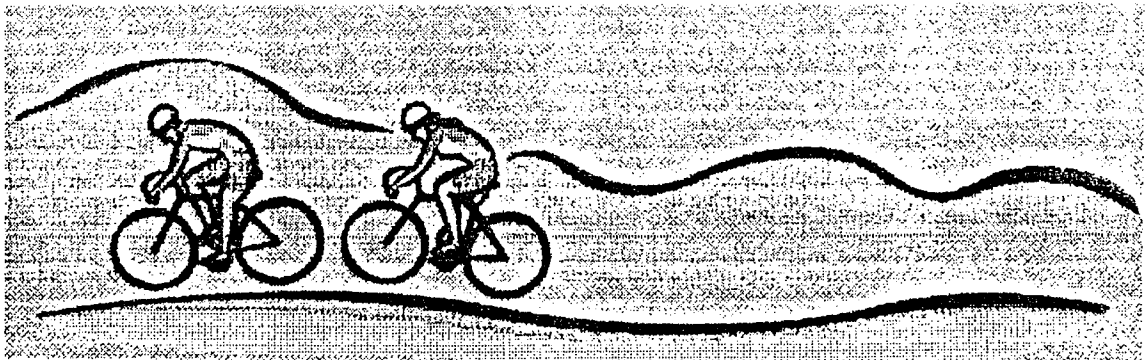


Photosynthesis in Twelve Sessions

by

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Collation sponsored by :
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All you wanted to know about Photosynthesis but were afraid to ask: From Photons to Sugar for your Darjeeling Tea

"Believe nothing
merely because you have been told it,
or because it is traditional, or because
you yourself have imagined it,
Do not believe what your teacher
tells you,
merely out of respect for the teacher,
But whatever after due examination
and analysis, you find to be
conductive to the good of the
benefit, the welfare of all beings, that
doctrine believe and cling to,
and take it as your guide."

Lord Gautam Buddha

Standards for inconsequential trivia
(PA Simpson, NBS Standard 15: January 1, 1970)

10 ⁻¹⁵ bismol	= 1 femto-bismol
10 ⁻¹² boos	= 1 picoboo
1 boo ²	= 1 boo boo
10 ⁻¹⁸ boys	= 1 attoboy
10 ¹² bulls	= 1 terabull
10 ¹ cards	= decacards
10 ⁻⁹ goats	= 1 nanogoat
2 gorics	= 1 paregoric
10 ⁻³ ink machines	= 1 millink machine
10 ⁹ los	= 1 gigalo
10 ⁻¹ mate	= 1 decimate
10 ⁻² mental	= 1 centimental
10 ⁻² pedes	= 1 centipede
10 ⁶ phones	= 1 megaphone
10 ⁻⁶ phones	= 1 microphone
10 ¹² pins	= 1 terapin

A Minicourse on Photosynthesis (November, 1996-February, 1997)

by

Govindjee

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URL: <http://www.life.uiuc.edu/govindjee/> [This site has many
slides and teaching tools related to this course.]

Schedule of Topics

[Class participation mentioned in # 12 will require selection of a
demonstration leader and practice directed by the instructor from the
second day of the lectures: after hours]

#1. Introduction to Photosynthesis: Conversion of Light Energy into Chemical Energy--Photochemistry and Photosynthesis

Photochemistry

- Dual Nature of Light
- Absorption Spectroscopy: Energy level diagram (Joblanski diagram) and absorption spectra of pigments
- Franck-Condon Principle and the Stokes' shift
- Multiplicity of States: singlets, doublets and triplets
- What is Primary Photochemistry?

Photosynthesis

- Introduction to Photosynthesis: definition and overall reaction
- Brief History of Photosynthesis
- Classification of Photosynthetic Organisms
- Key Component: chlorophyll or bacteriochlorophyll
- Principles of Photosynthetic Energy Transformation
- The Quantum Yield of Photosynthesis: Warburg versus and Emerson
- Nobel Laureates in Photosynthesis

#2. Light Absorption and Energy Transfer: Pathways of Excitation and Deexcitation--Fluorescence, Energy Transfer and Chemistry

- Molecules and Absorption
- Pathways of Deexcitation
- Fluorescence ; relationship to photosynthesis/demonstration
- Excitation Energy (or Exciton) Transfer
- Concept of "Photosynthetic Unit"

- Evidence for Excitation Energy Transfer
- Mechanism of Excitation Energy Transfer: The Foerster Theory
- Review of Primary Photochemistry: charge separation
- Difference Absorption Spectroscopy

#3 Reaction Centers and Primary Photochemistry: In Photosynthetic Bacteria, In Cyanobacteria and in Plants

- Reaction Centers of Plants and Bacteria: observation by difference absorption spectroscopy; evidence that reaction center chlorophyll is oxidized in light; isolation in a test tube
- Crystallization of Reaction Centers
- X-ray crystallography: structure of protein and arrangement of chromophores
- Anoxygenic Photosynthesis: purple bacteria; green bacteria; green gliding bacteria
- Primary Photochemistry and "Two Electron Gate" in Bacteria
- Intraprotein Electron Transfer: Electron tunnelling; Marcus theory
- Photosystem I : X-ray structure
- Photosystem II: A knowledge based model

#4 Oxygenic Photosynthesis: Membrane Organization and Components

- Chloroplast Structure
- Thylakoid Membranes
- Particles in Membranes
- Components: Proteins and Lipids
- The Four Major Protein Complexes
- Mobile Electron Carriers
- Genetic Coding of the Various Polypeptides

#5 The Two Photosystems and Two Light Reactions: in Plants and in Cyanobacteria, but not in Anoxygenic Photosynthetic Bacteria

- Review of Oxygenic Photosynthesis: light absorption; simultaneous primary photochemistry in photosystem I and II; electron transport
- Experiments and Ideas that Led to the Discovery of the "Two Light Reaction" Scheme: "The Red Drop in Photosynthesis"; "The Emerson Enhancement Effect"
- Experimental Evidence: antagonistic effect of light I and II on the redox state of cytochrome f; physical separation of the photosystems; chemical manipulations; genetic approach; and kinetic spectroscopy

#6 Electron Transport Pathways-- From Water to Plastoquinone: Water-Plastoquinone Oxido-reductase (Photosystem II): Oxygen Evolution in Plants and Cyanobacteria, but not in (Anoxygenic) Photosynthetic Bacteria

- Introduction to Oxygenic Photosynthesis in Cyanobacteria and Algae
- Review of 4 Major Protein Complexes; mobile carriers; genetic coding presented in # 4.
- Review of Source of Oxygen in Photosynthesis
- The S-states and the Oxygen clock
- Proton Release
- Evidence that S-states refer to the redox states of Manganese: XAS; EPR Measurements
- The Two Electron Gate: how herbicides kill plants?
- The Stimulatory Effect of Bicarbonate: current perceptions
- Impact of Molecular Biology

#7 Plastoquinol-Plastocyanin Oxido-Reductase (Cyt b6/f complex) :Intersystem Electron Transport; The "Q" Cycle

- Creation of a Proton Gradient
- Components and their Function
- Cytochrome f: structure
- Rieske Fe-S : structure
- Linear Electron Flow Scheme
- The "Q" cycle: How it doubles the proton gradient per electron transferred to Photosystem I?
- Transport of Electrons to Photosystem I: Plastocyanin

#8 Plastocyanin-ferredoxin Oxido-reductase (Photosystem I): Components and Electron Transport Pathways

- Function of Photosystem I
- Composition of Photosystem I
- X-ray Structure of Photosystem I
- Use of Molecular Biology
- Discussion of E. Greenbaum's experiments: a mutant of Chlamydomonas can live with just Photosystem II

#9 ATP Synthesis: Proton Gradient and Membrane Potential; Mitchell's Chemiosmotic Hypothesis; ATP Synthase and Boyer's Binding Change Hypothesis

- Synthesis of ATP by ATP Synthase
- Various Systems
- Mitchell's Chemiosmotic Theory
- Key Experiments in Support of Mitchell's Theory:

- Equivalence of proton gradient and membrane potential
- Boyer's Alternate Binding Change Hypothesis
- Structure of Bovine Heart Mitochondrial ATPase
- Evidence of Rotation in ATPsynthase from Junge's Laboratory

#10 Carbon Fixation: The Calvin Cycle; RUBISCO; C-4 Pathway; and CAM

- The Major Steps in the Calvin Cycle
- The Enzyme RUBISCO
- The C-4 Pathway
- The CAM Pathway
- Comparison of C-3, C-4 and CAM plants
- Link to the Problem of Increasing [CO₂] in our Environment

#11 Efficiency and Global Aspects of Photosynthesis

- Efficiency of plants
- The Carbon Cycle
- The Greenhouse Effect
- Biomass and Climate
- Impact of [CO₂] Rise in our Environment
- Concluding Remarks

#12 Sunlight, Summary and Comparative Aspects

- About our "Suraj"
- Summary of Photosynthesis
- Comparative Aspects: Photosynthetic Systems
- Comparative Aspects: Antenna Systems
- Comparative Aspects: Arrangement of Complexes on the Membrane
- Comparative Aspects: Energetics and Redox Potentials
- Class Participation: Demonstration of Electron and Proton Transport; Oxygen Evolution; and possibly "Q-cycle"

The End.

Introduction to Photosynthesis: Conversion of Light Energy into Chemical Energy —Photochemistry and Photosynthesis

-the sun is not only the author of visibility in all visible things, but of generation and nourishment and growth -- " Plato, *The Republic*

Outline

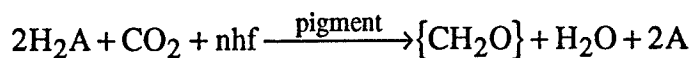
A. Photochemistry

1. Light has a dual nature: it has both wave and particle character; there is an inverse relationship between energy E and wavelength λ : $E = hc/\lambda$, where h = Planck's constant, and c = Velocity of light; also $E = hf$, where f = frequency of light.
2. You need to understand the relationship between Energy Levels (Jablonski diagram) and the absorption spectrum of a molecule based on #1, above.
3. Absorbance = $\log \frac{I_0}{I}$ where I_0 = incident light and I = transmitted light: should know absorption spectroscopy.
4. Electronic transition occurs in approximately a femtosecond (10^{-15} s); Franck-Condon Principle (electronic transitions are much faster than nuclear motions as nuclei are heavy) and the Stokes' shift (fluorescence is at a longer wavelength than absorption) must be understood. [NOTE: There are as many femtoseconds in a second as there are seconds in 31 million years.]
5. Excited states have different properties than ground states; molecules could be in different states; singlets, doublets and triplets; fates of excited states, that is, deexcitation pathways must be understood.

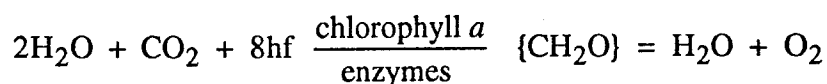
Multiplicity of a state = $2S + 1$, where S = total, spin; spin of an electron = $+ 1/2$ or $- 1/2$
6. Primary Photochemistry (rapid; first; occurs at very low temperatures); charge separation in photosynthesis: electron transfer from a "reluctant" donor to a "reluctant" acceptor using the absorbed light energy. This process occurs in approximately few picoseconds (10^{-12} s).

B. Photosyntheses

1. *Definition*: "Photosynthesis is a series of processes in which electromagnetic energy is converted to chemical free energy which can be used for biosynthesis" (Martin Kamen, 1963).
2. Overall reaction in plants, cyanobacteria or the so-called photosynthetic bacteria (purple; green sulfur: *Chlorobium*; green gliding: *Chloroflexus*) can be written as (C.B. Van Niel, 1930):



3. Time scale: from femtoseconds to seconds, learn the difference between oxygenic ($2\text{A} = \text{O}_2$) and anoxygenic ($\text{A} = \text{S}$, etc.) photosynthesis.
4. Some important components are chlorophylls (or bacteriochlorophylls), cytochromes, quinones, protein complexes, etc.
5. Quantum yield of O_2 Evolution in oxygenic Photosynthesis: Controversy between Nobel laureate (1931 in Physiology and Medicine) Otto Warburg ($10_2/4$ photons) and his student Robert Emerson ($10_2/8$ photons). Emerson was right! This number 8 replaces "n" in the above equation:



Sources

1. R.K. Clayton: Light and Living Matter, Vol.1 : The Physical Part, McGraw Hill, 1970.
2. Govindjee and Rajni Govindjee: The Primary Events of Photosynthesis. Scientific American, December, 1974.
3. Taiz and Zieger: Plant Physiology (Chapter 8).

The Photosynthetic Process by J. Whitmarsh and Govindjee: an appropriate portion is reproduced below.

Summary

The primary source of energy for nearly all life is the Sun. The energy in sunlight is introduced into the biosphere by a process known as photosynthesis, which occurs in plants, algae and some types of bacteria. Photosynthesis can be defined as the physico-chemical process by which photosynthetic organisms use light energy to drive the synthesis of organic compounds. The photosynthetic process depends on a set of complex protein molecules that

are located in and around a highly organized membrane. Through a series of energy transducing reactions, the photosynthetic machinery transforms light energy into a stable form that can last for hundreds of millions of years. This introductory text focuses on the structure of the photosynthetic machinery and the reactions essential for transforming light energy into chemical energy.

A. INTRODUCTION

Photosynthesis is the physico-chemical process by which plants, algae and photosynthetic bacteria use light energy to drive the synthesis of organic compounds. In plants, algae and certain types of bacteria, the photosynthetic process results in the release of molecular oxygen and the removal of carbon dioxide from the atmosphere that is used to synthesize carbohydrates (oxygenic photosynthesis). Other types of bacteria use light energy to create organic compounds but do not produce oxygen (anoxygenic photosynthesis). Photosynthesis provides the energy and reduced carbon required for the survival of virtually all life on our planet, as well as the molecular oxygen necessary for the survival of oxygen consuming organisms¹. In addition, the fossil fuels currently being burned to provide energy for human activity were produced by ancient photosynthetic organisms. Although photosynthesis occurs in cells or organelles that are typically only a few microns across, the process has a profound impact on the earth's atmosphere and climate. Each year more than 10% of the total atmospheric carbon dioxide is reduced to carbohydrate by photosynthetic organisms. Most, if not all, of the reduced carbon is returned to the atmosphere as carbon dioxide by microbial, plant and animal metabolism, and by biomass combustion. In turn, the performance of photosynthetic organisms depends on the earth's atmosphere and climate. Over the next century, the large increase in the amount of atmospheric carbon dioxide created by human activity is certain to have a profound impact on the performance and competition of photosynthetic organisms. Knowledge of the physico-chemical process of photosynthesis is essential for understanding the relationship between living organisms and the

¹ Some organisms derive their energy from electron donating inorganic molecules such as hydrogen gas or sulfur compounds and are not dependent on current or past photosynthesis for their survival. Examples include the bacterium *Methanobacterium thermoautotrophicum*, which grows in sewage sludge living on hydrogen gas and carbon dioxide and the bacterium *Methanococcus jannaschii*, which grows in the ocean near hot vents.

atmosphere and the balance of life on earth. Several books on photosynthesis are available for the uninitiated (Hall and Rao, 1994; Lawlor, 1993; and Walker, 1992) or advanced student (Govindjee, 1982; Amesz, 1987; Briggs, 1989; Barber, 1992; Scheer, 1991; Bryant, 1994; Blankenship et al. 1995; Amesz and Hoff, 1996, Baker, 1996; and Ort and Yocum, 1996). Taiz and Zeiger (1991) place the photosynthetic process in the context of over all plant physiology, and Cramer and Knaff (1991) describe the bioenergetic foundation of photosynthesis.

