dominant and often masks the IDPS transient (Mohanty and Govindjee, 1973a). A relatively long (15 min) period of dark adaptation and a relatively high light intensity were required in order to see a clear DPS transient, and the slow S-M rise was paralleled by an increase in the rate of O_2 evolution (Mohanty and Govindjee, 1973a).

Porphyridium cruentum shows a clear OIDPS (Kautsky) transient (Fig. 9b) in the yield of Chl *a* fluorescence on excitation with green light absorbed by the phycobilins (Mohanty *et al.*, 1971a). In this alga the P level is the maximum yield attained during the transient. Although not shown in Fig. 9b, the S level is followed by a slow rise to another maximum (M) and by a final decline to a terminal (T) steady-state level. The

blue-green algae also show a very slow decline from M to T (Papageorgiou and Govindjee, 1967a, 1968).

The OIDP transient is, in all likelihood, related to changes in Q, where Q ($\equiv Q_A$) is the first quinone electron acceptor of PSII: Chl *a* fluorescence is high when Q is reduced and low when Q is oxidized. For a further discussion of this point, the reader should consult the chapters by Duysens, van Gorkom, Briantais *et al.*, and Govindjee and Satoh (Chapters 1, 10, 18, and 17, respectively).

The herbicide DCMU abolishes the OIDP transient. In the presence of this inhibitor there is a rapid rise on illumination from an initial (F_0) level to a higher maximum level (F_m) as the traps of PSII are reduced (see Briantais *et al.*, Chapter 18, this volume; Mohanty and Govindjee, 1973b). After the F_0-to-F_m rise is complete, a further, slow increase of fluorescence is seen on a time scale of about 1 min (Papageorgiou and Govindjee, 1967a; 1968; Mohanty and Govindjee, 1973a,b). Since this rise is seen after all PSII traps are closed, it is not related to redox changes of Q_A. The slow fluorescence rise occurring in the presence of DCMU is characteristic of blue-green algae but is also seen in other algae as well. The suggestion was made that this slow rise in DCMU represented a state II–state I transition (Mohanty and Govindjee, 1973b; Williams *et al.*, 1980; Satoh and Fork, 1983a; Catt *et al.*, 1984).

The effects of uncouplers and inhibitors of photophosphorylation on the slow fluorescence increase in the presence of DCMU have been studied by Papageorgiou and Govindjee (1968), Mohanty and Govindjee (1973b), and Satoh and Fork (1983a) and are illustrated in Fig. 10 for *Synechococcus*. Uncouplers such as CCCP and methylamine (Figs. 10b and 10c) reduced the increase, but inhibitors of phosphorylation such as phlorizin had no effect (Fig. 10d). Antimycin A, which inhibits cyclic electron flow, decreased the rate but not the extent (Fig. 10e). These results suggest that the state II–state I transition is related to proton translocation caused by PSI-driven cyclic electron flow (see Section XI). In agreement with this idea, light absorbed by PSI is more effective than light absorbed by PSII in both *Schizothrix calcicola* (Duysens and Talens, 1969) and *S. lividus* (Satoh and Fork, 1983a).

Rather than an increase of fluorescence in the presence of DCMU, *Porph. perforata* exhibits a large (~50%) *decrease* of fluorescence in the light, lasting several minutes (Satoh and Fork, 1983b). This decrease was produced not by a state I–state II transition but apparently by an accumulated back reaction of PSII. This reaction may be related to one of the mechanisms used by this intertidal red alga to avoid damage to its photochemical apparatus by high salt and light conditions in its natural habitat (see Sections XI and XII,C).

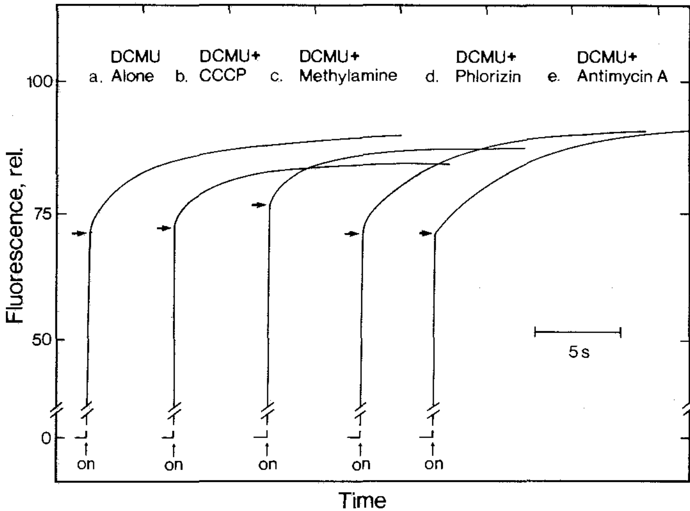


FIG. 10. Time courses of the slow fluorescence increase in the presence of DCMU and DCMU plus uncouplers and inhibitors of photophosphorylation in *Synechococcus lividus* measured at 52°C. The arrowheads mark the F_m level attained before the onset of a further, slow fluorescence increase (see Section IX). (Adapted from Satoh and Fork, 1983a.)

In spite of the complexity, much useful information can be gained from studies of fluorescence yield changes, both in intact algae and in subcellular preparations. The challenge is to correctly attribute changes of fluorescence to biophysical and physiological events. Some of the difficulties of this task are outlined by Govindjee and Satoh in Chapter 17 of this volume.

Unlike the situation with isolated chloroplasts, there are only a few studies of cation-induced fluorescence yield changes in the intact cells of blue-green and red algae. Schreiber (1979) showed monovalent–divalent antagonistic cation-induced changes in the fluorescence of PC. Brand *et al.* (1983) found that depletion of Ca^{2+} from growth medium that lacked Na^{+} reversibly lowered the fluorescence of variable yield in intact cells of *A. nidulans*. This quenching appears to result from damage to the electron donor side of PSII or a loss in the ability to stabilize charges at the PSII reaction centers. Depletion of Ca^{2+} has also been shown to affect energy transfer from PC to Chl *a* as well as the so-called state transitions (see Section XI and Briantais *et al.*, Chapter 18, this volume) in *Anacystis* (Mohanty *et al.*, 1985b). Brand and Becker (1984) reviewed the overall role of Ca^{2+} in O_2 evolution, and Govindjee *et al.* (1985) reviewed the electron donor side, including O_2 evolution, of PSII.

Although Ca^{2+} appears to be very closely associated with charge stabilization in *Anacystis,* its role in the primary photochemistry of PSII remains to be elucidated.

X. Delayed Light Emission

Delayed light emission (DLE), or delayed luminescence or fluorescence (see Jursinic, Chapter 11, this volume), is primarily a property of PSII, at least at physiological temperatures. PSI emits only in very specialized situations (see, e.g., Shuvalov, 1976). Many excellent reviews have appeared that detail the experimental phenomenology of various types of delayed light emission, their kinetics, yield, and mechanism (see Lavorel, 1975; Malkin, 1977; Govindjee and Jursinic, 1979; Lavorel *et al.,* 1982). DLE spans a time interval from nanoseconds to several minutes and beyond. In fact, prompt fluorescence has been considered a fast DLE (see Chapters 11 by Jursinic, 7 by Moya *et al.,* and 10 by van Gorkom in this volume).

Murata (1977) reported that the DLE spectrum has a component of APC emission, which clearly suggests that delayed fluorescence originates from the PSII antenna Chl as a result of back transfer of excitation from the reaction center pigments to antenna Chl and APC (see Section VII,A,1). Similar results have been observed in *Porph. perforata,* where substantial DLE is seen at the emission wavelength of PC and APC (R. Ruby, personal communication). Sonneveld *et al.* (1980) compared emission spectra of delayed fluorescence in the microsecond region for *Cyanidium caldarium, P. cruentum,* and *A. nidulans.* In the case of *A. nidulans,* but not *Cyanidium* or *P. cruentum,* there is distinct microsecond emission in the 620–655-nm region, indicating uphill energy transfer from the reaction center Chl *a,* via the antenna Chl, to the phycobilins (Fig. 11). DLE from the PBsome has also been reported by Kovalev and Krasnovsky (cited by Krasnovsky, 1982).

Like fluorescence, the millisecond DLE measured by repetitive flashes shows complex Kautsky-type transients; some three to five distinct phases in DLE transients have been noted in blue-green and green algae (Ono and Murata, 1977; Satoh and Fork, 1983c). Blue-green and red algae show a distinct initial rise to the I level followed by a distinct dip and then a subsequent rise to peak (P level). Just as with fluorescence transients, dark periods have a very large influence on DLE induction kinetics (Mimuro and Fujita, 1977).

DLE shows remarkable sensitivity to temperature and inhibitors (see, e.g., Jursinic and Govindjee, 1972; Ono and Murata, 1977; Satoh and

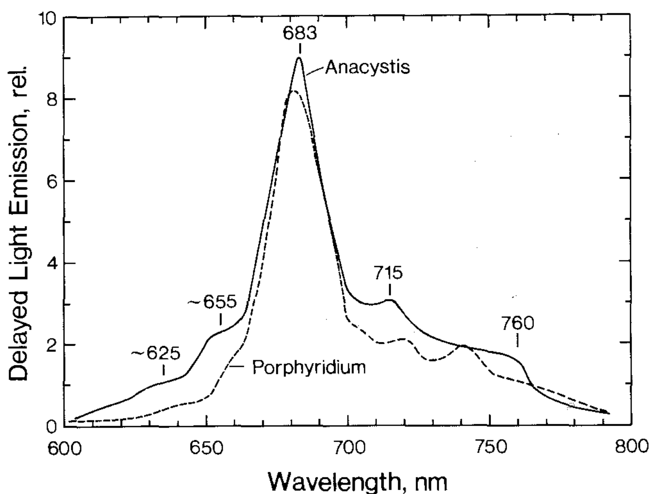


FIG. 11. Emission spectra of microsecond delayed light emission in *Anacystis nidulans* (solid line) and *Porphyridium cruentum* (dotted line). For *Anacystis* the delayed emission measured had a lifetime of 1 μs and for *Porphyridium*, 15 μs. (Adapted from Sonneveld *et al.*, 1980.)

Fork, 1983c). Ono and Murata (1977) showed that the millisecond decay kinetics of *A. nidulans* contained two exponentially decaying components; the fast component remained independent of temperature, while the slow component was temperature-dependent. Furthermore, they found that this slow component reflected phase transitions (see e.g., Havaux and Lannoye, 1983; Mohanty *et al.*, 1985a).

Although diverse and conflicting results on the effect of DCMU on DLE exist, NH_2OH shows a consistent effect (see Malkin, 1977). As in chloroplasts and green algae, NH_2OH suppresses millisecond DLE in *P. cruentum* and the decay of DLE assumes a single exponential (Mohanty *et al.*, 1971b). The loss of millisecond DLE along with reversibility of variable fluorescence in the presence of NH_2OH and DCMU has been taken as evidence that millisecond DLE originates as a result of recombinations of charges at PSII reaction centers (see Govindjee and Jursinic, 1979; Jursinic, Chapter 11, this volume).

We are not aware of any report on the DLE from PSI in red or blue-green algae. Björn *et al.* (1983) found that resting spores or akinetes (see Section XII,B) from *An. variabilis,* which have no PSII activity, produce a small amount of DLE. It will be of interest to use this material, which has only PSI functioning *in vivo,* for the investigation of DLE from PSI.

XI. Energy Distribution between Photosystems I and II (State Changes)

As described in Section VIII,A, light absorbed by phycobilins is transferred mainly to PSII, while light absorbed by Chl *a* is delivered to PSI (see, e.g., Ried *et al*, 1977; Ley and Butler, 1980a,b). Blue-green and red algae have evolved mechanisms that allow them to adapt to conditions of changing light intensity and quality (color).

One mechanism that algae use to cope with changes in light quality has been termed the state I–state II transition (Bonaventura and Myers, 1969; Murata, 1969; Wang and Myers, 1974; see Briantais *et al.*, Chapter 18, and Govindjee and Satoh, Chapter 17, this volume). State I has been proposed to develop after algae have been in light I for a considerable period. In state I there is reduced energy transfer from PSII to PSI resulting in higher Chl fluorescence at room temperature and, by definition, higher PSII activity and lower PSI activity compared to those in state II.

An impediment to the study of state changes exists in many plants because fluorescence transients seen during a dark-to-light transition (the so-called Kautsky transients; see Govindjee and Papageorgiou, 1971, and Briantais *et al.*, Chapter 18, this volume) overlap fluorescence changes produced by the state transitions. This situation complicates the study of state changes in many plants. Fortunately, in blue-green algae the variable (Kautsky) component of fluorescence is small, usually amounting only to about 10% of the total fluorescence signal, while state transitions are large.

In the thermophilic blue-green alga *Synechococcus,* the dark state was identified as state II. Regulation by state transitions was induced by absorption of system I light, which produced a state II to state I transition (Fork and Satoh, 1983).

As mentioned in Section IX, the slow fluorescence rise in the presence of DCMU that is clearly seen in blue-green algae represents a state II–state I transition (Mohanty and Govindjee, 1973b; Williams *et al.*, 198[illegible]; Satoh and Fork, 1983a; Catt *et al.*, 1984). Uncouplers, but not inhibitors of phosphorylation suppress the slow fluorescence rise produced in *A. nidulans* and *S. lividus* as a result of the state II–state I transition (Mohanty and Govindjee, 1973b; Satoh and Fork, 1983a), suggesting that proton transport through cyclic electron flow was related to the transition (cf. Fig. 10). Moreover, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), methyl viologen, and antimycin A inhibited the transition, indicating that cyclic electron flow around PSI, but not oxidation of electron carriers such as plastoquinone (PQ), was necessary in order

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to see the state II–I transition (Satoh and Fork, 1983a). (See Fig. 1 in Duysens, Chapter 1, or in Sane and Rutherford, Chapter 12, of this volume for components of PSI and PSII.) Biggins *et al.* (1984a) have shown that in a transition to state I, *P. cruentum* requires cyclic electron transport and coupled proton transport mediated by PSI.

It has been suggested that reversible phosphorylation and dephosphorylation of the Chl *a*/*b* light-harvesting protein complex regulate state transitions in higher plants via kinase-mediated phosphorylation of LHCP, which is controlled by the redox state of the PQ pool: over reduction of PQ by PSII leads to phosphorylation of LHCP and causes an increased excitation flow to PSI (state II); see Briantais *et al.*, Chapter 18, this volume. The oxidized state of PQ inactivates the kinase and a phosphatase-mediated dephosphorylation shifts the equilibrium back to state I (see, e.g., Bennett, 1979; Chow *et al.*, 1981; Horton *et al.*, 1981).

Blue-green algae lack LHCP, although they contain light-harvesting PBsomes. The redox state of PQ seems not to have a controlling influence on state transitions in *Synechococcus;* in this alga PQ connects both the respiratory and the photosynthetic electron transport chains (Hirano *et al.*, 1980). In starved cells PQ is mostly in the oxidized state, while in normal cells it is in the reduced state. However, in both kinds of cells, state transitions could still be observed. Fluorescence yield changes induced by state transitions in red and blue-green algae where PSII turnover was blocked by NH_2OH and DCMU were observed earlier by Mohanty and Govindjee (1973b) and by Satoh and Fork (1983a).

Biggins *et al.* (1984b) reported that no reversible protein phosphorylation accompanied state transitions in *P. cruentum* or *A. nidulans*. It is clear that the blue-green and red algae use a mechanism to control state transition different from that proposed for green algae and higher plants.

A new state (state III) has been observed in the intertidal red alga *Porph. perforata* (Satoh and Fork, 1983d,e) in which light energy reaching PSII is decreased with no attendant increase of PSI activity (see Section IX). In *Porphyra* as in other red algae (Ried and Reinhardt, 1977) the dark state is state II. Illumination of *Porphyra* in state I or state II with light II produces state III, which seems to be related to formation of ΔpH, while the state III to state II transition appears to be related to the formation of membrane potential (Satoh and Fork, 1983d,e). In state III the initial fluorescence level (F_0), see immediately after illumination, was diminished considerably and both F685 and F695 measured at 77°K decreased in parallel. There was no change in the distribution of light energy between the two photosystems as a result of the state II to state III transition; however, there was a decrease in the amount of energy

delivered to the reaction centers of PSII from the light-harvesting pigments. Perhaps this state represents an adaptation that allows *Porphyra* to thrive in the intertidal zone, where it is exposed to high light intensities often in combination with high salinity and extreme desiccation. Such a mechanism would protect the alga against photodamage by limiting the amount of quanta coming to PSII under conditions where normal photosynthesis is precluded.

For a complete review on state changes, see Fork and Satoh (1986).

XII. Specialized Adaptation

A. Cyanelles

This term has been applied to structures seen within the biflagellate *Cyanophora paradoxa* first isolated by Korschikoff (1924). These structures are apparently endosymbiotic blue-green algae (Hall and Claus, 1963). Cyanelles contain Chl *a*, C-PC, APC, β-carotene, and zeaxanthin (Chapman, 1966). The photosynthetic properties of these endosymbionts have been characterized and found to be like those of blue-green algae (Klein *et al.,* 1981). The genome size of cyanelle DNA is smaller than that in cyanobacteria and more like that of chloroplast DNA (Herdman and Stanier, 1977). Also, Floener and Bothe (1982) could not detect KCN-sensitive respiratory cytochrome oxidase in cyanelles, and the respiratory activity of crude extracts was supported by NADH and not by NADPH, which is the preferential electron donor in cyanobacteria (Biggins, 1969). Floener and Bothe (1982) thus suggest that cyanelles are not related to cyanobacteria or chloroplasts of cryptomonads or red algae, but have more resemblance to eukaryotic chloroplasts. (For a brief discussion of evolution of various photosynthetic systems, see Govindjee and Satoh, Chapter 17, this volume.) In any event, the occurrence of phycobilins in cyanelles remains an attractive feature for the study of prompt and delayed fluorescence in these endosymbionts. A model of the thylakoid of the cyanelle of *C. paradoxa* has been proposed by Giddings *et al.* (1983) in which the PBsomes on the thylakoid surface are in direct contact with PSII centers (composed of two subunits) which are located within the membrane. The PBsomes and PSII centers are thought to be aligned in rows, facilitating energy transfer between adjacent PSII complexes or their accessory pigments.

B. Heterocysts and Akinetes of Blue-Green Algae

Heterocysts of filamentous blue-green algae are usually only slightly larger than vegetative cells, have thickened walls, and may be found at

the ends of filaments or between vegetative cells along the filaments. Heterocysts are the site of nitrogen fixation, which requires an anaerobic environment that is provided by the thickened walls surrounding the heterocysts (Haselkorn, 1978; Alberte *et al.*, 1980). Thomas (1970) found no PSII pigments (Chl *a*-670, C-PC, C-PE, or APC) in young heterocysts of *Anabaena*. On aging, however, these pigments appeared (Thomas, 1972).

PSI is the only photosystem that is functional in heterocysts (Donze *et al.*, 1972). Heterocysts do not show reactions characteristic of PSII (Donze *et al.*, 1972; Almon and Böhme, 1980). They have a low yield of Chl *a* fluorescence, no variable fluorescence or delayed fluorescence, and no Hill reaction or O_2 evolution.

Heterocysts have been shown to contain phycobiliproteins that are effective in sensitizing P700 oxidation (Peterson *et al.*, 1981a,b). The heterocyst phycobiliproteins are apparently localized in PBsomes (Ke *et al.*, 1983).

Under certain conditions (Wolk, 1973), certain filamentous blue-green algae form resting spores, termed akinetes, that have thick cell envelopes, are usually larger in diameter and length than vegetative cells, and contain large amounts of the protein cyanophycin. Akinetes are very resistant to desiccation and temperature and are viable for long periods (Fritsch, 1945; Fogg *et al.*, 1973).

Björn *et al.* (1983) made a comparative analysis of *in vivo* absorption and fluorescence of both vegetative cells and akinetes of *Anabaena variabilis*. Their results suggest efficient energy transfer at room temperature between the various phycobilin pigments and Chl *a* in akinetes. However, at low temperature there may be a more direct energy transfer in akinetes from PC to Chl *a* bypassing APC and APC-B, as suggested for *Anacystis* by Csatorday *et al.* (1978). akinetes produced very feeble delayed fluorescence compared to vegetative cells. The photosystem II activity of akinetes seems to be quite low (Björn *et al.*, 1983).

Akinetes, like heterocysts, may prove to be valuable material for the study of PSI photochemistry. Although heterocysts have been used for analysis of cyclic electron flow around PSI (Houchins and Hind, 1983), no such comparable studies are available with akinetes. Analysis of fluorescence transients may provide interesting information on the development of PSII in germinating akinetes.

C. Tolerance to Salt, High Light, and Desiccation Stress

Some blue-green algae occupy a wide range of habitats where they can be exposed to wide temperature fluctuations, extreme desiccation, high light intensities, and salt concentrations. Most submerged marine red

algae experience only moderate changes in environmental conditions; however, certain intertidal species can tolerate environmental extremes. A case in point is the red alga *Porph. perforata,* whose flat, expanded blade in some species is only one cell layer thick and is routinely exposed to air drying during low tides. The salt concentration in the water remaining around the algal tissue can increase up to 10 times as a result of evaporation. Smith (1983) found that this alga can lose up to 90% of its fresh weight during desiccation at low tide and that dehydration either by air drying or by high salt concentration causes inhibition of photosynthesis. Wiltens *et al.* (1978) studied the fluorescence induction of high and low intertidal species of algae. Tolerant red algae such as *Porph. perforata* and *Porph. sanjuanensis* recovered their photosynthetic competence rapidly on rehydration of desiccated samples, while the low subtidal red algae *Porph. miniata, Porph. latissima, Porph. fascia,* and *Nitophyllum notti* did not recover when rehydrated from a loss of only a small fraction of their water content (see also Satoh *et al.*, 1983).

It is well known that photoinhibition can occur as a result of inhibition of photosynthesis under high light intensities (Powles *et al.*, 1979, 1980; Fork *et al.*, 1981; Osmond, 1981; Satoh and Fork, 1982); nevertheless, dehydrated *Porph. perforata* does not become photoinhibited under high light intensity when its photosynthesis is inhibited. This alga has apparently evolved several mechanisms to protect it during severe environmental conditions. Analysis of fluorescence at 77°K, using the model of Butler (1978, 1979) for *Porph. perforata* that had been severely desiccated (91% water loss), showed an increased distribution of quanta to PSI (Öquist and Fork, 1982). This increase was produced by an increase in α, the absorption cross section of PSI, as well as by an increased "spillover" of energy from PSII to PSI (Butler, 1978). These changes may represent a protective mechanism whereby quanta absorbed by nonfunctional PSII reaction centers are transferred to PSI, where they are perhaps disposed of harmlessly as heat. Measurements made at physiological temperatures (Fork and Öquist, 1981) also showed that desiccation induced an increased transfer of energy to PSI compared to that in wet samples. The PSI component of fluorescence with a lifetime of 150 ps (see Section VIII,A) was found by Karukstis and Sauer (1984) to be dramatically increased on desiccation of *Porphyra.* The fluorescence lifetime of APC was also increased on drying. Dehydration increased the lifetime of F690, suggesting a less well coupled association of the Chl *a* antenna with PSII reaction centers. The increased lifetime of the F730 band on drying agrees with the suggestion mentioned above that one of the mechanisms by which desiccated *Porph. perforata* avoids photodynamic damage in strong light is transfer of excess excitation energy to PSI.

Porphyra perforata dried in the dark could photoreduce PSII traps at about half the capacity of normally hydrated plants. This decrease was probably caused by an increase in α, mentioned above, as was seen from measurements made at 77°K. *Porphyra perforata* dried in strong light seems to have all of its PSII traps closed. P700 changes can still be seen in light-dried *Porph. perforata,* however (Fork and Hiyama, 1973). Since light drying affected not the initial fluorescence level (F_0), but only variable fluorescence (F_v), it appears that fluorescence quenching by reaction centers of PSII occurs, since under desiccated conditions these centers would most probably be in the state ($P680^+Q_A^-$) where P680 is oxidized and Q_A (the primary quinone electron acceptor) is reduced. Butler (1978) has found this state to be an effective quencher of Chl *a* fluorescence.

XIII. Concluding Remarks

This chapter has outlined how the blue-green and red algae as well as the cryptomonads have been employed to study the basic mechanisms of photosynthesis, how diverse pigments are ordered for efficient absorption and transfer of light energy, and how these organisms have become adapted to life in extremely diverse habitats.

One of the great advantages of using these algae is that the absorption bands of their photosynthetic pigments are quite widely separated, allowing almost exclusive excitation of phycobilins but not Chls and vice versa. Since the photosynthetic pigments of these organisms are so widely separated, it is often easier to interpret action spectra for photosynthesis and fluorescence as well as fluorescence emission spectra than it is in plants containing only various Chls having relatively closely spaced absorption bands.

The fact that these algae can be readily cultured in the laboratory is also a great advantage. Many of them are unicellular or filamentous and are the starting material for preparations of membrane fragments, PSI and PSII particles having high activities, as well as Chl proteins.

Unlike other algae, the red and blue-green algae have their accessory phycobiliproteins organized into PBsomes that are located on the exterior surface of the thylakoid membrane and can be easily removed and studied. The PBsome has proved to be an excellent model in which to study the assembly of pigments for efficient capture and transfer of light energy to reaction centers of photosynthesis.

Finally, many of these algae have adapted to live in habitats where they experience severe desiccation (either by air drying or by hypersaline conditions), high light intensities, and high or low temperatures,

often in combination. Study of the mechanisms employed by these plants to avoid damage to their photosynthetic apparatus can yield interesting and valuable new results and, in turn, help to gain a better understanding of the basic mechanisms of photosynthesis.

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17

Fluorescence Properties of Chlorophyll *b*- and Chlorophyll *c*-Containing Algae

GOVINDJEE

Departments of Physiology and Biophysics and Plant Biology
University of Illinois at Urbana–Champaign
Urbana, Illinois 61801

KAZUHIKO SATOH

Department of Pure and Applied Sciences
College of Arts and Sciences
University of Tokyo
Tokyo 153, Japan

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ABBREVIATIONS AND SYMBOLS

BChl	Bacteriochlorophyll
Chl	Chlorophyll
Cyt *f*	Cytochrome *f*
DCPIP	2,6-Dichlorophenolindophenol
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea; diuron
F_λ	Emission band at wavelength λ, in nanometers (nm)
FCCP	Fluorocarbonyl cyanide phenylhydrazone
I	Primary electron acceptor of PSII, pheophytin
kD	kilodalton
LDS	Lithium dodecyl sulfate

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LHC	Light-harvesting complex
LHCP	Light-harvesting Chl *a*/Chl *b* protein complex
M_r	Molecular weight
O → I → D → P → S → M → T transient	Sequence of fluorescence levels during continuous illumination with time (O, origin; I, inflection; D, dip; P, peak; S, semisteady state; M, maximum; T, terminal steady state)
P680	Primary electron donor of PSII; reaction center Chl of PSII
P700	Primary electron donor of PSI; reaction center Chl of PSI
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

ABSTRACT

Light energy absorbed by photosynthetic pigments is used in photochemistry or lost as heat (internal conversion) or light [fluorescence; phosphorescence; delayed light emission (DLE)]. In this chapter we shall provide a brief review on the fluorescence properties of greenish [chlorophyll (Chl) *b*-containing] and brownish (Chl *c*-containing) algae and their possible relevance to photosynthesis. In Section I we mention the origin and phylogeny of photosynthesizing organisms, including the prokaryotes, whose fluorescence is discussed in the accompanying chapters. In Section II we describe the light-harvesting, i.e., the antenna, system; this includes a description of the photosynthetic pigments (Chl *a*, Chl c_1, Chl c_2, fucoxanthin, peridinin, etc.) and the pigment–protein complexes [light-harvesting complex (LHC), Chl *a*–Chl *c*–carotenoid complexes, etc.]. Except for some of the peridinin–Chl *a* complexes, the pigment–protein complexes are intrinsic proteins embedded in the thylakoid membrane. The transfer of excitation energy from Chl *b* and Chl *c* to Chl *a* is highly efficient, but that from the various carotenoids to Chl *a* is of variable efficiency. In Section III the significance of the various measured fluorescence parameters for photosynthesis is discussed. These discussions include: (a) the lifetime, τ, of fluorescence, which provides information on the excited states involved and relates the quantum yield of fluorescence, ϕ_f, to the natural lifetime of fluorescence, τ_0, which in turn is related to light absorption; (b) the quantum yield of fluorescence, which is a measure of the probability of light emission with respect to the probabilities of all other deactivation pathways of the excited state; (c) emission spectra, which provide information on the composition and the character of the emitting species; (d) the excitation spectrum of fluorescence, which provides information on the composition of the pigment systems and on the efficiency of the excitation energy transfer; (e) fluorescence induction under continuous illumination (also called fluorescence transients or the Kautsky phenomenon), which provides information on the electron flow on the electron donor and acceptor side of photosystem II (PSII) and its interaction with PSI; (f) flash-induced changes, which provide information on the primary photochemistry and the subsequent reactions in PSII, such as the electron flow that restores P680 from P680$^+$, where P680 is the reaction center Chl *a* of PSII, and the electron flow that leads to the recovery of Q_A from Q_A^-, where Q_A is the first quinone

electron acceptor of PSII; and (g) fluorescence polarization, which provides information on the excitation energy migration and on the orientation of pigments *in vivo*. Recent studies cited in Section IV show that fluorescence studies in algae can also provide information on the regulation of excitation energy distribution and redistribution between the two photosystems or photosynthesis.

I. Introduction

This chapter deals with the fluorescence properties of Chl *b*- and Chl *c*-containing greenish (Chlorophyta, Euglenophyta) and brownish (Chromophyta, Dinophyta) algae. Mention will also be made of the prokaryotic greenish chloroxybacterium *Prochloron,* since it contains Chl *b*. Fluorescence characteristics of the phycobilin-containing algae (Rhodophyta, Cyanophyta (or Cyanobacteria), and Cryptophyta) and of *Prochloron* are discussed by Fork and Mohanty in Chapter 16, of the photosynthetic bacteria (both purple and green) by Amesz and Vasmel in Chapter 15, and of the primitive halobacteria by R. Govindjee and Ebrey in Chapter 14 of this volume. The bioluminescence properties of dinoflagellates (Dinophyta) are discussed by Hastings in Chapter 13.

This book deals with the *fluorescence* of various organisms, but we shall digress somewhat and discuss the *evolution* of some of these organisms. It is now generally believed that eukaryotic algae have evolved, in several parallel lines, by symbiosis of prokaryotic photosynthesizing bacteria with other bacteria or protozoa (see, e.g., Margulis, 1981). The details of the process are unknown, and the origin and phylogeny of eukaryotic algae are highly controversial. However, it is not too far-fetched to consider that the prokaryotic *Prochloron* (Chloroxybacterium), which is closely related to cyanobacteria, may be an ancestor of the green algae (Chlorophyta) and/or the euglenids (Euglenophyta) in view of the presence of Chl *b* in all these three groups. In the same way, one could speculate that cyanobacteria may be an ancestor of red algae (Rhodophyta) since they both contain phycobilins and phycobilisomes. Cryptomonads (Cryptophyta) are an interesting group since they contain phycobilins and Chl *c* but no phycobilisomes; their origin is uncertain. We speculate that all the Chl *c*-containing brownish algae (Chromophyta) may have evolved from as yet undiscovered Chl *c*-containing bacteria. [The reader should note that Chromophyta include the brown algae (Chrysophyceae), yellow-green algae (Xanthophyceae), and diatoms (Bacillariophyceae), and contain the carotenoid fucoxanthin.] On the other hand, many of the Chl *c*-containing dinoflagellates (Dinophyta), which contain the carotenoid peridinin instead of fucoxanthin, must have evolved separately as they are quite distinct from the

Chromophyta in their morphology. Wilcox and Wedemayer (1985) suggested that the "blue-green" chloroplast of the dinoflagellate *Amphidinium wigrense* has evolved from an endosymbiotic eukaryote (a cryptomonad).

In contrast to algae, the origin and phylogeny of prokaryotes are somewhat better known (see Fig. 1, based on discussions with C. Woese and D. Blubaugh) from the nucleotide sequences of their RNAs (ribonucleic acids) (Fox *et al.*, 1980). There are 10 (almost parallel) lines of evolution from a "common ancestor." We speculate the existence of an eleventh Chl *c*-containing line (see dashed line, Fig. 1). Out of the 10 lines, there are 5 photosynthesizing lines: (1) *Heliobacterium chlorum* (a gram-positive, BChl *g*-containing bacterium); (2) chloroxybacteria (e.g., *Prochloron*) and cyanobacteria (the only Chl *a*-containing O_2-evolving line); (3) purple bacteria (BChl *a*- or BChl *b*-containing), which are divided into three subgroups: the α and β groups (both are purple nonsul-

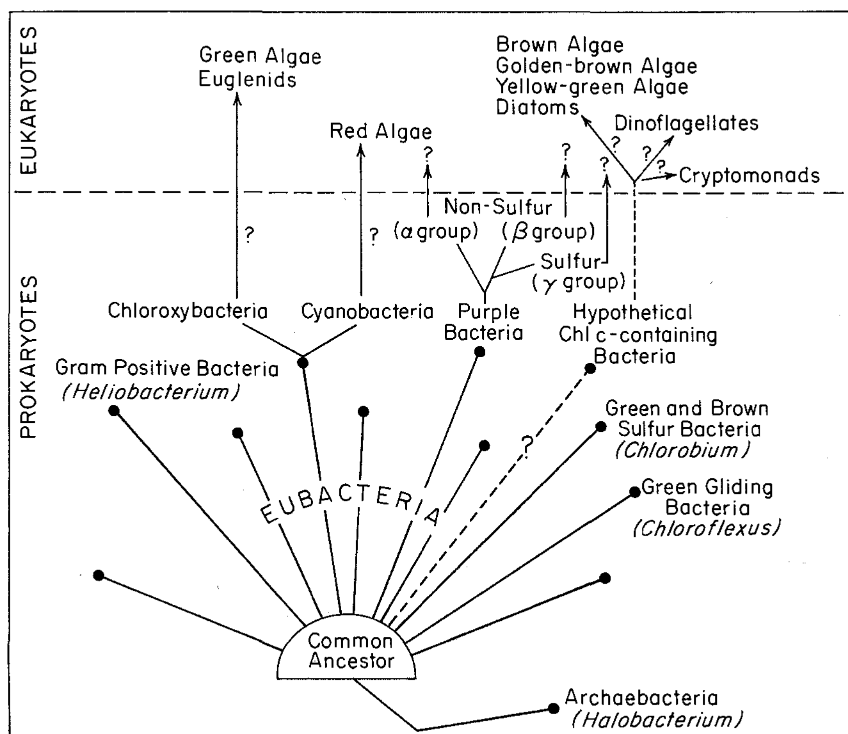


FIG. 1. Working hypothesis for the evolution of algae, cyanobacteria, and photosynthetic bacteria. See text.

fur bacteria, Rhodospirillaceae) and the γ group (purple sulfur bacteria, Chromatiaceae) (see Woese *et al.*, 1984a,b); (4) green and brown sulfur bacteria (Chlorobiaceae), which, in addition to BChl *a*, contain BChl *c* or *d* or *e*; like PSI of green plants, they possess FeS centers as secondary electron acceptors; and (5) green gliding bacteria (e.g., *Chloroflexus*), which are like the green sulfur bacteria in their pigment content but resemble the purple bacteria in their photochemical reactions (see Amesz, 1983; Blankenship, 1984, 1985). All the bacteria mentioned thus far are evolutionarily as far removed from the eukaryotes as they are from the so-called archaebacteria, which contain, among others, methanogens, and the light-transducing, bacteriorhodopsin-containing, H^+-pumping, and ATP-synthesizing *Halobacterium halobium.*

In the following, we shall first discuss the light-harvesting systems and then fluorescence from greenish (Chl *b*-containing) and brownish (Chl *c*-containing) algae. However, Chl *b*-containing *Prochloron* and Chl *c*-containing cryptomonads are discussed more fully by Fork and Mohanty (Chapter 16, this volume). For earlier reviews on Chl *a* fluorescence, see Govindjee and Papageorgiou (1971), Govindjee and Mohanty (1972), Goedheer (1972), Govindjee *et al.* (1973), Govindjee and Braun (Zilinskas) (1974), Mohanty and Govindjee (1974), Papageorgiou (1975), Lavorel and Etienne (1977), and Krause and Weis (1984). For a background on general aspects of photosynthesis, the reader is referred to Clayton (1980) and earlier volumes edited by Govindjee (1975, 1982a,b).

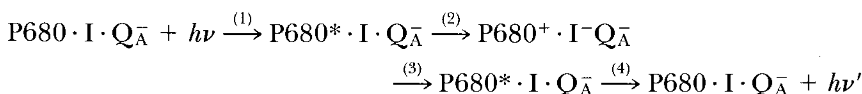
II. Light-Harvesting Systems

A. General

In photosynthesis of all plants and photosynthesizing bacteria, a collection of pigment molecules act as an antenna in absorbing light energy and transferring the excitation energy to specialized reaction center Chl *a* (or BChl) molecules, where the primary photochemistry occurs. This assemblage of pigments is referred to as the *photosynthetic unit,* and it contains the light-harvesting system of photosynthesis. It is now generally accepted that there are two light-harvesting pigment systems (PSI and PSII; Duysens *et al.*, 1961), with PSI being weakly fluorescent and PSII being somewhat more strongly fluorescent (see, e.g., Lavorel and Etienne, 1977). The electron transport pathway and the electron carriers involved in PSI and PSII reactions are shown in Fig. 1 in Duysens, Chapter 1, and Sane and Rutherford, Chapter 12, in this volume.

Before we begin a discussion of the light-harvesting system, we shall first review some well-known aspects of Chl *a* fluorescence in order to be

able to discuss the data in a rational fashion in this chapter. (For original references and a detailed description of this phenomenon, see Chapter 1 by Duysens and Chapter 10 by van Gorkom in this volume.) Fluorescence originating during the energy transfer process, i.e., before the photochemistry occurs, is labeled O level fluorescence (or, simply F_0); PSI contributes very little to F_0. As photochemistry occurs in PSII, and the quinone electron acceptor Q_A is reduced, the fluorescence yield of PSII increases. This fluorescence is known as the "variable" fluorescence, and is suggested (see Klimov *et al.,* 1978) to originate as



$$P680 \cdot I \cdot Q_A^- + h\nu \xrightarrow{(1)} P680^* \cdot I \cdot Q_A^- \xrightarrow{(2)} P680^+ \cdot I^- Q_A^- \xrightarrow{(3)} P680^* \cdot I \cdot Q_A^- \xrightarrow{(4)} P680 \cdot I \cdot Q_A^- + h\nu'$$

where P680 is the reaction center Chl *a* of PSII and I is the primary electron acceptor pheophytin. The exciton created in reaction (3) is transferred back to the antenna molecules, and it appears from there as fluorescence, more appropriately as delayed fluorescence since the exciton was created by charge recombination in reaction (3) above (cf. van Gorkom, Chapter 10, and Jursinic, Chapter 11, this volume, for a critical discussion of this point). (Also see Note Added in Proof.)

Mauzerall (1985) recently succeeded in observing a lag or a rise time of 150–200 ps in the variable Chl *a* fluorescence yield from the green alga *Chlorella.* This finite rise time demonstrates an intermediate step between excitation and emission of the variable yield. One of the simplest interpretations is that 150–200 ps is the time for reaction (2) above, and that step (3), i.e., charge recombination, produces the variable fluorescence. The emission yield is low under the conditions $P680^+Q_A^-$, $P680 \cdot Q_A$, and $P680^+Q_A$, and is high only when both P680 and Q_A are in the reduced state: $P680 \cdot Q_A^-$.

The following is a description of the light-harvesting system in several Chl *b*- and Chl *c*-containing algae. We shall first describe the pigments, and then the pigment–protein complexes.

B. Photosynthetic Pigments

Chl *a* is present in all O_2-evolving photosynthetic organisms in the antenna as well as in the reaction center complexes (see, e.g., Prézelin and Alberte, 1978; Satoh and Butler, 1978; Anderson and Barrett, 1979; Green *et al.,* 1982). Chl *b* and Chl *c* serve only as light-harvesting pigments. Chl *b* is different from Chl *a* in only one group (see Fig. 1 in Chapter 5 by Seely and Connolly, this volume): CH_3 on ring II is replaced by CHO. Chl *c* is different from Chl *a* in that (1) it does not

contain the phytol side chain, (2) it has an unsaturated ring IV, and (3) it has an acrylic acid instead of a propionic acid side chain and a phytol on ring IV. These differences make Chl *c* more polar than both Chl *a* and Chl *b*. Compared to Chl *a* and Chl *b*, the absorption spectra of the two types of Chl *c* (c_1 and c_2; see, e.g., Jeffrey, 1969) are characterized by (1) weaker absorption in the red region relative to that in the blue region, and (2) a shift of the red band to shorter wavelengths and of the blue band to longer wavelengths (Fig. 2).

Chl *b* is found in green algae (Chlorophyta), in Euglenophyta, and in the green prokaryote *Prochloron*, whereas Chl *c* of two types, c_1 and c_2, is

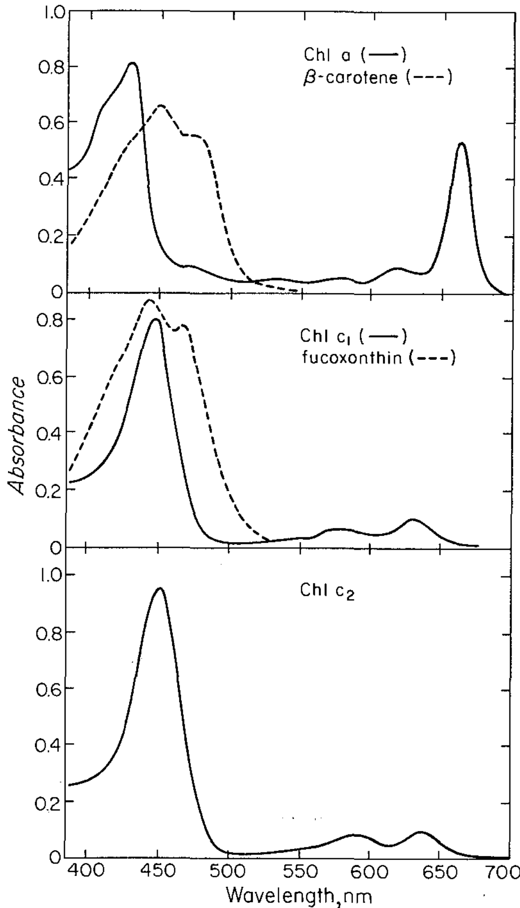


FIG. 2. Absorption spectra of several photosynthetic pigments *in vitro*. Spectra for Chl c_1 and Chl c_2 were redrawn from Jeffrey (1969).

TABLE I
Major Photosynthetic Pigments in Green and Brown Algae, Euglenids, Diatoms, and Dinoflagellates

Source	Chlorophylls	Main carotenoids
Dinoflagellates	a, c_2	Peridinin
	a, c_1, c_2	Fucoxanthin
Diatoms	a, c_1, c_2	Fucoxanthin
Brown algae	a, c_1, c_2	Fucoxanthin
Green algae	a, b	β-Carotene
Euglenophyta	a, b	β-Carotene

widely distributed in various marine brownish algae, diatoms, brown aglae, and dinoflagellates (Table I). Most cryptomonads (Cryptophyta) have only Chl c_2 (Jeffrey, 1969, 1976; see Fork and Mohanty, Chapter 16, this volume). Dinoflagellates (Dinophyta) that have the carotenoid fucoxanthin contain both Chl c_1 and c_2, but most of those that have the carotenoid peridinin possess only Chl c_2 (Jeffrey, 1976).

Like the chlorophylls, carotenoids [e.g., fucoxanthin (also called fucoxanthol) and peridinin] have an important role in collecting light energy (Tanada, 1951; Duysens, 1952; Haxo, 1960; Goedheer, 1970). The absorption peaks of fucoxanthin and peridinin in cells are usually shifted to about 20 nm to longer wavelengths compared to those of the same pigments in organic solvents (Fig. 2).

C. Pigment–Protein Complexes

It is generally believed that almost all photosynthetic pigments are associated with proteins; i.e., they exist as pigment–protein complexes. In plants there are essentially six light-harvesting complexes: two reaction center (RC) Chl *a* complexes (RC I or RC II complex) containing the reaction center Chl *a* (P700 or P680) and possessing several molecules of Chl *a* serving as the antenna; another set of two Chl *a* complexes (core Chl *a* complexes, CC-I or CC-II) containing only antenna molecules; and finally, two light-harvesting Chl *a*/Chl *b* protein complexes (LHCP-I or LHCP-II), again containing only antenna molecules. All six complexes act as the light-harvesting system of photosynthesis. The major difference between PSI and PSII is that the LHCP-II contains much more Chl *b* than the LHCP-I, and the Chl *a*-containing antenna of PSI possesses additional long-wavelength absorbing forms of Chl *a*.

The reaction center complexes of PSI and PSII have been isolated from various algae; they all contain *only* Chl *a*. In the following we shall

mainly discuss the peridinin–Chl *a*, Chl *a*/Chl *c*, and Chl *a*/Chl *b* complexes. For an excellent background on the photosynthetic pigments and models for their organization *in vivo*, the reader is referred to a review by Thornber and Barber (1979).

1. PERIDININ–CHLOROPHYLL *a* PROTEINS

Although it had already been known in the nineteenth century that at least part of peridinin is bound to a protein *in vivo* in dinoflagellates (Shütt, 1890),the nature of this pigment–protein was not studied until Haidack *et al.* (1966) isolated a water-soluble peridinin–Chl *a* protein from *Gonyaulax polyedra*. Further studies (Prézelin and Haxo, 1976) showed that it is composed of a single polypeptide of 32 kD and that it contains four peridinins and one Chl *a* per protein. By contrast, *Glenodinium* sp. has two peridinin–Chl *a* proteins which are similar in M_r (about 35,000) but different in isoelectric point (pI, 7.4 and 7.3, respectively). For further details, see Siegelman *et al.* (1977).

The excitation spectrum of Chl *a* fluorescence in the chromoprotein from *Amphidinium carterae* (Fig. 3) suggests that light energy absorbed by peridinin is transferred efficiently to Chl *a* (Haxo *et al.*, 1976). Song *et al.* (1976) and Koka and Song (1977) have shown that each Chl *a* is surrounded by two pairs of peridinin molecules with an appropriate orientation to ensure efficient energy transfer from peridinin to Chl *a*. These peridinin–Chl *a* proteins are the light-harvesting pigment–proteins and

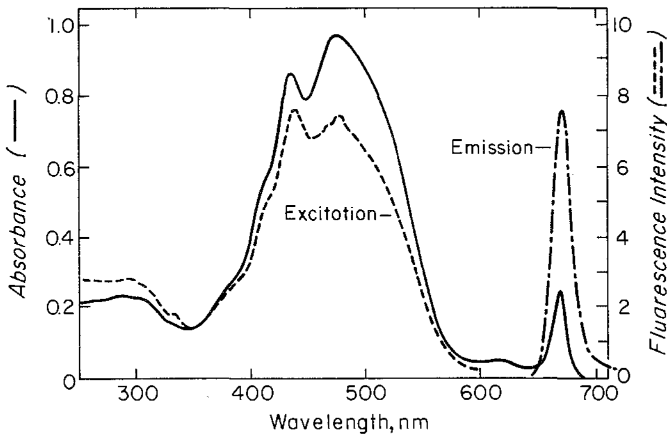


FIG. 3. Absorption, action, and emission spectra of fluorescence of a peridinin–chlorophyll *a*-protein complex from *Amphidinium carterae*. Solid line, absorbance; dashed line, action spectrum; and dash-dotted line, emission spectrum. Redrawn from Haxo *et al.* (1976).

are, perhaps, located peripherally on the photosynthetic membrane (Prézelin and Alberte, 1978) since they are easily extracted from the system (Haidack *et al.*, 1966; Prézelin and Haxo, 1976). These pigment–protein complexes are highly variable in amount, depending on the culture conditions (Prézelin, 1976; Prézelin and Haxo, 1976). In addition to the water-soluble proteins discussed above, there are also intrinsic membrane pigment–proteins which contain peridinin and Chl *a* (see, e.g., Boczar *et al.*, 1980).

2. CHLOROPHYLL *a*/CHLOROPHYLL *c* PROTEINS

All Chl *c* seems to be bound to intrinsic proteins to form light-harvesting Chl *a*/Chl *c* proteins. A comparison of the absorption and fluorescence excitation spectra of Chl *a*/Chl *c* proteins suggests efficient energy transfer from Chl *c* to Chl *a* (Anderson and Barrett, 1979; Alberte *et al.*, 1981). Most of the Chl *a*/Chl *c* proteins also contain the carotenoids (peridinin or fucoxanthin), which also function as light-harvesting pigments. Like other light-harvesting pigment–proteins (Prézelin and

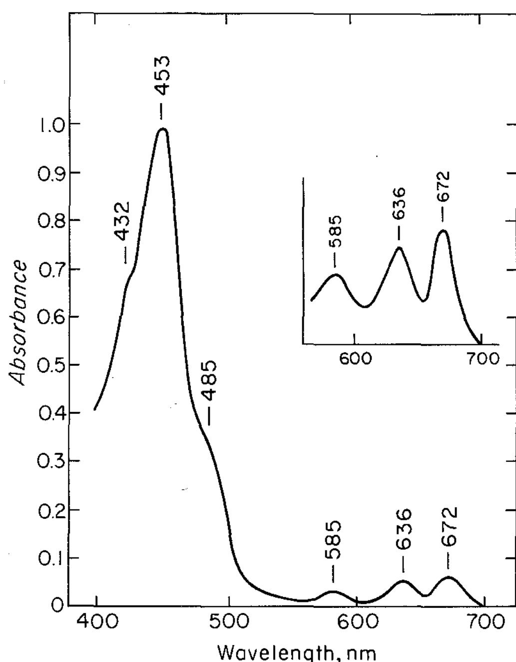


FIG. 4. Absorption spectrum of a chlorophyll *a*–chlorophyll *c*–protein complex from *Glenodinium* sp. Inset shows an enlarged portion of the absorbance bands in the red region of the spectrum. Redrawn from Boczar *et al.* (1980).

Haxo, 1976; Boardman *et al.*, 1978), the amounts of Chl *a*/Chl *c* proteins vary with the culture conditions (Anderson and Barrett, 1979).

Among the four Chl proteins obtained by Boczar *et al.* (1980) from the dinoflagellates *Gonyaulax polyedra* and *Glenodinium* sp., one was enriched in Chl *c*, having a Chl *c*/Chl *a* ratio of about 4.8, and contained xanthophylls (fucoxanthin, etc.). Figure 4 shows the absorption spectrum of this Chl *c*-enriched protein, where the 636-nm band is due to Chl *c* and the 672-nm band to Chl *a*. For a recent description of a Chl *a*/*c* protein containing fucoxanthin from the yellow-green alga *Synura petersenii*, see Wiedemann *et al.* (1983). A number of similar pigment–proteins have been isolated from brown algae and diatoms, and their properties vary considerably depending on the algal species and the isolation methods used. Table II summarizes the results on the various Chl *a*/Chl *c*–carotenoid proteins from four different algae (*Hormosira, Acrocarpia, Lami-*

TABLE II
Light-Harvesting Pigment–Proteins in Brown Algae and Diatoms

Species	Ref.	Methods	Pigment–proteins	Fluorescence maximum (nm)
Hormosira sp. (brown alga)	Kirk (1977)	0.05% Triton X-100, hydroxyapatite chromatography	Chl *a*, *c*, fucoxanthin, β-carotene–protein (5.7 : 1.1 : 3.0 : 0.6)[a]	—
			Chl *a*, *c*, fucoxanthin, violaxanthin, β-carotene–protein (6.4 : 0.7 : 3.0 : 1.2 : 0.8)	—
Acrocarpia paniculata (brown alga)	Barrett and Anderson (1977, 1980)	1% Triton X-100, sucrose density gradient	Chl *a*, c_2, fucoxanthin–protein (2 : 1 : 2)	680
			Green complex: Chl *a*, c_1, c_2, violaxanthin–protein (8 : 1 : 1 : 1)	683
			Orange complex: Chl *a*, c_2, fucoxanthin–protein (2 : 1 : 2)	683
Laminaria saccharinia (brown alga)	Alberte *et al.* (1981)	SDS–PAGE	Chl *a*, *c*–protein (2 : 1)	680
			Chl *a*, fucoxanthin–protein (1 : 4–6)	676
Phaeodactylum tricornutum (diatom)	Gugliemelli *et al.* (1981)	Broken cells treated with sodium lauryl sarcosinate	Chl *a*, *c*, fucoxanthin–protein (1 : 1 : 4)	690

[a] Numbers in the parentheses are the ratios of the component pigments.

naria, and *Phaeodactylum*). Examination of this table shows that it is difficult to draw a general picture.

3. CHLOROPHYLL *a*/CHLOROPHYLL *b* PROTEINS

Such proteins have been isolated from several greenish algae (Table III). What is important to note is that Chl *a*/Chl *b* proteins are present not only in PSII, but also in PSI (CPO in *Chlamydomonas* and LHC I in *Codium*).

Following Apel *et al.* (1975) and Apel (1977), who obtained only two Chl proteins from *Acetabularia,* Green and Camm (1981, 1982) and Green *et al.* (1982) resolved seven Chl-containing bands by using the detergent β-octyl glucoside as a solubilizing agent. Of these seven bands, four contained both Chl *a* and Chl *b* (see Table III).

Following the early work of Kan and Thornber (1976) on the light-harvesting Chl *a*/Chl *b* protein from *Chlamydomonas,* Delepelaire and Chua (1981) obtained several Chl *a*/Chl *b* proteins from photosystem II (CP-II-a, -b, -c, -d, and -e, which had several polypeptides of M_r between 25,000 and 33,000 in various ratios). Wollman and Bennoun (1982) found another Chl protein (CPO), which also had several polypeptides between 19 and 28 kD. This complex emits fluorescence at 705 nm at 77°K, and was proposed to be the antenna complex of PSI.

TABLE III
Chlorophyll *a*/*b* Protein Complexes in Several Algae

Genus	Ref.	Chl *a*/*b* proteins	Characteristics
Acetabularia	Green *et al.* (1982)	CP29 (a : b ratio, 3.4)	Internal antenna of PSII (apoprotein, 29 kD)
		CPII (a : b ratio, 1.0)	Apoprotein, 27 kD
		CPII*	Oligomeric form of CPII
		D	Oligomeric form of CP29
Chlamydomonas	Delepelaire and Chua (1981); Wollman and Bennoun (1982)	CP0	Part of antenna of PSI (27.5, 27, 25, 23, 19 kD)
		CPII-a,-b,-c,-d,-e	LHCPII (33, 30.5, 27.5, 27, 26.5, 25 kD)
Chlamydobotrys	Brandt *et al.* (1982)	LHCPb	LHCPII (several polypeptides)
Euglena	Brown (1980)	LHCP	LHCPII (several polypeptides)
Codium	Anderson (1983); Chu and Anderson (1985)	LHCPI	Oligomeric form of LHCP 3
		LHCP3	LHCPII (several polypeptides)
		LHCI	LHC of PSI

Light-harvesting Chl *a*/Chl *b* proteins have also been isolated from *Chlamydobotrys* (Brandt *et al.*, 1982), *Euglena* (Brown, 1980), *Codium* (Anderson, 1983; Chu and Anderson, 1985), *Chlorella fusca* (Wild and Urshel, 1980), and *Ulva mutabilis* (Huskovd *et al.*, 1982). In a first approximation, these complexes are similar to those from higher plants (see Briantais *et al.*, Chapter 18, this volume). However, Chl *a*/Chl *b* proteins and CPI from *Prochloron* (Hiller and Larkum, 1985) seem to be quite different from those in green algae and higher plants. Comparison of Chl proteins from a wide variety of algae is important not only from the evolutionary point of view, but also from the point of view of understanding the structure and function of Chl proteins.

III. Physical Parameters of Fluorescence

After a brief comparison of fluorescence of Chl *a* in solution and *in vivo,* we shall discuss some examples of fluorescence data obtained for the brownish (Chl *c*-containing) and the greenish (Chl *b*-containing) algae.

A comparison of the fluorescence characteristics of dilute solutions of Chl *a* and of Chl *a* in algae shows the following differences:

(a) The emission spectra are red-shifted by ~20 nm *in vivo* (685 nm) from that *in vitro* (665 nm) at room temperature; this emphasizes that Chl *a* in algae is in a distinctly different environment.

(b) The fluorescence yield (ϕ_f) (see Latimer *et al.*, 1956) and the measured lifetime of fluorescence (τ_f) are low (ϕ_f, 3–6%; τ_f, 1 ns, several components) compared to those *in vitro* (ϕ_f, 30%; τ_f, 5 ns), suggesting efficient energy utilization for photosynthesis in algae.

(c) Both the action and emission spectra of fluorescence in algae are complex and show the presence of several spectral forms of Chl *a in vivo;* under certain experimental conditions, especially at very low temperatures, the *in vivo* emission spectra of algae (e.g., *Chlorella*) may show up to six bands: F665 (a minor band from Chl *b*?), F680 (a minor band from Chl *a* of LHCP-II), F685 (Chl *a*-antenna of PSII, Chl *a*-II), F695 (reaction center complex II), F705 (an antenna of PSI), and F720 (another antenna of PSI).

(d) The degree of polarization of fluorescence in algae is low, and this may partly be due to extensive energy migration.

(e) Chl *a* fluorescence in algae undergoes characteristic changes with time of illumination (see, e.g., Govindjee and Papageorgiou, 1971; Papageorgiou, 1975; Briantais *et al.*, Chapter 18, this volume); such changes reflect the dynamic character of the photosynthetic system in algae; for

methods, see Lavorel *et al.* (Chapter 4, this volume) and Schreiber (1983).

A. Lifetimes and Yields

Lifetime of light emission provides information on the nature of the excited states involved. For example, if triplet states are involved, phosphorescence is observed which not only has a longer-wavelength emission than fluorescence, but also has a long lifetime (milliseconds) (see Hoff, Chapter 9, this volume). In photosynthesis, we usually deal with the singlet states (see Shipman, 1982), which last from picoseconds to nanoseconds (see Pearlstein, 1982). If the light emission is preceded by the creation of excitons by chemical back reactions (delayed fluorescence; see Jursinic, Chapter 11, this volume), its decay time reflects the time of the stabilization reactions and/or the actual recombination reactions. However, excitons created by light absorption decay by the prompt fluorescence pathway, basically, in competition with the excitation energy transfer, trapping at the reaction center, and other radiationless losses. The fluorescence decay and its lifetime can provide information not only on the reactions that cause it, but also on the heterogeneity of the pigment systems involved, their organization, and the excitation energy pathways and photochemical reactions of photosystems. We shall first briefly summarize the early work on the lifetime of fluorescence in algae, and then discuss our present understanding of this field, particularly in green algae. For references to early literature and for further details, the readers should consult Moya *et al.* (Chapter 7, this volume) and reviews by Lavorel and Etienne (1977), Govindjee and Jursinic (1979), and Karukstis and Sauer (1983).

The measured lifetime of fluorescence (τ_f) is related to the quantum yield of fluorescence (ϕ_f) by the simple relationship: $\tau_f = \phi_f \tau_0$, where τ_0 is the natural lifetime of the excited state, calculated from the integration of the absorption spectrum of the fluorescent state. Conclusions drawn in the early work (see, e.g., Brody and Rabinowitch, 1957; Müller and Lumry, 1965; Merkelo *et al.*, 1969; Müller *et al.*, 1969; Briantais *et al.*, 1972) on the green alga *Chlorella*, by both the flash and the phase methods, were: (1) some nonfluorescent (or weakly fluorescent) Chl *a* exists *in vivo*, (2) τ_f increases from a value of ~0.6 to ~2.0 ns as the reaction centers close, and (3) an exciton finding a reaction center closed migrates to pigment beds with open reaction centers. It was generally assumed that there is only one τ_f component. However, the existence of more than one τ_f component in photosynthetic systems, at room temper-

ature, was demonstrated by Malkin *et al.* (1980) from their data on leaves of higher plants. What was measured in the older work was an average τ_f and new measurements with refined techniques now show several different values for τ_f, which can easily explain the older results (see Moya *et al.,* Chapter 7, this volume).

Haehnel *et al.* (1983) measured three lifetime components in the green algae *Chlorella vulgaris* and *Chlamydomonas reihardtii* by the single-photon direct flash method (Table IV). The interpretation of the three observed τ_f components (labeled τ_1, τ_2, and τ_3, Table IV) is not yet certain, but it is clear that τ_2 and τ_3 belong to PSII. The fastest component, τ_1, is in all likelihood made up of a PSI component and a PSII component (energy transfer from core Chl *a* II complex to RC II complex). On the basis of data with several mutants of *Chlamydomonas,* Gulotty *et al.* (1985) suggested that τ_1 of ~90 ps is composed of a 50-ps PSI and a 150-ps PSII component. Emission spectra for τ_1 in *Chlorella* by Kushida *et al.* (1981) and Wendler *et al.* (1984) support the PSI nature of τ_1. The τ_2 could reflect energy transfer either from LHCP-II to RC II or from one PSII to another independent PSII unit (called a "puddle" model). The τ_3 has been suggested to originate in charge recombination at the reaction center of PSII, with excitons created as $P680^+ \cdot I^- \rightarrow P680^*I$, where I refers to pheophytin; these excitons probably migrate to the antenna before being emitted (see Moya *et al.,* Chapter 7, this volume, and Karukstis and Sauer, 1983, for further discussion). Another explanation, which does not involve the charge recombination hypothesis, is that τ_3 simply originates in PSII α units where there is no barrier in energy transfer among different units (called a "lake" model) (see, e.g., Magde *et al.,* 1982, for interpretations concerning higher plant chloroplasts).

Further experiments on detergent-free PSI and PSII particles from algae are needed to answer questions regarding the identification of all the τ_f components.

TABLE IV
Lifetime of Fluorescence of Green Algae

Algae	Fluorescence level	τ_1	τ_2	τ_3	Ref.
Chlorella	F_0	0.13	0.5	1.4	Haehnel *et al.* (1983)
vulgaris	F_{max}	0.10	1.2	2.2	Haehnel *et al.* (1983)
Chylmydomonas	F_0	0.11	0.6	1.4	Haehnel *et al.* (1983)
reinhardtii	F_{max}	0.06	1.1	2.3	Haehnel *et al.* (1983)
	F_0	0.09	0.4	1.4	Gulotty *et al.* (1985)

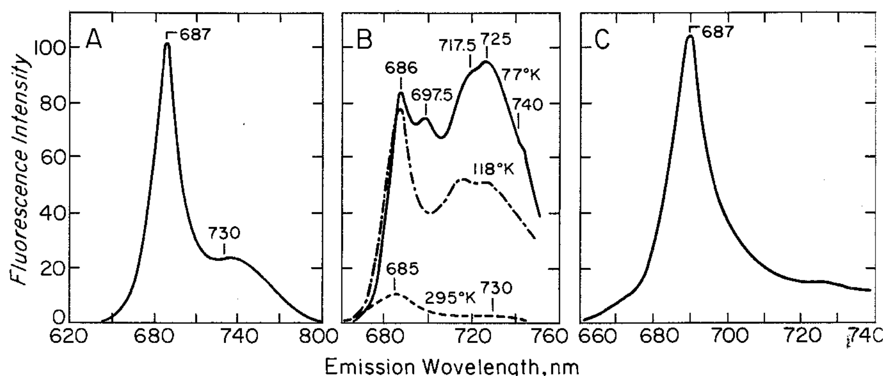


FIG. 5. Emission spectra of algae. (A) *Chlorella vulgaris* at room temperature (Ghosh *et al.*, 1966). (B) *Chlorella pyrenoidosa* at 77 and 118°K; for comparison, the room temperature spectrum is also shown (Cho and Govindjee, 1970a). (C) *Gonyaulax polyedra* (cultured in a light–dark cycle) at 77°K (Govindjee *et al.*, 1979).

At 77°K, τ_f is 2.3 and 1.4 ns, respectively, for F730 and F685 in *Chlorella* (Mar *et al.*, 1972; Hervo *et al.*, 1975). (See Moya *et al.*, Chapter 7, this volume, for further references and a detailed discussion of this topic.)

B. Emission and Excitation Spectra

1. EMISSION SPECTRA

a. Room Temperature: Multiplicity of Bands. In green algae, the room temperature fluorescence spectrum appears generally as a single major band at ~685 nm, accompanied by a vibrational band at ~740 nm (Fig. 5A; Duysens, 1952; Ghosh *et al.*, 1966). In brown algae and diatoms, the main emission band is often at 681 nm. In spite of the simplicity of this spectrum, it has been known for some time, based on a "matrix analysis"* (Weber, 1961) of fluorescence, that there is more than one emitting species even at room temperature in *Euglena* (Brody and Brody, 1963) and in *Chlorella* (Williams *et al.*, 1969). Recently, Marchiarallo and Ross (1985) extended this analysis to its ultimate form ("factor analysis") and provided emission spectra of the various emitting

* If fluorescence intensities at several wavelengths are measured after excitation with various wavelengths, the results can be set as an $m \times n$ matrix, with m columns determined by the wavelengths of excitation and n by the wavelengths of emission. The number of emitting components can be obtained from an analysis of this matrix (for details, see Weber, 1961).

components in several algae. More direct evidence for the existence of a band (or bands) around 700 nm in green algae was obtained from the following observations: (1) a band around 712 nm was inferred to exist when the emission spectrum at the maximum fluorescence (P level) was compared with that at the minimum fluorescence (O level) in *Chlorella,* the latter predominating in the 712-nm band (Lavorel, 1963), and (2) a small band around 695–700 nm was observed in the difference emission spectrum obtained when *Chlorella* cells were treated with different light regimes or with high and low light intensities (Papageorgiou and Govindjee, 1968b; Govindjee and Briantais, 1972).

Another small band at 665 nm was also noted in the difference spectrum, and was suggested to be due to Chl *b* emission (Govindjee and Briantais, 1972). To our knowledge, no emission from Chl *c* has been observed in intact cells of brown algae or diatoms since experiments similar to those on green algae have not yet been done with these organisms.

As surmised above, the green alga *Chlorella* has its main emission peak at ~685 nm in addition to at least one or more bands around 700 nm, excluding the vibrational band at 740 nm. In all likelihood, the 685-nm band is the strongly fluorescent PSII band and the 700-nm band is the weakly fluorescent PSI band. In other algae, however, distinct emission bands around 700–710 nm have been observed (for *Euglena* and *Ochromonas,* see Brown, 1966; Goedheer, 1981; for the green alga *Scenedesmus,* see Brown, 1967; Goedheer, 1981; for the diatom *Phaeodactylum,* see Brown 1967; Goedheer, 1973, 1981; and for the diatom *Detonula,* see Jupin, 1973). The band at 710 nm is often destroyed when the cells are broken (Brown, 1966). Several of these long-wavelength bands show fluorescence induction (see Section III,C), indicating that they may belong to PSII (Brown, 1967; Goedheer, 1973, 1981). However, a detailed analysis is not available to show that there was no significant contribution from the 685-nm band under the measuring conditions used and/or that the induction was not due to excitation energy transfer from PSII to PSI.

Mende *et al.* (1983) have shown that fluorescence spectra of *Chlorella* change during its life cycle, suggesting changes in energy distribution. It is important, therefore, that samples always be compared with cells of the same physiological age. Furthermore, stress effects can also be studied by measuring emission spectra [see, e.g., Harnischfeger and Jarry (1982) for changes in emission spectrum of *Chlorella* induced by cold treatment].

Chl *a* of PSI is only weakly fluorescent at room temperature. In addition to the emission bands mentioned above, some algae show distinct PSI fluorescence at ~720 nm (for *Chlorella vulgaris,* see Goedheer, 1981).