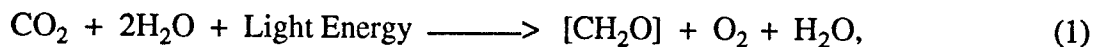
The overall equation for photosynthesis is deceptively simple. In fact, a complex set of physical and chemical reactions must occur in a coordinated manner for the synthesis of carbohydrates. To produce a sugar molecule such as sucrose, plants require nearly *30 distinct proteins* that work within a complicated membrane structure. Research into the mechanism of photosynthesis centers on understanding the structure of the photosynthetic components and the molecular processes that use radiant energy to drive carbohydrate synthesis. The research involves several disciplines, including physics, biophysics, chemistry, structural biology, biochemistry, molecular biology and physiology, and serves as an outstanding example of the success of multidisciplinary research. As such, photosynthesis presents a special challenge in understanding several interrelated molecular processes.

B. BRIEF HISTORY

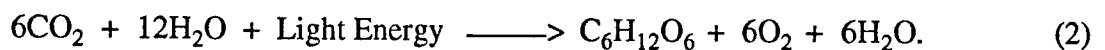
In the 1770s Joseph Priestley, an English chemist and clergyman, performed experiments showing that plants release a type of air that allows combustion. He demonstrated this by burning a candle in a closed vessel until the flame went out. He placed a sprig of mint in the chamber and after several days showed that the candle could burn again. Although Priestley did not know about molecular oxygen, his work showed that plants release oxygen into the atmosphere. It is noteworthy that over 200 years later, investigating the mechanism by which plants produce oxygen is one of the most active areas of photosynthetic research. Building on the work of Priestley, Jan Ingenhousz, a Dutch physician, demonstrated that sunlight was necessary for photosynthesis and that only the green parts of plants could release oxygen. During this period Jean Senebier, a Swiss botanist and naturalist, discovered that CO₂ is required for photosynthetic growth and Nicolas-Théodore de Saussure, a Swiss chemist and plant physiologist, showed that water is required. It

was not until 1845 that Julius Robert von Mayer, a German physician and physicist, proposed that photosynthetic organisms convert light energy into chemical free energy. An interesting time line of the history of photosynthesis has been presented by Huzisige and Ke (1993).

By the middle of the nineteenth century the key features of plant photosynthesis were known, namely, that plants could use light energy to make carbohydrates from CO_2 and water. The empirical equation representing the net reaction of photosynthesis for oxygen evolving organisms is :

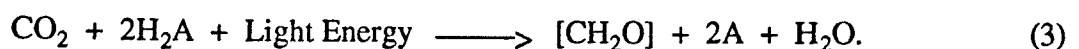


where $[\text{CH}_2\text{O}]$ represents a carbohydrate (e.g., glucose, a six-carbon sugar). The synthesis of carbohydrate from carbon and water requires a large input of light energy. The standard free energy for the reduction of one mole of CO_2 to the level of glucose is +478 kJ/mol. Because glucose, a six carbon sugar, is often an intermediate product of photosynthesis, the net equation of photosynthesis is frequently written as :



The standard free energy for the synthesis of glucose is +2,870 kJ/mol.

Not surprisingly, early scientists studying photosynthesis concluded that the O_2 released by plants came from CO_2 , which was thought to be split by light energy. In the 1930s comparison of bacterial and plant photosynthesis lead Cornelis van Niel to propose the general equation of photosynthesis that applies to plants, algae and photosynthetic bacteria (discussed by Wraight, 1982). Van Niel was aware that some photosynthetic bacteria could use hydrogen sulfide (H_2S) instead of water for photosynthesis and that these organisms released sulfur instead of oxygen. Van Niel, among others, concluded that photosynthesis depends on electron donation and acceptor reactions and that the O_2 released during photosynthesis comes from the oxidation of water. Van Niel's generalized equation is :



In oxygenic photosynthesis, 2A is O₂, whereas in anoxygenic photosynthesis, which occurs in some photosynthetic bacteria, the electron donor can be an inorganic hydrogen donor, such as H₂S (in which case A is elemental sulfur) or an organic hydrogen donor such as succinate (in which case, A is fumarate). Experimental evidence that molecular oxygen came from water was provided by Hill and Scarisbrick (1940) who demonstrated oxygen evolution in the absence of CO₂ in illuminated chloroplasts and by Ruben et al. (1941) who used ¹⁸O enriched water.

The biochemical conversion of CO₂ to carbohydrate is a reduction reaction that involves the rearrangement of covalent bonds between carbon, hydrogen and oxygen. The energy for the reduction of carbon is provided by energy rich molecules that are produced by the light driven electron transfer reactions. Carbon reduction can occur in the dark and involves a series of biochemical reactions that were elucidated by Melvin Calvin, Andrew Benson and James Bassham in the late 1940s and 1950s. Using the radioisotope ¹⁴C, most of the intermediate steps that result in the production of carbohydrate were identified. Calvin was awarded the Nobel Prize for Chemistry in 1961 for this work (see Calvin, 1989).

In 1954 Daniel Arnon and coworkers discovered that plants, and A. Frenkel discovered that photosynthetic bacteria, use light energy to produce ATP, an organic molecule that serves as an energy source for many biochemical reactions (discussed by Frenkel, 1995). During the same period L.N.M. Duysens showed that the primary photochemical reaction of photosynthesis is an oxidation/reduction reaction that occurs in a protein complex (the reaction center). Over the next few years the work of several groups, including those of Robert Emerson, Bessel Kok, L.N.M. Duysens, Robert Hill and Horst Witt, combined to prove that plants, algae and cyanobacteria require two reaction centers, photosystem II and photosystem I, operating in series (Duysens, 1989; Witt, 1991).

In 1961 Peter Mitchell suggested that cells can store energy by creating an electric field or a proton gradient across a membrane. Mitchell's proposal that energy is stored as an electrochemical gradient across a vesicular membrane opened the door for understanding energy transformation by

membrane systems. He was awarded the Nobel Prize in Chemistry in 1978 for his theory of chemiosmotic energy transduction (Mitchell, 1961).

Most of the proteins required for the conversion of light energy and electron transfer reactions of photosynthesis are located in membranes. Despite decades of work, efforts to determine the structure of membrane bound proteins had little success. This changed in the 1980s when Johann Deisenhofer, Hartmut Michel, Robert Huber and co-workers determined the structure of the reaction center of the purple bacterium *Rhodospseudomonas viridis*. (Deisenhofer et al., 1984, 1985; Deisenhofer and Michel, 1993). They were awarded the Nobel Prize for Chemistry in 1988 for their work, which has provided insight into the relationship between structure and function in membrane-bound proteins .

A key element in photosynthetic energy conversion is electron transfer within and between protein complexes and simple organic molecules. The electron transfer reactions are rapid (as fast as a few picoseconds) and highly specific. Much of our current understanding of the physical principles that guide electron transfer is based on the pioneering work of Rudolph A. Marcus (Marcus and Sutin, 1985), who received the Nobel Prize in Chemistry in 1992 for his contributions to the theory of electron transfer reaction in chemical systems.

C. CLASSIFICATION OF PHOTOSYNTHETIC ORGANISMS

All life can be divided into three domains, Archaea, Bacteria and Eucarya, which originated from a common ancestor (Woese *et al.*, 1990). Historically, the term *photosynthesis* has been applied to organisms that depend on chlorophyll (or bacteriochlorophyll) for the conversion of light energy into chemical free energy (Gest , 1993). These include organisms in the domains Bacteria (photosynthetic bacteria) and Eucarya (algae and higher plants). The most primitive domain, Archaea, includes organisms known as halobacteria, that convert light energy into chemical free energy. However, the mechanism by which halobacteria convert light is fundamentally different from that of higher organisms because there is no oxidation/reduction chemistry and halobacteria cannot use CO₂ as their carbon source. Consequently some biologists do not consider halobacteria

as photosynthetic (Gest 1993). This chapter will follow the historical definition of photosynthesis and omit halobacteria.

C.1 Oxygenic Photosynthetic Organisms

The photosynthetic process in all plants and algae as well as in certain types of photosynthetic bacteria involves the reduction of CO_2 to carbohydrate and removal of electrons from H_2O , which results in the release of O_2 . In this process, known as oxygenic photosynthesis, water is oxidized by the photosystem II reaction center, a multisubunit protein located in the photosynthetic membrane. Years of research have shown that the structure and function of photosystem II is similar in plants, algae and certain bacteria, so that knowledge gained in one species can be applied to others. This homology is a common feature of proteins that perform the same reaction in different species. This homology at the molecular level is important because there are estimated to be 300,000-500,000 species of plants. If different species had evolved diverse mechanisms for oxidizing water, research aimed at a general understanding of photosynthetic water oxidation would be hopeless.

C.2 Anoxygenic Photosynthetic Organisms

Some photosynthetic bacteria can use light energy to extract electrons from molecules other than water. These organisms are of ancient origin, presumed to have evolved before oxygenic photosynthetic organisms. Anoxygenic photosynthetic organisms occur in the domain Bacteria and have representatives in four phyla - Purple Bacteria, Green Sulfur Bacteria, Green Gliding Bacteria, and Gram Positive Bacteria.

D. PRINCIPLES OF PHOTOSYNTHETIC ENERGY TRANSFORMATION

The energy that drives photosynthesis originates in the center of the sun, where mass is converted to heat by the fusion of hydrogen. Over time, the heat energy reaches the sun's surface, where some of it is converted to light by black body radiation that reaches the earth. A small fraction of the visible light incident on the earth is absorbed by plants. [It takes about 8 minutes for light to reach from the Sun to the Earth; please check.] Through a series of energy transducing reactions, photosynthetic organisms are able to transform light energy into

chemical free energy in a stable form that can last for hundreds of millions of years (*e.g.*, fossil fuels). A simplified scheme describing how energy is transformed in the photosynthetic process is presented in this section. The focus is on the structural and functional features essential for the energy transforming reactions. For clarity, mechanistic and structural details are omitted. A more highly resolved description of oxygenic and anoxygenic photosynthesis will be given later.

The photosynthetic process in plants and algae occurs in small organelles known as chloroplasts that are located inside cells. The more primitive photosynthetic organisms, for example oxygenic cyanobacteria, prochlorophytes and anoxygenic photosynthetic bacteria, lack organelles. The photosynthetic reactions are traditionally divided into two stages - the "light reactions," which consist of electron and proton transfer reactions and the "dark reactions," which consist of the biosynthesis of carbohydrates from CO_2 . The light reactions occur in a complex membrane system (the photosynthetic membrane) that is made up of protein complexes, electron carriers, and lipid molecules. The photosynthetic membrane is surrounded by water and can be thought of as a two-dimensional surface that defines a closed space, with an inner and outer water phase. A molecule or ion must pass through the photosynthetic membrane to go from the inner space to the outer space. The protein complexes embedded in the photosynthetic membrane have a unique orientation with respect to the inner and outer phase. The asymmetrical arrangement of the protein complexes allows some of the energy released during electron transport to create an electrochemical gradient of protons across the photosynthetic membrane.

Photosynthetic electron transport consists of a series of individual electron transfer steps from one electron carrier to another. The electron carriers are metal ion complexes and aromatic groups. The metal ion complexes and most of the aromatic groups are bound within proteins. Most of the proteins involved in photosynthetic electron transport are composed of numerous polypeptide chains that lace through the membrane, providing a scaffolding for metal ions and aromatic groups. An electron enters a protein complex at a specific site, is transferred within the protein from one carrier to another, and exits the protein at a different site. The protein controls the pathway of electrons between the carriers by determining the location and environment of the metal ion complexes and aromatic groups. By setting the distance between electron carriers and

controlling the electronic environment surrounding a metal ion complex or aromatic group, the protein controls pairwise electron transfer reactions. Between proteins, electron transfer is controlled by distance and free energy, as for intraprotein transfer, and by the probability that the two proteins are in close contact. Protein association is controlled by a number of factors, including the structure of the two proteins, their surface electrical and chemical properties and the probability that they collide with one another. Not all electron carriers are bound to proteins. The reduced forms of plastoquinone or ubiquinone and nicotinamide adenine dinucleotide phosphate (NADPH) or NADH act as mobile electron carriers operating between protein complexes. For electron transfer to occur, these small molecules must bind to special pockets in the proteins known as binding sites. The binding sites are highly specific and are a critical factor in controlling the rate and pathway of electron transfer.

The light reactions convert energy into several forms (*see diagram after the text*). The first step is the conversion of a photon to an excited electronic state of an antenna pigment molecule located in the antenna system. The antenna system consists of hundreds of pigment molecules (mainly chlorophyll or bacteriochlorophyll and carotenoids) that are anchored to proteins within the photosynthetic membrane and serve a specialized protein complex known as a reaction center. The electronic excited state is transferred over the antenna molecules as an *exciton*. [*An exciton is nothing else than an excited electron with its hole.*] Some excitons are converted back into photons and emitted as fluorescence, some are converted to heat, and some are trapped by a reaction center protein. (For a discussion of the use of fluorescence as a probe of photosynthesis, see e.g., Govindjee et al., 1986 and Krause and Weis, 1991, Govindjee, 1995.) Excitons trapped by a reaction center provide the energy for the primary photochemical reaction of photosynthesis - the transfer of an electron from a donor molecule to an acceptor molecule. Both the donor and acceptor molecules are attached to the reaction center protein complex. Once primary charge separation occurs, the subsequent electron transfer reactions are energetically downhill.

In oxygenic photosynthetic organisms, two different reaction centers, known as photosystem II and photosystem I, work concurrently but in series. In the light photosystem II feeds electrons to photosystem I. The electrons are transferred from photosystem II to the

photosystem I by intermediate carriers. The net reaction is the transfer of electrons from a water molecule to NADP^+ , producing the reduced form, NADPH. In the photosynthetic process, much of the energy initially provided by light energy is stored as redox free energy (a form of chemical free energy) in NADPH, to be used later in the reduction of carbon. In addition, the electron transfer reactions concentrate protons inside the membrane vesicle and create an electric field across the photosynthetic membrane. In this process the electron transfer reactions convert redox free energy into an electrochemical potential of protons. The energy stored in the proton electrochemical potential is used by a membrane bound protein complex (ATP-Synthase) to covalently attach a phosphate group to adenosine diphosphate (ADP), forming adenosine triphosphate (ATP). Protons pass through the ATP-Synthase protein complex that transforms electrochemical free energy into a type of chemical free energy known as phosphate group-transfer potential (or a high-energy phosphate bond) (Klotz, 1967). The energy stored in ATP can be transferred to another molecule by transferring the phosphate group. The net effect of the light reactions is to convert radiant energy into redox free energy in the form of NADPH and phosphate group-transfer energy in the form of ATP. In the light reactions, the transfer of a single electron from water to NADP^+ involves about 30 metal ions and 7 aromatic groups. The metal ions include 19 Fe, 5 Mg, 4 Mn, and 1 Cu. The aromatics include quinones, pheophytin, NADPH, tyrosine and a flavoprotein. The NADPH and ATP formed by the light reactions provide the energy for the dark reactions of photosynthesis, known as the Calvin cycle or the photosynthetic carbon reduction cycle. The reduction of atmospheric CO_2 to carbohydrate occurs in the aqueous phase of the chloroplast and involves a series of enzymatic reactions. The first step is catalyzed by the protein Rubisco (D-ribulose 1,5-bisphosphate carboxylase/oxygenase), which attaches CO_2 to a five-carbon compound. The reaction produces two molecules of a three-carbon compound. Subsequent biochemical reactions involve several enzymes that reduce carbon by hydrogen transfer and rearrange the carbon compounds to synthesize carbohydrates. The carbon reduction cycle involves the transfer and rearrangement of chemical bond energy.