The PSI character of the 720-nm band at room temperature was confirmed by the fact that it did not show fluorescence induction and did not increase in intensity on addition of DCMU (Goedheer, 1981). DCMU is known to enhance PSII, not PSI, fluorescence by keeping Q_A in its reduced form Q_A^-, where Q_A is a quencher of fluorescence and Q_A^- is not (Duysens and Sweers, 1963).

b. Low Temperature. For low-temperature studies the reader is referred to reviews by Harnischfeger (1977) and by Amesz and Rijgersberg (1981). Brody (1958) discovered that, in addition to an emission band at 685 nm (F685), *Chlorella* cells have a new band at 725 nm (F720) at 77°K. Bergeron (1963), Brody and Brody (1963), Govindjee (1963), and Kok (1963) independently observed another new band at about 696 nm (F695) in the blue-green alga *Anacystis*, the green algae *Chlorella*, and *Scenedesmus*. It is now generally agreed that F685 and F695 belong to PSII and F720 to PSI. It was suggested that F695 originates in the reaction center complex of PSII (Govindjee, 1963); Breton (1983) suggested that F695 originates in pheophytin of the reaction center of PSII. Cho and Govindjee (1970a) showed that F720 is composed of at least two emission bands at 718 and 725 nm in *Chlorella* (see Fig. 5B), both of which originate in PSI. For studies on low-temperature spectra of mutants of *Chlorella sorokiniana*, the reader is referred to Lacambra *et al.* (1984).

In contrast to the broad band at 720 nm in most green and brown algae, this band in higher plants appears at 735 nm (see Briantais *et al.*, Chapter 18, this volume). Furthermore, in most cases the F735/F685 ratio is much higher in higher plant chloroplasts than the F720/F685 ratio in algae. This is often not due to a real difference; sometimes, it is due to higher reabsorption of short-wavelength components in either thick samples or samples having multiple path lengths (see Govindjee and Yang, 1966, for a relatively low F735/F685 ratio in a thin suspension of higher plant chloroplasts). Low-temperature fluorescence spectroscopy must be interpreted carefully because of the problems of artifacts. Several precautions must be taken (for a full discussion, see Govindjee, 1972) to reduce the possibility of reabsorption of the short-wavelength fluorescence bands: use of dilute samples; use of faster versus slower cooling (see, e.g., Fig. 1 in Cho and Govindjee, 1970a; Harnischfeger, 1977); use of front-surface optics; use of dimethyl sulfoxide as a protectant; and use of clear glass. Kramer (1984) has shown that excellent excitation and emission spectra can be obtained even with scattering samples. Kramer *et al.* (1985) have presented highly resolved 4°K emission and excitation spectra of *Chlorella vulgaris* and *Dunaliella salina*.

Furthermore, inclusion of a pigment that fluoresces at wavelengths different from that of the sample as a fluorescence standard is very useful in obtaining quantitative data at low temperatures.

Two fluorescence bands, F695 and F720, are almost absent in the dinoflagellates (Govindjee *et al.*, 1979) and in diatoms (Caron *et al.*, 1983) (Fig. 5C). We do not know whether the PSI pigment–protein complex in these algae is devoid of Chl *a* 695 responsible for F720, or whether there is another reason for the quenching of this band. The relative lack of F695 is more difficult to explain because it is predicted to arise from reaction center II. Further research is needed to understand the relationship of the four emission bands (F685, F695, F718, F725) to the various Chl *a* complexes in algae.

Cho *et al.* (1966) observed that as the temperature was lowered from 77 to 4°K, the F685/F695 ratio increased in *Chlorella* (Fig. 6). This suggested that the efficiency of excitation energy transfer from the core Chl

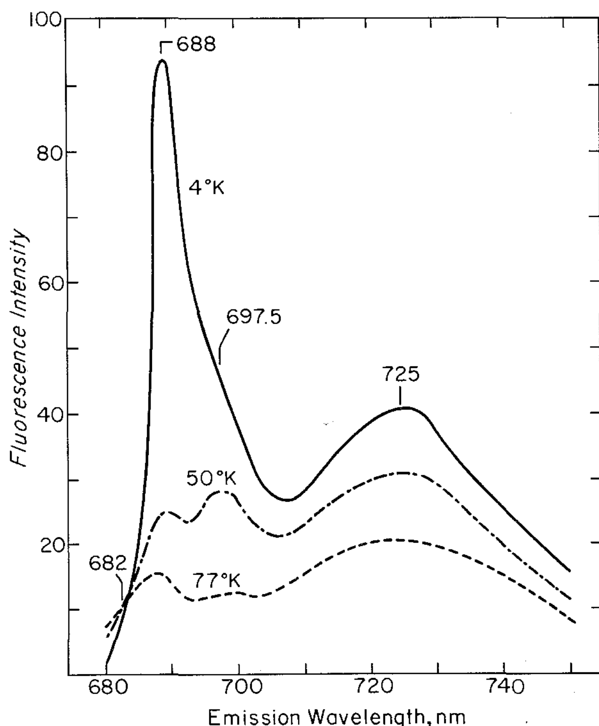


FIG. 6. Emission spectra of *Chlorella pyrenoidosa* at 4 and 50°K; for comparison, 77°K spectrum is also shown (Cho *et al.*, 1966).

a II complex to the reaction center II complex decreases at the lowered temperature. Experiments on the temperature dependence of emission spectra of *Chlorella* in the range 295 to 77°K revealed that the F720 band increased rather slowly up to 180°K, and then rapidly as the sample was cooled, whereas the F685 band increased rapidly up to 180°K, and then slowly (Cho and Govindjee, 1970a). One interpretation of these data is that the efficiency of energy transfer from PSII to PSI may decrease when the sample is cooled from 240 to 180°K, and then increase at still lower temperatures. Unfortunately, such conclusions are difficult to prove because changes in fluorescence intensity could arise for several other reasons as well. However, it is tempting to accept the notion of decreased energy transfer at lower temperatures due either to uncoupling of one Chl *a*–protein complex with the other, and/or to the reduction in the Förster overlap integral (see van Grondelle and Amesz, Chapter 8, this volume) between absorption and fluorescence due to sharpening of the bands at lower temperatures.

2. ACTION SPECTRA OF FLUORESCENCE

Action (excitation) spectra of fluorescence have been used extensively to study excitation energy transfer in photosynthesis of algae (see, e.g., the historical papers by Dutton *et al.*, 1943; Wassink and Kersten, 1946; Duysens, 1952). For an earlier review on action spectra, see Fork and Amesz (1969). In the well-known technique of sensitized fluorescence, excitation of a molecule B leads to fluorescence from another molecule A; this shows excitation energy transfer from B to A. The efficiency of this energy transfer can be calculated by measuring the action spectrum of the fluorescence of A, i.e., the fluorescence intensity of A per incident number of photons at different wavelengths of excitation, and the fractional absorbance spectrum of the system. The wavelength-dependent quantum yield spectrum of fluorescence of A, calculated from the above measurements, provides information on the efficiency of energy transfer. For example, if the quantum yield in the region of absorption of B is 50% of that in the region of absorption of A, then the efficiency of energy transfer is 50%. The earliest evidence for efficient (70–80%) excitation energy transfer in photosynthesis was from the carotenoid fucoxanthin to Chl *a* when fucoxanthin-sensitized Chl *a* fluorescence was observed in a diatom by Dutton *et al.* (1943). Goedheer (1970) reported a similarly high efficiency of excitation energy transfer, at 77°K, from fucoxanthin and from Chl *c* to Chl *a* in the diatom *Phaeodactylum tricornutum*.

a. Action Spectra at Room Temperature. The wavelength-dependent quantum yield spectrum of Chl *a* fluorescence at >690 nm in the green alga *Chlorella* shows a "red drop" around 685 nm suggesting the presence of nonfluorescent or weakly fluorescent Chl *a* molecules which preferentially absorb light beyond 685 nm (see, e.g., Szalay *et al.*, 1967; R. Govindjee *et al.*, 1968). The red drop in the fluorescence can be eliminated by aerobic sonication of *Chlorella* cells, and it can be related to the presence of a minor Chl *a* form absorbing around 695 nm (in all likelihood, a PSI component) which is weakly fluorescent at room temperature, but which is responsible for the fluorescence band at 720 nm (F720) at 77°K (Das and Govindjee, 1967). A consequence of the presence of a nonfluorescent or weakly fluorescent form of Chl *a*, in Chl *b*-containing algae, is that the ratio of Chl *b* to Chl *a* peaks is higher in the action spectrum of fluorescence than in the absorbance spectrum (see, e.g., Butler and Bishop, 1963; Goedheer, 1966).

A typical action spectrum of Chl *a* fluorescence at 740 nm at room temperature (mostly from PSII) shows peaks at about 440 nm (Chl *a*), 480 nm (Chl *b*), 650 nm (Chl *b*), and 670 nm (Chl *a*) in the green alga *Chlorella vulgaris* (see, e.g., Ghosh *et al.*, 1966; Fig. 7). A comparison of the action spectrum of fluorescence with the absorbance spectrum in

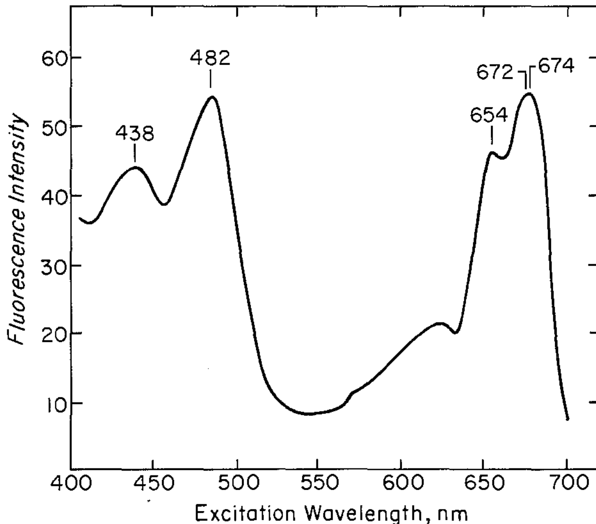


FIG. 7. Action spectrum of fluorescence at 740 nm at room temperature from *Chlorella vulgaris* (Ghosh *et al.*, 1966).

Chlorella reveals that the efficiency of energy transfer from Chl *b* to Chl *a* is 100%, but the efficiency of energy transfer from the various carotenoids (absorbing in the 400–540-nm region; see Govindjee, 1960) to Chl *a* is somewhat lower (~60%). For a discussion of efficient energy transfer from the carotenoid siphonoxanthin to Chl *a* in the seaweed *Ulva japonica,* see Kageyama *et al.* (1977).

b. Action Spectra at Low Temperatures. The action spectra of Chl *a* fluorescence in Chl *b*- or Chl *c*-containing cells at 77°K are narrower than those obtained at room temperature in each case due to the sharpening of the absorption bands.

In the dinoflagellate *Gonyaulax polyedra,* the action spectrum of Chl *a* fluorescence at 750 nm, in the presence of DCMU, and at 77°K (F_{max}, maximum fluorescence intensity) shows peaks at 672 nm (Chl *a*) and 636 nm (Chl *c*) and a broad but low band in the 400–560-nm region (due to carotenoids diadinoxanthin, dinoxanthin, and peridinin) (Govindjee *et al.,* 1979). The efficiency of energy transfer from Chl *c* to Chl *a* has been shown to be very high (~100%). Furthermore, the efficiency of energy transfer from peridinin to Chl *a* in isolated complexes has been shown to be very high. (However, there are many carotenoids *in vivo* that are not present in the photosynthetic membranes, but are in the cell walls, and are thus responsible for an apparently low calculated efficiency of energy transfer from carotenoids to Chl *a*.)

At low temperatures (77°K), the green alga *Chlorella* shows four emission bands: F685 (PSII), F695 (PSII), F718 (PSI), and F725 (PSI) (see, e.g., Fig. 5B; Cho and Govindjee, 1970a,b). Thus, action spectra for Chl *a* fluorescence can be measured for the various components in *Chlorella.* Figure 8A shows the absorbance spectrum at 4°K with peaks at 440 (Chl *a*), 464 (carotenoids), 477 (carotenoids + Chl *b*), 491 (carotenoids), 650 (Chl *b*), 670 (Chl *a*), and 678 nm (Chl *a*). Action spectra of fluorescence for F685, F695 (Fig. 8B), and F725 (Fig. 8C) measured at various temperatures from 77 to 4°K shows two important features: (1) the contribution of Chl *b* (peaks at ~480 and ~650 nm) with respect to Chl *a* (670, 678 nm) is greater for F685 and F695, suggesting that they are from PSII, in contrast to that for F725, and (2) the ratio of the Chl *b*/Chl *a* peaks is almost independent of temperature (Cho and Govindjee, 1970b). Furthermore, action spectra measured with narrow slit widths (~1 nm) for F685 (Fig. 8D) and F695 show that the pigments funneling energy to these components are almost identical and that both Chl *a* 670 and Chl *a* 678 belong to the complexes fluorescing at these two wavelengths. Action spectra measured at several long wavelengths show clearly that F725 is sensitized by pigments absorbing at longer wave-

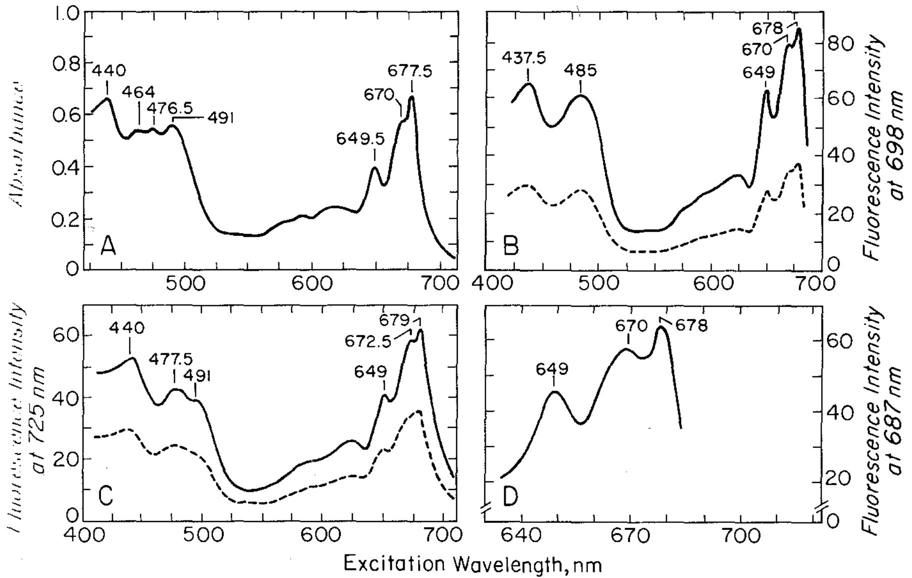


FIG. 8. **Absorption spectrum and action spectra of fluorescence at 4–77°K from *Chlorella pyrenoidosa.* (A) Absorption spectrum at 4°K (spectrum at 77°K overlapped with that at 4°K). (B) Action spectra at 4 (solid line) and 77°K (dashed line) for fluorescence at 698 nm (F695). (C) Action spectra at 4 (solid line) and 77°K (dashed line) for fluorescence at 725 nm (F725). (D) Action spectrum at 4°K in the red region of the spectrum for fluorescence at 687 nm. (Redrawn from Cho and Govindjee, 1970b.)**

lengths than the fluorescence at ~760 nm (which arises from the vibrational band of the short-wavelength Chl *a* forms).

C. Induction of Chlorophyll a Fluorescence

Among algae, the unicellular green species *Chlorella* has been most extensively used for the study of induction of Chl *a* fluorescence. An advantage of green algae is that they show a rather simple fluorescence induction (Kautsky transient—see, e.g., Kautsky *et al.*, 1960; Munday and Govindjee, 1969a,b). However, the transients become complicated because state I–state II transitions (see, e.g., Williams *et al.*, 1980; Satoh and Fork, 1983; Catt *et al.*, 1984; Section IV, this chapter), high-energy-state-induced fluorescence quenching (Mohanty and Govindjee, 1973a,b; Krause *et al.*, 1983), or transients related to carbon dioxide fixation (Walker *et al.*, 1983) may begin to overlap the late stage of the Kautsky transients. We will summarize here mainly information on the

early stage of the fluorescence transients in green algae (for earlier reviews, see Govindjee and Papageorgiou, 1971, Mohanty and Govindjee, 1974; Papageorgiou, 1975; Krause and Weis, 1984). For studies on brown algae, see Berkaloff and Duvall (1980) and Bruce *et al.* (1983), and for studies on leaves and chloroplasts from higher plants, see Briantais *et al.*, Chapter 18, this volume.

Characteristic transients (Fig. 9) of the fluorescence induction in green algae are referred to as OIDPSMT following the terminology discussed in Govindjee and Papageorgiou (1971), but they are referred to here as $OIDPS_1M_1S_2M_2T$, as modified by Yamagishi *et al.* (1978) to include three peaks; this is based partially on the alternative terminology of Bannister and Rice (1968). A brief description of the terminology follows. The O (for origin) level, the so-called constant fluorescence, is the instantaneous fluorescence level obtained within the opening time of the shutter used to start the illumination (<1 ms). The level I stands for an inflection or an intermediate peak or hump, whereas the OI phase is photochemical and dependent on exciting light intensity. The level D is used for the dip which follows I. The highest level is P, which stands for peak or plateau; the IDP rise occurs within ~1 s, when the intensity of light just saturates photosynthesis. The PS decay occurs within 5 to 10 s. The S stands for semi-steady state. The SM rise occurs within 0.5 min; the symbol M stands for a maximum. The MT decline occurs within 2 min. The symbol T stands for terminal steady state. Sometimes there are two maxima M_1 and M_2 and then S is referred to as S_1 and the lower fluorescence point between M_1 and M_2 as S_2 (see Fig. 9). We emphasize that it is important for the interpretation of the data that the O level not be confused with the I level or with the first measured point (F_i), which may

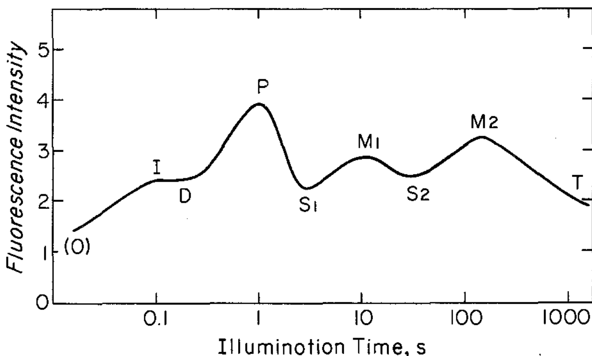


FIG. 9. **Chlorophyll *a* fluorescence intensity as a function of time of illumination in *Bryopsis* cells. See text for definition of symbols. (Redrawn from Yamagishi *et al.*, 1978.)**

lie anywhere between O and P depending on the opening time of the shutter and the exciting light intensity.

It is well established that the intensity of Chl *a* fluorescence *in vivo* is controlled by the redox state of Q_A, the first quinone electron acceptor of PSII; the fluorescence yield is high when Q_A is reduced and low when Q_A is oxidized (Duysens and Sweers, 1963). In addition, as noted above, the emission intensity is modulated by state transitions, the high-energy state of the thylakoid membranes, and cation transport across the membranes at physiological temperatures (see reviews by Papageorgiou, 1975; Lavorel and Etienne, 1977; Briantais *et al.*, Chapter 18, this volume).

1. THE O → I RISE

The initial fluorescence (O) is thought to be emitted from PSII during the time when excitation energy is transferred among antenna Chl *a* molecules and from the weakly fluorescent Chl *a* of PSI (Munday and Govindjee, 1969a) (see Table V). Thus, it represents the fluorescence of the system with open reaction centers, i.e., before photochemical trapping. Its intensity is linearly proportional to the incident light intensity, but is dependent on the distribution and redistribution of excitation energy between the two photosystems. Since this fluorescence originates as one of the deactivation pathways of antenna molecules, its level is also dependent on the connection between the antenna and the reaction

TABLE V
Explanation of Chlorophyll *a* Fluorescence Induction

Fluorescence levels and/or transients	Current interpretation
O level	Due to loss of excitation energy during transfer (PSII and PSI) to the reaction centers
OI rise	Reduction of Q_A to Q_A^- by PSII
ID decline	Reoxidation of Q_A^- to Q_A by PSI
DP rise	Accumulation of reducing equivalents in the electron transport chain up to PSI acceptors
PS decline	Complex—mostly explained by Q_A^- reoxidation. Current hypothesis includes role of O_2 (decline is absent under anaerobic conditions); see Fig. 10 and text
SM rise	Independent of redox state of Q_A; regulated by structural (state) changes. Alternatively, (1) ΔpH-dependent effect on electron flow; (2) regulation by Calvin cycle reactions
MT decline	Indirectly linked to ATP synthesis and utilization; structural (state) changes

center. In view of the fact that the energy trap of PSII, P680, and the antenna Chl *a* of PSII are of approximately the same energy, the trap is not irreversible, and the so-called constant fluorescence is not truly constant since the rate constants of various pathways at the reaction center would control this fluorescence yield. The OI rise and the ID plateau (or decline) are reflections of the initial rapid reduction of Q_A by PSII and the subsequent oxidation of reduced Q_A by PSI, respectively (Munday and Govindjee, 1969b; Satoh and Katoh, 1981). The I level increases with increasing intensity and is enhanced when the electron flow from Q_A^- to Q_B is blocked. Since there is a large plastoquinone (PQ) pool between PSI and Q_A, it takes several tens of milliseconds for PSI to oxidize Q_A^-. Thus the OI rise precedes the ID plateau or decline. In fact, the extent of the OI rise and the ID decline depend strongly on the redox state of the PQ pool (Jennings and Forti, 1975; Satoh and Katoh, 1981), and the ID decline is increased by PSI light (Munday and Govindjee, 1969a,b; Schreiber and Vidaver, 1974). The requirement of light for D is shown by the delay in its occurrence when it is measured with flashing light (with dark times between flashes) compared to that with continuous light. Furthermore, the ID decline is very pronounced under anaerobic conditions.

2. THE D → P RISE

It seems that during the D-to-P transition all electron carriers up to X (a PSI acceptor) are reduced, as suggested by the following observations: (1) intersystem electron carriers Cyt *f* (Satoh *et al.*, 1977) and P700 (Maxwell and Biggins, 1977) become fully reduced during the DP rise following the initial rapid oxidation during the OI rise, and (2) simultaneous measurements of O_2 evolution and Chl *a* fluorescence in *Chlorella* show that electron flow is greatly inhibited at the P stage (Delosme *et al.*, 1959; Joliot, 1965, 1968; Bannister and Rice, 1968; Govindjee and Papageorgious, 1971).

The classical explanation of the DP rise was that as the PSII reactions occur, the PQ pool is filled, which leads to accumulation of Q_A^-. This explanation assumes that the PQ pool is oxidized in dark-adapted samples, but is apparently contradicted by data suggesting that the PQ pool is in the reduced state in dark-adapted samples (see, e.g., discussion by Rutherford *et al.*, 1984, for intact leaves). Another possibility is that the electron transport on the acceptor (reducing) side of PSI is blocked in the dark-adapted state, most likely between ferredoxin and $NADP^+$. (The reader may consult Fig. 1 in Duysens, Chapter 1, or Sane and Rutherford, Chapter 12, this volume.) In this picture, the reduction of

Q_A apparently occurs subsequent to the reduction of intermediates between P700 and the blocked site. This idea seems to be consistent with the following observations. The specific PSI electron acceptors which remove electrons before ferredoxin (e.g., methyl viologen—Munday and Govindjee, 1969b; Lavergne, 1974; Satoh *et al.*, 1977; or nitrite—Kessler and Zumft, 1973; Satoh and Katoh, 1980) eliminate the DP transient in green algae. By contrast, oxidants that accept electrons after ferredoxin–$NADP^+$ reductase ($NADP^+$ in *Bryopsis*—Satoh, 1981; oxaloacetate, 3-phosphoglycerate in intact spinach chloroplasts—Satoh and Katoh, 1980) have little effect on the fluorescence transient. Furthermore, the DP rise seen in dark-adapted algal cells is decreased or abolished by preillumination (Duysens and Sweers, 1963; Mohanty and Govindjee, 1973a, 1974; Satoh *et al.*, 1977), possibly because the block mentioned above is removed during preillumination and thus Q_A^- does not accumulate and the P level is low.

For a quantitative analysis of fluorescence induction curves (O → I → P) in isolated chloroplasts from higher plants, see Renger and Schulze (1985); application to algae remains to be made.

3. THE P → S DECLINE

A complete explanation of the subsequent fluorescence decline from P to S is still more complex. For a long time it was thought that the P-to-S phase in *Chlorella* cannot be simply explained by the reoxidation of Q_A^- to Q_A even though the rate of O_2 evolution is complementary to fluorescence changes during this time (Lavorel, 1959; Duysens and Sweers, 1963; Mohanty and Govindjee, 1974). The P-to-S phase is a thermal phase since it can continue in darkness (Lavorel, 1959; Lavergne, 1974; Mohanty and Govindjee, 1974). Furthermore, it can be shown that the S level increases on preillumination with light I but decreases on preillumination with light II (Munday and Govindjee, 1969b; Mohanty and Govindjee, 1974), in contrast to the antagonistic effect of light I and light II on Chl *a* fluorescence yield, which is reduced by light I and increased by light II (Govindjee *et al.*, 1960; Butler, 1962; Duysens and Sweers, 1963; Mohanty *et al.*, 1970). The susceptibility of P-to-S decay to uncouplers of photophosphorylation, the absence of the decay under anaerobiosis, and its possible relationship to the redox state of the PSI acceptor side indicate that it is a complex phenomenon. Mohanty and Govindjee (1974) suggested that the PS decline in algae is due to an increase in the rate constant of internal conversion and/or energy transfer to weakly fluorescent Chl *a* molecules (state changes), which is a consequence of the structural changes in the membrane, which in turn are due to the

buildup of a "phosphorylation or ΔpH potential" because of increased noncyclic electron flow. Satoh *et al.* (1977) proposed a different working hypothesis to explain the PS decline (Fig. 10). They suggested that the block on the electron acceptor side of PSI (labeled "Dark-inactivated site" in the diagram) is first removed during the illumination (see section on D → P rise). Then, during the P-to-S phase, the reduced PSI acceptor is oxidized, which leads to subsequent reoxidation of Q_A^- to Q_A and thus lowered fluorescence at the S level. The observation that the activity of ferredoxin–$NADP^+$ reductase changes on transfer of algal cells from a dark to a light cycle (Satoh, 1981) suggests that this enzyme may be the site of light-dependent modulation of electron transport of the reducing (electron acceptor) side of PSI. The reactivation of PSI is also suggested by the observation that this P-to-S phase of the fluorescence induction is accompanied by reoxidation of Cyt *f* (see right side of Fig. 10). The PS decline is too rapid to be explained in terms of activation of the CO_2 fixation enzyme system. Furthermore, KCN, at concentrations where carbon fixation is completely inhibited, has no effect on the PS decline (Wassink and Katz, 1939; Satoh *et al.,* 1977).

The sensitivity of the PS decline to uncouplers of photophosphorylation (see Tables 1 and 2a in Mohanty and Govindjee, 1974) may be explained by the fact that uncouplers like CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) decrease the internal pH of the chloroplasts, and the PS decline is highly sensitive to pH (see Briantais *et al.,* 1979, for isolated chloroplasts; Yamagishi *et al.,* 1981).

Oxygen seems to play an important role in the PS phase because the PS fluorescence decay is strongly suppressed under anaerobiosis

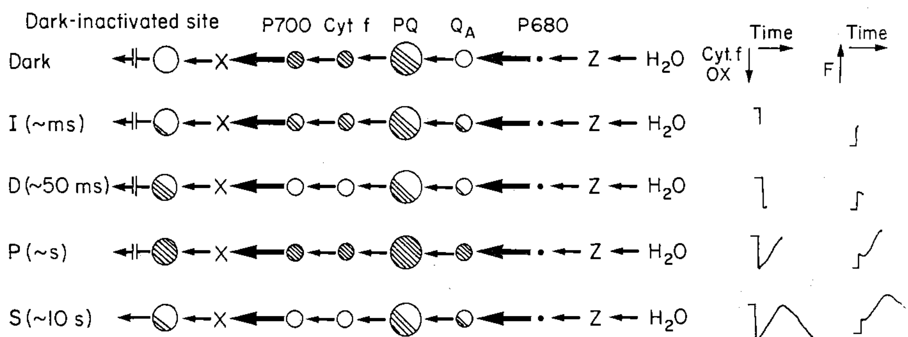


FIG. 10. Working hypothesis to explain the fluorescence transient. Z, electron donor to the reaction center chlorophyll *a* of photosystem II (P680); Q_A, electron acceptor of photosystem II; PQ, plastoquinone pool; cyt *f*, cytochrome *f*; P700, reaction center chlorophyll *a* of photosystem I. See text for details.

(Kautsky and Frank, 1943; Shiau and Franck, 1947; Bannister and Rice, 1968; Munday and Govindjee, 1969a; Papageorgiou, 1975; Satoh, 1982). However, preillumination lowers the P level when measured under aerobic conditions, but not under anaerobic conditions, indicating that even after the preillumination, electron flow through PSI is limited when O_2 is absent (Satoh, 1982). This suggests that O_2 serves as an electron acceptor (also see Radmer and Ollinger, 1980). Perhaps during this pseudocyclic electron flow the system is being prepared for the initiation of CO_2 fixation.

For the relationship of fluorescence transients to photoacoustic signal measurements in *Bryopsis,* see Katoh and Yamagishi (1984). Results reported in that paper are apparently consistent with the hypothesis for P-to-S decay shown in Fig. 10. Further research is necessary to fully understand the mechanism of P-to-S decay in algae and to relate it to similar, although quite distinct, changes in higher plants (see Briantais *et al.,* Chapter 18, this volume).

4. THE S → M RISE

The S to M portion of the fluorescence transient seems to be independent of the redox state of Q_A since the rate of O_2 evolution and the SM phase increase in parallel in *Chlorella* (Papageorgiou and Govindjee, 1968a). Slovacek and Bannister (1973) showed that in CO_2-depleted *Chlorella* cells the SM phase is suppressed, but the addition of NH_4Cl revives a large SM rise. Although the latter authors argued against the SM rise being related to the state changes, structural changes facilitating the transformation of the system to the highly fluorescent state I cannot be ignored (see Section IV). On the other hand, Walker *et al.* (1983), working with higher plants, found an antiparallel relationship of the SM rise with O_2 evolution (although out of phase) and related these changes to decreased NADPH reoxidation and increased utilization of ATP in the Calvin cycle.

In the alga *Bryopsis,* which shows two distinct peaks, M_1 and M_2, the fluorescence increase up to M_1 was shown to be related to the formation of ΔpH across the thylakoid membranes (Yamagishi *et al.,* 1978). Uncouplers that dissipate ΔpH suppress the SM_1 rise, whereas energy transfer inhibitors and valinomycin, which increase ΔpH, magnify the fluorescence rise. The transient was, therefore, ascribed to partial reduction of Q_A caused by control of electron transport by ΔpH. However, in *Chlorella* (1) Papageorgiou and Govindjee (1968a) showed that atabrin, but *not* FCCP and phloridzin, had a significant effect on the SM rise, (2) Papageorgiou and Govindjee (1971) showed that the SM rise in *Chlorella*

was unaffected by the pH of the culture medium, and (3) the formation of ΔpH is expected to decrease the electron flow, but O_2 evolution was found to increase in parallel to the SM rise. Obviously, further work is needed to understand the SM rise in algae.

5. THE M → T DECLINE

The M-to-T decline in *Chlorella* is strongly affected by the presence of the uncoupler FCCP (Papageorgiou and Govindjee, 1968a; Mohanty and Govindjee, 1974) and by the pH of the external medium, being slower at alkaline pH (Papageorgiou and Govindjee, 1971). Thus, it appears that the MT phase may be related to the synthesis and utilization of ATP in *Chlorella*. Of course, the effects of fluorescence yield are indirect (again, via structural changes of the membrane in which the pigment–protein complexes are embedded).

6. PHYSIOLOGICAL CHANGES

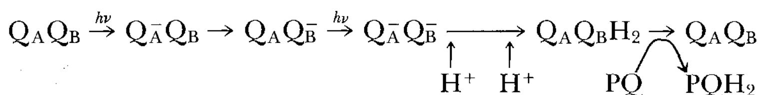
Chl *a* fluorescence transients are being extensively used as monitors of the physiological status of samples (also see Briantais *et al.*, Chapter 18, and Renger and Schreiber, Chapter 19, this volume). Because of the complexities of the various phases of the fluorescence transient, it is difficult to make meaningful interpretations, but in spite of this fluorescence can serve as a quick and sensitive indicator of changes in physiological parameters, which can then be investigated later by parallel measurements on partial reactions. We refer here only to two examples on green algae: the first deals with the effect of salts on *Chlorella* (Mohanty *et al.*, 1974) and *Chlamydomonas* (Wollman and Diner, 1980) and the second with the effect of the CO_2 concentration during growth of *Chlamydomonas* (Spalding *et al.*, 1984).

D. *Flash-Induced Changes*

Flash-induced Chl *a* fluorescence yield changes have provided important information on the primary photochemical and the associated reactions of PSII (see, e.g., Amesz and Duysens, 1977; Lavorel and Etienne, 1977; van Gorkom, 1985; and Chapter 10, this volume). For completeness, we shall mention a few key experiments on this topic on the green alga *Chlorella*.

The decay of Chl *a* fluorescence yield, after a 30-ms exciting flash, was monitored by Lavorel (1965), who showed two components of $t_{1/2}$ of ~10 ms and 0.1–1 s. The latter slow component was explained as due to a back reaction of Q_A^- with an oxidized component on the electron donor

side of PSII, an idea revived by van Best and Duysens (1975). The use of a shorter flash (microsecond range) allowed Zankel (1973) to resolve a fast ($t_{1/2} \sim 200\ \mu s$) component. This is now recognized as the $t_{1/2}$ of electron flow from Q_A^- to the second quinone acceptor, Q_B (for *Chlorella*, see, e.g., van Best and Duysens, 1975). The current electron transfer scheme on the acceptor side is



Mauzerall (1972) measured the rise and the decay of the yield of Chl *a* fluorescence in *Chlorella* after 2-ns flashes. He observed that after the first flash, the fluorescence yield rise was in the 30-ns range, but after the second flash the rise was slower. Butler (1972) suggested that this rise was a measure of electron flow from the electron donor Z to $P680^+$ (the latter was proposed to be a quencher of Chl *a* fluorescence). (For an elegant and quantitative study, see Sonneveld *et al.*, 1979, also for *Chlorella*.)