In anoxygenic photosynthetic organisms, water is not used as the electron donor. Electron flow is cyclic and is driven by a single photosystem, producing a proton electrochemical gradient

that is used to provide energy for the reduction of NAD^+ by an external H-atom or e-donor (e.g., H_2S or an organic acid) in a process known as "reverse electron flow". Fixation of CO_2 occurs via different pathways in different organisms.

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Questions

[1] How do photons interact with a pigment molecule?

What is Franck-Condon principle?

What is the molecular basis of the Stokes' shift?

[2] What is the relationship of energy levels of a molecule with its absorption spectrum?

Why is chlorophyll green?

[3] What is the definition of photosynthesis ?

What is the basic equation of photosynthesis?

[4] Where does photosynthesis occur?

What are the major components needed for photosynthesis?

How would you describe the basic steps involved in the conversion of light energy into chemical energy by oxygenic and by anoxygenic photosynthesizers?

[5] What are the different photosynthetic systems in Nature?

Why do all photosynthetic systems need light?

JE Walker
P. Boyer

Noble-laureates related to photosynthesis research : a personal list

- [1] **Rudolph Marcus** (1992, Chemistry): Electron transfer theory: included application to photosynthesis. [He was at UIUC, Urbana; had attended my course in "Bioenergetics in Photosynthesis"; is currently at Cal Tech in Pasadena, CA.]
- [2] **Hartmut Michel; Robert Huber; and Johannes Deisenhofer** (1988, Chemistry): X-ray structure of bacterial reaction center. [Michel has visited Urbana several times and I have had many conversations with him before and after his prize; I have casually met Deisenhofer, but never Huber. The work was done at Munich, Germany.]
- [3] **Peter Mitchell** (1978, Chemistry ..to check): Oxidative and photosynthetic phosphorylation: chemi-osmotic theory.[The work was done in England, UK]
- [4] **Robert Burns Woodward** (1965, Chemistry): Total synthesis of chlorophyll, vitamin B12, and other natural products.[He was at Harvard University]
- [5] **Melvin Calvin** (1961, Chemistry): Carbon-di oxide assimilation in photosynthesis.[The work was done at Berkeley; Professor Calvin is known to me as he has visited UIUC, and, he is one of the two professors I had applied to do PhD with; the person who discovered ^{14}C (Martin Kamen), that was crucial for Calvin's experiments, is known to me personally.]
- [6] **Richard Kuhn** (1938, Chemistry): carotenoids; vitamins [Germany]
- [7] **Paul Karrer** (1937, Chemistry): Carotenoid structure; flavins; vitamin B2[Germany]
- [8] **Hans Fischer** (1930, Chemistry): Chlorophyll chemistry; hemin synthesis[Germany]
- [9] **Richard Martin Wilstatter** (1915, Chemistry): Chlorophyll purification and structure, carotenoids, etc.[Germany]

Those who worked in Photosynthesis, but only after their Prize in another field:

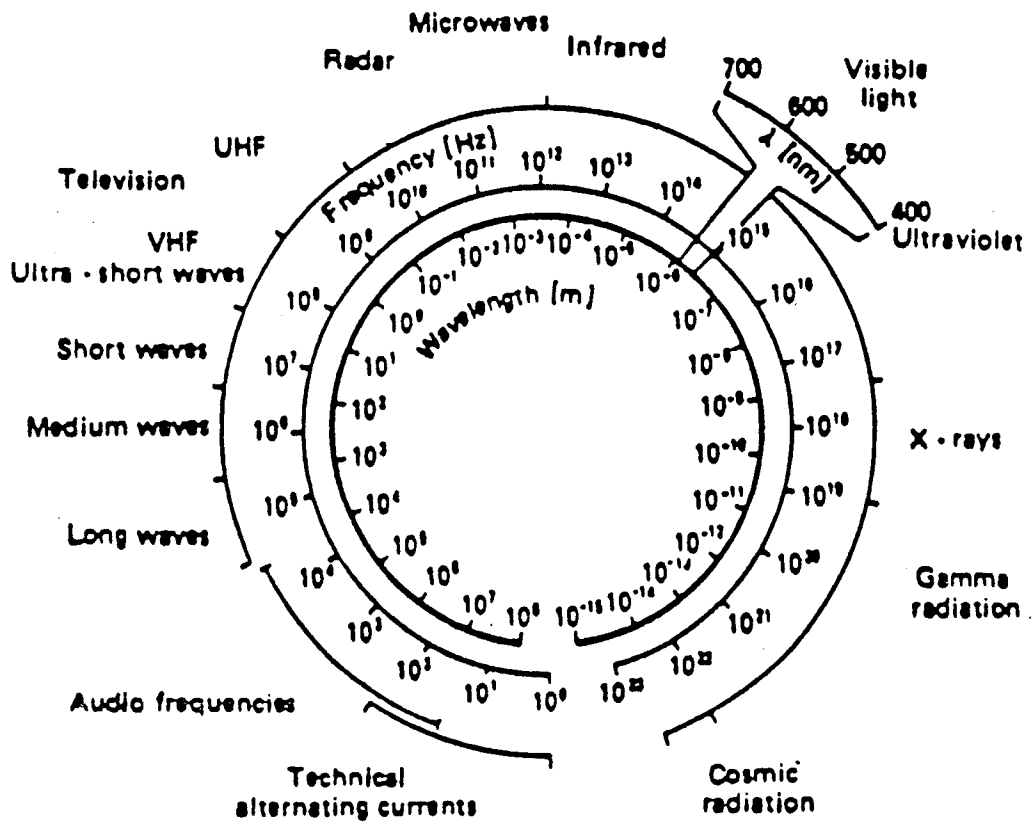
- [1] **James Franck** (with Hertz) (1925, Physics): Electron-atom collisions; later developed the principle known as the Franck-Condon principle; he later worked in photosynthesis; my PhD advisor Eugene Rabinowitch did his post-doctorate research with Franck on an effect known as the "cage effect" in photochemistry. Franck was brilliant, but most of his ideas on photosynthesis turned out to be wrong; he headed the Chemistry division of the Manhattan project on the "Atom bomb" at Chicago; later, he worked hard to get the US from not dropping the bomb in Japan.[Work done in Germany] I met Professor Franck only once in 1963.
- [2] **Otto Warburg** (1931, Physiology and Medicine): The respiratory Enzyme. He worked on the quantum yield of photosynthesis; discovered the role of chloride and bicarbonate in the "light reactions" of photosynthesis, among many phenomena in the "dark reactions" of photosynthesis. His PhD student Robert Emerson was my first PhD advisor from Sept. 1956- Feb. 1959. Warburg was proven wrong in the quantum yield controversy with Emerson. He visited UIUC

before I went there.[Work done in Germany] My current major research deals with the "bicarbonate effect", discovered by Warburg.

- [3] Sir (now Lord) **George Porter**, O.M.(with Norrish and Eigen) (1967, Chemistry): Flash photolysis, among other systems, he studied aromatic molecules and chlorophyll, energy transfer in photosynthesis and primary photochemistry of photosynthesis in femtosecond-picosecond time scale. [Work done in England, UK] One of my major research areas deals with femtosecond-picosecond spectroscopy in photosystem II reaction centers. I have met Professor Porter several times and had pleasant conversations; he has visited India.]

... the sun is not only the author of visibility in all visible things, but of generation and nourishment and growth ...

Plato, *The Republic*



1. Spectrum of the electromagnetic waves. Inner scale: wavelengths in meters; central scale: frequencies in Hertz; outer scale: name and usage of waves. The enlarged inset shows the range of visible light.

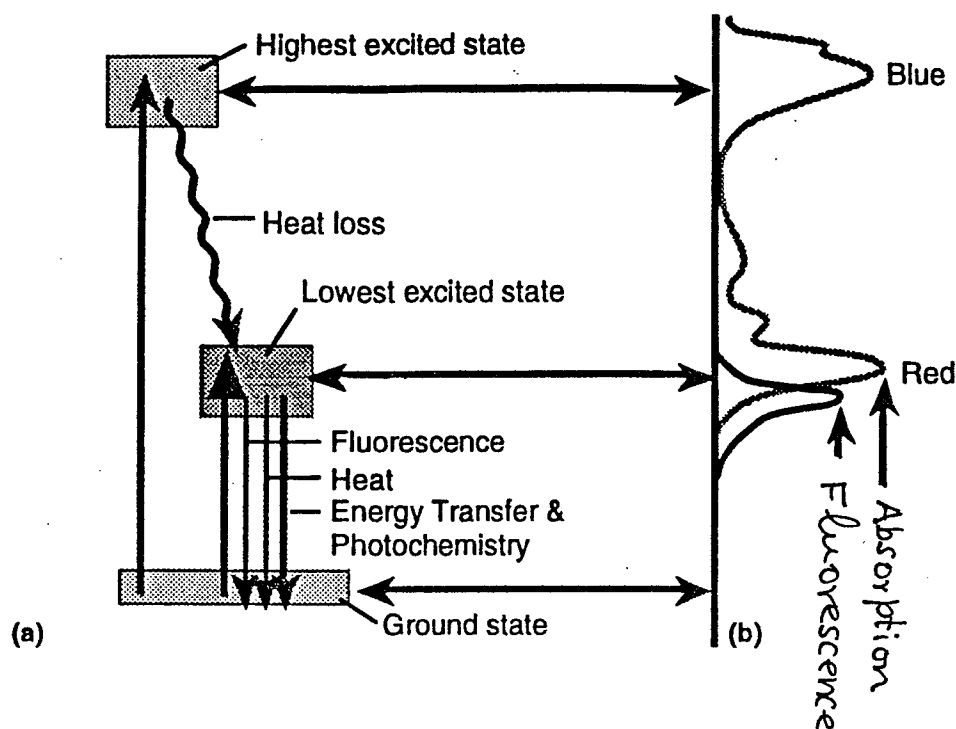


Figure 2

2. Absorption and fluorescence of chlorophylls. The illustration is for Chlorophyll *a*, but it applies qualitatively to Chl *b* as well. (a) Energy level diagram, showing spectral transitions (vertical arrows). The energy levels are broadened (shading) by vibrational sublevels. (b) Absorption spectrum corresponding to energy levels of part (a). This spectrum is turned 90° from the usual orientation in order to show the relationship to the energy levels. Note the red shift of the fluorescence compared with the corresponding absorption. This Stokes shift owes to vibrational relaxation in the excited electronic state prior to fluorescence emission and in the ground electronic state after emission.