The above discussion shows the versatility of Chl *a* fluorescence in monitoring electron flow from Z to $P680^+$ and from Q_A^- to Q_B in algal cells. In view of the known effects of various herbicides on the Q_A-to-Q_B reaction, the application of Chl *a* fluorescence to herbicide research by using algal cells as model systems has promising future possibilities.

E. Polarization

If polarized light is used to excite Chl *a* molecules, those with absorption dipoles parallel to the polarization of the incident light are preferentially excited. As energy is transferred among the Chl *a* molecules, the "memory" of the initial polarization (p) is lost if the Chl *a* molecules are arranged in a random fashion within the thylakoid membrane. (Note that the $p = (F_{\parallel} - F_{\perp})/(F_{\parallel} + F_{\perp})$, where $F_{\parallel}$ and $F_{\perp}$ are intensities of fluorescence parallel and perpendicular to the polarization of the exciting light, the direction of the measured fluorescence being perpendicular to the direction of the exciting beam.) In the discussion of fluorescence polarization, we should make a clear distinction between oriented and unoriented samples (see Amesz and Vasmel, Chapter 15, this volume). In the first case, with polarized excitation, one obtains information on the angles between exciting and emitting dipoles, but mixed with depolarization by energy transfer, as noted above. In the second case, with nonpolarized excitation, one learns about the orientation of emission dipoles with respect to the membrane; however, with polarized

excitation, the excitation spectrum gives information on the orientation of absorption dipoles. Mixed cases are difficult to analyze.

The earlier studies on unoriented samples showed low degrees (0.01 to 0.06)* of polarization of fluorescence of Chl *a in vivo* (Arnold and Meek, 1956; Goedheer, 1957, 1966; Weber, 1958; Teale, 1960; Govindjee, 1966; Cederstrand and Govindjee, 1966) in contrast to that *in vitro* ($p = 0.40$) (Goedheer, 1957; Teale, 1960). These low values were taken as evidence of energy transfer. The value of p is even lower for the variable ($F_p - F_0$) fluorescence than for the F_0 level (Lavorel, 1964) in *Chlorella*. Mar and Govindjee (1972) showed that addition of DCMU, which inhibits the electron flow, also reduces p; this was taken, in a first approximation, as evidence for further excitation migration in *Chlorella* (for a detailed and elegant study, see Whitmarsh and Levine, 1974).

In chloroplasts from higher plants, support for cation-induced changes in excitation energy distribution and redistribution between the two photosystems has come from observations of the cation-induced changes in the degree of polarization of Chl *a* fluorescence in unoriented samples (Wong *et al.*, 1979; Wong and Govindjee, 1981). Similar experiments on polarization of fluorescence are needed to study state changes in algae (see Section IV).

Light-harvesting Chl *a*/Chl *b* proteins from higher plants have been examined by Van Metter (1977a,b) for polarization of fluorescence. The small value (+0.02) observed for p on excitation at 650 nm (in Chl *b*) is indicative of a nearly spherical symmetry for the exciton states of the three Chl *b* molecules present there. A model was proposed (Knox and Van Metter, 1979), based on data on p as well as circular dichroism and absorption spectra, for the arrangement of the three Chl *a* and three Chl *b* molecules in LHCP. This model has been refined by Shepanski and Knox (1981). The basic picture is that the three Chl *a* molecules are arranged at the periphery of a core of three Chl *b* molecules whose dipoles are not parallel to each other. We suspect that the LHCP of green algae (see Section II,C) will show similar results.

During the 1970s several papers appeared on polarized fluorescence of oriented photosynthetic systems (Geacintov *et al.*, 1971, 1972, 1974; Breton *et al.*, 1973; Breton, 1975; Becker *et al.*, 1976; Garab and Breton, 1976), but the conclusions regarding the orientation of the various pigments are quite complex (see Breton and Vermeglio, 1982, for a sum-

* J. Amesz (personal communication) suggests that these low values are due partly to overlap of absorption and emission bands at room temperature, and that low-temperature data, where the overlap is minimal, should give better results (cf. Kramer and Amesz, 1982, for studies on oriented spinach chloroplasts).

mary, and consult the original papers for details). Using *Chlorella* cells oriented in a magnetic field (Geacintov *et al.,* 1971), Geacintov *et al.* (1974) were able to show that the ratio (FP) of fluorescence polarized parallel to that polarized perpendicular to the thylakoid membrane in *Chlorella* ranges from 1.2 to 1.9, especially at wavelengths >690 nm; this ratio is, however, closer to 1.0 at wavelengths <690 nm. The picture has emerged that the Q_y absorbance bands of the long-wavelength forms of Chl *a* (e.g., Chl *a* 695–712) are oriented approximately parallel to the plane of the membrane, that of Chl *a* 680 is also oriented somewhat (<30°C) parallel, whereas that of Chl *a* 670 is little oriented. Furthermore, the pigment responsible for F695 (at 77°K) is oriented more or less perpendicular to the membrane plane. (For a polarization study of magnetically oriented *Chlorella vulgaris* cells at 77°K, see Vasin and Verkhoturov, 1979.)

Gulyayev *et al.* (1982) found that the maximum polarization of fluorescence at room temperature was for F685, followed by F705 and then F680. However, also at room temperature, these authors observed that the polarization of fluorescence was low for F_0 and high for variable fluorescence. In unoriented samples, the reverse is true (Lavorel, 1964; Mar and Govindjee, 1972). Further research is needed to understand these different results. If it is proved that Chl *a* is significantly oriented at room temperature *in vivo,* then energy transfer will not cause complete depolarization of fluorescence, as was inferred in earlier studies (*vide supra*).

An application of measurements of polarization of fluorescence to the physiology of algal cells (i.e., effects of growth cycle) may be found in a paper by Chemeris and Venediktov (1980).

IV. State I–State II Changes

Bonaventura and Myers (1969), working on the green alga *Chlorella,* and Murata (1969), working on the red alga *Porphyridium,* reported light-driven changes in the distribution of excitation energy between PSI and PSII. Bonaventura and Myers (1969) coined the term (light) state I as the state created by exposure to light I (i.e., light preferentially absorbed by PSI) and (light) state II as the state created by exposure to light II. State I is recognized by a higher Chl *a* fluorescence yield at room temperature, a higher ratio of F685 and F695 to F720 at 77°K, and a higher quantum yield of PSII reactions at low light intensities. Conversely, state II is recognized by a lower Chl *a* fluorescence yield at room temperature, a lower ratio of F685 and F695 to F720 at 77°K, a lower

quantum yield of PSII, and a higher quantum yield of PSI reactions. For studies on state I–state II transitions in green algae, see Salamon (1980), Williams *et al.* (1980), Saito *et al.* (1983), Sane *et al.* (1982), and Hodges and Barber (1983). In the case of chloroplasts from higher plants, these state changes are linked to the phosphorylation of LHC II (see Briantais *et al.*, Chapter 18, this volume); for a discussion of state changes in phycobilin-containing algae, see Fork and Mohanty (Chapter 16, this volume).

Light I and light II induced changes in Chl *a* fluorescence properties, which were unrelated to the Q_A hypothesis of Duysens and Sweers (1963), were recognized earlier in algae by Papageorgiou and Govindjee (1967, 1968a,b). They were interpreted in terms of conformational changes in the membrane leading to changes in the orientation/distance between different Chl *a* molecules; the increase in the intensity of the S level by PSII preillumination (Munday and Govindjee, 1969a,b) was a manifestation of a similar phenomenon.

A redistribution of energy between PSII and PSI could cause the system to become more or less balanced and thus change the interaction of PSI light with Chl *a* fluorescence from PSII, explaining the different quenching efficiencies at different times of the transient (Mohanty *et al.*, 1970). Canaani *et al.* (1984) have shown clearly that in leaves of higher plants both the quenching of Chl *a* fluorescence by PSI light and the Emerson enhancement effect in O_2 evolution are high only in state I; in state II, the system is well balanced. [These results could explain the "old" controversies between investigators who could observe or not observe Emerson enhancement in the same experimental system; see discussions in Govindjee and Govindjee (1975) and Govindjee and Whitmarsh (1982).]

The state I → state II transition has been described (see, e.g., Allen *et al.*, 1981; see Fig. 7, Briantais *et al.*, Chapter 18, this volume) in terms of the following sequence: PSII light accumulates PQH_2; a kinase is activated; phosphorylation of LHC II occurs; phosphorylated LHC moves from a stacked to an unstacked region; and a balanced absorption cross section of PSI and PSII is achieved. Conversely, the state II → state I transition is described as follows: PSI light oxidizes the PQ pool; a phosphatase is activated; dephosphorylation of LHC II occurs; LHC II moves back to the appressed regions; and an unbalanced absorption cross section of PSII and PSI is attained. In addition to the above phenomenon, green algae show changes in excitation energy distribution or redistribution on dark adaptation (see, e.g., Catt *et al.*, 1984). Both the dark state and state II are poised in favor of PSI, whereas state I is poised in favor of PSII (see, e.g., Williams *et al.*, 1980; Satoh and Fork,

1983; Catt *et al.*, 1984). However, it was suggested that the dark state is different from state II. Catt *et al.* showed that low intensities of light II have a similar effect to light I (also see earlier observations of Murata and Govindjee, 1969b); i.e., the dark state is driven to an intermediate state between the dark state and state I. This low-intensity wavelength-independent change in green algae has been attributed to changes in the local ionic environment, while the high-intensity induced state I–state II changes are related to phosphorylation of LHC II (Catt *et al.*, 1984).

The state I → state II transitions and their relationship to phosphorylation of LHC II has been shown by Wollmann and Delepelaire (1984) in *Chlamydomonas* thylakoids and suggested by Saito *et al.* (1983) in *Chlorella* thylakoids. Further work is needed to prove that state transitions are related to LHC II phosphorylation in intact algal cells, although the current data favor this mechanism. The only experiment to date relating state changes to phosphorylation of LHC II *in vivo* is that of Canaani *et al.* (1984) on leaves, but even this conclusion is dependent on acceptance of the idea that sodium fluoride treatment of leaves specifically affected the LHC II phosphorylation in their system.

For a complete review on state changes, see Fork and Satoh (1986).

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Note Added in Proof

Evidence against the idea that variable fluorescence is delayed fluorescence is mounting: (1) Using an instrument with a 60-ps response time, Moya *et al.* (1987) were unable to confirm the results of Mauzerall (1985) in *Chlamydomonas;* in addition, these authors observed the presence of the slow component in a *Chlamydomonas* mutant lacking the reaction center II. (2) On the basis of an analysis of a model correlating the exciton decay kinetics in picosecond fluorescence studies with the primary processes of charge separation in the reaction center of PSII, Schatz and Holzwarth (1987) have also concluded that variable fluorescence is prompt, not delayed, fluorescence.

Moya, I. Hodges, M., Briantais, J.-M., and Hervo, G. (1987). *Photosynth. Res.* (in press).
Schatz, G. H., and Holzwarth, A. R. (1987). *Photosynth. Res.* (in press).

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Chlorophyll *a* Fluorescence of Higher Plants: Chloroplasts and Leaves

JEAN-MARIE BRIANTAIS
CLAUDIE VERNOTTE
Laboratoire de Photosynthèse
Centre National de la Recherche Scientifique
91190 Gif-sur-Yvette, (C.S.) Recherche, France

GOTTHARD HEINRICH KRAUSE
ENGELBERT WEIS
Botanisches Institut der Universität Düsseldorf
D4000 Düsseldorf 1, Federal Republic of Germany

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ABBREVIATIONS AND SYMBOLS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Chl	Chlorophyll
Cyt	Cytochrome
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea (diuron)
EDTA	Ethylenediaminetetraacetate

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F680, F685, etc.	Fluorescence bands with maxima at 680, 685 nm, etc.
Fd	Ferredoxin
F_0	Initial or constant fluorescence
F_v	Variable fluorescence
F_m	Maximum fluorescence
HES	High-energy state
I	Pheophytin *a*, primary electron acceptor of PSII
kD	Kilodalton
LHC I	Light-harvesting complex of PSI
LHC II	Light-harvesting complex of PSII
M_r	Molecular weight
MV	Methyl viologen
$NADP^+$	Nicotinamide-adenine dinucleotide phosphate
P	Chlorophyll *a* of photochemical reaction center
P680	Chlorophyll *a* of photochemical reaction center II
P700	Chlorophyll *a* of photochemical reaction center I
PC	Plastocyanin
PMS	Phenazine methosulfate
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
Q_A	Primary stable acceptor of PSII; a plastoquinone molecule
Q_B	Secondary acceptor of PSII; a plastoquinone molecule
RC	Reaction center
X	Primary PSI electron acceptor
Z	Electron donor of photosystem II reaction center

I. Introduction

Fluorescence, one of the ways of deactivating excited Chl *a* molecules, is in competition with other processes such as the photochemical reaction, thermal deactivation, and transfer of excitation energy to nonfluorescing pigments. Thus Chl *a* fluorescence changes can indicate variations in photosynthetic activity. In 1874 Müller, using a combination of colored glasses, visually observed Chl *a* fluorescence changes in green leaves and recognized a correlation between Chl *a* fluorescence and photosynthesis (see Schreiber, 1983). A systematic study of Chl *a* fluorescence variations occurring upon illumination of photosynthetic material began with the work of Kautsky and Hirsch (1934) and Kautsky and Eberlein (1939). McAlister and Myers (1940) observed an inverse relationship between the time course of Chl *a* fluorescence induction and photosynthesis. This was followed by the work of Kautsky and Franck (1943), Kautsky *et al.* (1960), and many others (discussed by Duysens, Chapter 1, this volume). In the past 20 years, progress in the analysis of fluorescence characteristics has been the subject of several reviews (Gov-

indjee *et al.*, 1967; Govindjee and Papageorgiou, 1971; Mohanty and Govindjee, 1974; Papageorgiou, 1975; Barber, 1976; Lavorel and Etienne, 1977; Butler, 1977; Schreiber, 1983; Krause and Weis, 1984).

Before discussing Chl *a* fluorescence it is necessary to give a brief description of the photosynthetic apparatus. In higher plants, the Chl–protein complexes and electron carriers from H_2O to $NADP^+$ are integrated in a membrane, the thylakoid. Two photosystems, PSII and PSI, operate in series as shown in Fig. 1. (Also see Figs. 1 in Duysens, Chapter 1, and Sane and Rutherford, Chapter 12, this volume.) The thylakoids are enclosed in an organelle called the chloroplast; they are embedded in the stroma, which contains the enzymes of carbon fixation.

Chl *a* fluorescence emitted by PSII depends on the redox state of Q_A, the primary stable PSII electron acceptor (Duysens and Sweers, 1963). However, Chl *a* fluorescence can also be influenced by other parameters, e.g., pH, ionic strength, and the spatial distribution of Chl–protein complexes. Furthermore, these parameters are susceptible to changes due to the photosynthetic activity itself. Thus, when dark-adapted material is illuminated, Chl *a* fluorescence intensity undergoes variations due to the onset of different photosynthetic processes. Figure 2 shows typical examples of the fluorescence induction produced by a dark–light transition of a leaf. One can distinguish rapid transients (O-I-D-P) and slow transients (P-S-M), leading to the "terminal" level, T. (Compare with transients in green algae—see Fig. 9 in Govindjee and Satoh, Chapter 17, this volume.) The rapid transients, in the range of 1 s, depend mainly on the rate of reduction of Q_A and the PQ pool by PSII. Slower transients, in the range of minutes, express secondary processes such as reoxidation of the electron transport chain via PSI, as affected by Calvin cycle reactions, and the buildup of a proton gradient. Analysis of these complex variations has been made possible by the accumulation of data on fluorescence variations in simpler systems such as isolated thylakoids

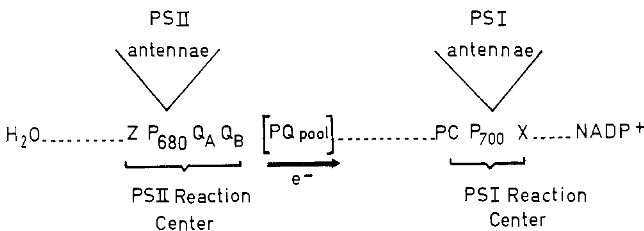


FIG. 1. Schematic representation of photosynthetic electron transfer. See list of abbreviations.

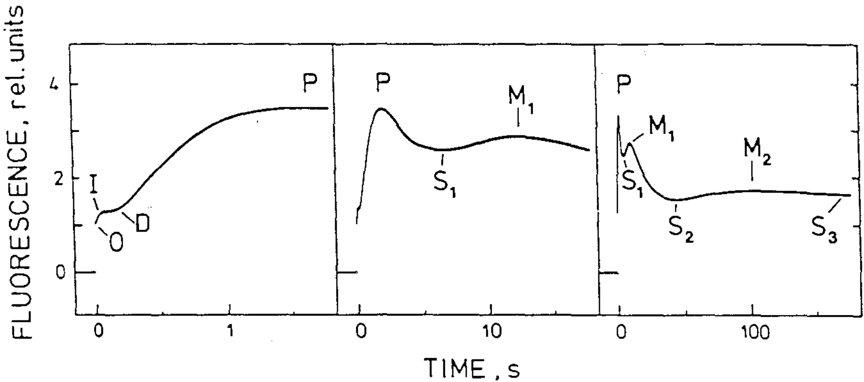


FIG. 2. Chlorophyll *a* fluorescence induction curves from a tomato leaf. At time zero a dark-adapted sample was illuminated with continuous blue light at 15 W/m^2. Fluorescence was measured at wavelengths greater than 650 nm. Temperature: 20°C. Induction at three different time scales is presented. The characteristic fluorescence levels are: O, initial level (nonvariable part of fluorescence); I, intermediary level or inflection point; D, dip level; P, peak level; S_1, S_2, S_3, quasi-stationary levels following the peak or relative maxima M_1, M_2, ... The final S level (S_3) may be labeled T (terminal level). (From Schreiber, 1983.)

and intact chloroplasts. For example, Horton (1983a) was able to reconstitute the *in vivo* transients by using thylakoids supplemented with various substrates.

Fluorescence spectroscopy at a low temperature (77°K) was also found to be very useful because it allowed us to "fix" and characterize the photosynthetic material at various times after the onset of illumination.

This chapter is concerned with fluorescence phenomena in isolated chloroplasts and in whole leaves of higher plants. The reader is referred to other chapters of this volume for fluorescence studies on other organisms: see Amesz and Vasmel, Chapter 15, for photosynthetic bacteria; Fork and Mohanty, Chapter 16, for phycobilin-containing algae; Govindjee and Satoh, Chapter 17, for Chl *b*- and Chl *c*-containing algae; and Renger and Schreiber, Chapter 19, for practical applications of fluorometric methods. For a thorough discussion of the relationship between Chl *a* fluorescence yield and the photochemical reactions, see Duysens, Chapter 1, and van Gorkom, Chapter 10.

II. Fluorescence Phenomena of Isolated Chloroplasts

In this section we discuss Chl *a* fluorescence characteristics of isolated chloroplasts. When chloroplasts still possess their outer double mem-

brane (envelope) and stroma we speak of *intact chloroplasts;* when the envelope is broken, we refer to them as *thylakoids.* Thylakoids, when maintained in the presence of a sufficient cation concentration, retain their *in situ* structural differentiation; membranes are partly unpaired (stroma lamellae) and partly stacked to form grana. When thylakoids are suspended in a low-salt medium, grana stacks are lost and all lamellae are unappressed (stacking–unstacking phenomenon).

Each photosystem consists of a reaction center complex, a Chl *a* core antenna, and a light-harvesting Chl *a*/Chl *b* protein complex, LHC I and LHC II, associated with PSI and PSII, respectively. Part of LHC II constitutes a third structural entity, which is sometimes called "free LHC II" to distinguish it from the LHC linked to the PSII core complex (see Kaplan and Arntzen, 1982).

At room temperature Chl *a* fluorescence is emitted mainly by PSII. We will try to explain schematically why PSI does not contribute significantly to fluorescence emission. In both photosystems, light energy absorbed by the antennae is trapped by the reaction center (RC), where charge separation occurs:

$$DPA + h\nu \rightarrow DP^{*}A \rightarrow DP^{+}A^{-} \quad (1)$$

where P is the Chl of the reaction center (P680 and P700 for PSII and PSI, respectively), D an electron donor, A an electron acceptor, and P* the excited state of chlorophyll. The state $DP^{+}A^{-}$ is very quickly transformed into either $D^{+}PA^{-}$ (in PSII) or $DP^{+}A$ (in PSI).

When the RC is in the state DPA, it is called an *open* center, i.e., a center capable of photochemistry. This state is nonfluorescent because the main pathway for deactivation of the excited Chl is charge separation.

In the other states, centers are considered to be *closed,* i.e., unable to carry out charge separation. It has been shown (Butler *et al.,* 1973) that the RC, when in the P^{+} state, is a quencher of fluorescence. Thus, neither in the transitory state $DP^{+}A^{-}$ nor in the state $DP^{+}A$ can fluorescence be emitted. Only the state $D^{+}PA^{-}$, where both P and A are reduced, does fluorescence emission take place.

Consequently, for PSI, whether the center is open or closed, the fluorescence intensity is identical and low. Only under very strong reducing conditions can a high fluorescence intensity be demonstrated for PSI at room temperature (Ikegami, 1976; Telfer *et al.,* 1978). At low temperature (77°K), a high fluorescence emission by PSI appears because a special fluorescing Chl species, absorbing at 710–720 nm, competes with P700 in trapping the energy (Butler *et al.,* 1973). However, as at room

temperature, the fluorescence intensity does not depend on the open or closed state of the PSI center.

In contrast, for PSII at room temperature and at 77°K, the fluorescence will be low when the RCs are open and high when they are closed, because excitation trapped in the state DPA^- (giving DP^*A^-) has a high probability of being emitted as fluorescence; this causes the phenomenon of *variable fluorescence.*

A. Chlorophyll Fluorescence at Low Temperatures

At low temperatures, PSI, PSII, and LHC can be distinguished by their characteristic fluorescence bands (for a review, see Bose, 1982). Measurements have been made at temperatures as low as 4°K (Kramer *et al.,* 1981), but in most studies spectra are recorded at 77°K. Emission and excitation spectra and fluorescence induction (i.e., fluorescence transients occurring at the beginning of illumination of dark-adapted materials) at various wavelengths have been reported (see, e.g., Murata *et al.,* 1966b; Harnischfeger, 1977; Kramer *et al.,* 1981; Kramer and Amesz, 1982).

1. EMISSION SPECTRA

Figure 3 depicts a fluorescence emission spectrum of intact chloroplasts recorded in the presence of fluorescein as an internal standard at 77°K. The emission spectrum consists of three bands: F685 and F695, attributed to PSII, and F735, attributed to PSI (Govindjee and Yang, 1966; Butler and Kitajima, 1975b; Satoh and Butler, 1978).

F685: This band had previously been attributed to LHC II (Strasser and Butler, 1977b). However there are now many arguments in favor of the suggestion that F685 originates from the PSII core antenna, while the LHC II fluoresces at 680 nm. First, both F685 and F695 are present in chloroplasts of mutants deficient in LHC II (Vernotte *et al.,* 1976; Rijgersberg *et al.,* 1979). Second, a weak band F680 (better seen at 4°K) is present in wild-type spinach, barley, maize, and tobacco plants, but is absent in all mutants deficient in LHC II (Rijgersberg *et al.,* 1979). Third, a maize mutant containing a normal amount of LHC II but no PSII RCs exhibits an emission at 680 nm with a shoulder at 696 nm, but it does not show the F685 band (Leto and Arntzen, 1981). Emission spectra of various isolated Chl *a*–protein complexes confirm the above assignment. Gasanov *et al.* (1979) assigned F685 to emission of the core antenna of PSII, not to LHC II. Isolated LHC II presents an emission maximum at 680 nm; a shoulder at 695 nm appears only when LHC II is aggregated

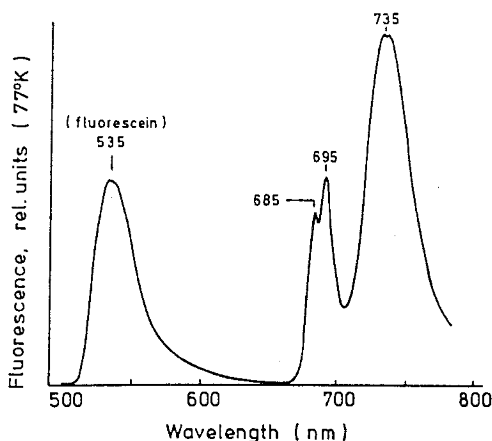


FIG. 3. Fluorescence emission spectrum at 77°K of intact isolated spinach chloroplasts in the presence of fluorescein (2 μM) as internal standard. Exciting light: wavelength, 480 nm (half-bandwidth, 20 nm); intensity, 4 W/m^2. The spectrum is corrected for the wavelength dependence of the photomultiplier sensitivity. Fluorescence was recorded with a bandpass of 1 nm. (From Krause and Weis, 1984; altered.)

by the addition of Mg^{2+} (Mullet and Arntzen, 1980). Furthermore, when LHC II is disconnected from the PSII core antenna by lipid supplementation of thylakoids (Siegel *et al.*, 1981), a large F680 band becomes apparent.

F695: The emission at 695 nm may originate from the PSII core antenna or from the PSII RC complex. The excitation spectrum of F695 (Kramer *et al.*, 1981) and its presence in mutants of algae and higher plants that do not contain LHC II support the conclusion that the contribution of LHC II to F695 is small. Govindjee and Yang (1966) suggested that F695 could arise from Chl *a* of the PSII RC complex. Breton (1982, 1983) attributed this emission to pheophytin (I), an intermediate acceptor between P680 and Q_A in the RC complex. According to Breton's hypothesis, the recombination of charges in the state $P^+I^-Q_A^-$ may produce either $P^*IQ_A^-$ or $PI^*Q_A^-$, the latter fluorescing at 695 nm and the former either fluorescing at 685 nm or transferring its energy to the PSII antenna that fluoresces at 685 nm. Nakatani *et al.* (1984) have isolated from spinach two Chl *a*-binding proteins associated with PSII: (1) a Chl protein of M_r 47,000 that displays a 695-nm fluorescence emission at 77°K and light-induced absorption changes characteristic of the PSII RC, and (2) a Chl protein of M_r 43,000 that displays a fluorescence emission at 685 nm and probably serves as an antenna.

F735: The F735 band is an emission from PSI. Mullet *et al.* (1980a,b) prepared "native" PSI particles, which contained 110 Chls per P700 and fluoresced at 735 nm. Extraction of this preparation with Triton X-100 caused removal of a peripheral Chl–protein complex. This complex has been isolated (see, e.g., Anderson *et al.,* 1983; Haworth *et al.,* 1983; Remy and Ambard-Bretteville, 1984) and called the light-harvesting Chl *a*/*b*–protein complex I (LHC I). "PSI-65" particles (65 Chls per P700) that are devoid of this LHC I, thylakoids from mutants deficient in LHC I, or immature thylakoids (Mullet *et al.,* 1980a,b; Argyroudi-Akoyunoglou *et al.,* 1984) exhibit an emission maximum at 722 nm, instead of 735 nm. PSI particles more extensively depleted of antenna Chl have been prepared (see Kaplan and Arntzen, 1982). The "PSI-40" particles (40 Chls per P700) show an emission maximum at 694 nm (Mullet *et al.,* 1980b). Very immature thylakoids have a high ratio of F685 and F695 to F730. It is possible that another band in the 695-nm region, labeled F694, which originates from PSI is present in these thylakoids and is responsible for the high ratio observed. Thus the F694 emission may reflect the presence of Chl in a low organizational state, which in the PSI-40 particles may be caused by the presence of detergents.

Butler *et al.* (1979) attributed the F735 band to a Chl *a* absorbing around 705 nm (C705) that acts as a trap at 77°K for the energy coming from the PSI antennae. This species is present in LHC I at room temperature as well as 77°K, but it is less fluorescent at the higher temperature because of increased energy transfer to P700. The above picture of PSI fluorescence, i.e., a core Chl *a* antenna fluorescing at 722 nm and a peripheral antenna (LHC I) containing a long-wavelength component which absorbs at 705–725 nm and fluoresces at 735 nm, has been confirmed by polarized absorption and fluorescence measurements of PSI particles and thylakoids from peas (Tapie *et al.,* 1984).

For a discussion of F685, F695, F718, and F725 in green algae, see Govindjee and Satoh, Chapter 17, this volume.

2. FLUORESCENCE INDUCTION

When dark-adapted samples are frozen at 77°K and then illuminated, Chl *a* fluorescence transients are observed. The fluorescence intensity increases from an initial level, F_0, to a maximum level, F_m (Murata, 1968). The ratio of the variable fluorescence ($F_v = F_m - F_0$) to F_m is about 0.8 at 695 nm and 0.3 at 735 nm in thylakoids suspended in a high-salt medium (Murata, 1968) and in intact chloroplasts (Krause and Behrend, 1983). At 685 and 695 nm, the rise from F_0 to F_m is linked to the reduction of Q_A (Butler and Kitajima, 1975a; see also van Gorkom,

Chapter 10, this volume). At 77°K no appreciable Q_A^- reoxidation is possible except in a very long dark time (Murata *et al.*, 1973; Bonnet *et al.*, 1977). At 735 nm, the variable fluorescence is attributed to excitons that have migrated from closed PSII RCs (Q_A reduced) via PSII antennae to PSI. A relationship between the different Chl–protein complexes was formulated by Butler and Kitajima (1975c) and Butler and Strasser (1977) in their "tripartite model." In this model there are three complexes: LHC II, the PSII core complex (including the PSII RC and Chl *a* core antenna), and PSI (including the PSI RC and its antennae). The equations describing the fluorescence properties include terms representing the distribution of absorbed quanta between the complexes and rate constants for excitation energy transfer between them, photochemistry, thermal deactivation, and fluorescence.

Low-temperature fluorescence measurements have been extensively used to study changes in excitation energy distribution between PSII and PSI (see, e.g., Butler, 1977; Haworth *et al.*, 1982; Krause and Behrend, 1983; Satoh and Fork, 1983a), to characterize fluorescence quenching (Briantais *et al.*, 1979; Krause *et al.*, 1982, 1983; Satoh and Fork, 1983b), and to analyze alterations of the photosynthetic pigment system due to changes in environmental parameters (Schreiber and Armond, 1978; Öquist and Fork, 1982; Powles and Björkman, 1982; Weis, 1984a).

B. Chlorophyll Fluorescence at Room Temperature

At room temperature, the emission spectrum of Chl *a* fluorescence of thylakoids and intact chloroplasts has a main band at 685 nm and a minor band at 720–740 nm. The excitation spectra for the various bands indicate that most of the fluorescence has its origin in PSII, but some fluorescence in the region 710–720 nm originates from PSI (see a review by Papageorgiou, 1975; also see, e.g., Wong and Govindjee, 1979). When dark-adapted material is illuminated, the fluorescence emission shows intensity transients, the various successive levels being labeled (Fig. 2) O,I,P,D,S,M,T. Alternatively, the terms F_0, F_i, F_p, and F_s are used for the O, I, P, and S levels. The F_0 level is also termed constant fluorescence, as opposed to variable fluorescence, which reflects time-dependent changes in fluorescence intensity.

In thylakoids in which electron transport is uncoupled from phosphorylation, only levels O, I, D, and P can be observed. In coupled thylakoids supplemented with PSI electron acceptors, as well as in intact chloroplasts, levels O, I, D, P, S, M, and T can be observed, giving a fluorescence induction curve resembling that of a leaf presented in Fig.

2. In the presence of DCMU, which blocks electron transfer between Q_A and Q_B (see Fig. 1), the fluorescence induction is simpler because it reflects only electron transfer to Q_A.

1. "CONSTANT" FLUORESCENCE, F_0 OR O LEVEL

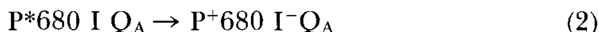
The light energy absorbed by the antenna Chl *a* molecules migrates to the RC. Some of this energy is lost and appears as a fluorescence emission, giving rise to the F_0 level (see, e.g., Mathis and Paillotin, 1981). Thus the quantum yield of F_0 is independent of photochemical events. The F_0 level increases linearly with light intensity except at very high intensities, where exciton annihilation processes occur. The true F_0 level is seen only when, at the onset of illumination, the first quinone electron acceptor Q_A is fully oxidized; this can be achieved by dark preincubation for several minutes. The yield of F_0 emitted from PSII antennae depends on the initial density of excitons within the PSII pigments. It is therefore affected by the distribution of excitation energy between PSI and PSII. For a further discussion of the O level, see Govindjee and Satoh, Chapter 17, this volume.

The F_0 level is also known to be affected by environmental stress (see Renger and Schreiber, Chapter 19, this volume). High-temperature incubations (Krause and Santarius, 1975; Schreiber and Berry, 1977; Schreiber and Armond, 1978) and addition of free fatty acids to thylakoids (Golbeck *et al.*, 1980; Vernotte *et al.*, 1983) increase F_0 drastically. This has been interpreted as reflecting decreased efficiency of energy transfer from the antenna Chl *a* to the RCs and/or perturbation of the functioning of the PSII RCs.

2. VARIABLE FLUORESCENCE, F_v

The fluorescence rise from F_0 is usually considered to reflect reduction of the electron acceptor of PSII, Q_A. According to the "classical" hypothesis of Duysens and Sweers (1963), oxidized Q_A acts as a fluorescence quencher, because in the state P^*680Q_A (where P^*680 is the excited PSII RC Chl) photochemical energy conversion (i.e., formation of $P^+680Q_A^-$) is the favored pathway of deactivation. Once Q_A is reduced, the probability of fluorescence emission from the state $P^*680Q_A^-$ is increased. Butler and Kitajima (1975b) suggested that there is no direct fluorescence emission by $P^*680Q_A^-$; instead, a rapid back transfer of excitons from these closed RCs to the antenna pigments is assumed to cause an increase in the fluorescence emission from Chl *a* antenna molecules of PSII. This explains the high levels of variable fluorescence. In the state $P^*680Q_A^-$, no photochemical energy conversion can occur.

Since pheophytin *a* (I) acts as an electron mediator between P680 and Q_A (Klimov *et al.*, 1977; see van Gorkom, Chapter 10, this volume), the hypothesis outlined above requires modification; the primary photochemical event is the following reaction:



$$P^*680\ I\ Q_A \rightarrow P^+680\ I^-Q_A \quad (2)$$

Klimov and Krasnovskii (1981) proposed that when Q_A is reduced (high fluorescent state), excitation of P680 leads to a primary charge separation, giving $P^+680\ I^-Q_A^-$, which then leads to recombination of charges and production of the excited state ($P^*680\ I\ Q_A^-$). The latter state deactivates, as in the preceding hypothesis, by energy transfer to the PSII antenna pigments (see further discussion by van Gorkom, Chapter 10, Moya *et al.*, Chapter 7, and Jursinic, Chapter 11, this volume).

3. INDUCTION IN THE PRESENCE OF DIURON

Reoxidation of Q_A^- via the electron transport chain is blocked by diuron (DCMU), and therefore the maximum fluorescence level F_m is rapidly reached on illumination. However, some fluorescence quenching may still take place in the presence of DCMU. This is ascribed to the quenching by the oxidized PQ pool (Vernotte *et al.*, 1979). The rise of variable fluorescence observed in the presence of DCMU reflects the kinetics of "closure" of the normal PSII chemistry. At the F_0 level all the centers are open, and at the F_m level they are all closed to normal chemistry. In a model of separate PSII units (see Duysens, Chapter 1, this volume) a progressive reduction of Q_A with first-order kinetics can be predicted, resulting in an exponential increase in fluorescence. However, in most cases a sigmoidal increase in fluorescence is observed; according to Joliot and Joliot (1964) and Joliot *et al.* (1973), this is due to energy transfer between PSII units. In this theory, the energy absorbed by a unit when its center is closed (Q_A^-) has a certain probability of being transferred to a neighboring unit, where it can be utilized for a photochemical reaction, rather than being dissipated as fluorescence (for a quantitative approach, see Mathis and Paillotin, 1981).

The time necessary for the reduction of all Q_A is inversely proportional to the number of photons reaching the centers per unit time. This rate of photon arrival is determined by the exciting light intensity and by the size of the antenna associated with each center. The approximate size of the antenna can be estimated as the inverse of the half rise-time of the variable fluorescence.

At a constant light intensity, the area delineated by the fluorescence rise curve and its asymptote is proportional to the number of excitons

that have not been dissipated by fluorescence but have been used for photochemistry (also consult Lavorel *et al.*, Chapter 4, this volume). The area is proportional to the concentration of the electron acceptor Q_A. It can be used to calculate the number of PSII centers per unit Chl (Murata *et al.*, 1966a; Malkin and Kok, 1966), but it is generally used as a relative measure, either to estimate the relative size of the photosynthetic units of a material under various conditions, or to compare various materials. Experimental or physiological conditions can cause F_m to vary; thus it is necessary to normalize the area above the fluorescence rise curve to the PSII fluorescence F_m.

Melis and Homann (1975) showed that an analysis of the fluorescence induction of DCMU-treated thylakoids revealed an additional complication, i.e., the existence of a fast nonexponential (α) phase and a slow exponential (β) phase. This has been interpreted in terms of the existence of connected α units and disconnected β units, i.e., two types of PSII units differing in their quantum efficiency of photochemistry (Melis and Homann, 1978; Melis and Duysens, 1979). The two components of fluorescence induction have been characterized with respect to their different sensitivities to cations and to the enzymatic phosphorylation of the LHC II (Melis and Ow, 1982; Hodges and Barber, 1983b; Horton and Black, 1983; Telfer *et al.*, 1983). Indications of heterogeneity of the PSII acceptor side came from redox titration of fluorescence (Horton and Croze, 1979; Horton, 1981). Schreiber and Pfister (1982) assumed that the β phase arises from a population of PSII centers in the thylakoids with a low affinity for DCMU. Joliot and Joliot (1979) proposed a different model. They suggested that one type of PSII centers can alternately reduce two different acceptors, Q_1 and Q_2. However, the nature of the two phases of fluorescence induction is still controversial. In particular, it is not clear whether the α and β phases are related to structurally distinct forms of PSII or to interconvertible organizational states of PSII. The latter possibility was discussed by Percival *et al.* (1984), who argued that the α characteristic reflects the proportion of LHC II that is physically or energically connected to PSII. For reviews on the heterogeneity of PSII, see Vermaas and Govindjee (1981), and Black *et al.* (1985).

4. INDUCTION IN THE ABSENCE OF DIURON

We will consider uncoupled thylakoids and then intact chloroplasts.

In uncoupled thylakoids without the addition of PSI electron acceptors, the fluorescence induction curve describes only the O-I-P transients; P comes close to the maximum level, F_m, obtained in the presence of DCMU. Electrons are transferred from Q_A via Q_B to the PQ pool,

which is reduced in the light when electron transport via PSI is limiting (see Fig. 1). Because of this electron transfer, the fluorescence rise, which reflects Q_A reduction, is much slower than in the presence of DCMU. The biphasic kinetics of the fluorescence rise, the rapid O-I phase and slow I-P phase, have been well explained by Lavergne (1974) in terms of PSII heterogeneity for Q_A reoxidation. It is known that increasing the rate of Q_A reduction by increasing the light intensity, or decreasing the rate of reoxidation of Q_A^- by the PQ pool by using inhibitors such as DCMU, will give a higher I level. Lavergne (1974) was able to resolve the I level light-intensity curve into 3 components in algae which reflect 3 rate-constants for Q_A reoxidation (assigned to 3 "fluorescence states" with lifetimes of 1 msec, 100 msec and several seconds). In low incident light intensity, the O-I transient will correspond to centers which are loosely or not connected to plastoquinones. In high light (5000 photons per second) all centers are in the state Q_A^- at the I level; the I-P phase is then purely thermal and corresponds to the release of quenching by oxidized plastoquinones (Delosme, 1967; Vernotte *et al.,* 1979).

The area above the fluorescence curve is proportional to the electrons accumulated in the light on the electron acceptor side of PSII, thus to the amount of PQ which has been reduced. By comparing the area with that obtained in the presence of DCMU (which reflects only Q_A reduction), one can estimate the size of the PQ pool. A ratio of the two areas of about 15–20 was observed in thylakoids isolated from spinach or peas. As the molecules of the PQ pool are two-electron acceptors and Q_A is a one-electron acceptor, an estimate of 7–10 PQs per PSII reaction center was found (Murata *et al.,* 1966a; Forbush and Kok, 1968; see also Renger and Schreiber, Chapter 19, this volume). One should note that the size of the PQ pool can be overestimated using this method if a part of the PSII centers have their donor side inactivated. Then reduction of PQ and Q_A of inactive centers by active ones occurs through redox interactions within the PQ pool (Siggel *et al.,* 1972). This situation can be detected by a stimulation of the I-P rate upon hydroxylamine addition (Briantais *et al.,* 1977).

In intact chloroplasts, the fluorescence induction curve is more complex than that in thylakoids. It shows the successive O, I, D, P, and S levels similar to those of a leaf (Fig. 2) or of intact cells of green algae (see Govindjee and Satoh, Chapter 17, this volume). The relative height of the P level depends on the intensity of the exciting light. Under strong illumination P may come close to F_m, and the ratio F_v/F_0 may reach values of 4–5. At very low light intensities, the fluorescence emission remains near the F_0 level. The variable fluorescence, reflecting Q_A reduction and reoxidation, depends on the redox level of the PQ pool,

which in turn is modulated by the relative rates of the PSI and PSII reactions. The P level reflects accumulation of electrons between the two photosystems due to a transient block of electron transport through PSI (as suggested by Munday and Govindjee, 1969, for green algae). The transient decline of PSI activity may result from lack of efficient electron acceptors of PSI, i.e., from exhaustion of the $NADP^+$ pool. Carbon dioxide, a final substrate in intact chloroplasts, mediates efficient reoxidation of NADPH only after a lag phase during which the carbon reduction cycle is activated. Reduction of O_2, instead of $NADP^+$ may occur (Mehler reaction), but its rate appears insufficient for fast reoxidation of the PQ pool. In fact, when algae or intact chloroplasts are supplied with an efficient mediator of O_2 reduction, such as methyl viologen, the fluorescence peak P is lowered drastically, and a strong transient reduction of electron carriers is avoided (Munday and Govindjee, 1969; Lavergne, 1974; Krause *et al.*, 1981).

The fluorescence decline from P to S can be only partly explained by enhanced reoxidation of Q_A^- due to increased activity of PSI (Duysens and Sweers, 1963); several other possible mechanisms of fluorescence quenching may be involved, as discussed below (also see Govindjee and Satoh, Chapter 17, this volume, for a discussion of a similar transient in green algae).

C. *Effect of Intrathylakoid H^+ Concentration on Fluorescence Emission*

Thylakoids treated with DCMU and illuminated in the presence of phenazine methosulfate (PMS), which promotes cyclic electron flow around PSI, show a reversible lowering of the F_m level (Govindjee *et al.*, 1967; Murata and Sugahara, 1969). This fluorescence quenching was ascribed to the formation of a high-energy state (HES), because it was independent of the redox state of Q_A and was inhibited by uncouplers. The PMS-induced effect was further studied and compared to the Mg^{2+} effect on fluorescence (see Section II,D) by Mohanty *et al.* (1973). In contrast to the Mg^{2+} effect, the PMS effect does not significantly modify the shape of the fluorescence emission spectrum at 77°K. This suggests that the PMS effect is not related to a change in excitation energy distribution between the two photosystems.

Energy-dependent quenching during the P-S phase, in the absence of DCMU and PMS, was described for intact chloroplasts by Krause (1973). It was also observed with thylakoids, where the P-S phase could be restored by addition of Mg^{2+} (Krause, 1974, 1975). Fluorescence quenching and the apparent absorption change (ΔA) at 535 nm (reflecting a

scattering change and used as an indicator of the proton gradient across the thylakoid membrane) vary in a parallel fashion. The effect of uncouplers or electron transport inhibitors suggested that both fluorescence quenching and ΔA at 535 nm are related to the HES. It was proposed that photoinduced H^+ uptake into the thylakoid compartment induces cation displacement from the thylakoid compartment to the stromal space. Using specific ionophores, Barber *et al.* (1974a,b) demonstrated that Mg^{2+} was the main counterion involved in the fluorescence change during the P-S phase in intact chloroplasts. This was corroborated by measurements of cation transport across the thylakoid membrane (Hind *et al.*, 1974) and of light-induced Mg^{2+} movement within intact chloroplasts (Portis and Heldt, 1976; Krause, 1977, 1978).

The high and low fluorescence levels, P and S, correspond approximately to the F_m levels of thylakoids in the presence and absence of Mg^{2+}, respectively (see Section II,D). It was thus tempting to assume that illumination induces a state of the thylakoids equivalent to that in the absence of Mg^{2+}. However, analysis and characterization of these various states by electron microscopy and fluorescence emission measurements at 77°K showed that this is not the case. The transition from the P to the S state is not accompanied by major structural changes such as destacking of the thylakoids (Telfer *et al.*, 1976). Neither are there changes in the distribution of excitation energy between the two photosystems; either the ratio of PSI to PSII fluorescence is relatively unchanged (Briantais *et al.*, 1979) or, if an increase in the ratio is observed, it results from preferential quenching of F695 and not from enhancement of the F735 emission (Krause *et al.*, 1983).

Simultaneous measurements of the proton gradient and fluorescence intensity during the P-S phase of thylakoids show a linear relationship between the intrathylakoid proton concentration and fluorescence quenching (Briantais *et al.*, 1979, 1980; see also Garlaschi *et al.*, 1977).

As fluorescence is affected by both light-driven electron transport and concomitant proton translocation, we must consider two types of quenching of fluorescence in the P-S phase of chloroplasts—one dependent on the redox level of Q_A and the other dependent on the proton gradient. The relative importance of each kind of quenching can be determined by sudden addition of DCMU at various times during the P-to-S decline (Fig. 4). Addition of DCMU to illuminated intact chloroplasts increases the emission of Chl *a* fluorescence. This increase is usually biphasic, consisting of a fast phase with $t_{1/2} \sim 1$ s and a slow phase with $t_{1/2} \sim 15$ s. Figure 4 shows this increase for different physiological states of the chloroplast. The fast relaxation of quenching, R_{fast}, can be attributed to reduction of the part of Q_A that was in the oxidized state

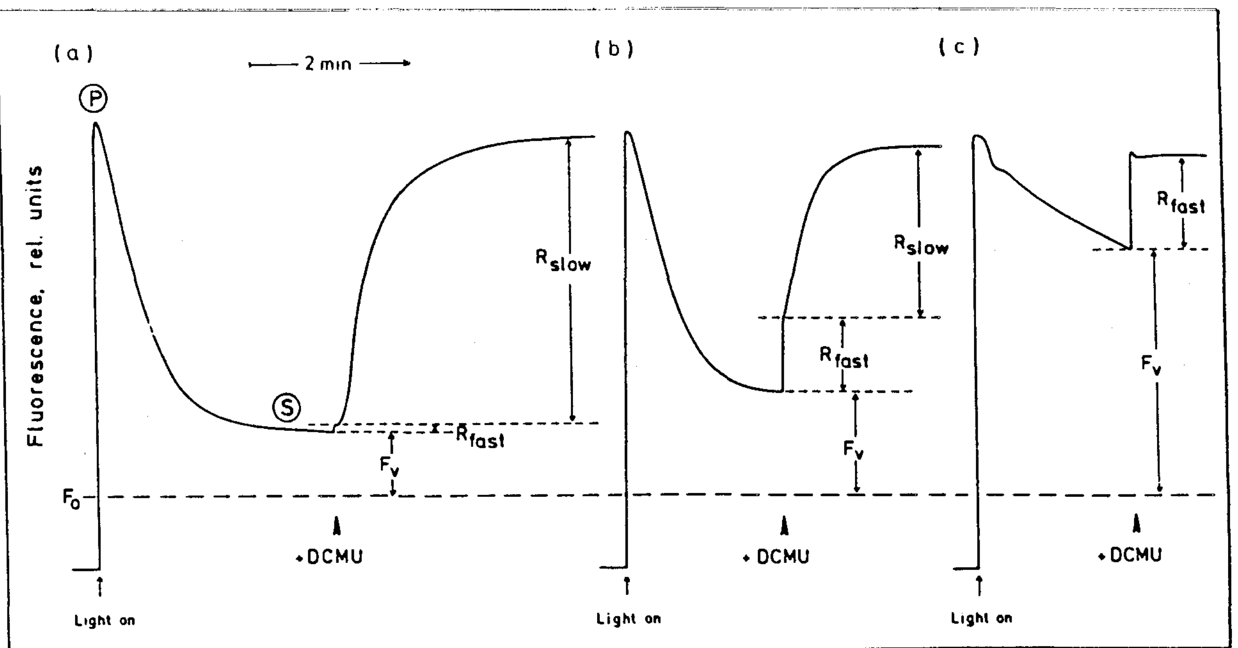


FIG. 4. Induction of chlorophyll *a* fluorescence in isolated intact chloroplasts at 20°C and relaxation of quenching by addition of DCMU (4×10^{-5} *M*). Illumination was started after 3 min of dark adaptation. DCMU was added in the light as indicated; R_{fast} and R_{slow} denote fast and slow phases of fluorescence increase on DCMU addition. (a) Absence of bicarbonate; net O_2 evolution was not detectable. (b) Presence of 2 m*M* $KHCO_3$; stationary rate of O_2 evolution, 28 μmol (mg chlorophyll)$^{-1}$ h^{-1}. (c) Presence of 2 m*M* $KHCO_3$ and 2 m*M* NH_4Cl; stationary O_2 evolution, 26 μmol (mg chlorophyll)$^{-1}$ h^{-1}. Light intensity (15 W/m^2, blue light) about 20% saturating for O_2 evolution. (From Krause *et al.*, 1982.)

before the electron transport was blocked by the inhibitor. The slower phase, R_{slow}, can be ascribed to relaxation of "energy-dependent" quenching, which is related to the intrathylakoid H^+ concentration. When chloroplasts are illuminated in the absence of efficient electron acceptors, Q_A stays in a largely reduced state and therefore the fast phase of relaxation is very small (Fig. 4a). Since in CO_2-depleted chloroplasts "phosphorylation energy" cannot be utilized in the Calvin cycle, an increased proton gradient develops which is responsible for the large energy-dependent fluorescence quenching represented by R_{slow}. The presence of bicarbonate (Fig. 4b) leads to a steady rate of photosynthesis after a few minutes of illumination. The electron acceptor Q_A is then partly reoxidized and the proton gradient at the thylakoid membrane is diminished as it is utilized by phosphorylation. Oxidized Q_A and the proton gradient then contribute about equally to the overall fluorescence quenching. The proton gradient can be further diminished by addition of NH_4Cl (2 m*M*) in the presence of bicarbonate, without inhibiting CO_2 fixation and the affiliated electron transport. Then only a fast phase of relaxation is visible on addition of DCMU (Fig. 4c). This means that ΔpH-dependent quenching is abolished by NH_4Cl, whereas Q_A^--dependent quenching remains.

D. Effect of Cations on Chlorophyll a Fluorescence

Izawa and Good (1966) demonstrated that the differentiation of thylakoids in grana lamellae, where thylakoids are stacked, and stroma lamellae, where thylakoids are unpaired, is controlled by the cation concentration of the suspending medium. When the thylakoids are maintained in a high-salt medium (100 m*M* NaCl or KCl or 5 m*M* $MgCl_2$) the *in situ* situation is preserved. If they are suspended in a low-salt medium (10 m*M* NaCl), grana stacks are lost and a system of unappressed thylakoids forms. Upon readdition of cations, the stacking is restored (see Kaplan and Arntzen, 1982). These observations have greatly helped in relating structure to function of the chloroplasts. Many results are now available which relate changes in photosynthetic parameters to stacking and unstacking of thylakoids. We shall discuss those involving changes of Chl *a* fluorescence.

1. VARIATIONS OF FLUORESCENCE AND PHOTOCHEMICAL ACTIVITIES

Homann (1969) and Murata (1969b) were the first to show that addition of cations, in the same concentration range as that used to induce

stacking of thylakoids, enhances the Chl *a* fluorescence at room temperature. They observed that F_v is much more stimulated than F_0. Interestingly, this enhancement was observed even when the electron flow was blocked by DCMU. Murata (1969b) also showed that emission spectra at 77°K are modified by addition of cations: F685 and F695 are enhanced, and F735 is decreased. The presence of Mg^{2+} accelerates the rate of photoreaction II and diminishes the rate of photoreaction I. To describe these results, Murata used the following equations:

$$F = caI_a\Phi_f \qquad (3)$$

$$\Phi_f = \frac{k_f}{k_f + k_i + k_p + k_t} \qquad (4)$$

where F is the fluorescence intensity, c a constant, I_a the total amount of light absorbed by the thylakoids, a the fraction of light transferred to and directly absorbed by the Chl *a* molecules of PSII (depending on the absorption cross section of PSII), Φ_f the fluorescence yield, [given by Eq. (4)], and k_f, k_i, k_p, and k_t the rate constants of deactivation by fluorescence, internal conversion (thermal deactivation), PSII photochemistry, and transfer to PSI, respectively.

A decrease in k_t, the rate constant of energy transfer from PSII to PSI, may be the dominant effect of Mg^{2+} addition. It explains why PSII activity is enhanced by Mg^{2+} while that of PSI decreases. Murata (1969b) concluded that, in addition to the change in the rate constant of energy transfer from PSII to PSI, a change in the value of a, the proportion of absorbed energy delivered to PSII, could occur.

Briantais *et al.* (1973) analyzed the effect of cation addition on fluorescence induction at room temperature, measuring the lifetime τ and intensity F of Chl *a* fluorescence simultaneously. According to Eqs. (3) and (5),

$$\tau = \tau_0\Phi_f \qquad (5)$$

where τ_0 is the natural lifetime of Chl *a* fluorescence, these authors concluded from the quasi-proportionality of τ and F observed when Mg^{2+} is added that the main change is in Φ_f and not in a. On the basis of parallel measurements of τ and F at 77°K, Wong *et al.* (1981) calculated that three concomitant changes, in order of importance, are: decrease in energy transfer from PSII to PSI, increase in radiationless losses, and a decrease in a. A more recent analysis (Nairn *et al.*, 1982) showed that there are three components of Chl *a* fluorescence with different lifetimes, instead of one as assumed in the above study. This led to the conclusion that the Mg^{2+}-induced absorption cross section changes

(changes of *a*) are much larger than those suggested previously (see Moya *et al.*, Chapter 7, this volume).

Briantais *et al.* (1973) also showed that the fluorescence induction in the presence of DCMU is sigmoidal in the presence of Mg^{2+} and nearly exponential in its absence. Therefore it was assumed that energy transfer between PSII units exists only in the presence of high cation levels.

Butler and Kitajima (1975b) studied the emission spectra and induction curves at 77°K in the presence and the absence of Mg^{2+}. They proposed a model of the various pathways of excitation energy distribution between the PSI and PSII Chl *a* complexes. They also concluded that Mg^{2+} influences the distribution of energy by exerting control on both the initial distribution of quanta [*a* in Eq. (3)] and the yield of energy transfer from PSII to PSI [depending on k_t in Eq. (4)].

Wydrzynski *et al.* (1975) and Henkin and Sauer (1977) pointed out that the slight enhancement of the F_0 level by the addition of $MgCl_2$ to thylakoids is saturated at lower concentrations (0.5 to 0.7 m*M*) than the large stimulation of F_m. These results suggested that changes in *a* and k_t may not have the same origin. Loos (1976), Wong and Govindjee (1981), and Jennings (1984a,b) observed Mg^{2+}-induced modifications of the action spectra of PSII fluorescence. These variations may occur because the regulation of the initial distribution of excitation to PSII involves coupling of LHC II to Chl *a* molecules of the PSII reaction center complex. This coupling is stronger in the presence of Mg^{2+} than in its absence.

Evidence for PSII heterogeneity (see Section II,B,3; Black *et al.*, 1985), be it the existence of PSIIα and PSIIβ units or of several Q_A-type acceptors, has led to more complex interpretations of the effect of cations on Chl *a* fluorescence in thylakoids. Melis and Ow (1982) found that cation addition enhances the fluorescence of PSIIα much more than that of PSIIβ. Karukstis and Sauer (1983) observed Mg^{2+}-induced variations of the apparent midpoint redox potential of the high- and low-potential Q_A-type acceptors. Thus, several cation effects can modify PSII fluorescence. The most important effects of cations are (1) a decrease of energy transfer from PSII to PSI, (2) an increase in the PSII absorption cross section, and (3) an increase in energy transfer between PSII units.

2. STRUCTURAL IMPLICATIONS

Murata (1969b) suggested that conformational changes within the thylakoids might alter the mutual orientation of pigments and hence energy transfer between them. Biggins (1981) showed that cation-induced

stacking of thylakoids is accompanied by only slight variations of linear dichroism. Therefore energy transfer changes may not be based on a change of orientation between the pigments, but are probably based on the distance between the Chl *a* complexes. Lutz (1977) and Markwell *et al.* (1979) reported evidence that all Chls are localized in chromoprotein complexes. It is now well known (see Arntzen and Staehelin, 1979) that thylakoid stacking is associated with the segregation of the two photosystems: PSII is located predominantly in the grana stacks and PSI mainly in the stroma lamellae. When the thylakoids are unstacked (at low cation concentrations) the protein complexes are homogeneously distributed within the membrane (Arntzen and Staehelin, 1979; Andersson and Anderson, 1980; Miller, 1980). As hypothesized by Barber and Chow (1979) and Anderson (1981), lateral segregation of the two photosystems is the simplest explanation for the decrease in excitation energy transfer between the two when cations are added. Energy transfer can occur between PSI and PSII complexes only in unstacked thylakoids, where they are close to each other (Vernotte *et al.*, 1982). Barber and Malkin (1981) and Briantais *et al.* (1984) have shown that the fluorescence increase and lateral segregation of particles occur within the same time span. This is in contrast to the results of Garlaschi *et al.* (1984), who studied the kinetics of the decrease in light scattering, stacking, and fluorescence yield that occurs on removal of Mg^{2+} from thylakoids by the addition of EDTA. They observed that the scattering changes are faster than the fluorescence decrease (also see VanderMeulen and Govindjee, 1974). However, particle separation may not always occur simultaneously with membrane appression and related light scattering changes. When the fluidity of the membrane increases, the cation-induced particle segregation and fluorescence increase become faster (Chapman *et al.*, 1982). Incorporation of cholesterol into the membrane, to lower the fluidity, blocks the cation-induced fluorescence enhancement (Scoufflaire *et al.*, 1981; Yamamoto *et al.*, 1981) and particle segregation (Briantais *et al.*, 1984). The latter authors observed that this treatment abolishes neither the scattering increase nor the appression of adjacent membranes. Thus it is the lateral segregation which is responsible for the decrease of PS II → PSI energy transfer; the appression by itself does not change energy transfers. The same conclusion was reached by Garlaschi *et al.* (1984).

3. MECHANISM OF CATION-INDUCED CHANGES

a. Physicochemical Aspects. The results of Gross and Hess (1973, 1974), Gross and Prasher (1974), VanderMeulen and Govindjee (1974),

and Gross *et al.* (1975) on cation-induced Chl fluorescence and thylakoid structural changes showed that these phenomena are controlled by the electrostatic properties of the membrane. Barber and Mills (1976), Barber *et al.* (1977), Mills and Barber (1978), and Duniec *et al.* (1979) showed that thylakoid stacking and increased PSII fluorescence result from screening of negative surface charges on the thylakoid membrane by cations (for review, see Barber, 1980a,b). This is illustrated by schemes from Barber (1982a,b) in Fig. 5, which shows the general effects of charge screening, and Fig. 6, which represents the specific case of thylakoid membranes. Two theories support this interpretation: the Gouy–Chapman theory for diffuse ionic layers and the Verwey and Overbeck (1948) theory for the stability of hydrophilic colloids.

Another problem was to distinguish between electrostatic screening

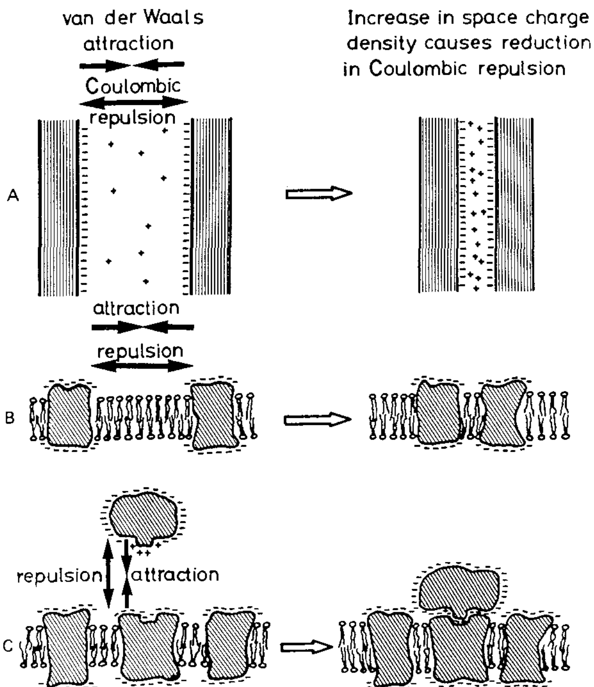


FIG. 5. Schematic representation of effects of the space charge density on spatial relationship between (A) two membranes, (B) intrinsic membrane proteins, and (C) an extrinsic protein and a membrane surface. In the latter case a Coulombic attraction occurring at short distances permits specific interactions between the extrinsic protein and a particular intrinsic protein. (From Barber, 1982a. Reproduced from the *Annual Review of Plant Physiology*, Volume 33, © 1982 by Annual Reviews, Inc.)

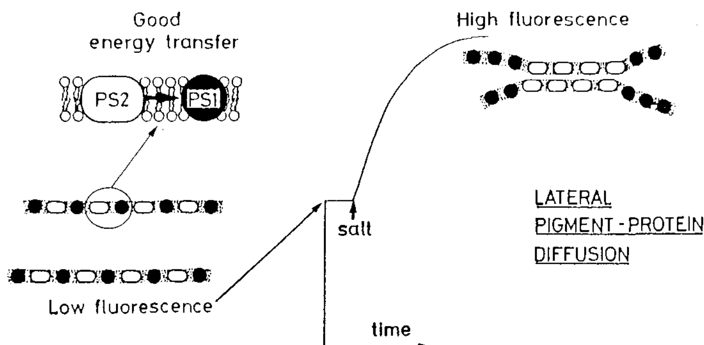


FIG. 6. Schematic representation of changes in thylakoid structure caused by the screening of electrical surface charges. The protein complex of PSII, including the LHC II (shown as an unshaded particle) is postulated to carry a low net electrical charge on its exposed surface. Under high-salt conditions, PSII preferentially aggregates in regions where there is close membrane interaction (strong van der Waals interactions between adjacent complexes and between adjacent membrane surfaces in the absence of significant short-range Coulombic repulsion). The pigment–protein complexes of PSI are shown as filled circles (●) and postulated to carry net negative charge on their exposed external surfaces. Therefore they aggregate less readily on addition of salt and are excluded from membrane regions where the Coulombic repulsion is sufficiently weak to favor membrane appression. Under low-salt conditions, Coulombic repulsion is large and prevents aggregation and membrane appression so that the PSII and PSI proteins are randomized in the plane of the membrane. The change from the randomized to the aggregated condition is reversible and can be readily monitored by changes in the yield of chlorophyll fluorescence which reflects alterations in the degree of energy transfer from the PSII to PSI complexes. (From Barber, 1982b. Reprinted by permission from *Bioscience Reports*, Volume 2, pp. 1–13. Copyright © 1982 The Biochemical Society, London.)

mechanisms (as described above) and electrostatic neutralization mechanisms. Both H^+ and La^{3+} ions bind to thylakoids and thus neutralize negative charges on the membranes. Two distinct effects can be discriminated. At low concentrations H^+ and La^{3+} mimic the action of cations such as K^+ and Mg^{2+} and cause an increase in stacking and fluorescence attributed to charge screening. At higher concentrations (below pH 5.1 for H^+ and above 20 μM for La^{3+}) binding of these cations causes gross membrane appression, but no lateral movements of Chl–protein complexes in the membrane occur (Briantais *et al.*, 1979; Gerola *et al.*, 1979; Jennings *et al.*, 1979, 1980a,b; Scoufflaire *et al.*, 1982).

Karukstis and Sauer (1985) have demonstrated specific cation effects as determined by the hydrated metal ionic radius. They suggest that the smaller the radius, the more effective is the electronic screening, and thus PSII and PSI on the same membrane and PSII and PSII on neighboring membranes are closer together.

b. Molecular Mechanism. Several lines of evidence show that the presence of LHC II is a requirement for cation-induced changes of light energy distribution. (1) The effect is very small in the *Su/su* mutant of tobacco deficient in LHC II (Vernotte *et al.*, 1976); there is no effect of cations on PSII units which do not possess LHC II (Melis and Homann, 1978; Thielen and van Gorkom, 1981). (2) The cation effect and biosynthesis of LHC II develop in parallel during continuous illumination of leaves previously greened in intermittent light (Armond *et al.*, 1976). In a maize mutant that lacks PSII RCs but synthesizes LHC II, cation-induced fluorescence increases and stacking still occur (Leto and Arntzen, 1981).

McDonnell and Staehelin (1980) and Mullet and Arntzen (1980) demonstrated appression of liposomes containing LHC II and the aggregation of these complexes. According to Jennings *et al.* (1978, 1981), mild trypsin treatment of thylakoids inhibits cation effects. Steinback *et al.* (1979) and Mullet *et al.* (1981) showed that a positively charged 2-kD segment of LHC II, which is exposed to the outer membrane surface, is involved in the cation effects. Mullet *et al.* (1981) suggested that these positively charged peptides interact with specific negative groups on an opposite membrane. After trypsin treatment of thylakoids, Nakatani and Barber (1980) measured an increase of negative surface charge density, and Jennings *et al.* (1981) showed that after such treatment both fluorescence enhancement and stacking require a larger cation concentration.

In conclusion, cations regulate the energy distribution between the two photosystems by changing the spatial organization of the various Chl–protein complexes. This is due not only to screening of negative charges but also to specific interactions dependent on LHC II.

E. Phosphorylation of Light-Harvesting Complex II and State I–State II Transitions

Working with algae, Bonaventura and Myers (1969) and Murata (1969a) demonstrated that photosynthetic organisms can regulate the partitioning of light energy between the two photosystems so that maximum efficiency of light energy utilization may be maintained (see Govindjee and Satoh, Chapter 17, and Fork and Mohanty, Chapter 16, this volume; also see Fork and Satoh, 1986). Illumination for a few minutes with light absorbed preferentially by PSII (light 2) brings about the production of state II, where there is increased energy distribution to PSI. This adaptation results in a decrease of PSII fluorescence. Conversely, light absorbed preferentially by PSI (light 1) produces state I, in

which excitation of PSII is favored. State I–state II transitions were also observed in higher plants (Vernotte *et al.,* 1975; see also Section III,D).

For several years these transitions were attributed to light-induced changes in the concentration of divalent cations surrounding the thylakoids (see Sections II,C and II,D). According to this hypothesis, state I and state II would be equivalent to the states of thylakoids in the presence of high and low cation concentrations, respectively. Light 1 would have to increase the cation concentration in the stroma of the chloroplast in order to promote thylakoid stacking and repression of energy transfer from PSII to PSI. Light 2 would have to reverse these effects. However, it is doubtful that light 1 and light 2 differ sufficiently in the degree to which they modify the Mg^{2+} concentration in the stroma by H^+ pumping into the thylakoids and passive countertransport of Mg^{2+}.

A mechanism for state I–state II transitions has been revealed as a result of work by Bennett *et al.* (1980), Horton and Black (1980, 1981), and Allen *et al.* (1981). Phosphorylation of LHC II was found to increase the distribution of energy to PSI at the expense of PSII, while dephosphorylation had the reverse effect (see Bennett, 1983, for a review).