Absorption spectroscopy

Govindjee

Cuvette containing sample

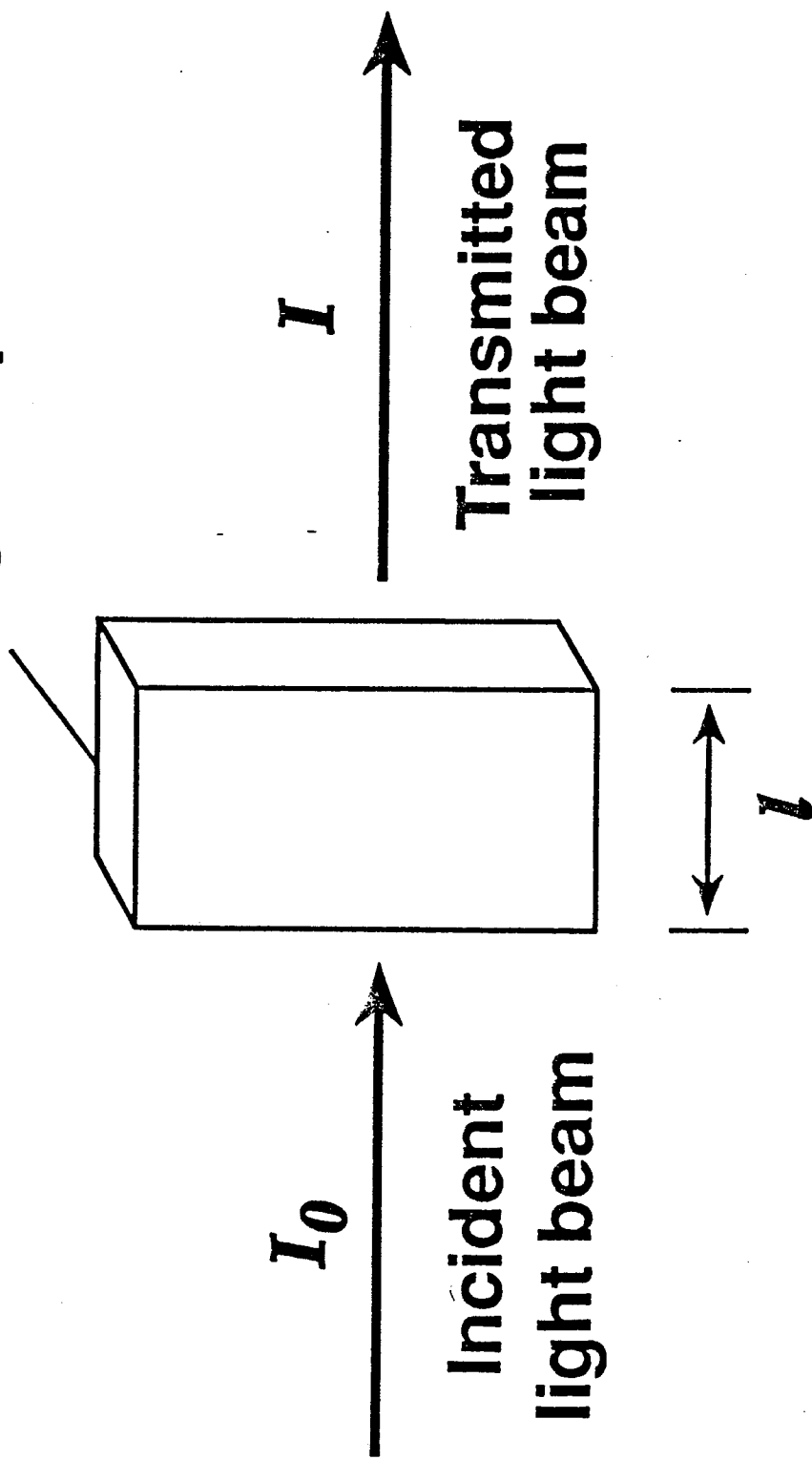


Figure 3

Absorption spectroscopy

Govindjee

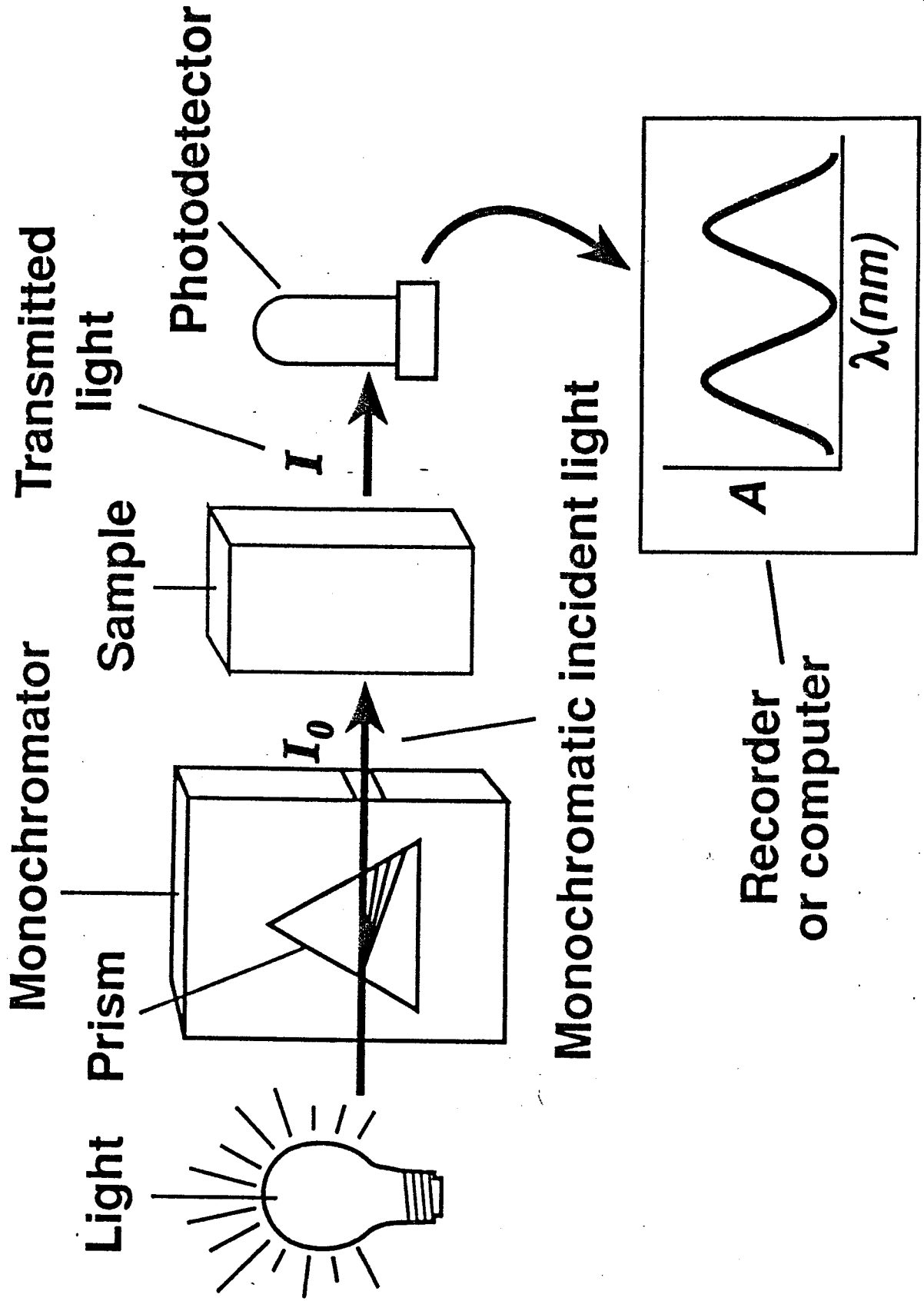
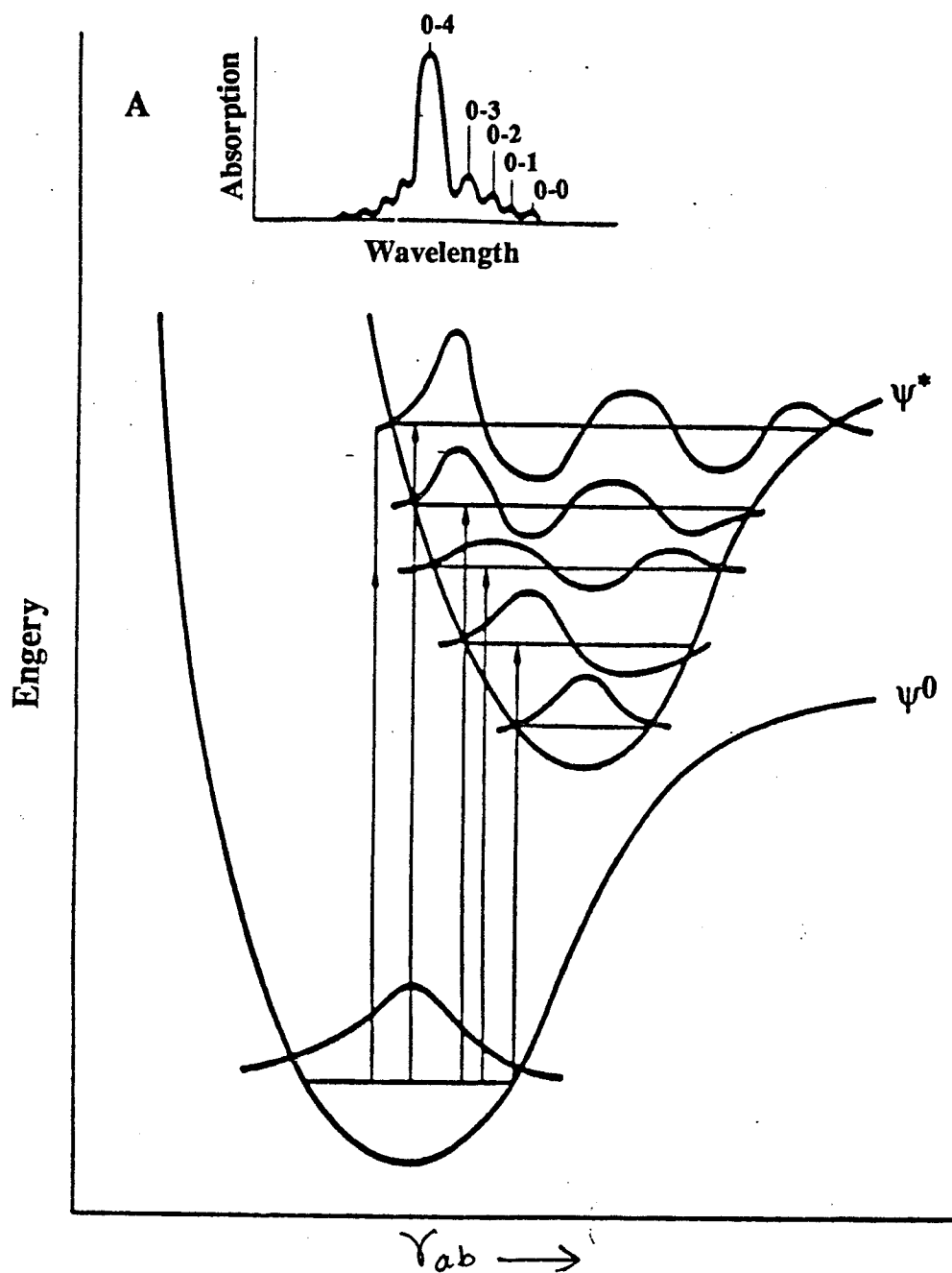


Figure 4

Franck-Condon Principle



5. Quantum mechanical interpretation of the Franck-Condon principle: potential diagram for two electronic states. The molecules can jump from the various energetic levels of the ground state ψ^0 to the various energetic levels of the excited state ψ^* . The transition probability depends on the time spent on each energetic level. Therefore some transitions are relatively improbable (weak absorption) while others occur frequently (strong absorption, see the schematic absorption (curve A) spectrum) (from Turro, 1981, modified). r_{ab} = distance between nuclei. (James Franck received the Nobel Prize in Physics in 1925 for electron-atom collision.)

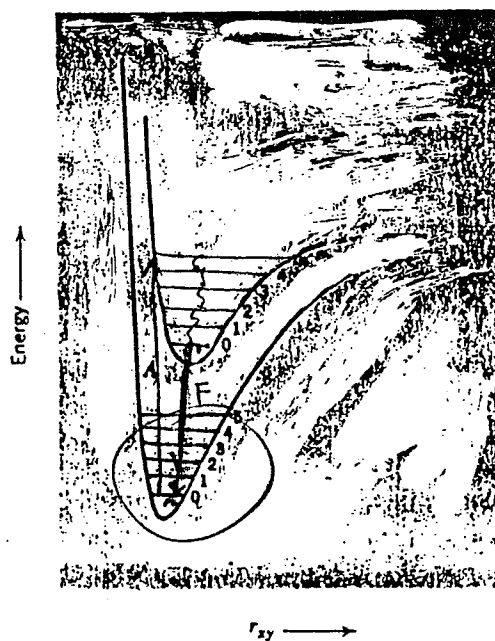
From Rabinowitch and Govindjee (1969): pp. 8-10.

When a photon is absorbed, the molecule usually is not merely transferred into an excited electronic state, but also acquires some vibrational energy. According to the so-called Franck-Condon principle, the absorption of a photon is a practically instantaneous process, since it involves only the rearrangement of practically inertia-free electrons. The much heavier atomic nuclei have no time to readjust themselves during the absorption act, but have to do it after it is over, and this readjustment brings them into vibrations. This is best illustrated by potential energy diagrams, such as that in Figures 5 and 6. It is an expanded energy level diagram, with the abscissa acquiring the meaning of distance between the nuclei, γ for example, distance $x-y$ in a diatomic molecule xy . The two potential curves show the potential energy of the molecule as function of this distance for two electronic states, a ground state and an excited state. Excitation is represented according to the Franck-Condon principle, by a *vertical* arrow (A). This arrow hits the upper curve, except for very special cases, not in its lowest point, corresponding to a nonvibrating state, but somewhere higher. This means that the molecule finds itself, after the absorption act, in a nonequilibrium state and begins to vibrate like a spring. This vibration is described, in Figure 5, by the molecule running down, up, down again, *etc.*, along with upper potential curve, like a pendulum. The periods of these vibrations are of the order of 10^{-13} or 10^{-14} seconds. Since the lifetime of excited electronic states are of the order of 10^{-9} sec (or longer), there is enough time during the excitation period for many thousands of vibrations. During this time much if not all of the extra vibrational energy . . .

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Govindjee

... can be lost by energy exchange (temperature equalization) with the medium. The molecule, while it remains extremely "hot" as far as its electronic state is concerned, thus acquires the ambient "vibrational temperature." Fluorescence, when it comes, originates from near the bottom of the upper potential curve, and follows a vertical arrow down (F), until it strikes the lower potential curve. Again, it does not hit it in its deepest point, so that some excitation energy becomes converted into vibrational energy. The cycle absorption-emission thus contains two periods of energy dissipation. Because of this, the fluorescence arrow (F) is always shorter (that is, the fluorescence frequency is lower) than that of absorption (A). In other words, the wavelengths of the fluorescence band are longer than those of the absorption band. This displacement of fluorescence bands towards the longer waves compared to the absorption bands ("Stokes' shift") was a long-established experimental ...



Arrow A is larger than arrow F. Thus, F is shifted to longer λ 's.

Figure 6. Potential energy curves for the ground state and an excited state of a diatomic molecule. (r , interatomic distance; A , absorption; F , fluorescence; numbers indicate vibrational states.)

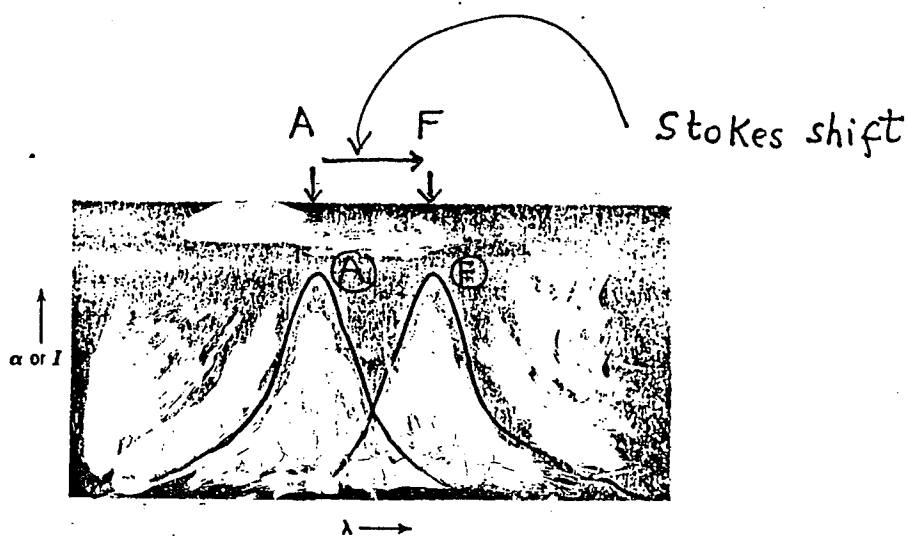
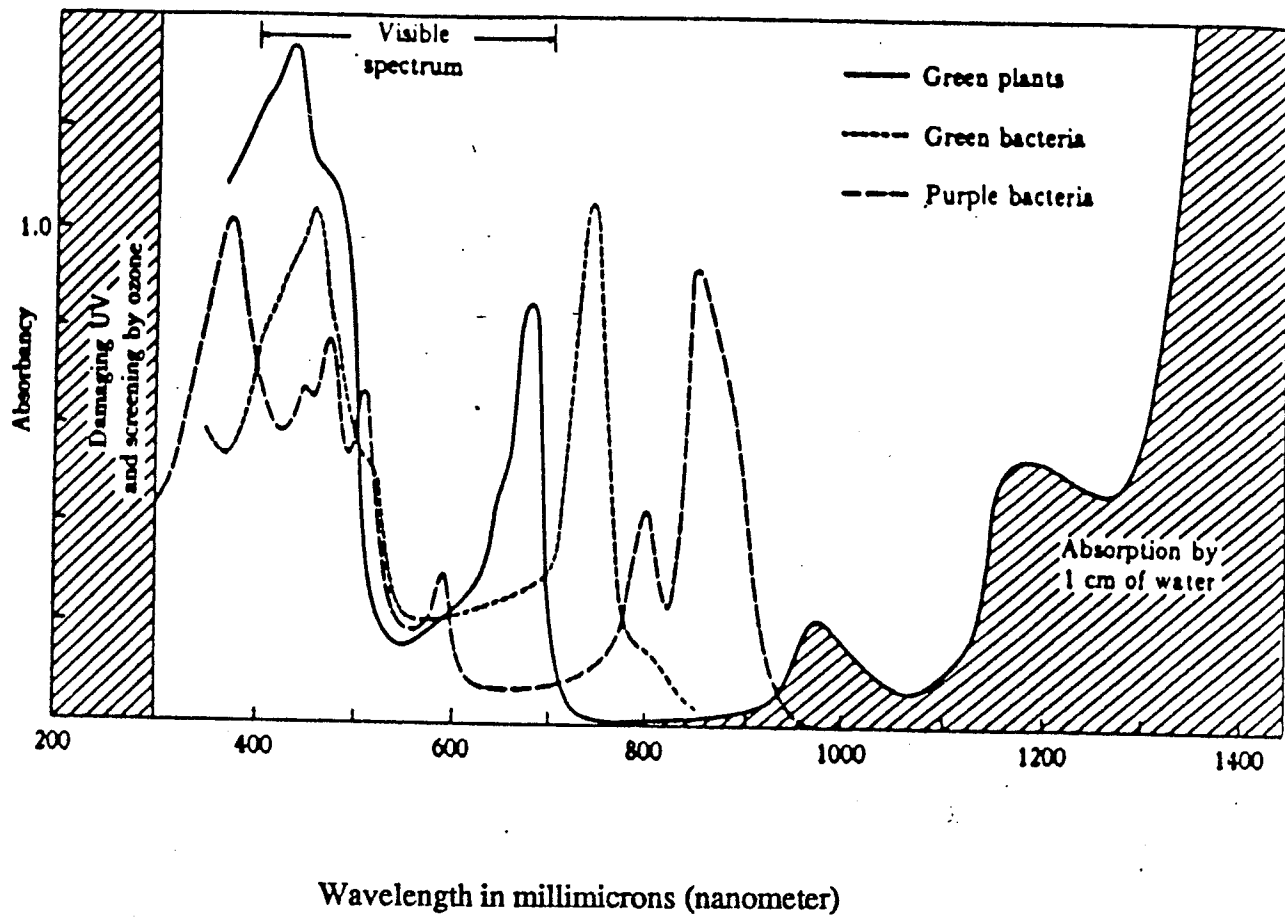


Figure 7. The Stokes' shift (displacement of fluorescence band compared to the absorption band of a molecule). Approximate mirror symmetry of the two bands exists when the shapes of the potential curves in the ground state and the excited state are similar.

fact (Figure 7), before the Franck-Condon principle provided its interpretation. Obviously, the extent of the shift depends on the difference between the two potential curves. The same difference determines the width of the absorption band. Chlorophyll *a*, the major pigment of all green plants, has (in organic solvents) a very narrow absorption band (half-band width, 18 nm) and a very small Stokes' shift (of the order of 7-10 nm).

ABSORPTION OF LIGHT

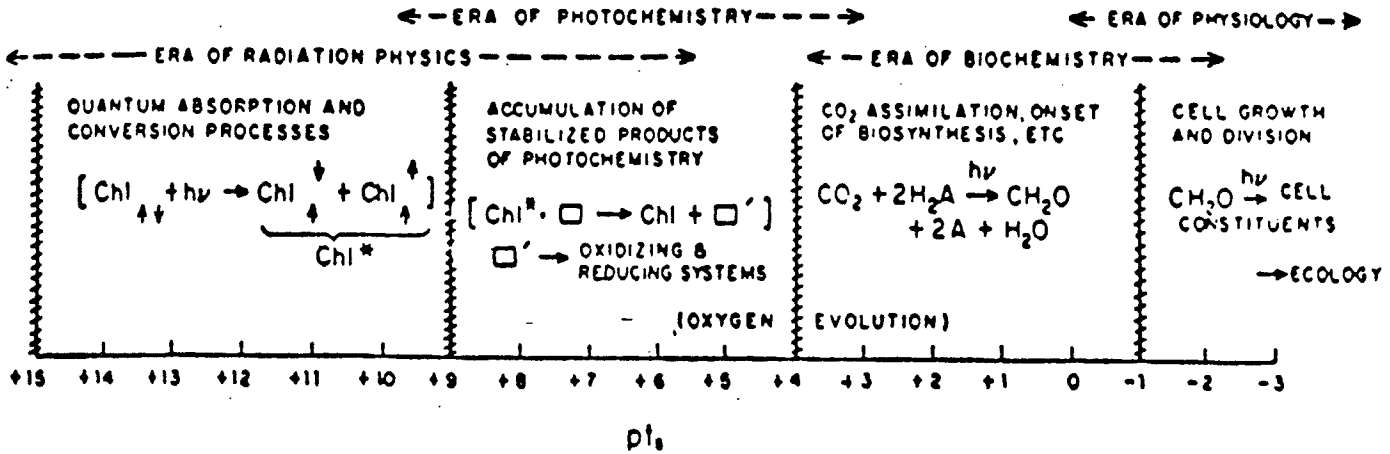


8. Absorption spectra of three kinds of photosynthetic organisms and of 1 cm of water, showing the regions of the spectrum that can be used for photosynthesis. (from a book by Clayton)

Kamen (1963):

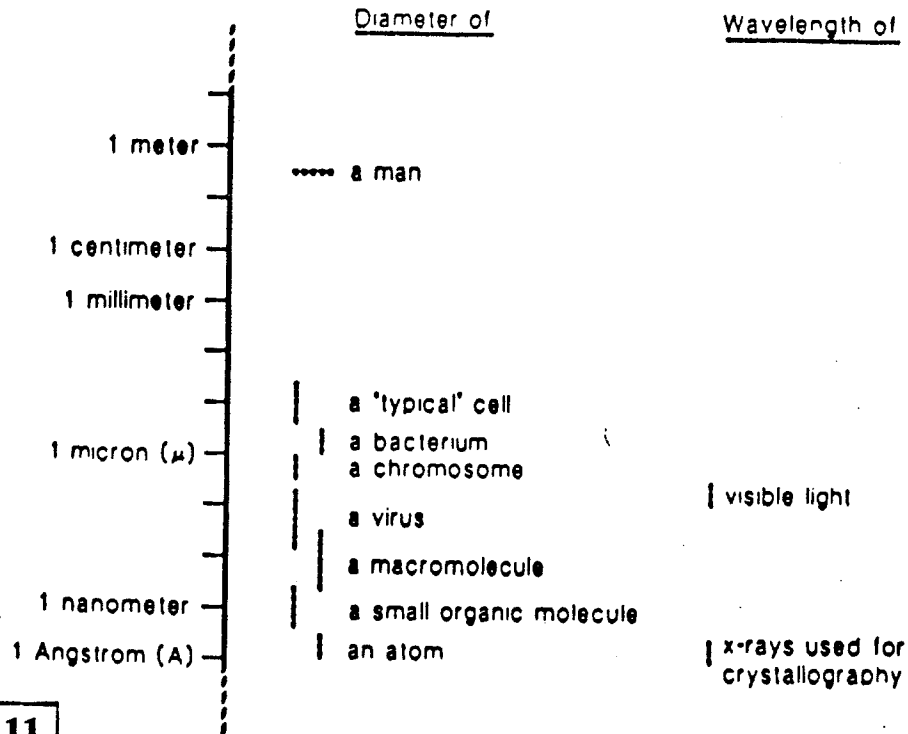


SPECTRUM OF IGNORANCE



10. The eras of photosynthesis

ON SIZES OF THINGS



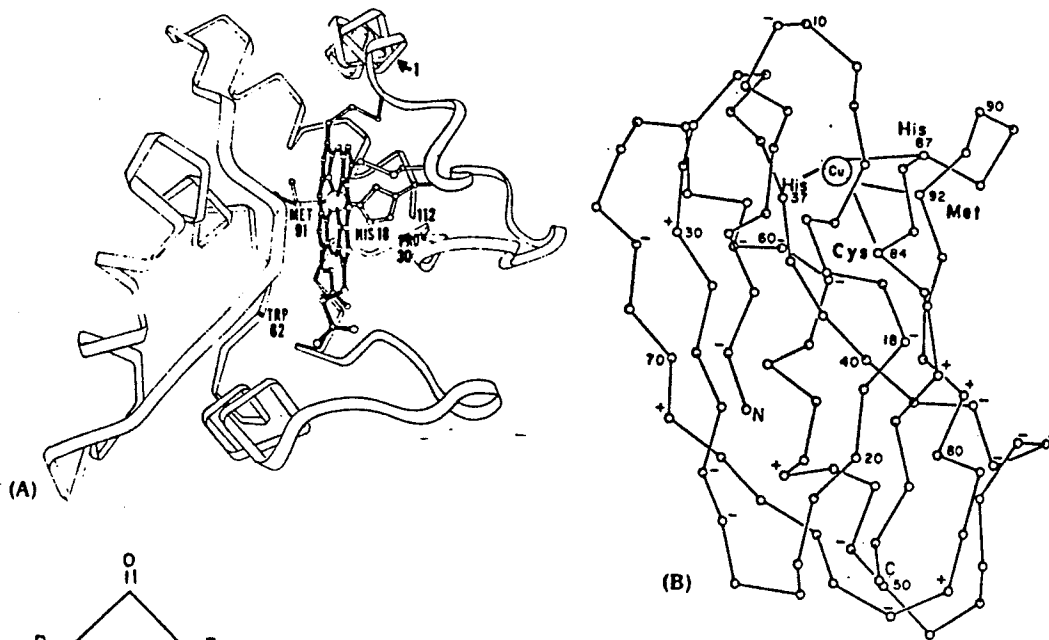
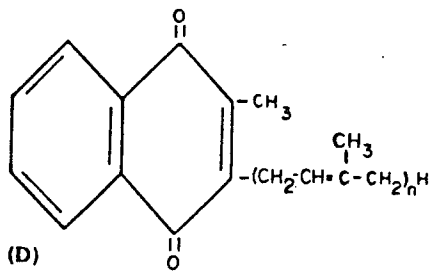


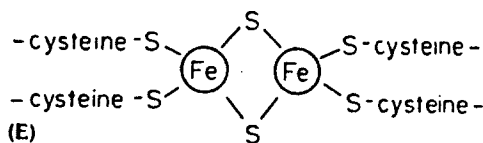
Figure 13

Structures of prosthetic groups of redox compounds involved in plant and bacterial photosyntheses. (A) Structure of Cyt C₂ from *Rp. rubrum*, viewed from the "front" of the protein showing the exposed heme edge. This figure is obtained from the work of F. R. Salemme, whose discussion of these data is found in Salemme (1977). (B) Structure of spinach plastocyanin. This figure was redrawn by D. W. Krogmann, using the original figure of Freeman and co-workers (Colman et al., 1978) and other sequence data. (C) Structure of ubiquinone and plastoquinone with substituent groups and isoprenoid chain length.

	R ₁	R ₂	R ₃	n
UQ	CH ₃	CH ₃ O	CH ₃ O	10
PQ	H	CH ₃	CH ₃	9



(D) Structure of menaquinone found in some photosynthetic bacteria. $n = 7$ in the purple nonsulfur bacterium *Chr. vinosum*, where the isoprenoid chain length is also seven units. (E) Structure of iron-sulfur linkage characteristic of 2Fe - 2S* cluster found in soluble low potential plant ferredoxins and perhaps the high potential Rieske iron-sulfur protein as well.



(From Cramer and Crofts in Govindjee, ed. Photosynthesis, 1982)

**Light Absorption and Energy Transfer: Pathways of
Excitation and Deexcitation—Fluorescence, Energy Transfer and
Chemistry**

Outline

(1.) Fates of Excited State; de-excitation is by:

fluorescence	(f)	(compare with phosphorescence)
heat and other losses	(h)	
energy transfer	(tr)	
chemistry	(chem)	

Relationship between the quantum yield (Φ) of one of the fates (fluorescence) to the rate constants (k_s : transitions per second) of the deexcitation of all the other processes is:

$$\Phi_f = \frac{k_f}{k_f + k_h + k_{tr} + k_{chem}}$$

Note the inverse relationship between fluorescence and chemistry.

$$\Phi_{chem} = \frac{k_{chem}}{k_f + k_h + k_{tr} + k_{chem}}$$

(2.) Fluorescence

- Démonstration (quinine water): excitation by ultra-violet light leads to emission of blue light
- is loss of energy, but it is an intrinsic, non-destructive and sensitive probe of chemistry
- Lifetime of fluorecence (time for one transition) is related to the quantum yield of fluorescence as:

$$\tau_f = \Phi_f(1/k_f) = \Phi_f \tau_0,$$

where τ_0 = intrinsic or natural lifetime of fluorecence when the **only** deexcitation process is fluorescence, it can be calculated from absorption spectra!

Heat loss - usually not too much is known.

(3.) Energy Transfer

Concept of Photosynthetic Unit (class as an example): an assembly of several hundred chlorophyll molecules serve to transfer energy to a reaction center molecule.

Concept of excitation energy transfer is shown by:

- *sensitized fluorescence* (excitation of a donor molecule and emission from an acceptor molecule);
- quenching of donor fluorescence; and
- high quantum yield of photochemistry when pigments other than chlorophyll *a* are excited

Other evidences for energy transfer are:

- steady state evidence from fluorescence emission and excitation spectra
- picosecond time “cascade” effect; and
- depolarization of fluorescence.

Mechanism of excitation energy transfer: *Förster resonance theory*

- \propto distance (R) to the power “-6” $\rightarrow (1/R^6)$;
- \propto orientation factor (κ^2); and
- \propto overlap between absorption of acceptor and emission spectra of donor molecule.

Note: Energy transfer **does not** take place by the trivial process of absorption of fluorescence.

(4.) *Primary Photochemistry: uphill charge separation using light energy.*

- (a) is very efficient : $\phi_{\text{chem}} = 0.95$ to 0.98 i.e., 95% to 98%;
- (b) is very rapid : few ps (ps = picosecond);
- (c) is the first chemical reaction to take place;
- (d) occurs at very low temperatures with high efficiency: at 4 degrees Kelvin : few ps; and
- (e) a “doublet” is produced (an “unpaired” electron, probed by Electron Resonance Spectroscopy, ESR)

(5.) A *light-induced difference absorption spectrum* is the difference between a spectrum measured in the presence of a bright light minus that in the absence of bright light, but both measured with weak light of different wavelengths. It is a plot of change in absorption as a function of wavelength of light. Such spectra are critical in identifying the reactions at the reaction centers to be discussed in the next lectures. They can measure the charge-separated state (see #4, above) if performed as a function of time after a brief (subpicosecond or femtosecond flash).

Sources

A. Basic Stuff

Govindjee and R. Govindjee. (1974). The Absorption of Light in Photosynthesis (reprinted as "The Primary Events of Photosynthesis"). *Scientific American*: 231(6)68-82. -

E.I. Rabinowitch and Govindjee. (1965). The Role of Chlorophyll in Photosynthesis. *Scientific American*: 213(1)74-83.

P.S. Nobel (1991) *Physico-chemical and Environmental Plant Physiology*. Academic Press, see Chapter 4 (pp. 191-243); chapter 5 (pp. 245-296).

A chapter by R. Blankenship (Chapter 8; pp. 179-216) in a book by Taiz/Zeiger "Plant Physiology".

Govindjee (1995) Sixty-three years since Kautsky: Chlorophyll a fluorescence. *Austr J Plant Physiol* 22: 131-160.

B. Chapters in Books

K. Sauer (1975) Primary Events and Trapping of Energy. In: Govindjee (ed.) *Bioenergetics of Photosynthesis*, Academic Press, N.Y., pp. 115-181.

L.L. Shipman (1982) Electronic Structure and Function of Chlorophylls and their Pheophytins. In: Govindjee (ed.) *Photosynthesis, Volume I. Energy Conversion by Plants and Bacteria*, Academic Press, N.Y., pp. 275-291.

✓ R. van Grondelle and J. Amesz (1986) Excitation Energy Transfer in Photosynthetic Systems. In: Govindjee (ed.) *Light Emission by Plants and Bacteria*, Academic Press, Academic Press, Orlando, pp. 191-223.

See Chapter 6 in Cramer and Knaff "Photosynthesis: Photons to Protons", pp. 239-298 (excellent write-up).