1. ENZYMATIC MECHANISM

Phosphorylation of LHC II and of several other thylakoid proteins is catalyzed by a kinase attached to the thylakoid membrane and requires the presence of 3–5 m*M* $MgCl_2$ (Bennett, 1979). Presumably the activity of the enzyme is sensitive to the redox state of the PQ pool. The kinase is activated when the PQ pool is reduced, either by illumination of the thylakoids (with no artificial electron acceptor to PSI added) or by addition of strong reducing agents such as reduced ferredoxin (Bennett, 1979) and dithionite (Allen *et al.,* 1981). Horton *et al.* (1981) demonstrated a close correlation between the redox titration curves of the PQ pool and the phosphorylation state of LHC II. For dephosphorylation of LHC II, thylakoids possess a phosphatase, but the activity of this enzyme apparently is not regulated by the plastoquinones.

For newer results, the reader is referred to papers by Black and Horton (1984), Black *et al.* (1984), and Horton and Lee (1984).

2. CHARACTERIZATION OF THE CHANGES IN ENERGY DISTRIBUTION

When uncoupled thylakoids are illuminated for a few minutes in the presence of ATP and 3–5 m*M* $MgCl_2$, a slow decrease of PSII fluorescence is observed. There is a linear relationship between the extent of LHC II phosphorylation and the extent of the slow ATP-induced

quenching (Horton *et al.*, 1981). The fluorescence decrease is reversed by a dark period of about 20 min when LHC II becomes dephosphorylated. To study thylakoids in the phosphorylated state it is necessary to inhibit the phosphatase by addition of sodium fluoride. Thus it is possible to compare the fluorescence characteristics of phosphorylated and nonphosphorylated thylakoids.

Some authors found that fluorescence induction in phosphorylated thylakoids at room temperature in the presence of DCMU showed a minor decrease of F_0 and a major decrease of F_v, compared to that in nonphosphorylated thylakoids (e.g., Bennett *et al.*, 1980; Kyle *et al.*, 1982). Other authors observed that phosphorylation caused equivalent decreases of F_0 and F_v (Horton and Black, 1981) so that the F_v/F_m ratio remained constant. A constant F_v/F_m ratio was also observed at 77°K (Krause and Behrend, 1983). According to Eqs. (3) and (4) in Section II,D, a preferential decrease of F_v compared to F_0 reflects an increase in energy transfer from PSII to PSI (k_t). In contrast, equivalent decreases of F_0 and F_v are indicative of a decrease of the cross section for light absorption by PSII (a). In both cases, emission spectra at 77°K of phosphorylated thylakoids, compared to spectra of nonphosphorylated ones, showed a decrease of F685 and F695 and an increase of F735. Horton and Black (1983) and Telfer *et al.* (1983) showed that the contradictory results observed on phosphorylation of thylakoids were due to the concentration of divalent cations surrounding the thylakoids. At high levels of Mg^{2+} ($\sim$5 mM), phosphorylation of LHC II results mainly in a decrease in the absorption cross section of PSII. At lower levels of Mg^{2+}, however, LHC II phosphorylation essentially increases energy transfer from PSII to PSI. These two kinds of modifications can be explained by the same mechanism, described below.

3. ELECTROSTATIC AND MOLECULAR MECHANISMS

The structural organization of the various Chl–protein complexes in the thylakoid results from the balance between hydrophobic attraction and Coulombic repulsion (see Section II,D, Figs. 5 and 6). LHC II is phosphorylated on the surface-exposed N-terminal segment, and the phosphorylation site consists of one or two threonyl residues (Bennett, 1980). Thus, phosphorylation will greatly enhance the negative charge on LHC II and antagonize the screening effect of cations in its vicinity. The enhanced Coulombic repulsion may be sufficient to drive phosphorylated LHC II out of the appressed regions. There are two possible hypotheses. In the first, phosphorylation of LHC II produces a departure of PSII from the appressed regions, and there is partial thylakoid

destacking and an increase of energy transfer from PSII to PSI. In this hypothesis, LHC II is associated only with PSII core complexes. The second hypothesis is represented in Fig. 7. LHC II complexes may be associated with the PSII core complex or exist in a "free" state. In the presence of high cation concentrations, phosphorylation of LHC II essentially "ejects" free LHC from appressed regions to nonappressed ones, causing a decrease in the absorption cross section of PSII and some decrease of thylakoid stacking. Conversely, at low cation concentrations, phosphorylation of LHC II induces movement of both LHC II and PSII units, causing changes in PSII-to-PSI energy transfer and significant destacking. The scheme in Fig. 7 has been confirmed by Kyle *et al.* (1984), Telfer *et al.* (1984), and Torti *et al.* (1984).

In conclusion, phosphorylation and dephosphorylation of LHC II have been shown to modify the distribution of light energy between the two photosystems. This can provide a mechanism for the *in vivo* regulation of electron transport by the state I–state II transitions. An imbalance in the rates of excitation of PSII and PSI can be detected and corrected as a result of the sensitivity of the thylakoid protein kinase to the redox state of the PQ pool. Furthermore, this redox state depends on the availability of electron acceptors to PSI, thus on the functioning of the Calvin cycle (Allen *et al.*, 1981; Allen and Bennett, 1981). Thus

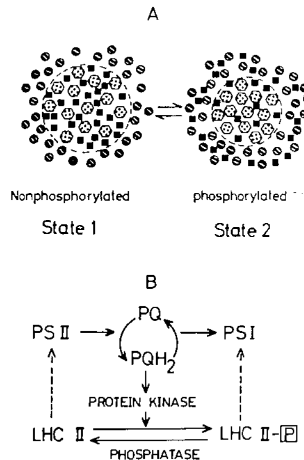


FIG. 7. Control of state transitions by the plastoquinol activated protein kinase. (A) Diagrammatic surface view of a thylakoid membrane. Dashed line denotes area of membrane appression; hexagons, LHC–PSII complex; circles, PSI complex; and squares, "free" LHC II. (B) Involvement of plastoquinone as a "meter" of PSII and PSI excitation rates and the function of the kinase in their control. Dashed lines indicate relative energy transfer probability. Also see list of abbreviations. (From Horton, 1983b.)

phosphorylation and dephosphorylation of LHC II adjust the rates of the light reactions with those of carbon assimilation. Furthermore, it has been suggested that phosphorylation of LHC II may regulate the balance between linear and cyclic electron transport and therefore may help to adjust the ATP/NADPH ratio during photosynthesis (Allen, 1983).

III. Fluorescence Phenomena of Intact Leaves

A. Methodological Problems

In general, fluorescence emission of leaves can be recorded by using the techniques applied for isolated chloroplasts (see Schreiber, 1983). However, special properties of leaves must be taken into account when measuring and interpretating fluorescence signals. Here, fluorescence kinetics also depend in a complex manner on the induction and regulation of carbon metabolism. Strict control of conditions such as the intensity of exciting light, duration of the dark phase prior to illumination, and gas phase composition is required for reproducible measurements. To maintain a defined gas phase, leaves should be enclosed in gassed cuvettes (Krause, 1973). A temperature-controlled cuvette in which Chl *a* fluorescence transients can be measured simultaneously with O_2 exchange of leaf disks has been described by Delieu and Walker (1983). A similar device that also allows recording of absorbance changes was developed earlier by Strasser (1974). For routine *in vivo* measurements, a portable fluorometer (Plant Productivity Meter models SF-10 and SF-20, Brancker, Ottawa; see Schreiber *et al.*, 1975) is commercially available. This may be linked to a microcomputer to facilitate data acquisition (Norrish *et al.*, 1983). Toivonen and Vidaver (1985) have described an integrating fluorometer for the measurement of Chl *a* fluorescence of leaves. For new techniques see also Renger and Schreiber, Chapter 19, this volume.

Compared to dilute chloroplast suspensions, intact leaves have a higher optical density and exhibit stronger scattering of incident light. The exciting light beam is attenuated as it passes the leaf, which results in weaker excitation of deeper chloroplast layers. Furthermore, a substantial part of the fluorescence is reabsorbed by the chloroplast pigment system. This applies particularly to wavelengths around 685 nm, i.e., those at the maximum of fluorescence emission at room temperature, whereas much less reabsorption occurs in the long-wavelength fluorescence beyond 700 nm. Thus, deeper layers contribute a relatively higher proportion of long-wavelength fluorescence to the measured signal.

The effects of differential excitation and reabsorption on the fluorescence induction kinetics of leaves have been evaluated by Malkin *et al.* (1981). Their analysis may explain differences in the induction measured simultaneously at short and long wavelengths. For example, the rise of variable Chl *a* fluorescence in the long-wavelength region is expected to be slower because a considerable proportion of this fluorescence originates from deeper layers of the leaf, which are exposed to weaker excitation. The authors state that reports which interpret such differences in terms of excitation energy redistribution between PSI and PSII (e.g., see Schreiber and Vidaver, 1976a,b) should be regarded with caution. Light-scattering effects also influence the fluorescence characteristics of leaves in a complex manner (Malkin *et al.*, 1981). In particular, scattering of incident light may increase excitation of the top layers by a given photon flux. Differences in fluorescence characteristics between the upper and lower sides of leaves (Schreiber *et al.*, 1977) may thus be caused in part by differential scattering. According to Malkin *et al.* (1981), scattering effects can be neglected when the intercellular space of the leaves has been infiltrated with water.

Effects caused by differential reabsorption of fluorescence and by scattering pose severe problems for low-temperature (77°K) fluorescence spectroscopy of leaves. Published fluorescence emission spectra for fully greened leaves are strongly distorted. The degree of self-absorption depends not only on the Chl content but also on the scattering properties of the leaf tissue, which may vary, e.g., with the water potential (Weis, 1985a). The short-wavelength bands related to PSII (F685 and F695) may be lowered more than 80% by self-absorption relative to the PSI band (F735). In addition, the profile of the bands of excitation spectra is flattened compared to that of spectra for dilute suspensions of isolated chloroplasts. A technique has been introduced by Weis (1984a, 1985a) to minimize effects of reabsorption by preparation of small fragments from the leaf tissue in the frozen state.

B. The Kautsky Phenomenon in Leaves

The fluorescence increase from the O level to the peak (P) in leaves, as in chloroplasts (see Fig. 2, and Section IV), is usually attributed mainly to reduction of the quencher Q_A. A second quenching mechanism may modify the O-P transient, as suggested by Bradbury and Baker (1983); however, the origin of this quenching, which is unrelated to the oxidoreduction state of Q_A, is not known. Fast changes in energy distribution were assumed by Schreiber and Vidaver (1976a,b) on the basis of changes in the F685/F735 ratio during the I-P transient of pea leaves.

One should keep in mind, however, that at room temperature F735 represents mainly PSII fluorescence, and only a small component of F735 originates from PSI. In addition, comparison of fluorescence transients measured at different wavelengths is complicated by reabsorption effects, as discussed in Section III,A.

The fluorescence decline in leaves from P to the terminal level (T) is usually more complex than the P-S decline in isolated chloroplasts. Its kinetics may vary widely with experimental conditions (Fig. 8). The first S level is usually followed by another maximum (M). Sometimes the M level is seen only as a shoulder in the decline of the induction signal. However, under certain conditions (high CO_2 concentration, high actinic light flux) a series of defined S and M levels in the shape of a damped oscillation is observed before the T level is attained (Walker, 1981; Walker *et al.*, 1983a,b, 1984; Sivak *et al.*, 1984). The Chl *a* fluorescence decline in leaves is probably governed by the same mechanisms as that in isolated intact chloroplasts. However, the interpretation is still much more uncertain because of difficulties in distinguishing between different components of quenching.

Measurements of the mean lifetime τ during the Kautsky transient revealed a correlation between τ and the Chl *a* fluorescence yield (Malkin *et al.*, 1980; Kotova and Il'ina, 1984). This correlation, however, is not linear. It has been proposed that there are at least two components of the lifetime (Malkin *et al.*, 1980). However, a refined analysis of differ-

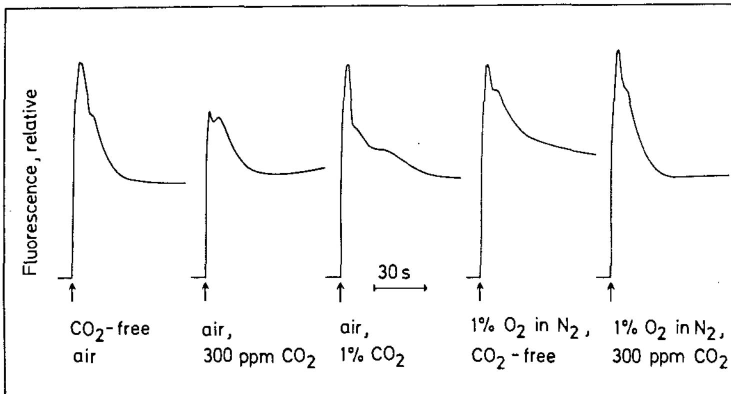


FIG. 8. Influence of gas-phase composition on the fluorescence induction signal of a spinach leaf. Exciting light, 45 W m^{-2}; half-bandwidth, 630–680 nm. The leaf was preilluminated in the indicated gas phase at room temperature until the terminal (T) level of fluorescence was reached; then the induction signal was recorded at 740 nm after 3 min of dark time. Upward arrows indicate onset of illumination. (Unpublished observations of the authors).

ent lifetime components, such as that published for isolated chloroplasts and algal cells (Haehnel *et al.*, 1982; see also Moya *et al.*, Chapter 7, this volume), is still lacking for leaves.

C. Evidence for Q_A-Dependent and ΔpH-Dependent Quenching of Chlorophyll *a* Fluorescence

During steady-state photosynthesis of spinach leaves in normal air and at a light flux limiting for CO_2 fixation, reoxidation of Q_A^- is apparently the predominant factor responsible for the low fluorescence level T (see Krause, 1973). Fast reoxidation of Q_A^- is accomplished by electron transport via PSI to $NADP^+$, forming NADPH, which in turn is reoxidized in the carbon assimilation cycle. Steady utilization of ATP keeps the ΔpH across the thylakoid membranes low and the ΔpH-dependent Chl *a* fluorescence quenching minimal. Additional quenching, probably caused by increased ΔpH, is observed when the oxygen partial pressure is reduced (Krause *et al.*, 1978), and thereby energy consumption by photorespiratory processes is diminished. Involvement of the proton gradient in fluorescence changes (see also Section III,C) was inferred from accompanying changes in light scattering (apparent absorbance at 535 nm) of the leaves (Krause, 1973; Krause *et al.*, 1978).

The fluorescence decline from P to T via S and M levels is obviously a complex process; contributions from different quenching mechanisms may vary with experimental conditions. According to Bradbury and Baker (1981, 1984), the P-S phase in bean leaves largely reflects reoxidation of Q_A^-. Possibly, reoxidation of Q_A^- is related to reduction of a pool of 3-phosphoglycerate present in dark-adapted leaves. In the S-M phase Q_A becomes more reduced again (see also Walker *et al.*, 1983b). If the full carbon reduction cycle is still largely inactive (lag phase of CO_2 fixation) and utilization of photosynthetic energy is slow, the ΔpH-dependent mechanism may dominate the further fluorescence decline. In fact, Bradbury and Baker (1984) suggested that Q_A^- reoxidation contributes little to the M-T transient. However, increasing activation of the carbon cycle during the M-T phase should diminish the ΔpH and related quenching and accelerate reoxidation of Q_A^-, which promotes Q_A-dependent fluorescence lowering. According to Andreeva and Tikhonov (1983), the M-T transient is correlated with changes in the electron paramagnetic resonance (EPR) signal I, which reflects oxidation of the RC of PSI, P700. They concluded that due to the onset of carbon metabolism (toward the end of the lag phase), linear electron transport is enhanced, leading to increased oxidation of P700 and thereby to fluorescence quenching by enhanced reoxidation of Q_A^-.

After brief dark periods (2–5 min) following activation of the carbon cycle in a preceding illumination period, fluorescence peaks observed in spinach leaves in normal air are low (Fig. 8). Apparently, fast onset of carbon reduction on illumination prevents sufficient net reduction of Q_a, keeping P at a low level (Krause, 1973; Krause and Weis, 1984). In CO_2-free air (Fig. 8), the P level is high after brief preceding dark periods, but the T levels are lower than in the presence of CO_2 (Krause, 1973; Walker *et al.*, 1984). Under such conditions, the P-T decline predominantly reflects a ΔpH-dependent quenching, as reoxidation of NADPH and consumption of ATP by carbon metabolism are restricted. Therefore, Q_A^- reoxidation will be low, while H^+ pumping by cyclic electron flow and by limited linear electron transport to O_2 via PSI (Mehler 1951; see Heber *et al.*, 1978) may still occur. Furbank and Walker (1985) investigated fluorescence induction and CO_2 uptake in leaf strips of a number of C_4 plants and discussed Q_A^-- and ΔpH-dependent fluorescence quenching with respect to particular aspects of the C_4 cycle.

Damped oscillations of Chl *a* fluorescence of leaves from C_3 plants, which are often observed at high levels of CO_2 in air (Fig. 2), have been interpreted in terms of overreactions of the regulatory system of carbon assimilation which affect the balance between turnover of ATP and NADPH (Walker *et al.*, 1983a,b). Photosynthetic O_2 evolution (Walker *et al.*, 1983b, 1984) or CO_2 fixation (Sivak *et al.*, 1984) was observed to oscillate with Chl *a* fluorescence in an approximately antiparallel mode (with a phase angle near 180°). In isolated mesophyll protoplasts, Q_A- and ΔpH-dependent fluorescence changes were found to participate in the oscillations (Quick and Horton, 1984a,b). It was suggested that cyclic electron flow is involved in these transients.

D. Evidence for Excitation Energy Transfer from Chlorophyll *a* Fluorescence Studies with Intact Leaves

Excitation energy transfer within the photosynthetic pigment system has been extensively studied with isolated thylakoids (see Section II,D,E). For intact leaves, interpretation of fluorescence phenomena in relation to energy transfer is often less definitive because of their complexity and the lack of direct biochemical data (e.g., determination of electron transport or protein phosphorylation) for comparison with the results of fluorescence measurements. Nevertheless, in a number of studies, fluorescence data obtained from leaves lend support to the hypotheses derived from investigations of chloroplasts. For energy transfer studies, fluorescence measurement at 77°K is of particular impor-

tance, because at that temperature F695 and F735 can be attributed, as in the case of isolated chloroplasts, to emission by pigments of PSII and PSI, respectively (see Strasser and Butler, 1977b). Problems arising from reabsorption of fluorescence were discussed in Section III,A.

1. ENERGY TRANSFER STUDIED DURING DEVELOPMENT OF THE PHOTOSYNTHETIC APPARATUS

Leaves with the photosynthetic apparatus at different stages of development have been important in the study of excitation energy transfer. Etiolated bean leaves greening in continuous light were used by Goedheer (1969) to investigate energy transfer from carotenoids to Chl *a*. From absorption and fluorescence excitation and emission spectra, he concluded that light energy absorbed by β-carotene is transferred to Chl of both photosystems, whereas no transfer occurs from xanthophylls to Chl! This is surprising in view of the efficient energy transfer from fucoxanthol to Chl *a* in brown algae and diatoms.

Although functional PSI and PSII units develop within 1 h of continuous illumination of etiolated leaves (see Baker and Butler, 1976), excitation energy transfer from PSII to PSI ("spillover") does not occur at this early stage of greening (Baker and Strasser, 1982). Variable fluorescence of PSII at 77°K appears within 30 min of continuous illumination of etiolated leaves. On the other hand, variable fluorescence in PSI, which is supposed to arise exclusively from energy transfer from PSII (see below), cannot be detected within 2 h. During further development, the rate constant of energy transfer gradually increases; this occurs approximately in parallel with the increase in the capacity of PSII photochemistry, as expressed by the ratio F_v/F_m of the PSII emission band. The onset of energy transfer is not correlated with Chl *b* synthesis, i.e., the assembly of LHC II. It was shown by Strasser and Butler (1976, 1977a) that energy transfer from PSII to PSI does not require the presence of LHC II, as it occurs in "flashed" bean leaves (etiolated leaves that are greened by repetitive 1-ms flashes separated by 12-min dark periods), which are devoid of LHC II. Strasser and Butler (1977a) demonstrated by a 77°K fluorescence analysis that the F_0 level of F735 is related to energy absorbed directly by PSI pigments, as well as to energy absorbed by PSII and LHC II pigments and transferred to PSI (see Fig. 9). In contrast, the variable part of F735 solely represents energy transferred from PSII to PSI. This is in accordance with the model of energy transfer derived from studies of isolated chloroplasts (Butler and Kitajima, 1975a). Direct energy transfer from LHC II to PSI was assumed to be involved in the maintainance of an almost constant initial distribution of energy be-

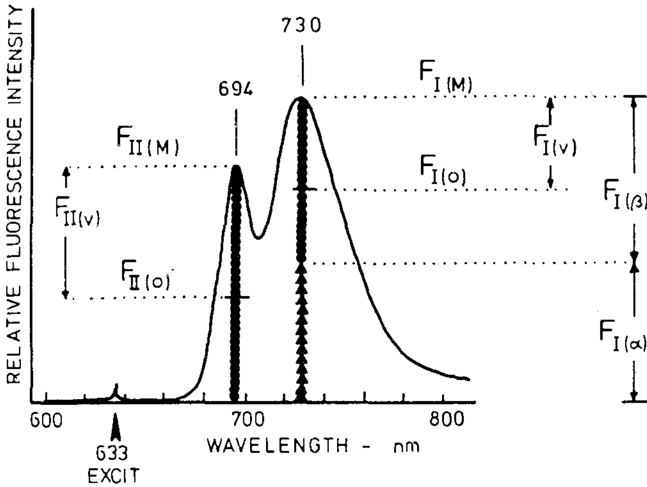


FIG. 9. Chlorophyll *a* fluorescence emission spectrum at 77°K of a "flashed" bean leaf lacking the light-harvesting complex II. F_0 (initial), F_v (variable), and F_m (maximum) levels of PSII (F_{II}) and PSI (F_I) bands are indicated; $F_{I(\alpha)}$ and $F_{I(\beta)}$ denote PSI fluorescence excited by energy initially distributed to PSI and PSII, respectively. (From Strasser and Butler, 1977a.)

tween PSI and PSII in the spectral range 400–675 nm. In leaves devoid of LHC II, the initial energy distribution varied considerably as a function of wavelength of the exciting light.

According to the tripartite model of the photosynthetic apparatus, which is based on quantitative measurements of constant and variable 77°K fluorescence at different wavelengths (Butler and Strasser, 1977), LHC II also mediates transfer of excitation energy among PSII units. This was confirmed with bean leaves which had been greened in intermittent light and then continuously illuminated to induce synthesis of LHC II (Armond *et al.*, 1976; Strasser and Butler, 1978). Fluorescence induction signals were measured at room temperature in the presence of DCMU. The sigmoidicity of the rise of variable fluorescence, which indicates energy transfer among PSII units (Joliot *et al.*, 1973), strongly increased as LHC II was formed (Fig. 10). In contrast to these reports, Melis and Akoyunoglou (1977) found, by an analysis of fluorescence induction in the presence of DCMU, that development of PSII cooperativity, as indicated by the so-called α component of fluorescence induction (see Section II,B,3), occurred in the absence as well as in the presence of LHC II. They concluded that both PSIIα and PSIIβ centers are formed during early stages of greening in intermittent or continuous light and that this development is independent of LHC II and related

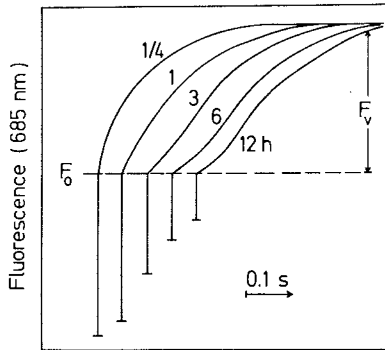


FIG. 10. **Room-temperature chlorophyll *a* fluorescence induction at 685 nm of "flashed" bean leaves after different times of greening in continuous light (as indicated). The leaves were infiltrated with 5 μM DCMU. (Modified after Strasser and Butler, 1978. Reprinted by permission from "Photosynthesis '77," pp. 527–536. Copyright ©1978 The Biochemical Society, London.)**

grana formation. This is in contradiction to Butler's model and it cannot be resolved at present.

2. EVIDENCE FOR STATE I–STATE II TRANSITION (REGULATION OF EXCITATION ENERGY DISTRIBUTION)

Regulation of the excitation energy distribution between PSII and PSI is assumed to optimize photosynthetic electron transport by balancing excitation of the two photosystems, which is important for photosynthesis in limiting light. Changes in the spectral composition of actinic light, which result in differential excitation of the photosystems, should be offset by changes in the energy distribution. Moreover, the ratio of cyclic (PSI) to noncyclic electron transport is thought to be controlled via distribution of excitation energy (Allen and Bennett, 1981). Such regulatory processes have been extensively studied in isolated chloroplasts and algae and are probably linked to changes in the redox state of the plastoquinone pool, which cause alterations in the phosphorylation of LHC II (see Section II,E). Canaani *et al.* (1984) provided evidence that energy distribution in leaves, as in chloroplasts, is controlled by the phosphorylation state of LHC II.

Analysis of room temperature fluorescence emission at short and long wavelengths has provided evidence for state transitions during the P-T decline (Bradbury and Baker, 1981; Lombard and Strasser, 1984; Graf *et al.,* 1984) in leaves. Problems with this experimental approach were discussed in Section III,A.

In studies by Chow *et al.* (1981), Hodges and Barber (1983a), and Canaani *et al.* (1984), state transitions in leaves were detected by means of "modulated" fluorescence. In the experiment depicted in Fig. 11, leaves were illuminated with a modulated light preferentially absorbed by PSII (called light 2) and with a superimposed nonmodulated beam of far-red light (light 1), and the fluorescence sensitized by the modulated light was recorded. It was suggested that the terminal fluorescence level represents state I. When light 1 is turned off, a strong transient increase in modulated fluorescence is seen. This is probably produced by reduction of Q_A, since in this state little of the excitation energy provided by light 2 is available to PSI. This would tend to keep Q_A in a reduced state. Continuing illumination with light then causes a fluorescence decline within several minutes to approximately the former T level; this decline seems to represent a transition to state II, in which sufficient excitation of PSI by light 2 balances the electron transport. In agreement with the current hypothesis, the state transition could not be detected in leaves of a barley mutant that lacks Chl *b* and LHC II.

Additional evidence for state transition in leaves was provided by 77°K fluorescence spectroscopy, which showed changes in the F735/F685 ratio (Chow *et al.,* 1981). It should be kept in mind, however, that such changes may also be due to a transthylakoid proton gradient existing at

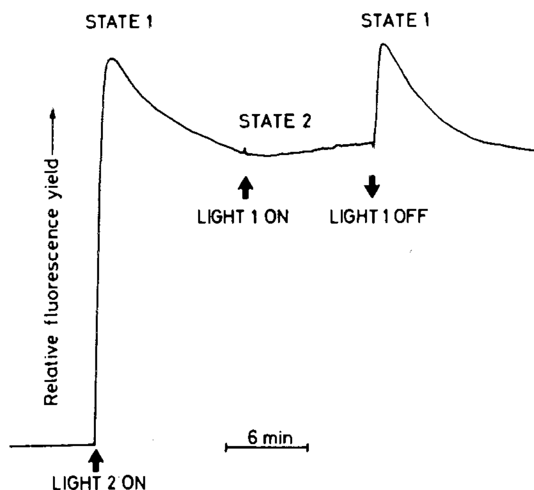


FIG. 11. Changes in chlorophyll *a* fluorescence of a pea leaf illuminated with light preferentially absorbed by PSII (light 2) or PSI (light 1). Only the fluorescence excited by the modulated light 2 was recorded. The leaf was preilluminated for 10 min with light 1 to obtain state I. Assumed state transitions are indicated. (From Hodges and Barber, 1983a.)

the time of freezing (see Krause *et al.*, 1983). This may apply to the study of bean leaves by Kitajima (1976), where fluorescence phenomena attributed to state transitions may, in fact, partly reflect changes of the intrathylakoid proton concentration (see Section II,A).

Satoh and Fork (1983a) investigated the fluorescence ratios F695/F735 at 77°K of spinach leaves frozen at various points of fluorescence induction. From the very different dark recovery kinetics (referred to the dark time between illumination and freezing), the authors postulated two distinct mechanisms for the changes observed at 77°K. It is not evident whether these are related to ΔpH-dependent and state transition changes. Satoh and Fork (1983a,c) assumed, in contrast to Chow *et al.* (1981), that dark-adapted leaves are not in state I but in state II. This discrepancy may be only apparent, because the dark state of spinach leaves varies with temperature (Weis, 1984a). At temperatures below 20°C leaves may attain a dark state close to state I, while at temperatures above 20°C the dark state tends to be near state II.

Changes in energy distribution could be mediated either by changes in the initial distribution of energy from LHC II to the two photosystems or by changes in the rate constant of energy transfer from PSII to PSI (see Section II,E). From photoacoustic and room temperature fluorescence measurements, Canaani and Malkin (1984) concluded that energy transfer ("spillover") changes are not involved in state I–state II transitions in tobacco, spinach, and barley leaves. Using 77°K fluorescence, Weis (1984b, 1985b) evaluated the relative contribution of energy transfer changes to state I–state II transitions in spinach leaves. No significant change in the rate constant of energy transfer was observed at temperatures lower than 20°C. However, when the leaf temperature was increased above 20°C a considerable increase (more than 30%) in the energy transfer constant could be detected during a state I–state II transition. Sane *et al.* (1984) have shown that heating favors the development of state II in canna leaves. This effect may be attributed to increased lateral mobility of pigment complexes within the thylakoid membrane at high temperatures. The changes in the initial distribution of energy (10–20%) were relatively unaffected by the leaf temperature.

E. *Influence of Physiological and Environmental Factors on Fluorescence Emission*

Chl *a* fluorescence emitted from leaves is strongly influenced by the physiological and developmental states of plants and the effects of environmental conditions. The various effects of leaf development on fluorescence emission have served to elucidate the characteristics of energy

transfer among the various pigment systems, as discussed in Section III,D,1. More generally, fluorescence gives insight into the assembly of the photosynthetic apparatus during different stages of development. The assembly of the pigment systems, PSI, PSII, and LHC II, on illumination of etiolated plants has been followed by means of fluorescence analysis (see, e.g., Strasser and Sironval, 1972; Armond *et al.*, 1976; Inoue *et al.*, 1976; Melis and Akoyunoglou, 1977; Lewandowska and Öquist, 1980a,b). The risetime of variable fluorescence, which can be used routinely to estimate the PSII unit size in leaves (Malkin *et al.*, 1981), was strongly shortened when the number of antenna pigments per RC increased during greening (Akoyunoglou, 1977). Variations in the F_m level and in the F_m/F_0 ratio have been attributed to changes in the organization of pigments and to grana formation during chloroplast development (Akoyunoglou, 1977; Argyroudi-Akoyunoglou and Akoyunoglou, 1977; Castorinis *et al.*, 1982).

Leaves adapted to sunny or shaded habitats exhibit pronounced differences in their fluorescence characteristics. Malkin and Fork (1980) and Fork and Govindjee (1980) calculated from the fluorescence rise distinctly larger photosynthetic unit sizes in shade than in sun plants. Leaves of seedlings grown in low light showed higher initial and variable fluorescence levels compared to those adapted to high light (Lichtenthaler *et al.*, 1981, 1982). According to fluorescence analyses by Hodges and Barber (1983a), plants grown in low light exhibit a higher PSIIα/PSIIβ ratio. Low-light plants also show a faster state I–state II transition than plants grown in high light. However systematic experiments, in which a distinction is made between changes in light intensity and wavelength of light, are not yet available.

Fluorescence intensities are also influenced by metabolic activities and their regulation in intact leaves, e.g., by the induction of CO_2 fixation (Walker *et al.*, 1983a,b) or by processes of the photorespiratory carbon cycle (Krause *et al.*, 1978; see also Section III,B). Fluorescence is also affected by responses of plants to environmental stress factors, as discussed by Renger and Schreiber (Chapter 19, this volume; see also Krause and Weis, 1984).

IV. Conclusions

In higher plants, there are three principal mechanisms that can lower the yield of chlorophyll fluorescence of PSII:

(1) Oxidation of the electron acceptor Q_A; in the light, this is caused by the electron transport through PSI.

(2) Acidification of the intrathylakoid space caused by light-dependent proton transport across the thylakoid membrane.

(3) Increased distribution of excitation energy to weakly fluorescent PSI at the expense of PSII excitation, regulated by enzymatic phosphorylation of LHC II.

The redistribution of energy in favor of PSI, mentioned above, may be achieved by an increase in the PSI and decrease in the PSII antenna size and/or by increased transfer of excitation energy from PSII to PSI. These effects may also depend on the cation level in the chloroplast stroma.

The manner in which the above events may determine the shape of fluorescence transients (the Kautsky effect) is schematically depicted in

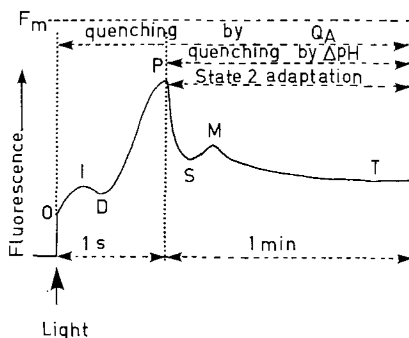


FIG. 12. Schematic representation of the Kautsky phenomenon in leaves and interpretation of fluorescence variations. O, Initial fluorescence (F_0); all PSII reaction centers are open. (The electron acceptor of PSII, Q_A, is fully oxidized.) O–I, Partial photoreduction of Q_A causes fluorescence rise to I ("inflection"). I–D, Transitory acceleration of reoxidation of Q_A^- by the plastoquinone pool lowers fluorescence to D ("dip"). D–P, Accumulation of NADPH and reduced plastoquinone (due to low rate of carbon assimilation) leads to reduction of Q_A; fluorescence rises to P ("peak"). F_m, Stationary fluorescence level in the presence of DCMU; it represents the maximum fluorescence yield, when all reaction centers of PSII are closed. F_m may be higher than P. P–S, Enhanced reoxidation of Q_A^- and buildup of a ΔpH across the thylakoid membranes causes fluorescence decline to S ("steady state"). S–M, Increased reduction of Q_A and decrease in ΔpH results in the fluorescence rise to M ("maximum") since a lag in CO_2 assimilation retards electron flow. M–T, Further decline of fluorescence to T ("terminal" level) is caused mainly by increase in ΔpH and/or by reoxidation of Q_A^-, as noncyclic electron flow is enhanced due to activation of the carbon reduction cycle.