Notes on Absorption

- Remember $\Delta\varepsilon$ (Delta epsilon) = $\varepsilon_1 - \varepsilon_0 = h\nu = hc/\lambda$, where epsilon 1 and epsilon 0 are energies of the excited and ground states, h = Planck's constant; greek new = frequency of light, c = velocity of light, and greek lambda = wavelength of light.
- The excited Chl a molecule has more energy (by Delta epsilon), has a different geometry and different pK, etc. It has a higher reducing capacity, i.e., ability to donate electrons than the unexcited Chl a.
- The intensity of absorption depends upon a summation of changes in charge density at various atoms and is different for the different states (first, second, third, etc) and in the x and y directions. It can be calculated by quantum mechanical methods (see Shipman, 1982, for example). In the Chl a, Q_y (first excited state, y direction) is more intense than Q_x (first excited state, x direction); the Soret (or the B band, summation of third, fourth, fifth, etc. excited states) band is also quite intense. On the other hand, the red absorption bands for Chl a⁺ are extremely weak, but additional weak near infra-red bands (around 820 nm) are formed. This allows us to measure the disappearance of Chl a * bands and appearance of Chl a⁺ bands during charge separation in photosynthesis. However, even this is not perfect as both the entities absorb there although to different degrees.
- In photobiological work, one deals often with quantum efficiency of a reaction in terms of number of quanta absorbed, and, the latter is calculated from the number of incident photons times percent of light absorbed, not optical density or absorbance; at very low optical densities, the two are linearly related.

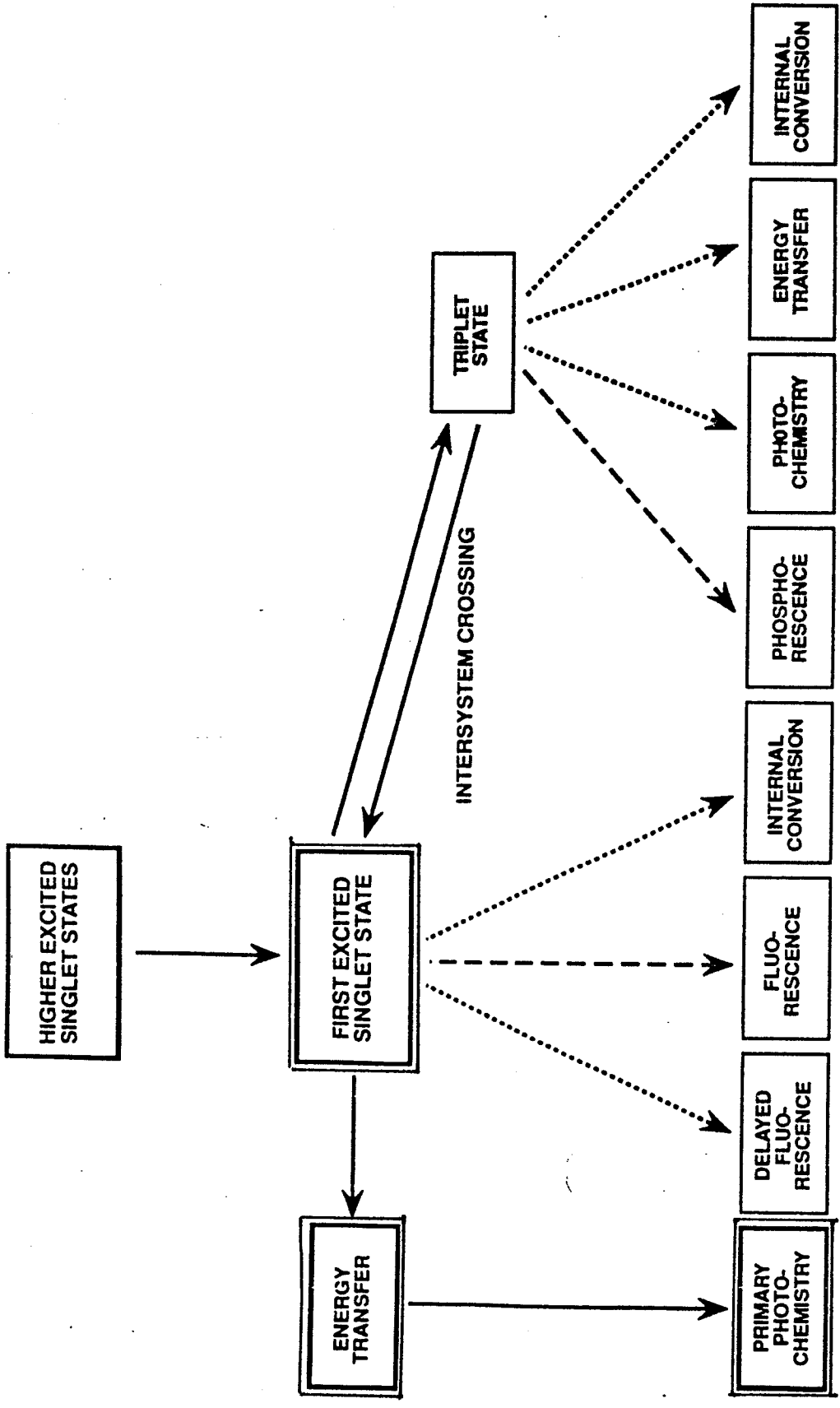
Questions

- [1] What are the de-excitation pathways of a molecule and how are they related to each other.
- [2] What is the quantitative relationship of fluorescence to photochemistry? Can you derive this relationship given: (1) at maximum quantum yield of fluorescence, photochemical yield approaches zero, and, at minimal quantum yield of fluorescence, photochemical yield is maximal.
- [3] What factors govern excitation energy transfer among pigment molecules?
- [4] What is Förster's mechanism of excitation energy transfer?
- [5] How can you visualize excitation energy transfer among a series of pigment molecules with varied absorption and fluorescence spectra?
- [6] How would you explain to your younger brother or sister why excitation energy transfer does not take place by absorption of fluorescence and re-emission of fluorescence followed by reabsorption, etc, and, so on.

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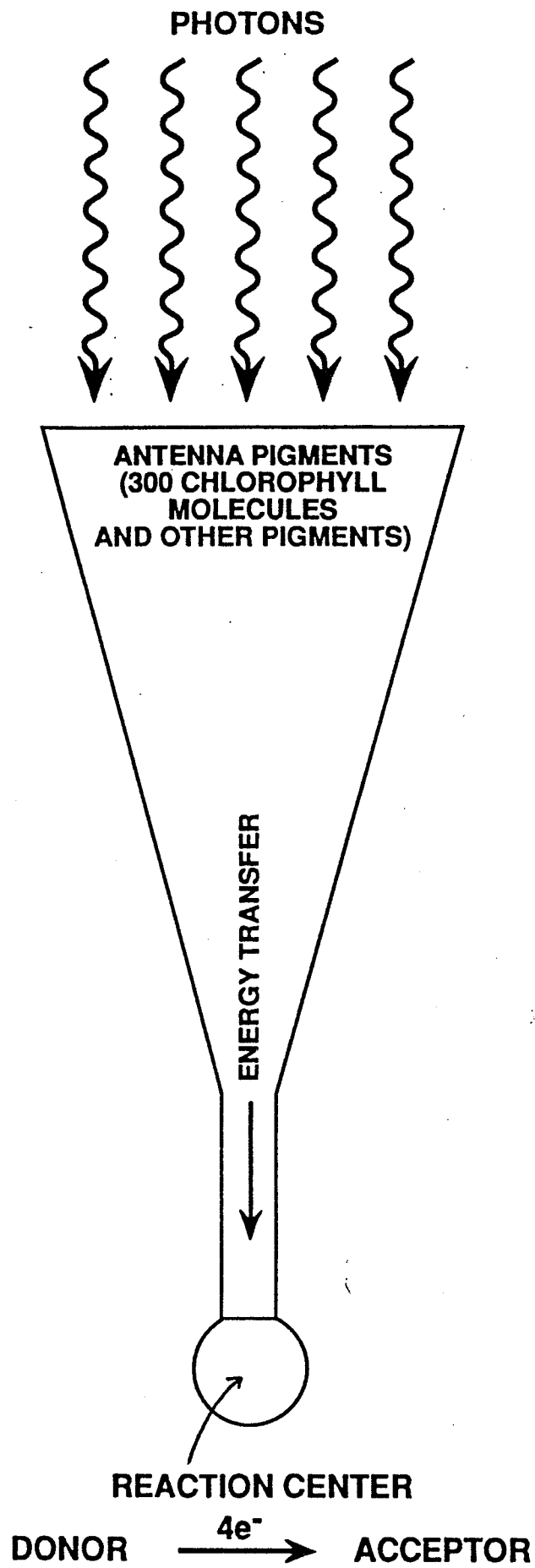
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Figure 1



Fates of the excited states.

Figure 2



Photosynthetic Unit Concept

Figure 3

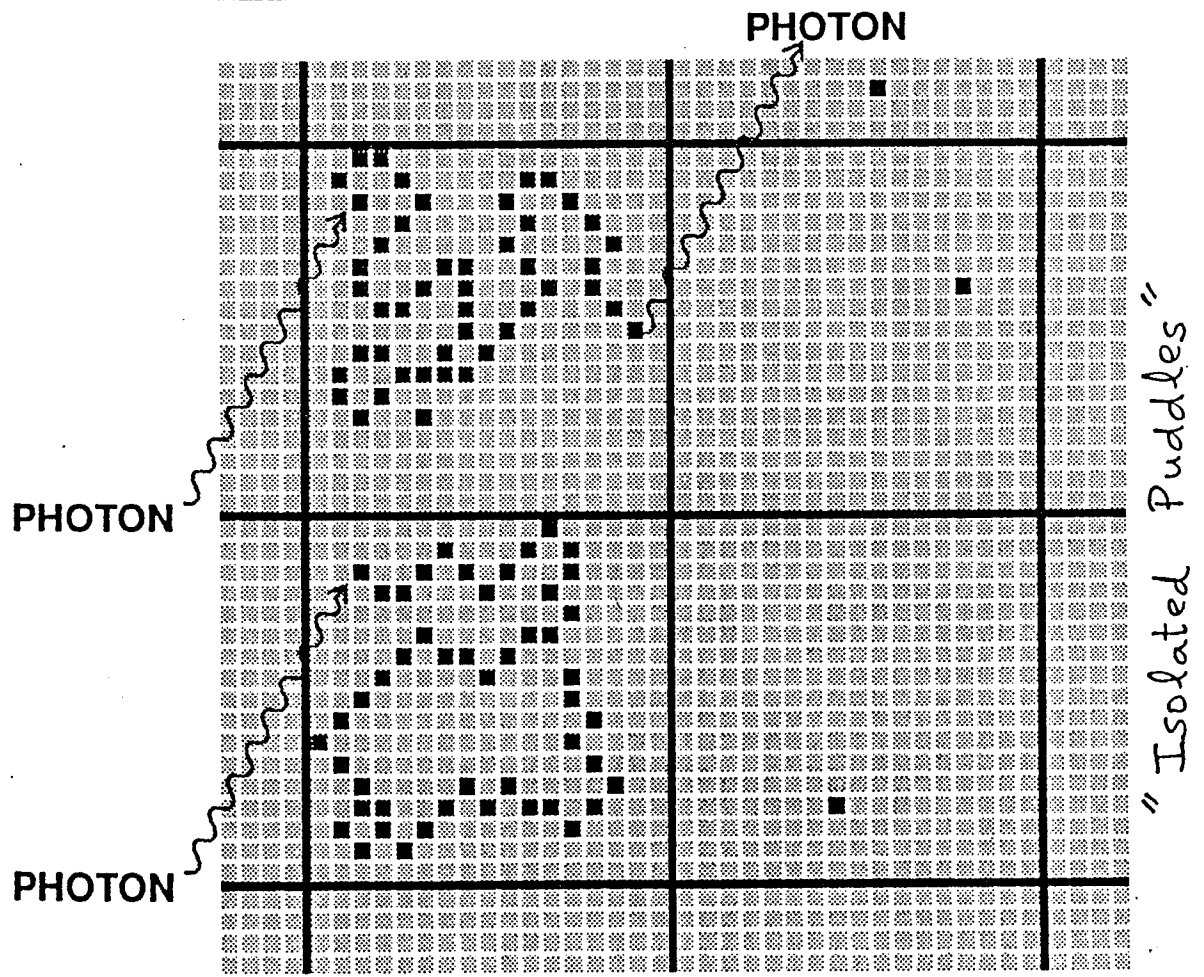
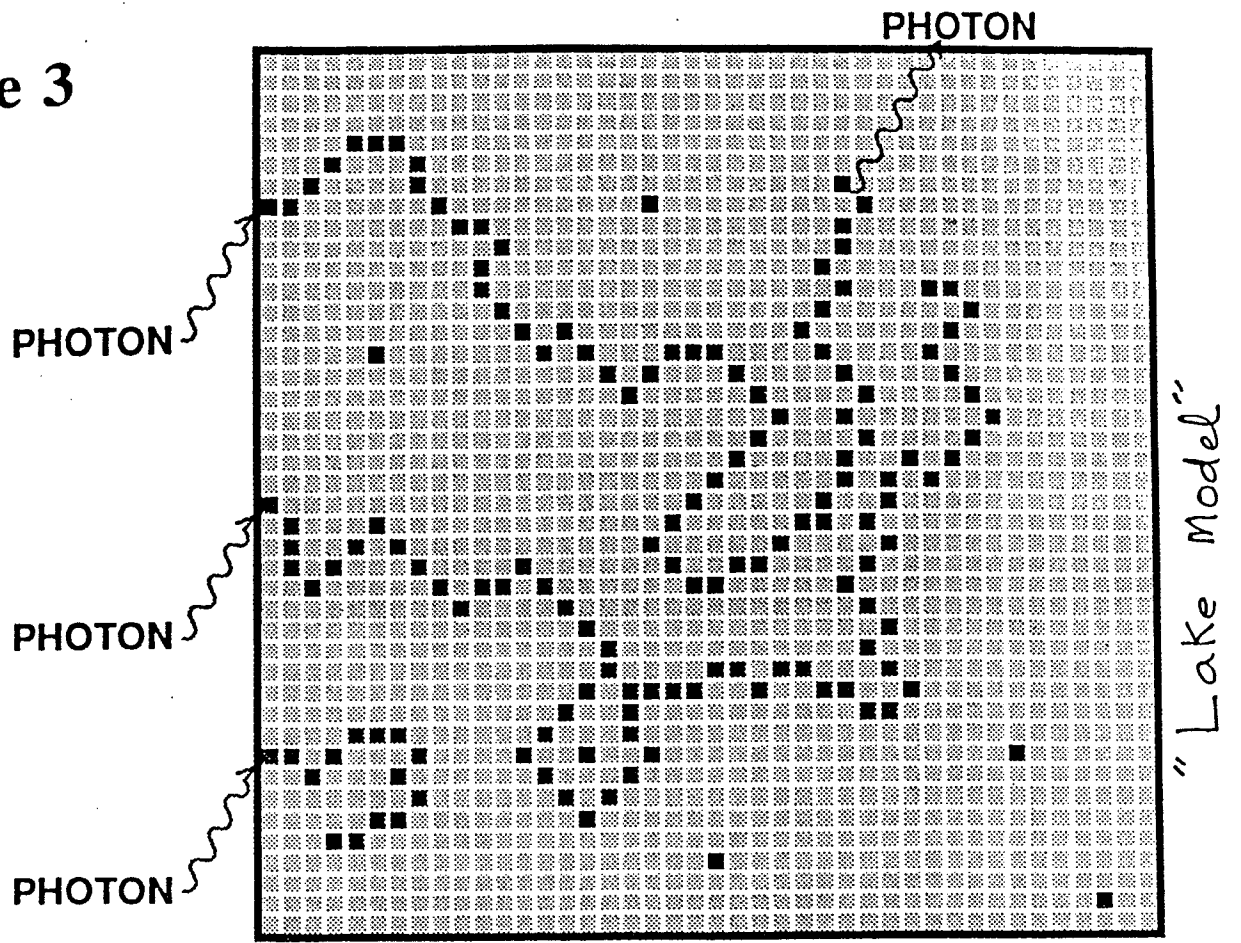
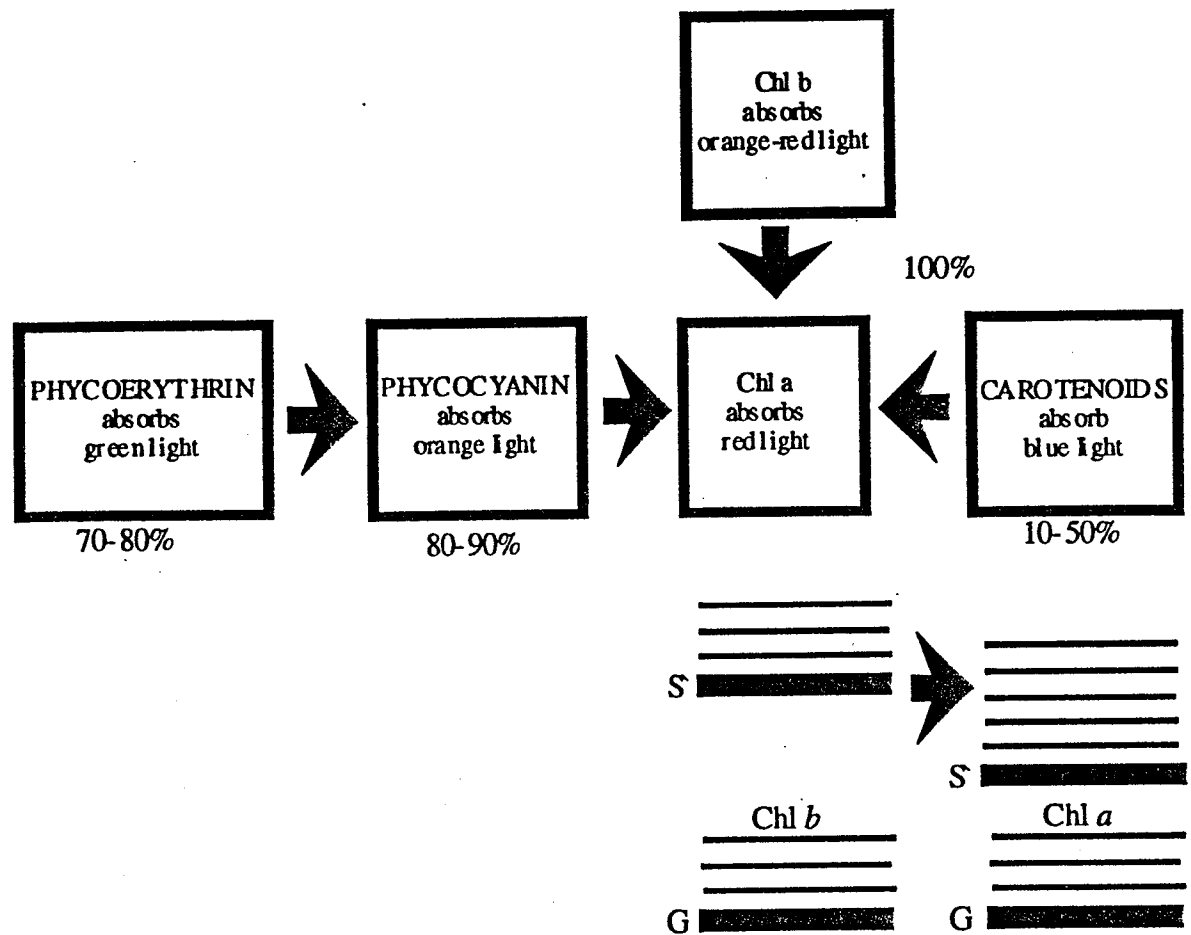


Figure 4



TRANSFER OF ENERGY from one kind of pigment to another follows an established sequence. In most photosynthetic organisms, the carotenoids, which absorb blue light, pass their energy of excitation on to chlorophyll *a* (see right side of diagram). In red algae, green light, absorbed by phycoerythrin, is transferred to phycocyanin. Phycocyanin absorbs orange light, and passes the accumulated energy to chlorophyll *a* (Chl *a*). G: ground state: S* excited singlet state. The numbers, in percent, refer to the efficiency of energy transfer from various pigments to Chl *a*. In each of these transitions energy is lost by internal conversion (IC, heat), and in some of the transfers energy is also dissipated as fluorescence (F).

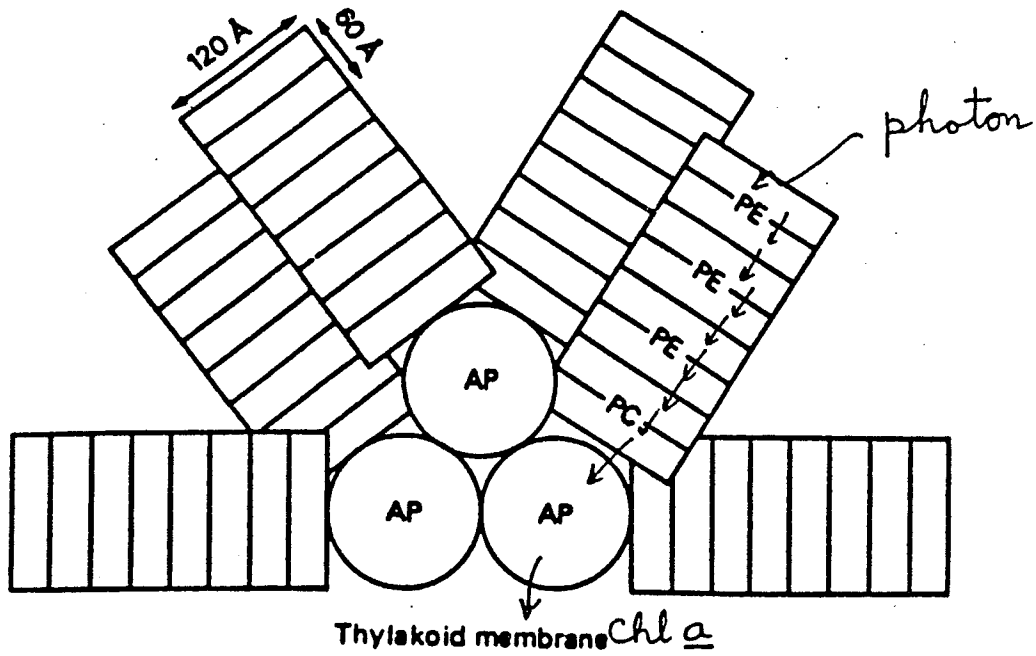
In higher plants and green algae, chlorophyll *b* (Chl *b*) contributes its energy directly to chlorophyll (Chl) *a* without loss. The efficiency of energy transfer is then 100%.

In *cyanobacteria*, there is no Chl *b* and in most no phycoerythrin. In higher plants and green algae, phycoerythrin and phycocyanin do not exist. Red algae contain phycoerythrin and phycocyanin, but no Chl *b*.

All oxygenic photosynthesizers contain Chl *a* and carotenoids.

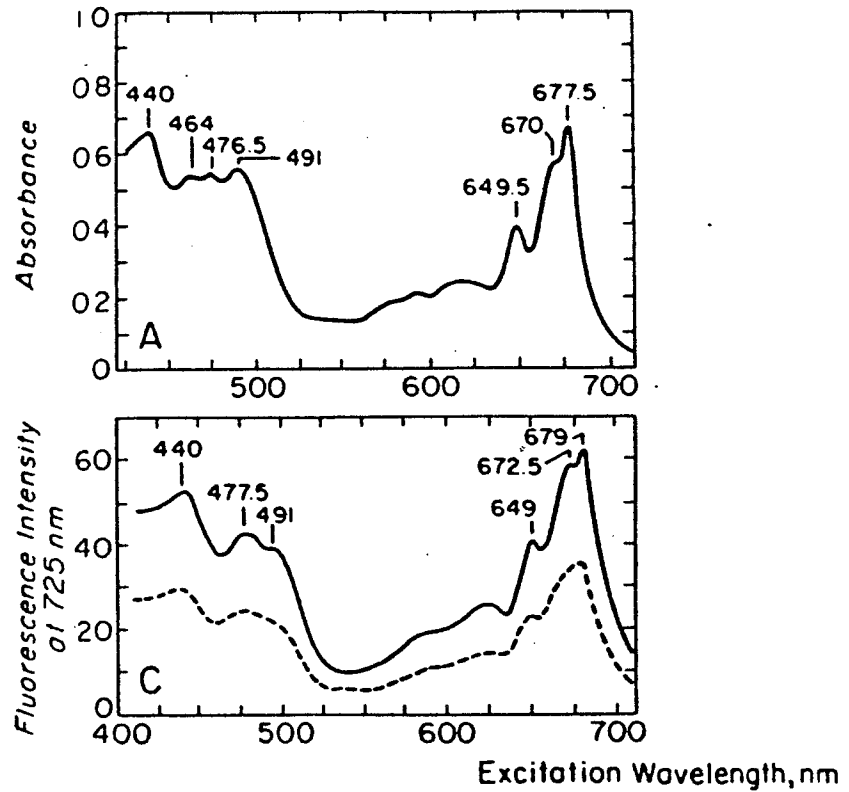
(Redrawn from Govindjee and Govindjee, 1975)

Figure 5

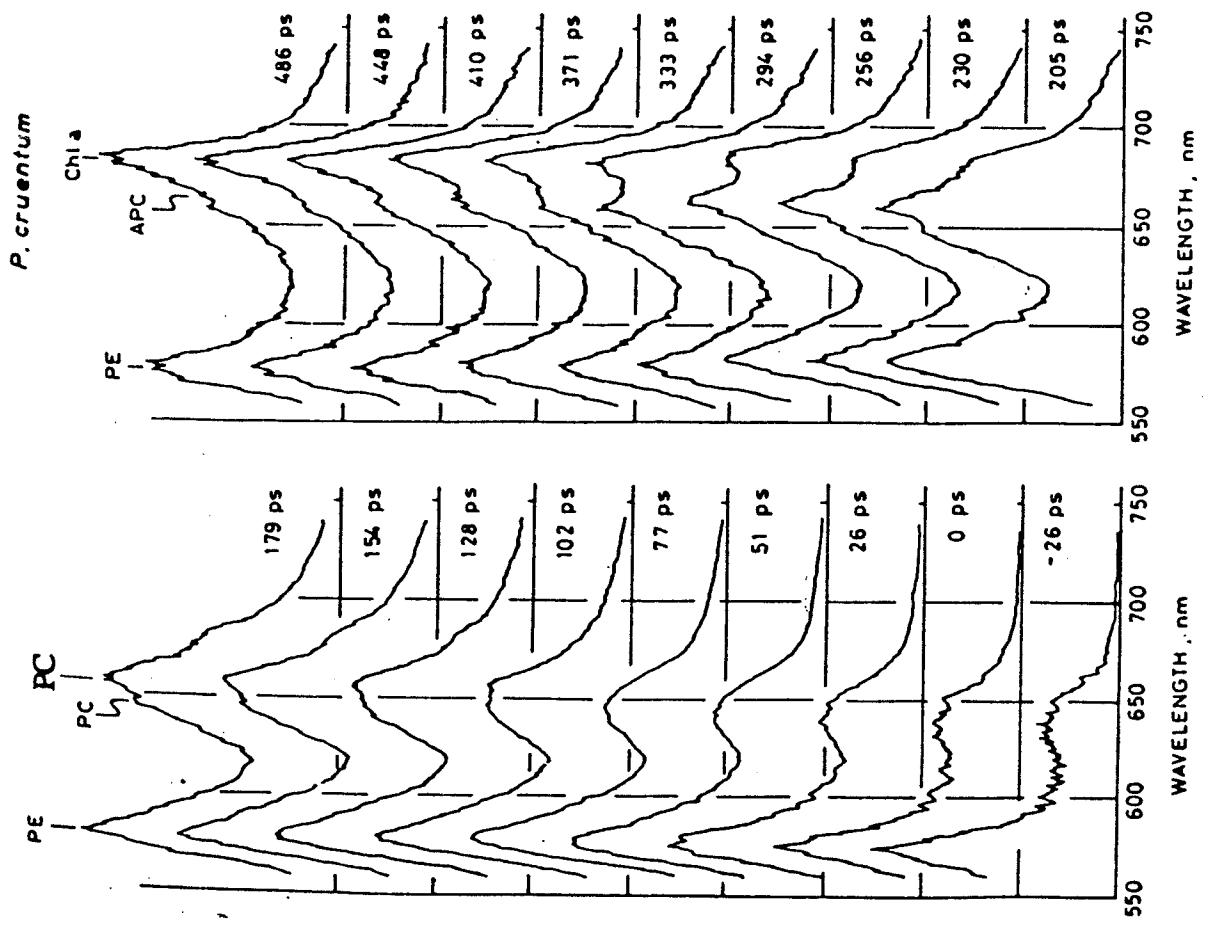


Schematic presentation of the cross-section through a phycobilisome of cyanobacterium LPP-7409, grown in green light. PE = phycoerythrin, PC = phycocyanin, AP = allophycocyanin (from Stumpf).

Figure 6

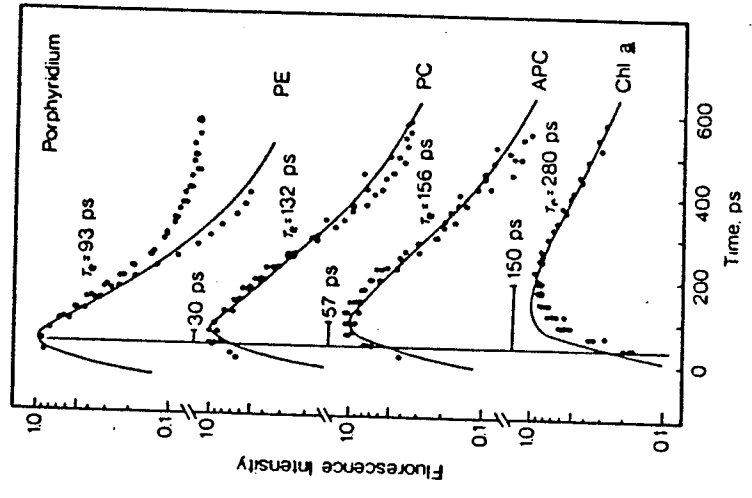


Absorption spectrum (top) and action spectrum of chlorophyll *a* fluorescence measured at 725 nm (bottom) at 4°K from *Chlorella pyrenoidosa*. (Redrawn from Cho and Govindjee, 1970). The bottom curve demonstrates that energy absorbed by Chlorophyll *b* (peaks at 491 and 649 nm) are transferred to Chlorophyll *a*. This will be explained in the class.