State II adaptation that optimizes electron flow may influence the P-S-M-T phases in two ways: (1) redistribution of exitation energy in favor of PSI decreases PSII excitation and thereby PSII fluorescence; (2) optimization of electron flow leads to increased ΔpH and reoxidation of Q_A^- and thus to fluorescence quenching.

Fig. 12. See Fig. 10 and Table IV in Govindjee and Satoh, Chapter 17, this volume, for an explanation of transients in green algae. The differences between the systems and the explanations proposed need to be reconciled. It must be noted, however, that both these pictures represent simplifications of the certainly more complex situation *in vivo*. The secondary fluorescence transients in higher plants depend on regulation of the dark reactions of photosynthetic CO_2 fixation. Furthermore, the characteristics of fluorescence phenomena vary with developmental stages of plants and their adaptation to environmental conditions.

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VI
Practical Applications

19

Practical Applications of Fluorometric Methods to Algae and Higher Plant Research

GERNOT RENGER
Max-Volmer-Institut für Biophysikalische und Physikalische Chemie
Technische Universität Berlin
D-1000 Berlin 12, Federal Republic of Germany

ULRICH SCHREIBER
Lehrstuhl für Botanik I
Universität Würzburg
D-8700 Würzburg, Federal Republic of Germany

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ABBREVIATIONS AND SYMBOLS

Atrazine	2-Chloro-4-(ethylamino)-6-(isopropylamine)-*s*-triazine
Chl	Chlorophyll
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea (diuron)
LIDAR	Laser-induced deduction and ranging
PSII (I)	Photosystem II (I)
Phenmedipham	3-Methoxycarbonylaminophenyl-*N*-(3-methylphenyl)carbamate

ABSTRACT

In this chapter the use of Chl *a* fluorescence as an intrinsic probe of the physiological state of plants is outlined. Although *in vivo* fluorescence is a complex function of a large number of environmental and physiological parameters, an appropriate choice of experimental conditions permits one to obtain reliable information on the functional properties

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which are of practical relevance. In practice, the induction kinetics of fluorescence in a dark–light transition (Kautsky effect) have proved most useful for rapid and nondestructive screening of photosynthetic performance. The parameters governing fluorescence yield and fluorescence induction kinetics *in vivo* are described, and the various mechanisms of fluorescence quenching are discussed (Section II). The basic principles of a simple setup for fluorescence measurements in whole plants are briefly outlined in Section III. The practical application of these fluorometric methods in plant physiological work is reviewed with regard to (a) algal productivity measurements in limnology and marine biology (see Section IV,A) and (b) the effects of environmental stress factors, e.g., temperature (IV,B,1), water (IV,B,2), salinity (IV,B,3), light (IV,B,4), air pollution (IV,B,5), and herbicide application (IV,C). The information obtained from fluorescence measurements can be improved by appropriate "calibration" against more direct assays of photosynthesis (O_2 evolution or CO_2 fixation rates). After such calibration, the fluorescence method is a reliable tool of extraordinary value because it is rapid, nondestructive, rather easily performed, and of high sensitivity.

I. Introduction

The transformation of light into chemical free energy, performed in algae and higher plants, does not occur with a 100% quantum yield of the absorbed radiation. A considerable part (2–10%) is reemitted as fluorescence from the lowest excited singlet state of chlorophyll *a* (Chl *a*) (for review see Murata and Satoh, Chapter 6, this volume). The basic observation on the applicability of fluorometric methods for practical use was made more than 100 years ago, when N. J. C. Müller (1874) discovered visually with the aid of colored glass filters that fluorescence changes occur in green leaves and are correlated with photosynthetic assimilation. Lack of the appropriate technical equipment prevented systematic investigations of these phenomena.

About half a century later, Kautsky and Hirsch (1931) again visually followed the time course of Chl *a* fluorescence and correlated it with that of photosynthetic CO_2 fixation. Later, Kautsky and co-workers (Kautsky and Eberlein, 1939; Kautsky and Franck, 1943; Kautsky *et al.*, 1960) presented detailed studies on the correlation between the time course of fluorescence emission (Kautsky effect) and photosynthetic activity. Even by our present standards, the quality of information obtained by Kautsky and Franck (1943) from fluorescence induction kinetics is remarkable. It should be emphasized that even in those early studies the existence of more than one light reaction became apparent, and finally Kautsky *et al.* (1960) formulated an explicit reaction model of photosynthetic electron transport with two different photoreactions. Later, Duysens and Sweers (1963) succeeded in showing by fluorescence measurements with modulated light that two light reactions are driven by two

types of pigment systems with different absorbance properties (see also Govindjee *et al.*, 1960; Butler, 1962). A key step in further development of this field was the discovery by Duysens and Sweers (1963) that variable fluorescence emission can be interpreted as reflecting (via a photochemical quenching mechanism) redox changes by the photosystem II (PSII) acceptor referred to as quencher Q (called Q_A in this volume). (See Fig. 1 in Duysens, Chapter 1.) This concept, which was found to be extremely useful during the past two decades, shifted the main interest in fluorescence measurements toward the more basic photophysical and photochemical aspects associated with the mechanism of photosynthesis (for reviews, see Govindjee and Papageorgiou, 1971; Govindjee *et al.*, 1973; Papageorgiou, 1975; Lavorel and Etienne, 1977; Govindjee and Jursinic, 1979). Development of picosecond laser pulse techniques and ultrafast detector systems further supported highly sophisticated mechanistic studies (see Alfano, 1982; Lavorel *et al.*, Chapter 4, and Moya *et al.*, Chapter 7, this volume). Only recently has the potency of Chl *a* fluorescence measurements been rediscovered for practical application in plant physiology research, and these measurements are now being increasingly used in different areas.

Studies of Chl *a* fluorescence have traditionally had two main characteristics. On the one hand, the extreme sensitivity and time resolution of the technique have made this method, at any given stage of technical progress, a pioneering tool in the discovery of fundamental mechanisms of photosynthesis. On the other hand, because of the complexity of the relationship between fluorescence emission and the physiological state of the photosynthetic apparatus, only indirect information was obtained, which needed to be substantiated by additional knowledge gathered by more direct analytical methods (e.g., pulse spectrophotometry).

The complexity mentioned above prevented widespread application of Chl *a* fluorescence measurements in plant physiological research, despite a wealth of information accumulated in the 1940s (McAlister and Myers, 1940; Franck *et al.*, 1941; Kautsky and Franck, 1943; van der Veen, 1949; see also reviews by Rabinowitch, 1951, 1956). These data received attention only rather recently, as a result of new insights into complex regulatory mechanisms governing interactions of light and dark reactions (Sivak and Walker, 1983). During the past decade, interest has been growing in the practical application of Chl *a* fluorescence measurements as a rapid, sensitive, and nondestructive method for the determination of photosynthetic activity in plants and their stress limitations under greenhouse and field conditions. These applications are based on solid information derived from studies discussed in earlier chapters in this volume. The purpose of the present chapter is to indi-

cate appropriate experimental approaches and conditions, and to show what kind of information with practical relevance may be obtained.

II. Fluorescence Emission as an Indicator of the Physiological State of Plants

A. Fundamental Aspects

Regardless of the excited states of the photosynthetic pigments created by light absorption, fluorescence emission always occurs from the lowest excited Chl singlet state. Plant leaves contain a very complex array of several types of pigment molecules associated with proteins in the thylakoid membrane (Murata and Satoh, Chapter 6, this volume). Because of the functional organization of the photosynthetic pigment complexes leading to efficient excitation energy transfer (Amesz and van Grondelle, Chapter 15, this volume), the fluorescence of green plants illuminated with visible light is almost exclusively emitted by Chl *a* molecules. At physiological temperatures, fluorescence is predominantly emitted from pigment system II (for a review see Papageorgiou, 1975; see Fork and Mohanty, Chapter 16, Satoh and Govindjee, Chapter 17, and Briantais *et al.*, Chapter 18, this volume). The fluorescence intensity F of leaves can be expressed in a generalized form by the function

$$F(n_{abs}, \lambda_{em}, p_1 \cdots p_n) \qquad (1)$$

where n_{abs} is the number of photons absorbed per unit area and per unit time, λ_{em} is the emission wavelength symbolizing the temperature-dependent normalized emission spectrum, and $p_1 \cdots p_n$ are modulating parameters of the fluorescence quantum yield that are dependent on the metabolic state of the chloroplasts (quenching mechanisms). Parameters $p_1 \cdots p_n$, in turn, depend on n_{abs}, temperature, and the illumination time, so that F (measured at constant excitation quantum flux) is a very complex function of emission wavelength λ, temperature, and time. In practice, a deconvolution of this function and a straightforward analysis of the physiological parameters $p_1 \cdots p_n$ is complicated by the complexity and interdependence of these parameters in whole leaves.

To obtain reliable information, appropriate experimental conditions must be selected in order to optimize the control of the physical and physiological parameters governing fluorescence. In most applications, the time dependence of fluorescence intensity, $[F(t)]_{T,n_{abs},\lambda_{em}}$, has been used in plant physiological studies. Figure 1 shows typical time courses of fluorescence induction (Kautsky effect) of an intact spinach leaf under standard conditions. At time zero a dark-adapted leaf was exposed

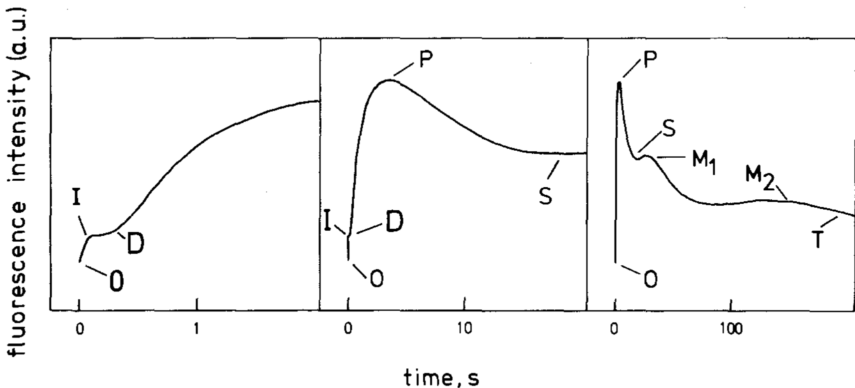

FIG. 1. Chlorophyll *a* fluorescence emitted from the upper surface of a spinach leaf as a function of time of illumination at three different time scales. At time zero, a dark-adapted (2 h) leaf was illuminated with blue light at 10 W/m^2 (Corning 4-96 filter). Fluorescence was detected at 680 nm (Schott RG 665 fiter). Temperature: 20°C. Symbols denote different characteristic levels of the fluorescence induction curve: O, initial; I, intermediary; D, dip; P, peak; S, quasi-stationary; M_1, M_2, relative maxima; T, terminal level.

to constant blue light of 10 W/m^2 at a constant temperature of 20°C. Before entering into a discussion of the detailed mechanisms controlling fluorescence *in vivo* (see Section II,B), the phenomenology of the effects caused by the most relevant extrinsic parameters on the induction kinetics will be described briefly. The experimental data, summarized in Fig. 2, reveal that light intensity, temperature, preillumination, and light-adaptational state have a decisive effect on the induction curves. Other important factors (not displayed in Fig. 2) are gas composition, humidity, leaf age, and the entire "prehistory" of the plant, including possible exposure to environmental stress (for further examples of induction phenomenology, see Kautsky and Franck, 1943; van der Veen, 1949; Rabinowitch, 1951, 1956; Franck *et al.*, 1969; Walker, 1981; Sivak and Walker, 1983; Horton, 1983a,b).

With all controllable factors kept at standard values, so-called control curves are obtained, which are modified in a characteristic way by application of any particular treatment affecting the state of the photosynthetic apparatus. In principle, even without a proper understanding of the underlying mechanisms, such an approach will provide empirical information on the extent of changes (damage) caused by such treatment. The reliability of the method depends on the reproducibility with which induction curves can be recorded, which in turn is determined by the control of the modulating parameters. Without going into the details

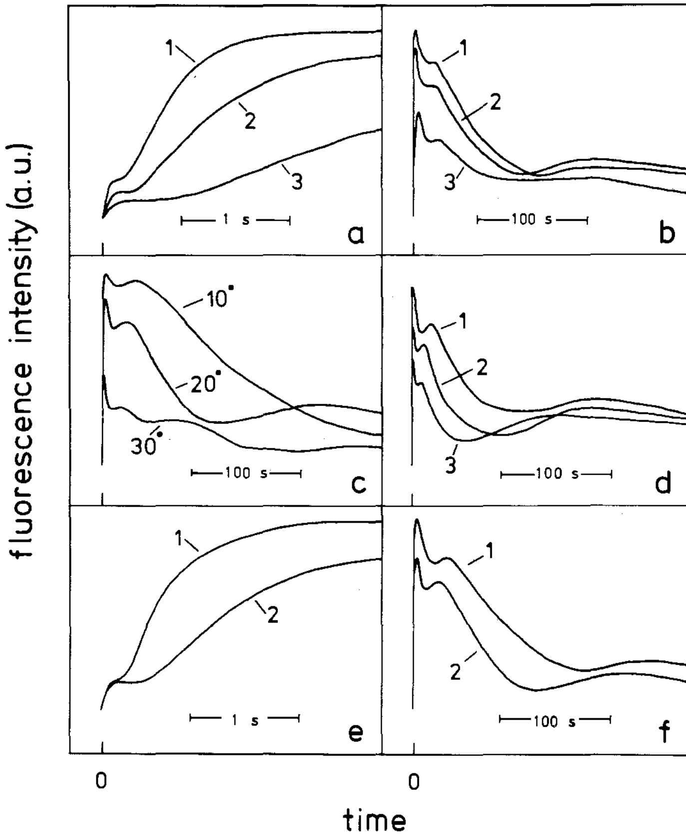

FIG. 2. Influence of various experimental parameters on the fluorescence induction curve of a spinach leaf. (a and b) Effect of light intensity on rapid (a) and slow (b) transients; curves 1, 15 W/m²; curves 2, 10 W/m²; curves 3, 5 W/m². (c) Effect of temperature. (d) Effect of dark-adapted state; curve 1, 4 h dark-adapted; curve 2, 30 min dark-adapted; curve 3, 10 min dark-adapted. (e and f) Rapid (e) and slow (f) transients from lower leaf side (curves 1) and upper leaf side (curves 2). Unless otherwise noted, the curves were recorded at 20°C and 10 W/m².

of the phenomenology apparent in the fluorescence curves of Fig. 2, the following aspects of practical relevance should be kept in mind:

(a) As even small temperature changes cause substantial modification of the induction kinetics, experimental conditions must be selected that avoid uncontrollable heating (e.g., due to strong blue actinic light) of the leaf.

(b) Homogeneous illumination of the leaf is required in order to elimi-

nate complications which might arise from monitoring an induction curve that represents a mixture of overlapping induction kinetics. This complication cannot be excluded completely because the recorded signal always contains some fraction of fluorescence emission originating from deeper layers of the leaf, where the actinic light intensity is necessarily lower than at the surface, thus giving rise to different induction kinetics. This effect can be minimized by detecting the fluorescence from the illuminated leaf surface and by selecting actinic light of an appropriate wavelength (Malkin *et al.,* 1981). A closely related factor is the wavelength-dependent fluorescence reabsorption, which lowers the 685-nm emission peak relative to the 735-nm satellite peak (see, e.g., Virgin, 1954; Malkin *et al.,* 1981; Briantais *et al.,* Chapter 18, this volume). For this reason, changes in the ratio of emission wavelengths (e.g., F685/F735) with time or those induced by special treatment should be analyzed with care. Differences reported for the induction kinetics at 685 and 735 nm in whole leaves (Schreiber and Vidaver, 1976; Bradbury and Baker, 1981) are likely, at least in part, to be caused by differences in effective light intensity. A larger portion of F685, seen by the fluorescence detector, originates from the high-irradiation surface layer, while relatively more F735 emerges from the low-irradiation deeper tissue layers. Hence, F685 induction will be faster than F735 induction.

(c) An appropriate protocol must be used for control of the dark and/or light adaptation state of the leaves. Dark adaptation for several hours guarantees reproducible results. However, this procedure is not successful for applications where the time dependence of some stress-induced damage is to be monitored. In this case, the sample can be exposed to several light–dark cycles until a stable induction pattern is reached and the change of this pattern by the applied treatment can be analyzed (e.g., for ozone stress see Schreiber *et al.,* 1978). Besides the aspect of short-term preillumination and dark adaptation, the long-term light adaptation during leaf growth must be taken into consideration (Schreiber *et al.,* 1977; Malkin *et al.,* 1981; Lichtenthaler *et al.,* 1981). This point is illustrated most convincingly by the distinctly different induction patterns observed at lower and upper surfaces of the same leaf (Figs. 2e and 2f).

The fluorescence induction phenomenon reflects dynamic changes within the chloroplast caused by a sudden dark–light transition when the photosynthetic apparatus starts to work. Another approach for obtaining information of physiological relevance may be a "static" method where special conditions are chosen (e.g., photosynthesis is maintained in an inactive state). Examples of the practical application of static fluorescence methods include the analysis of heat resistance of plants (see

Section IV,B,1) or the detection of herbicide effects by measuring the terminal steady-state fluorescence level (see Section IV,C).

B. Mechanisms Controlling Fluorescence Quenching

Different mechanisms are responsible for the modification of fluorescence emission. In general, three different types of effects can be distinguished (see also Seely and Connolly, Chapter 5, and Briantais *et al.*, Chapter 18, this volume): (a) photochemical quenching (via electron transfer reactions), (b) nonphotochemical quenching (e.g., via effects on the radiationless deexcitation rate constants of the excited pigment), and (c) exciton distribution and migration between pigment molecules.

Under common experimental conditions (dark-adapted system without serious modification of the redox state by pretreatment with chemicals, moderate actinic light intensity), the primary plastoquinone acceptor of PSII, referred to as Q_A, in its oxidized state appears to be the main photochemical quencher. [Recently it was shown for isolated class II chloroplasts that the assumption that Q_A is the only photochemical quencher is sufficient to describe the fluorescence induction curves quantitatively as a function of actinic light and different degrees of inhibition by DCMU (diuron) (Renger and Schulze, 1985).] Consequently, in respect to photochemical quenching the fluorescence intensity depends primarily on the redox state of Q_A (see Renger *et al.*, 1986b; van Gorkom, Chapter 10, this volume), which is a function of the rates of reduction by PSII and reoxidation by PSI. Thus, fluorescence quenching by Q_A reflects the state of photosynthetic electron transport, also under physiological conditions, provided that effects due to nonphotochemical quenching can be properly separated (see Section III). On the basis of the above-mentioned consideration, photochemical quenching will be referred to as *q*-quenching. Nonphotochemical quenching on the other hand, is caused by a modification of the rate constants for radiationless decay due relatively slow to changes in the microenvironment of the emitting Chl. It was found (see, e.g., Briantais *et al.*, 1980) that membrane energization by formation of a transmembrane pH difference leads to fluorescence quenching. For this reason, this type is referred to as energy-dependent quenching or *e*-quenching (see also Papageorgiou, 1975; see Briantais *et al.*, Chapter 18, this volume). It has to be emphasized that the term *e*-quenching does not necessarily reflect the molecular mechanism which is responsible for changes of the rate constants for radiationless deexcitation (see also Briantais *et al.*, Chapter 18, this volume). Distribution and migration of excitation energy depend strongly on the localization of the light-harvesting complexes (LHCs)

within the thylakoid membrane (see, e.g., Bennett *et al.,* 1980). Under physiological conditions, LHC phosphorylation and redistribution of LHC between PSII and PSI (Hodges and Barber, 1983; Kyle *et al.,* 1983) appear to be the predominant factors in excitation energy distribution between the photosystems. On the other hand, cation concentration changes which drastically affect fluorescence emission in isolated chloroplasts (for review, see Barber, 1976; Williams, 1977; Wong, 1979) seem to be of only minor importance in intact plants under physiological conditions (Krause *et al.,* 1983; see also Briantais *et al.,* Chapter 18, this volume).

In terms of the different types of modulating factors, the characteristics of fluorescence transients depicted in Fig. 2 are basically interpreted as follows (see, e.g., Table 2 in Mohanty and Govindjee, 1974; see also Papageorgiou, 1975; Krause and Weis, 1984; Briantais *et al.* and Govindjee and Satoh, this volume): as a result of Q_A reduction, the fast rise of O-I-D-P (origin–intermediate–dip–peak) is caused predominantly by removal of *q*-quenching. Maximum fluorescence, F_{max}, can be achieved only after complete Q_A reduction and exclusion of all other quenching mechanisms. Therefore, the P level in whole leaves does not necessarily reflect F_{max} (e.g., decreased P levels were recently shown to be caused also by quenching due to endogeneous O_2; Bruce *et al.,* 1983). The subsequent fluorescence decline to the terminal level (T) including intermediary maxima (see Fig. 1) reflects the complex interference of different factors that affect the magnitude of both *q*- and *e*-quenching. It is inferred that the P–S (peak–semisteady state) decline is dominated by an increase of *q*-quenching (Bradbury and Baker, 1981, 1984; Quick and Horton, 1984) brought about by faster electron flow through PSI, probably due to light activation of ferredoxin (Fd)–$NADP^+$ reductase (Carillo *et al.,* 1981; see further discussion in Govindjee and Satoh, Chapter 17, this volume). The conclusion about the dominance of *q*-quenching is supported by recent findings which reveal that in dark-adapted leaves *e*-quenching develops rather slowly even up to the M_1 level (Schreiber *et al.* 1986).

According to studies with a reconstituted chloroplast system (Horton, 1983a), the increase from S to the first intermediary maximum M_1 is believed to reflect a decrease in *q*-quenching caused by reduction of the $NADP^+$ pool. In addition, a ΔpH change resulting from a slow H^+ influx at constant H^+ efflux through the ATPase has been discussed as the contributing factor for M formation (Sivak and Walker, 1983). Finally, the decline from M_1 to the terminal steady-state level T (sometimes additional, rather shallow transient maxima M_2, M_3, . . . are observed) probably reflects activation of Calvin–Benson cycle enzymes, giving rise

to a higher CO_2 fixation rate. The dependence on the O_2 and CO_2 content of the air, as well as the differences between C_3 and C_4 plants and the effect of light–dark regimes before the start of measurements, supports the idea that there is a close interrelation between the reductive pentose phosphate pathway and the fluorescence transients (Sivak and Walker, 1983; Bradbury *et al.,* 1985; Ireland *et al.,* 1985; Sivak *et al.,* 1985). Accordingly, the damped oscillation in the P-T decline is assumed to reflect "overshoots" in the regulation of reductive power and energization of the thylakoids upon sudden changes of NADPH and ATP demand in the CO_2 fixation pathway (Walker *et al.,* 1983). These considerations imply that the actual fluorescence induction curves very likely depend on the pools of NADPH and ATP *in vivo* at the start of the measurements. Simulation studies performed on isolated chloroplasts as a function of ADP, Fd, $NADP^+$, etc. support the interpretation mentioned above (Horton, 1983a). Additional effects arise, as noted earlier, as a result of changes in excitation energy distribution predominantly caused by LHC phosphorylation (Kyle *et al.,* 1983; Briantais *et al.,* Chapter 18, this volume). Furthermore, significant diurnal variations in leaf fluorescence kinetics are caused by changes of cellular metabolic processes. In the case of CAM (crassulacean acid metabolism) plants, these effects are mainly produced by malic acid accumulation (Everson *et al.,* 1983). Circadian rhythms of photosynthesis and Chl *a* fluorescence were analyzed in field populations of phytoplankton (Prézelin and Ley, 1980). In a marine dinoflagellate (*Gonyaulax polyedra*) the Chl *a* fluorescence yield rhythm was inferred to be due to changes in the rate constant for radiationless decay (Sweeney *et al.,* 1979).

This brief discussion may give some insight into the complexity of the physiological parameters that modulate the fluorescence yield *in vivo* by more or less direct ways. As pointed out above (Section II,A), this complexity does not prevent the gathering of reliable information, provided the reproducibility of data recording is assured. Besides appropriate control of the physiological state of the plant material (e.g., culture conditions, gas environment, dark adaptation), reproducibility depends to a considerable extent on the performance of the measuring system. Hence, before going into the details of practical applications, it appears worthwhile to briefly describe the experimental setup that is appropriate for such measurements.

III. Fluorometers for Practical Use in Greenhouses and in the Field

The instrumentation for measuring Chl fluorescence changes is described in a review by Schreiber (1983; see also Paterson and Arntzen,

1982), so that only the essential points will be summarized here. Figure 3 shows a block diagram of a setup for measuring fluorescence induction. Depending on the analytical problem to be solved, more or less sophisticated components may be chosen. For field studies, portable systems with low power consumption have been developed on the basis of modern semiconductor technologies (Schreiber *et al.*, 1975a; Schreiber *et al.*, 1986). Light-emitting diodes and photodiodes in most applications can substitute for conventional lamp shutter systems and photomultipliers. For laboratory applications, bi- or polyfurcated fiber optics (Strasser, 1973; Schreiber *et al.*, 1976) have proved particularly useful because (a) they prevent interference with other light; (b) the system is flexible and can be used for different cuvette systems; (c) fluorescence is collected directly from the illuminated sample surface, thereby minimizing fluorescence reabsorption artifacts; and (d) other light signals, such as absorbance or scattering changes, can be measured readily in the same system.

In some applications it is advantageous to excite fluorescence by a measuring beam which by itself does not cause any significant induction phenomenon, and to monitor the changes in fluorescence yield at the O level caused by factors different from light, e.g., heat stress (Schreiber *et al.*, 1976; Schreiber and Berry, 1977), ozone stress (Schreiber *et al.*, and herbicide binding (Urbach *et al.*, 1984). Such measurements require a system of high sensitivity, with a modulated measuring beam, a photo-

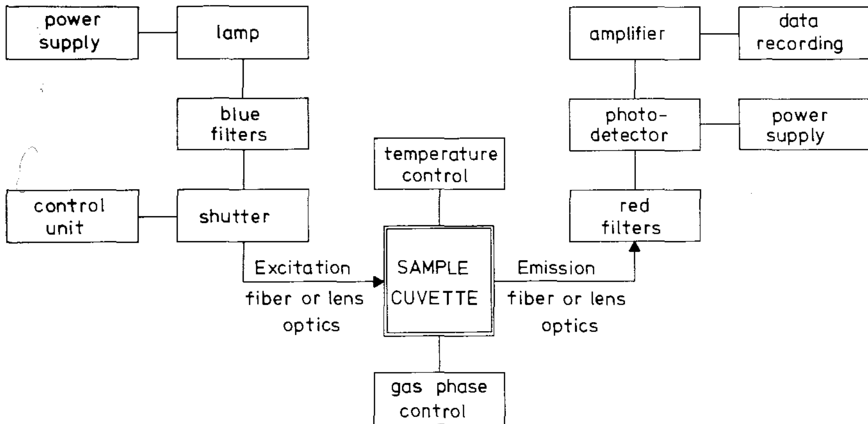

FIG. 3. Block diagram of the basic setup for measuring Chl fluorescence induction. For the recording of rapid changes, transient data storage (transient recorder, storage oscilloscope) is required. In standard applications a photodiode is sensitive enough for fluorescence detection. A stabilized high-voltage power supply is required to operate a photomultiplier tube.

multiplier or photodiode–operational amplifier (phot–op), and a lock-in amplifier. In principle, such a modulated fluorescence setup is also well suited for recording light-induced fluorescence changes (Duysens and Sweers, 1963; Schreiber and Vidaver, 1974; Chow *et al.,* 1981; Horton, 1983b; Ögren and Baker, 1985; Malkin *et al.,* 1986). As it directly measures fluorescence yield (instead of intensity), it is particularly useful in experiments where light intensity changes are essential. Very recently, a modulation fluorometer with a large dynamic range has been developed, tolerating a ratio of $1:10^6$ between measuring light and actinic intensities (Schreiber, 1986; Schreiber *et al.,* 1986). Hence, at a measuring light intensity which does not yet induce variable fluorescence (e.g., 10 mW/m^2), even saturating white light of 1000 W/m^2 may be applied and the change in fluorescence yield can be recorded. This system operates with 1 μs pulses of a light emitting diode (pulse amplitude modulation) at frequencies of up to 100 KHz, employing a photodiode as fast fluorescence detector. Because of its insensitivity to overlapping strong nonmodulated signals, this fluorometer is well suited for studies in the field with plants in their natural environment and direct sunlight for actinic illumination. Important additional information on the state of the photosynthetic apparatus may be obtained by a combination of the so-called light-doubling method (more appropriately called light-saturation method, Bradbury and Baker, 1981, 1984) and the modulation technique (Horton, 1983b; Quick and Horton, 1984; Schreiber *et al.,* 1986). Such a system allows simultaneous determination of q-quenching and e-quenching during the course of an induction curve or in the steady state, by use of superimposed saturating light pulses which cause transient, complete reduction of Q_A, correlated with the maximal fluorescence yield, F_{max}. The increase of the variable fluorescence due to a saturating light pulse, $\Delta F_v = (F_v)_s - F_v$, normalized to the maximum variable fluorescence at this point of the induction curve, $(F_v)_s$, can be used as a measure of q-quenching which in turn reflects the relative rate of photochemical energy conversion. Analogously, the difference between the saturating pulse induced maximum variable fluorescence in a dark adapted sample, $(F_v)_{max}$, and during the induction curve, $(F_v)_s$, related to $(F_v)_{max}$ provides information about nonphotochemical quenching, primarily on e-quenching. Theory and practical application of the saturation pulse method have been outlined in recent reports (Quick and Horton, 1984; Dietz *et al.,* 1985; Ögren and Baker, 1985; Bilger *et al.,* 1986; Schreiber and Bilger, 1986; Schreiber *et al.,* 1986). An example for the potency of this method will be presented in Section IV,B,2 (Fig. 7).

An integrating fluorometric probe system has been developed

(Toivonen and Vidaver, 1984) which allows measurement of fluorescence induction of whole plants and correlated determination of CO_2 fixation. Such a system may be particularly well suited for assessment of the overall photosynthetic productivity of plants. The authors reported a linear correlation between the relative amplitude of the M_1 peak and the capacity for CO_2 fixation.

Further progress in fluorescence instrumentation is linked to rapid developments in microcomputer techniques, which permit the construction of comparatively simple data processing systems at a reasonable price (see, e.g., Norrish *et al.*, 1983). These systems are particularly useful for routine screening experiments, e.g., testing the permeability of herbicide emulsions (G. Renger, unpublished).

IV. Application of Fluorometric Methods for Practical Use

Growth and cultivation of algae and higher plants are of utmost importance to humans. It is necessary to improve food production per unit area, to avoid detrimental effects due to parasite attack, etc. This goal can be reached by different methods (e.g., breeding and hardening of more efficient and better-adapted plants, application of fertilizers and pesticides) in agriculture, forestry, limnology, and marine biology. To test the success of these efforts, simple screening methods are required. Large-scale spectroscopic screening methods such as LIDAR (laser-induced detection and ranging) have been used to monitor the algal content of water or the degree of forest damage due to acid rain (for review, see Colwell *et al.*, 1983). But these methods provide only crude answers. For more detailed information, fluorometric methods can also be applied, but the fluorescence emission must be analyzed more thoroughly. This, however, requires measurements on individual plants or, in the case of limnological or marine biology studies, the use of rather small water samples. The present description is a brief review of a rapidly developing and diversified field.

A. *Productivity of Algae*

For different reasons (e.g., productivity and ecological aspects), the growth of algae is an important factor in marine biology and limnology. Accordingly, it has been desirable to develop a simple method for measuring the amount of algae in oceans, lakes, etc. Fluorometric methods have been widely tested for monitoring Chl content, and, in principle, they provide a reliable measure of the amount of algae. For precise

measurements, however, the dependence of fluorescence yield on the physiological state of the algae (photosynthetic activity, growth state) must be taken into account. An additional problem that can seriously affect (or even prevent) precise determinations is the turbidity of water probes. Application of herbicides that block electron transport and, therefore, induce the F_{max} state offers the possibility of using fluorescence also for rough productivity measurements by comparison of control samples with herbicide-treated probes. Different fluorometric methods have been developed (see, e.g., Lorentzen, 1966; Samuelson and Öquist, 1977; Neveaux and Jupin, 1981; Ernst and Schulze, 1982) that depend on the problems involved (e.g., large-scale screening or continuous recording) and the precision required for the data obtained. Probing presents the essential problem. If comparatively large areas are to be screened, LIDAR systems appear to be appropriate. The principle of the method is shown in Fig. 4. Probe fluorescence is excited by a laser system emitting high-power pulses directed from a helicopter toward the sea surface. The fluorescent light is collected by a telescope and monitored via photomultipliers. Data processing by computer provides the profiles of the fluorescence emission (for details of the method, see, e.g., Franz *et al.*, 1982; Gehlhaar, 1982; Diebel-Langohr *et al.*, 1984). Extension of this method should allow global screening by satellites (e.g., spacelab; for a recent review, see Colwell *et al.*, 1983). Of course, this rapid scanning procedure yields only qualitative information.

A more expensive and time-consuming but more precise method is the use of portable fluorometers on boats. This makes it possible to collect samples from lakes or the sea and to analyze them by measuring fluorescence curves in the absence and presence of the PSII herbicide DCMU (diuron), which blocks electron flow between Q_A and Q_B (for review, see Fedtke, 1982; Pfister and Urbach, 1983). In this case rather precise data can be obtained for an algal content down to a level of 1 μg Chl/liter (Neveux and Jupin, 1981; Ernst and Schulze, 1982; Öquist *et al.*, 1982). Furthermore, from the difference in fluorescence yield in the presence and absence of herbicides, a rough estimation of the photosynthetic capacity of algae and other marine phytoplankton is also possible. So far, the correlation with data obtained by more direct assays (measurements of oxygen evolution or CO_2 fixation rates) still appears to be inferior to that obtained by concentration determination methods. It has been shown that measurements of delayed fluorescence (monitored 0.1–2 s after excitation) provide a more appropriate method because rapid and continuous screening can be performed by using a special flux cuvette system. Computer processing of the numerical data readily gives information about different algal concentrations (diatoms, green-red,

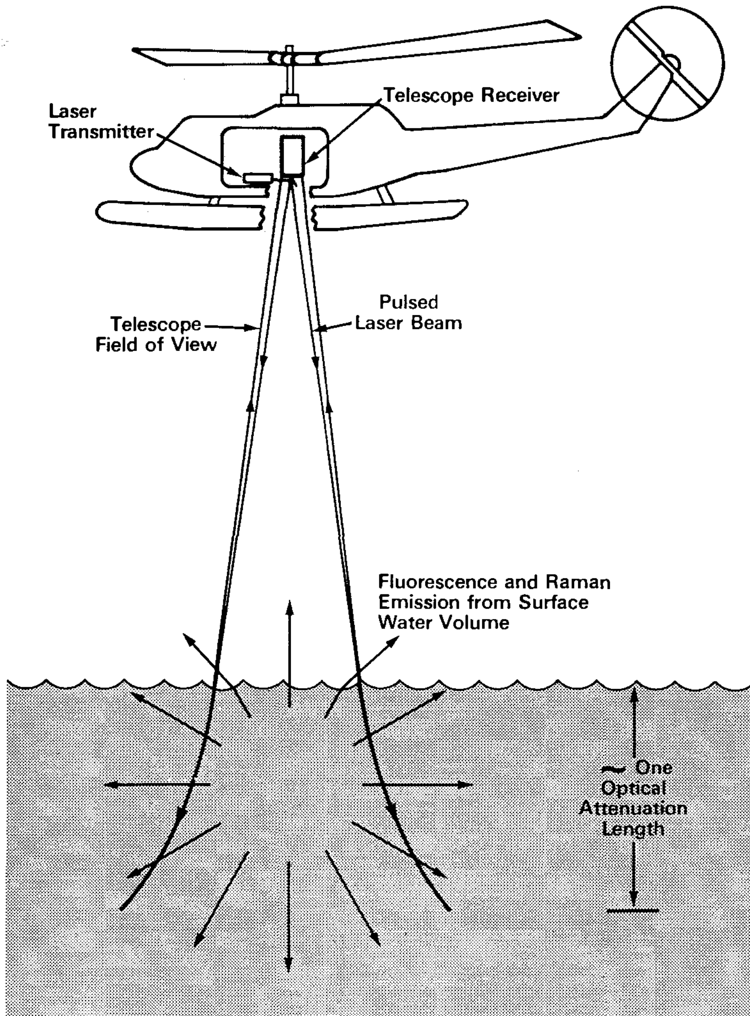

FIG. 4. Principle of a LIDAR system (Bristow *et al.*, 1981). Depending on the type of water screening (Chl fluorescence, tracer detection, oil pollution monitoring, etc.), fluorescence is excited with a pulse laser of appropriate emission wavelength. Scattered and fluorescent light from the water column is collected by a telescope. Detection wavelengths are spectrally selected and the signals are fed to a fast transient recorder. Data processing is performed with microcomputers (for computer simulation and theory of oceanographic fluorescence LIDAR signals, see Gehlhaar, 1982).

and blue-green algae) and productivity in oligotrophic and eutrophic waters (Krause *et al.*, 1982; G. Krause, unpublished.)

B. Monitoring of Algal and Higher Plant Physiology under Stress Conditions

Normally, wild-type plants are well adapted to their environmental conditions. The need for increased food and biomass production requires the development of more efficient plants, which should also be resistant to various stress conditions. As climates differ in different parts of the world, it is necessary to take into consideration various kinds of stress such as temperature stress (chill and heat), water and salt stress, as well as stresses that are caused by technological development (air and soil pollution arising mainly from exposure to emissions from cars and industry and applications of herbicides, insecticides, and fungicides, as well as possible increases of UV-B irradiation, etc.). Among these different factors, application of effectors (especially of herbicides) will be discussed separately because of the different aspects arising from their application (e.g., resistance and internal detoxification mechanisms). The environmental factors affecting photosynthesis have been reviewed by Berry and Downton (1982).

1. TEMPERATURE STRESS

It has been known for a long time that algae and higher plants grown under different temperature regimes exhibit different sensitivities to temperature stress. Therefore, efforts have been made to combine high plant productivity with high resistance so that these plants can grow under less favorable conditions. Screening methods for the selection of plants with the appropriate properties are required and must be as simple and rapid as possible. More than a decade ago, the first experiments were performed to analyze systematically the mechanisms underlying temperature resistance (Kniper, 1970; Berry *et al.*, 1975; Schreiber *et al.*, 1975b).

It was found that fluorescence measurements provide a very useful analytical tool for attacking this problem (Murata and Fork, 1975; Schreiber *et al.*, 1975b; Björkman *et al.*, 1978). With respect to high-temperature stress, it was shown that the dark fluorescence yield (F_0 level) more than doubled in the temperature region which is critical for heat-induced damage to the photosynthetic apparatus (Schreiber *et al.*, 1975b, 1976). There is a satisfactory correlation between the critical temperature, ϑ_c, where fluorescence begins to rise (at a constant heating

rate of about 1°K/min) and the temperature at which photosynthetic activity begins to decline (Björkman *et al.,* 1978; Monson and Williams, 1982). Typical traces of normalized $F_0(\vartheta)$ curves are shown in Fig. 5. It is apparent that the temperature threshold in the $F_0(\vartheta)$ curves reflects the heat tolerance of different plants. Similar results have been obtained with plants adapted to alpine, temperate, or tropical climates (Smillie and Nott, 1979; Smillie and Gibbons, 1981; Hetherington and Smillie, 1982; Downton *et al.,* 1984). In an investigation by Bilger *et al.* (1984), ϑ_c values from $F_0(\vartheta)$ curves were compared with ϑ_{LD50} values derived from the conventional leaf necrosis test (30 min heating at constant temperature and estimation of necrotic leaf area after 2–3 weeks of postculture). The outcome was a surprisingly close correlation between the results of these two vastly differing methods for a large variety of plant species. It was concluded that the increase of F_0 is a satisfactory indicator of the accumulation of heat dosage causing lethal damage to the overall leaf tissue (Bilger *et al.,* 1984).

Another important parameter for heat stress studies is the variable fluorescence (F_v = P minus O levels) of the O-I-D-P rise. It was found that F_v declines after heat injury. A thorough analysis of different plants

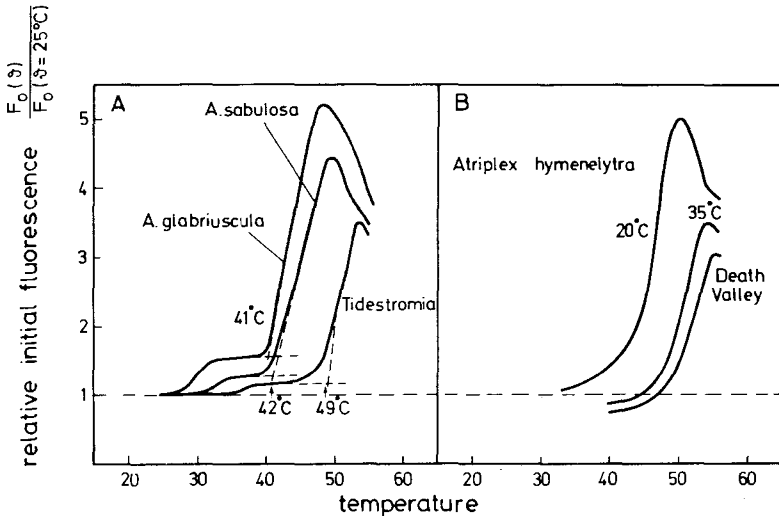

FIG. 5. Relative initial Chl *a* fluorescence, F_0 (ϑ)/F_0 (ϑ = 25°C) in leaves of different plants (A) and of *Atriplex hymenelytra* grown at different temperatures (B) as a function of treatment temperature ϑ during slow heating rates (ca. 1°K/min). Fluorescence was monitored with an extremely weak measuring beam ($I \sim 300\ \mu W/m^2$). Plants were grown under controlled temperature conditions (except for plants collected from Death Valley). Experimental data redrawn from Schreiber and Berry (1977).

led to the conclusion that variable fluorescence in leaves also provides a suitable basis for screening tests of heat tolerance (Smillie and Gibbons, 1981; Weis, 1982). Before irreversible damage is indicated by changes in F_0 and F_v, heat-induced limitation of carboxylation efficiency already becomes apparent (Weis, 1981). This reversible type of heat-inactivation is also reflected by fluorescence measurements if contributions due to *q*- and *e*-quenching can be separated, e.g., by application of the saturation pulse method (Bilger *et al.,* 1986).

Another essential temperature stress factor is tolerance to chilling. Different lines of evidence support the idea that heat and frost injuries to the photosynthetic apparatus are caused by different mechanisms (Klosson and Krause, 1981), although the capacity for protection against temperature stress (high- and low-temperature resistance) seems to be a specific property of a certain cultivar (Baker *et al.,* 1983). How the chilling mechanism affects degradation of photosynthetic activity is not yet completely resolved. It involves impairment of CO_2 assimilation rates coupled with a loss of activity in the noncyclic electron flow (Hetherington, *et al.,* 1983) and reduction of quantum efficiency (Baker *et al.,* 1983). It appears that tolerance to chilling is related to the lipid level of the thylakoid membrane rather than specific proteins (Havaux *et al.,* 1983).

Fluorometric methods have also been used to monitor chilling injuries in isolated chloroplasts and whole leaves (Smillie, 1979; Klosson and Krause, 1981). In contrast to the F_0 rise at higher temperatures (Fig. 5), leaves exposed to chilling stress do not show a significant F_0 variation. However, a drastic decline of variable fluorescence (O-I-D-P rise) is observed. This effect has been interpreted as meaning the chilling stress affects the electron donor side of PSII primarily (impairment of the donor side prevents elimination of *q*-quenching; Q_A remains oxidized). However, this idea is not confirmed by more recent studies (Martin and Ort, 1982), which clearly show that the chilling-induced impairment of the CO_2 fixation rate in tomato plants cannot be due primarily to destruction of the PSII donor side. By using a portable fluorometer, the fluorescence induction curves in detached leaves from different intolerant plants (cucumber, maize, soybean, guava, mango, grapefruit, etc.) have been measured at 0°C (Hetherington and Smillie, 1984). Here the maximum rate of fluorescence rise, $(dF_v/dt)_{max}$, referred to as R_F (see Fig. 6), was logarithmically related to the PSII activity. Furthermore, R_F measured in detached leaves exposed for different incubation times at 0°C correlated with the increase in plant fresh weight when plants were returned to a normal growth protocol after certain exposures to a temperature of 0°C (Smillie and Hetherington, 1984). Accordingly, at 50%

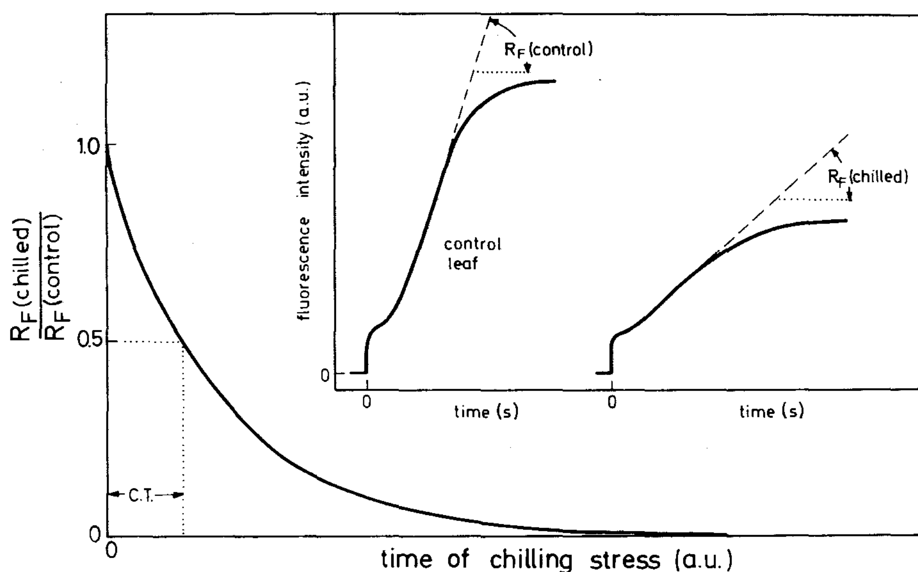

FIG. 6. Schematic representation of the relative maximum rate of O-I-P fluorescence rise. $(dF_v/dt)_{max}$, R_F (chilled)/R_F (control) as a function of chilling stress duration. Insert: Chl *a* fluorescence induction of control and chill-injured leaves. C.T., chill tolerance; a.u., arbitrary units. Scheme redrawn from Hetherington and Smillie (1984).

decrease of R_F can be used as a relative measure in screening different plants for chill tolerance. Typical curves are presented schematically in Fig. 6. The 50% value of R_F is also shown to reflect the hardening of maize plants by short-term adaptation of seedlings at low temperature and to reflect the different degrees of chill tolerance of tomato and potato plants grown at different altitudes (Hetherington and Smillie, 1984). In addition, R_F measurements have been shown to be appropriate for characterization of plant responses to other stress factors (Smillie and Hetherington, 1983) such as heat stress or light stress (see Section IV,B,4).

A relatively simple but somewhat nonspecific approach to monitoring low-temperature effects is the observation of steady-state fluorescence during temperature lowering (Sundbom *et al.*, 1982). The increase of fluorescence emission observed between the freezing point and −10°C can be used as a measure in screening plants for frost tolerance.

2. WATER STRESS

Water stress is another important factor that affects the photosynthetic apparatus of green plants in various ways (for review, see Boyer.

1976; Kriedemann and Downton, 1982). As in the case of temperature stress, plants adapted to different climates respond differently to drought conditions. Chl fluorescence has proved to be a suitable indicator for monitoring the changes in photosynthetic capacity on dehydration and rehydration of plants (Wiltens *et al.,* 1978; Govindjee *et al.,* 1981; Havaux and Lannoye, 1983). As a general pattern, dehydration first affects the P-S decline (block of Fd–$NADP^+$ reductase and/or Calvin cycle activation), then variable fluorescence (inhibition at the PSII donor side), and finally the F_0 level (disturbance of energy transfer at the pigment level). This pattern is observed for susceptible as well as tolerant plant species. However, only the tolerant species display a reversal to normal induction kinetics on rehydration (Wiltens *et al.,* 1978). For any given species, there is a threshold limit of water potential below which dehydration results in irreversible damage. Very recently, the saturation pulse method of modulated fluorescence (see Section III) was applied for the investigation of water stress effects on *Arbutus unedo* leaves (Schreiber and Bilger, 1986). As with high temperature stress, water stress causes a pronounced increase in *e*-quenching and a decrease in *q*-quenching before there arises any loss in variable fluorescence. Figure 7A shows fluorescence induction curves of *Arbutus unedo* leaves with repetitive application of saturation pulses at different relative water contents of the leaves. Figure 7B shows a plot of the relative assimilation rate versus the extent of *q*-quenching as a function of decreasing water content. The data reveal a close correlation between assimilation rate and *q*-quenching. It should be emphasized, however, that such correlation can be expected only if electron flow to acceptors different from CO_2 may be neglected (i.e., at high CO_2 concentration or at low O_2 concentration when irradiance levels are relatively high; see Schreiber and Bilger, 1986; Winter *et al.,* 1986).

Important parameters for the extent of irreversible damage at any given value of lowered water potential are the physiological age of the leaf (Hetherington *et al.,* 1982), the rate of dehydration (Downton and Millhouse, 1984), and the state of illumination (Björkmann, 1981; Downton and Millhouse, 1984). Illuminated leaves experiencing water stress display a pattern of fluorescence induction similar to that observed in control plants exposed to excessive light intensities that cause photoinhibition (for review, see Björkmann, 1981). Certain plant species such as grapevine (*Vitis vinifera*) (Downton and Millhouse, 1984) are capable of maintaining turgor during slow desiccation by osmotic adjustment. In such plants, variable fluorescence, indicating efficient water-splitting activity, remains high over a wide range of lowered water potentials even at relatively high irradiance levels.

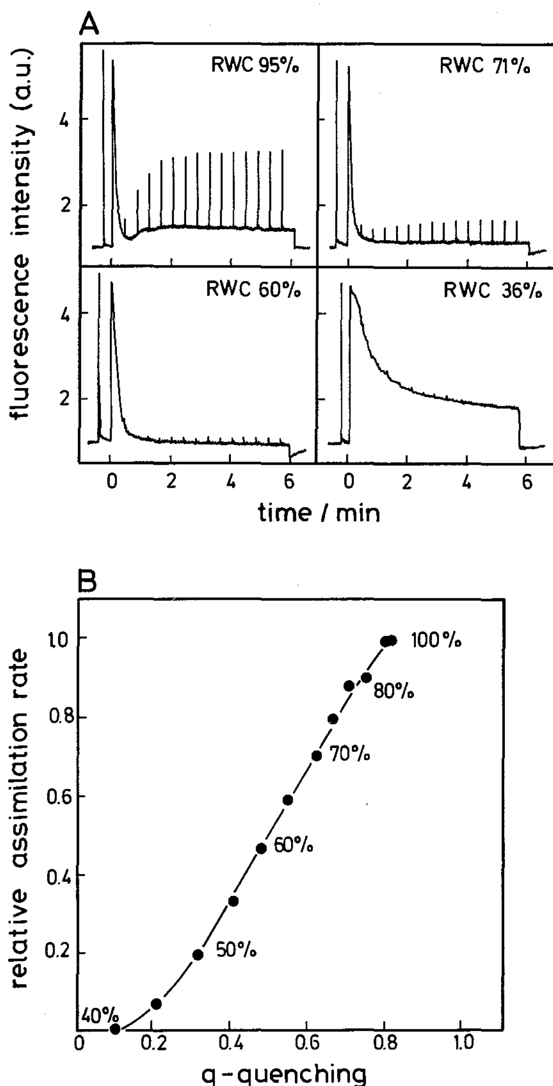

FIG. 7. Effect of water stress on fluorescence-quenching properties of *Arbutus unedo* leaves. (A) Induction curves upon illumination with 125 $\mu E/m^2s$ white light with repetitive application of 500 ms saturation pulses of heat-filtered white light of 10000 $\mu E/m^2s$. Relative water content (RWC) is varied as indicated in the figure. (B) Correlation between relative assimilation rate and *q*-quenching. Assimilation rate and fluorescence were measured simultaneously at high CO_2 concentration using a leaf disc electrode (Hansatech LD2) and a modulation fluorometer (Walz, PAM 101). The given values apply for steady state illumination. The varying parameter of relative water content is indicated in percent. Data from Schreiber and Bilger (1986).

3. SALINITY STRESS

Closely related to water stress are detrimental effects caused by increased salt concentration upon dehydration of cells. Concentration increases of certain salts beyond a critical level can limit photosynthetic capacity (Boyer, 1976; Downton, 1977). Salt stress is becoming a problem with growing relevance (especially for agriculture in regions which depend on irrigation) because increased salinity in soil and water affects crop plants. Therefore, the development of salt-tolerant species has become an important aim of plant breeding using techniques of modern genetics (Epstein *et al.,* 1980). Fluorescence methods were also shown to be appropriate for detection of salt effects in plants. Comparative measurements in salt-tolerant (sugar beet), moderately salt-tolerant (sunflower), and salt-intolerant (bean) plants revealed that the fluorescence rise O-I-D-P can be used as an indicator for the characterization of salt tolerance (Smillie and Nott, 1982). Only in salt-intolerant plants (e.g., *Phaseolus vulgaris*) was variable fluorescence suppressed by salt application to potted plants. An initial decrease of leaf turgor pressure upon salt treatment of grapevine leaves was accompanied by a decrease in fluorescence; with osmotic adjustment, the turgor and the variable fluorescence were observed to increase again.

4. LIGHT STRESS

Inhibition of photosynthesis can result when plants are exposed to light intensities that markedly exceed those of their normal growth conditions. This effect, referred to as photoinhibition (for review, see Björkman, 1981; Powles, 1984), depends on the interaction of high light levels and other stress factors. It has been shown that Chl fluorescence is a suitable indicator for the resulting damage of the photosynthetic apparatus (Critchley and Smillie, 1981; Kyle *et al.,* 1984; Krause *et al.,* 1985). Low-temperature fluorescence studies suggested (Fork *et al.,* 1981; Powles and Björkman, 1982; Björkman and Powles, 1984; Ögren and Öquist, 1984; Samuelsson *et al.,* 1985) that the observed suppression in photoinhibited leaves is caused by damage to the PSII reaction center complex. Detailed mechanistic studies on algae (*Chlamydomonas reinhardtii* y-1), however, led to the conclusion (Arntzen *et al.,* 1984) that the primary target of photoinhibition is the rapidly turned over 32-kD polypeptide, which is essential for the reoxidation of Q_A by the secondary plastoquinone (Q_B). It was suggested that reaction of Q_B^- with O_2 could lead to radical formation which results in damage to the 32-kD polypeptide. This idea seems to be at variance with the reported independence of photoinhibition on the presence of molecular oxygen (Powles and

Björkman, 1982). However, low internal O_2 partial pressures seem to be hardly maintainable at high irradiance levels. Therefore, the rather low concentrations of photosynthetically generated molecular oxygen might be sufficient for generation of toxic species (singlet oxygen, superoxide, hydroxyl radicals, H_2O_2) when the physiological acceptor system is overloaded. Latest experimental findings do not support the idea that the above-mentioned 32-kDa polypeptide is the primary target for photoinhibition (Arntz and Trebst, 1986; Cleland and Critchley, 1985). The results rather suggest an attack on the reaction center itself without detectable changes in the polypeptide pattern.

Another light effect of growing concern is the response of plants to UV-B irradiation (for review, see Gold and Caldwell, 1983), and information is needed on the consequences of an average increase in solar UV irradiation due to a diminished ozone layer. As in the case of injuries caused by other stress factors, experiments revealed that fluorometric methods are an appropriate tool for analyzing the effects of UV irradiation (Smillie, 1983; Björn *et al.*, 1986; Renger *et al.*, 1986a) in whole leaves, thereby allowing screening experiments for these properties. The mechanistic details of these effects in whole leaves and possible defense mechanisms by higher plants (e.g., formation of UV-B-absorbing pigment layers in the cuticle) are not yet completely resolved.

5. AIR POLLUTION

Among the anthropogenic stress factors leading to severe limitations of plant growth are the various forms of air pollution (for review, see Heath, 1980). The biochemical and physiological events occurring on exposure of plants to air pollutants are extremely complex. Depending on the type of pollutant, the photosynthetic capacity of leaves is affected in a more or less direct way. Accordingly, the resulting effects on Chl fluorescence are manifold. So far, systematic fluorescence studies have been carried out primarily for damage caused by ozone (Schreiber *et al.*, 1978) and SO_2 (Shimazaki *et al.*, 1980; Schiele *et al.*, 1981; Shimazaki *et al.*, 1984). As with most other stress parameters, ozone and SO_2 cause a suppression of variable fluorescence yield, which might indicate a primary attack on the water-splitting activity. With increasing interest in the mechanisms which lead to the death of forests over large parts of the world, it may be foreseen that Chl fluorescence methods will play an important role in the detection and analysis of such damage. Even without definite insights into the mechanisms involved, fluorescence induction kinetics can readily provide information on injury levels at vastly varying conditions of interacting environmental parameters (pollutant doses, temperature, light, water potential, mineral supply).

C. Herbicide Effects

One of the most important topics in modern agriculture is the application of chemicals that act as pesticides (herbicides, insecticides, fungicides, and compounds attacking other targets of minor importance; for reviews, see Geisbühler, 1978; Moreland, 1980; Fedtke, 1982; Pfister and Urbach, 1983). Within the framework of this chapter, only herbicide effects will be considered. Regardless of the various modes of application, different aspects of practical relevance must be considered: (a) penetration of herbicides into plants and transport to the site(s) of action (see Section IV,C,1), (b) tolerance to herbicides by internal detoxification (IV,C,1), and (c) development of resistance to herbicides in plants (IV,C,2).

Among the great variety of herbicides that interfere at different stages with plant metabolism, a large number effectively block photosynthetic electron transport between the primary and secondary plastoquinones (referred to as Q_A and Q_B; see Fig. 1, in Duysens, Chapter 1, this volume) at the PSII electron acceptor side (for reviews, see Fedtke, 1982; Pfister and Urbach, 1983; also see Paterson and Arntzen, 1982; Renger, 1986). Therefore, fluorometric methods seem to be most promising in analyzing these herbicide effects. Analysis of herbicide effects in whole leaves requires detection of the average inhibition degree without leaf destruction. Most of the fluorescence methods applied so far are based on measurements of the O-I-D-P rise in sufficiently dark-adapted (5 min) leaves. Typical fluorescence inductions observed in the absence and presence of diuron, as the prototype of herbicides that block Q_A^- reoxidation by Q_B, are shown in Fig. 8 for isolated chloroplasts and whole leaves. The normalized variable fluorescence, referred to as $F_v(t_m)$ (see Fig. 8), provides a satisfactory measure of the inhibitory degree in isolated chloroplasts, because the derived I_{50} values from fluorescence data agree well with those obtained by direct electron transport measurements (Brewer *et al.*, 1979; Voss *et al.*, 1984c). Despite many interfering parameters in whole leaves (see Section II), reliable results can be obtained for whole leaves (Voss *et al.*, 1984a, b, c). Another approach has been described (Richard *et al.*, 1983) in which the terminal fluorescence level F_T (see Fig. 2) provided a means for detecting the effect of PSII herbicides on the electron transport in whole leaves.

1. HERBICIDE PERMEATION AND DETOXIFICATION

Herbicide penetration into plants is an important aspect of practical application because compounds are known which act as very efficient inhibitors in isolated chloroplasts, but are completely inactive as herbi-

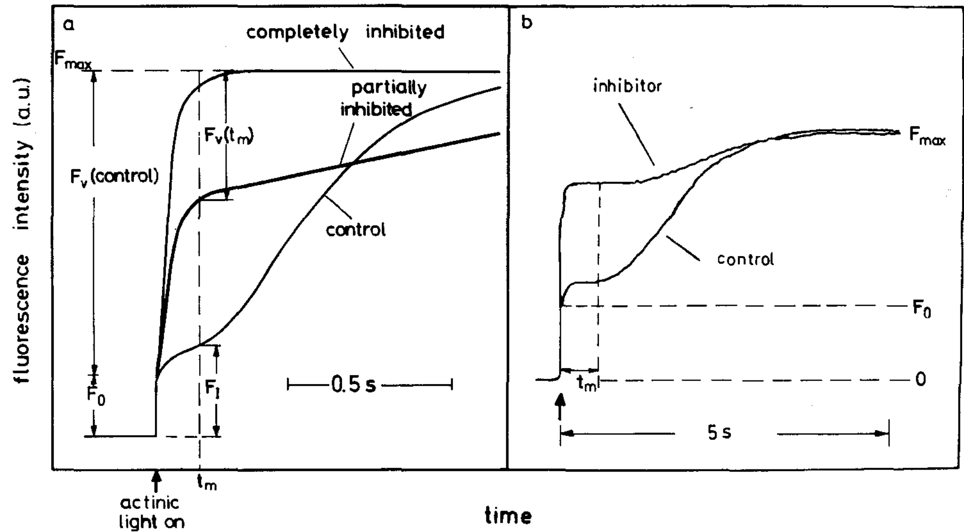

FIG. 8. Effect of PSII inhibitors on Chl *a* fluorescence induction in isolated chloroplasts and leaves. (a) Isolated spinach chloroplasts with intact, partially inhibited, and totally inhibited PSII electron transport [0, 13.10^{-8}, and 10^{-6} *M* diuron (DCMU), respectively] and (b) dwarf bean leaves without and after foliar application of the PSII herbicide phenmedipham in a commercial formulation (Voss *et al.*, 1984c).

cides (for reviews, see Fedtke, 1982; Pfister and Urbach, 1983). Accordingly, studies of the penetration mechanism are important for developing appropriate herbicide formulations. It has been shown that the penetration kinetics can be monitored by the normalized variable fluorescence, $F_v(t_m)$, as a function of time after herbicide application (Voss *et al.*, 1984b).

Fluorometric methods also provide an appropriate tool for the analysis of herbicide tolerance and resistance in plants. In plants susceptible to PSII herbicides, rapid Q_A^- reoxidation by Q_B is prevented and therefore the normalized variable fluorescence $F_v(t_m)$, measured at time t_m (see Fig. 8), remains low after herbicide penetration. A markedly different pattern, however, should arise in herbicide-tolerant plants, depending on the mechanism allowing survival. If the herbicide cannot reach the functional site, because of either transport barriers or a detoxification mechanism that operates faster than transport, then no effect on Chl *a* fluorescence transients can be observed. The same phenomenon arises in herbicide-resistant plants (see below). On the other hand, if the detoxification is slower than transport, then a transient inhibition would be expected, which should be reflected by a transient decrease in $F_v(t_m)$

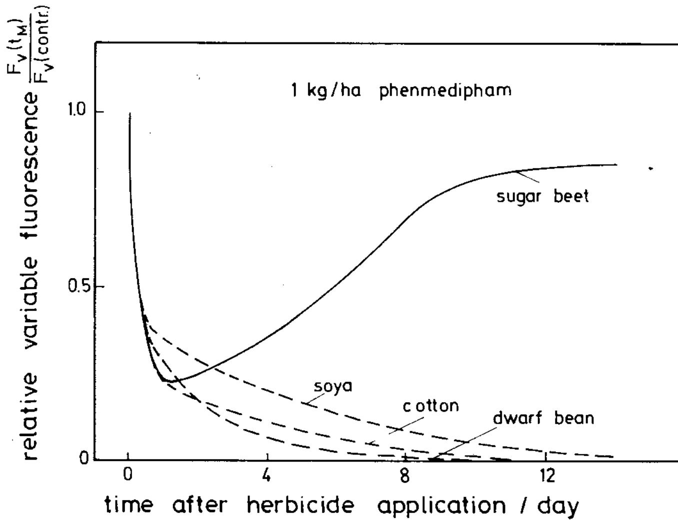

FIG. 9. **Relative variable Chl *a* fluorescence, $F_v(t_M)/F_v$ (control) as a function of time after foliar application of the PSII herbicide phenmedipham in different plant leaves (experimental data redrawn from Voss *et al.*, 1984a).**

followed by recovery to the control value. Experiments with various types of plants and herbicides showed that the normalized variable fluorescence does provide a useful tool for the detection of herbicide detoxification (Cadalia *et al.*, 1982; Ducruet *et al.*, 1984; Voss *et al.*, 1984b,c). Typical results for the PSII herbicide phenmedipham are depicted in Fig. 9. It was found that the fluorescence decline from the P to the T level can also be used for monitoring herbicide detoxification (Lansac *et al.*, 1984).

2. HERBICIDE-RESISTANT BIOTYPES

Development of herbicide resistance is important for two reasons: it is a desirable property in plants that we would like to cultivate, but an undesirable property in plants (e.g., weeds) that we would like to destroy. The extensive use of herbicides in modern agriculture has led to the selection of herbicide-resistant weeds. The most extensively analyzed examples are atrazine-resistant biotypes, which were first characterized biochemically by Arntzen *et al.* (1979, 1982). Since then, there have been confirmed reports of triazine-resistant biotypes in 38 species in 18 genera in 23 states of the United States, 4 Canadian provinces, 9 European countries, and 1 Middle Eastern country (July 1983; see Gressel, 1984). So far, triazine resistance in weeds seems to be the only case creating a

temporary agricultural problem, which occurs in areas of maize monocultures after a rather long application of this herbicide.

As in the case of other stress conditions, large-scale screening methods are required to analyze extended plant populations. Again, fluorometric methods appear to be an appropriate tool, because the fluorescence induction curves of biotypes resistant to PSII herbicides generally differ from those of the corresponding susceptible biotypes. The O-I-D-P rise of fluorescence becomes modified through changes in the electron transfer kinetics between Q_A and Q_B (Bowes *et al.*, 1980), which are regulated by proteinaceous components (Renger, 1976) that also provide the binding environment for PSII herbicides, so that small variations of the polypeptide structure can cause large changes in PSII herbicide binding (for recent data, see Vermaas *et al.*, 1984). Accordingly, in susceptible plants, the fluorescence induction curve is drastically modified by herbicide addition (see Section IV,C,1), whereas in resistant plants no significant change is observed after application of the herbicide to which the plant is resistant. Other herbicides, however, can still be active or exert an even stronger influence than they do on susceptible varieties. These differences make Chl *a* fluorescence measurements a powerful tool for screening of herbicide resistance.

Robinson (1985) has devised a new instrument, which uses flash lamps, to monitor the kinetics of the primary quinone acceptor of photosystem II, Q_A, in intact leaves. This instrument has allowed the measurement, in *Amaranthus hybridus* leaves, of the altered kinetics of the functioning of the two-electron gate of photosystem II (see van Gorkom, Chapter 10, this volume) in atrazine-resistant plants.

V. Concluding Remarks

This brief review has described the wide range of practical applications of Chl fluorescence measurements in plant physiological work. The basic phenomenon of the Kautsky effect contains the essential information on the state of the photosynthetic apparatus. The measuring principle is simple, nondestructive, and rapid, and therefore also suited for large-scale screening analysis of different stress conditions in plant populations. Portable instruments are available for field-oriented work, and further progress can be expected with application of low power-consuming microcomputers for data processing and storage. Such new developments will lead to more general application of the fluorescence method in applied plant sciences such as agriculture, forestry, marine biology, limnology, phytopathology, and pollution and pest control.

Earlier work on the practical applications of Chl fluorescence measurements in plant research, as summarized in this chapter, was to some extent empirical and phenomenological in nature. As has been pointed out, the method is based on a complex function of fluorescence emission, determined by a large number of partially interacting parameters. For any specific application, calibration of the method against a more direct assay of photosynthetic activity (e.g., O_2 evolution or CO_2 fixation) is essential. After such calibration, the inherent advantages of the fluorescence method (speed, nondestructiveness, small sample requirement, high signal/noise ratio, portability, etc.) will provide a screening and test system that, presumably, will not be equaled by any other known method. With the recent development of the saturation pulse method of modulated fluorescence, a powerful tool has become available for determination of relative electron transport rates as well as of the energy status of the chloroplasts *in situ*. Further progress in this field of research will depend on interaction between investigators specializing in the fundamental aspects of Chl fluorescence as well as the modulating parameters of the photosynthetic process, and the users of the fluorescence method in applied plant research.

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