Fluorescence spectra of *Porphyridium cruentum* taken at different delay times after the exciting light pulse. B-phycoerythrin was selectively excited at 540 nm (taken from Yamazaki et al., 1984).

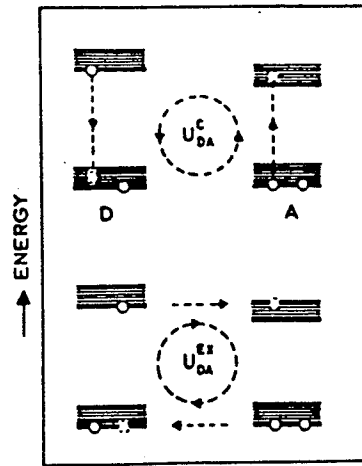
Figure 7



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).

Porphyridium cruentum is a red alga. These curves will be explained in the class. The ordinates on all of the curves are fluorescence intensities.

Figure 8



Schematic representation of the Coulomb (upper) and exchange (lower) contributions to excitation energy transfer.

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):

$$k_{DA} = k_f^D \left(\frac{R_0}{R_{DA}} \right)^6 \quad (1)$$

R⁻⁶

in which k_f^D is the rate of fluorescence of the donor D in the absence of A ($k_f^D \approx 6 \times 10^7 \text{ s}^{-1}$ 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):

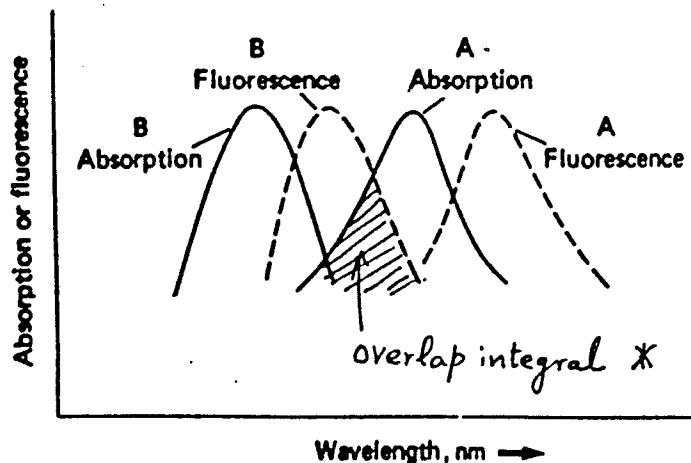
$$R_0^6 = 8.8 \times 10^{12} \kappa^2 n^{-4} \left(\int F_D(\nu) \epsilon_A(\nu) \nu^{-4} d\nu \right) \quad (2)$$

overlap integral

In Eq. (13) $\epsilon_A(\nu)$ 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. (3).

$$\kappa = \cos \alpha - 3 \cos \beta_1 \cos \beta_2 \quad (3) \quad (\text{See Fig. 9})$$

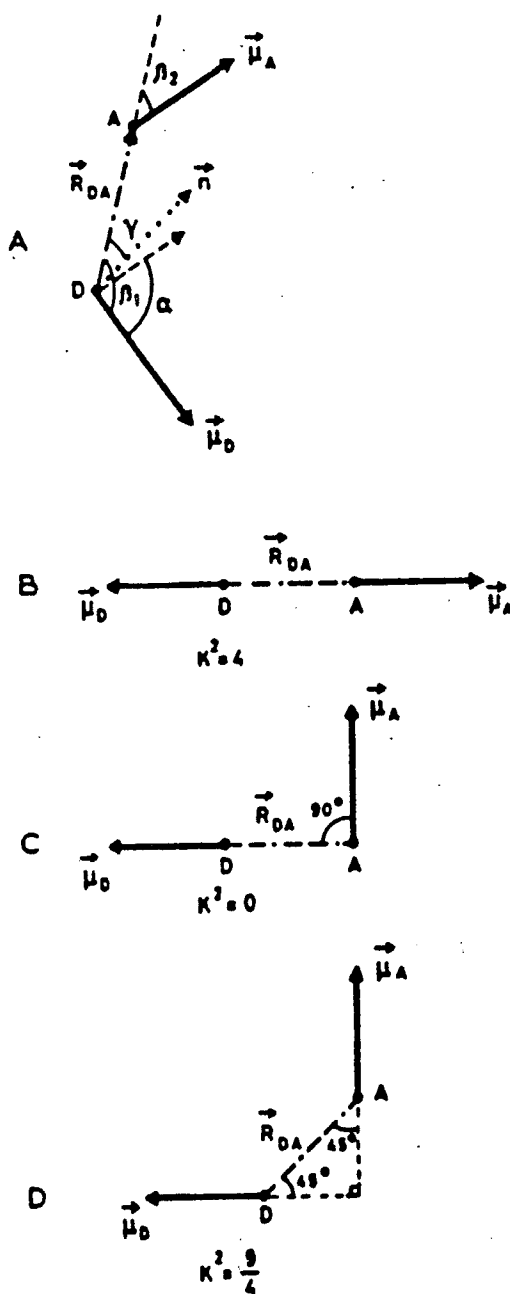
From Van Grondelle and Ames: in Govindjee (ed.) (1986.)



Diagrammatic representation of pigments having absorption and fluorescence bands close together as is the case, e.g., with Chl b and Chl a in green algae and higher plants. Since the fluorescence of B overlaps the absorption of A, there is a good probability that a photon absorbed by B will be transferred to A. This can be demonstrated by exciting B and observing only the fluorescence of A.

From Rabinowitch and Govindjee (1969.)

Figure 9

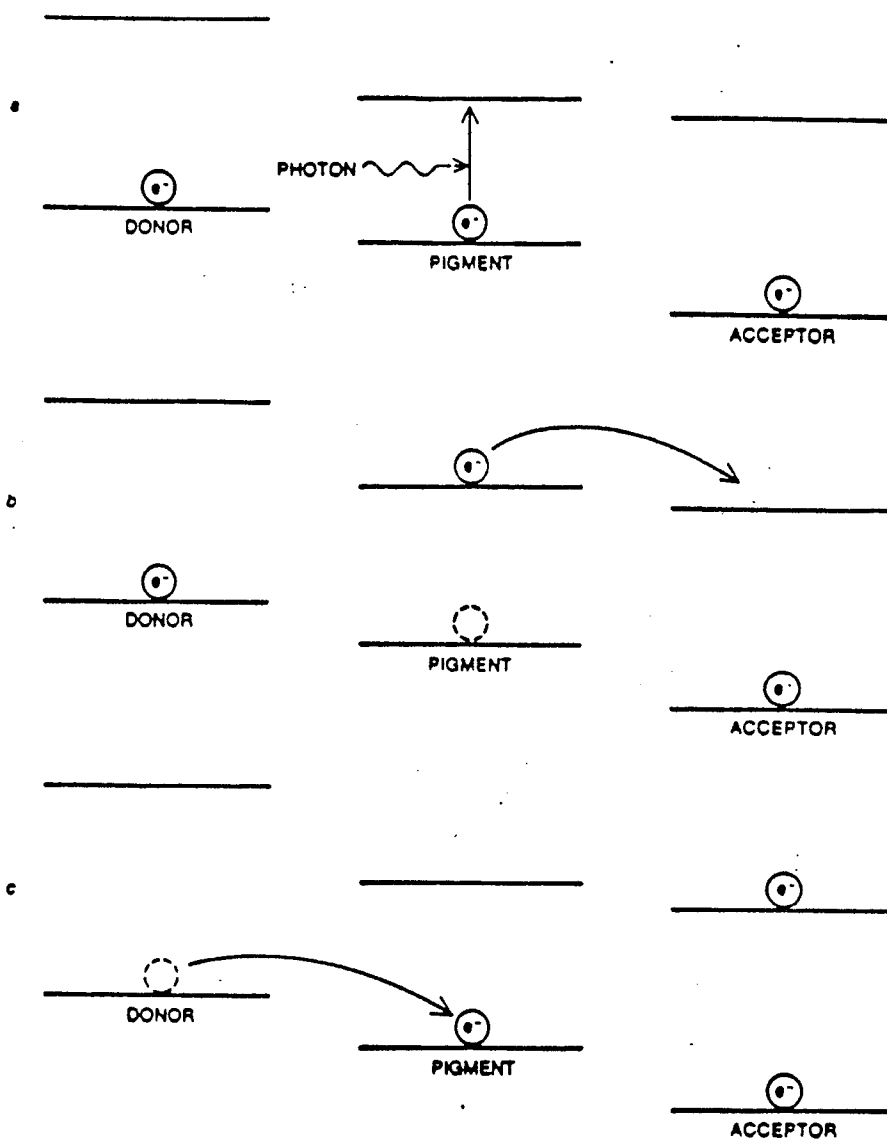


$$K = \cos \alpha - 3 \cos \beta_1 \cdot \cos \beta_2$$

(A) The donor-acceptor conformation that determines the geometric factor K in Eq. (2). The angle γ between the normal \vec{n} on the (μ_D, μ_A) plane and the vector R_{DA} connecting D and A is also shown. The lower diagrams illustrate a few simple cases: (B) $K^2 = 4$, (C) $K^2 = 0$, and (D) $K^2 = 9/4$. From Van Grondelle and Amez (1986).

Figure 10

CHEMISTRY



TRANSPORT OF ELECTRONS “uphill,” against an electrochemical gradient, in the reaction center of the chloroplast takes place in three stages. On absorbing a photon (a), an electron in a pigment molecule is promoted to an excited state. The electron can then be transferred to the acceptor molecule (b); because the pigment is in an excited state this process is “downhill,” that is, it is favored by the electrochemical gradient. In the final step (c), the pigment regains an electron from a donor molecule, another downhill process. (From Govindjee and Govindjee, 1974.)

Figure 11

Determination of the primary charge separation rate in isolated photosystem II reaction centers with 500-fs time resolution

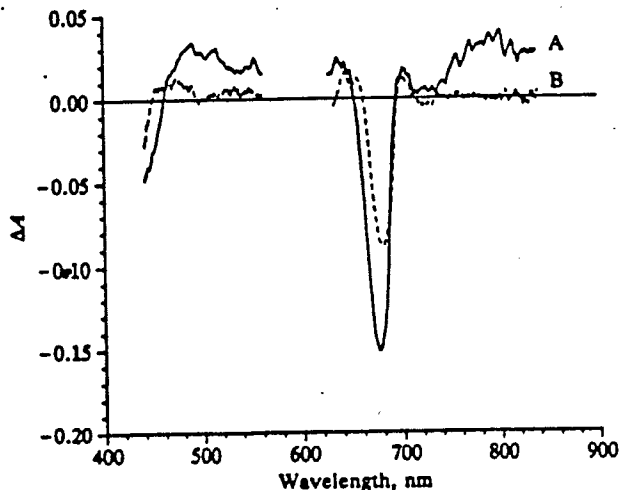
(electron transfer/ultrafast spectroscopy/photosynthesis/*Spinacia oleracea*)

MICHAEL R. WASIELEWSKI[†], DOUGLAS G. JOHNSON[†], MICHAEL SEIBERT[‡], AND GOVINDJEE[§]

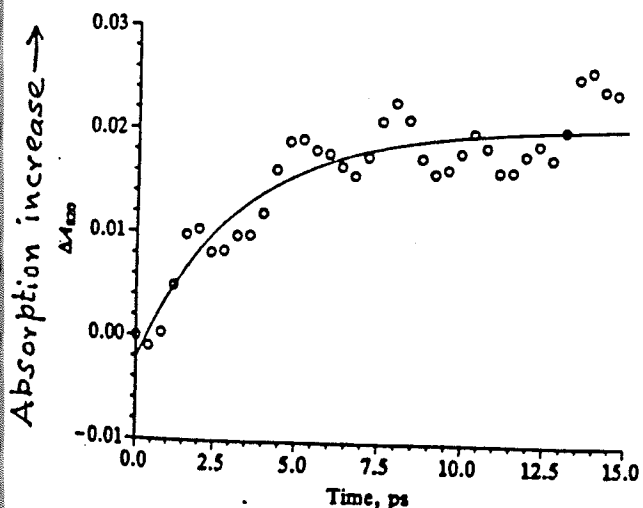
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Communicated by Joseph J. Katz, October 3, 1988

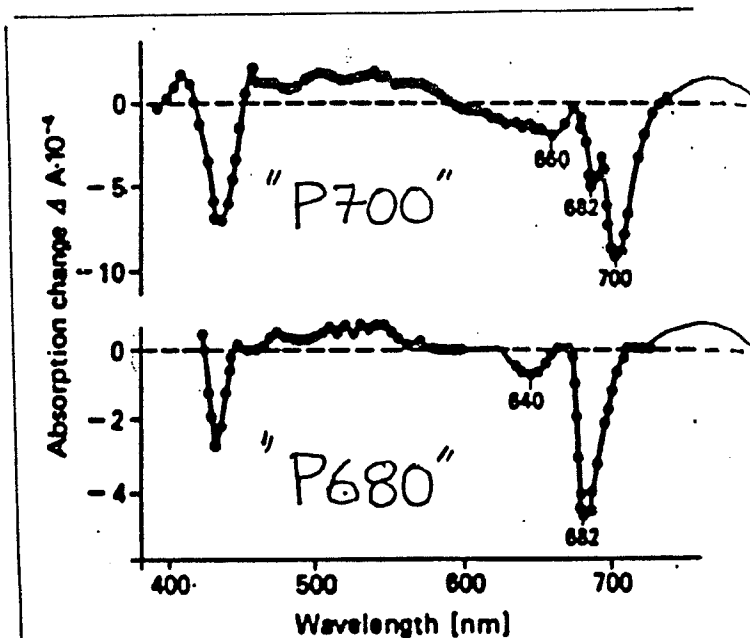
ABSTRACT We have measured directly the rate of formation of the oxidized chlorophyll a electron donor (P680⁺) and the reduced electron acceptor pheophytin a⁻ (Pheoa⁻) following excitation of isolated spinach photosystem II reaction centers at 4°C. The reaction-center complex consists of D₁, D₂, and cytochrome b-559 proteins and was prepared by a procedure that stabilizes the protein complex. Transient absorption difference spectra were measured from 440 to 850 nm as a function of time with 500-fs resolution following 610-nm laser excitation. The formation of P680⁺-Pheoa⁻ is indicated by the appearance of a band due to P680⁺ at 826 nm and corresponding absorbance changes at 505 and 540 nm due to formation of Pheoa⁻. The appearance of the 820-nm band is monoexponential with $\tau = 3.0 \pm 0.6$ ps. The time constant for decay of ¹P680, the lowest excited singlet state of P680, monitored at 650 nm, is $\tau = 2.6 \pm 0.6$ ps and agrees with that of the appearance of P680⁺ within experimental error. Treatment of the photosystem II reaction centers with sodium dithionite and methyl viologen followed by exposure to laser excitation, conditions known to result in accumulation of Pheoa⁻, results in formation of a transient absorption spectrum due to ¹P680. We find no evidence for an electron acceptor that precedes the formation of Pheoa⁻.



Spectrum A (solid trace), transient absorption spectrum of PSII RCs; spectrum B (dashed trace), transient absorption spectrum of PSII RCs treated with 1.5 mM sodium dithionite and 15 μ M methyl viologen. Both traces occur 10 ps following a 100- μ J, 500-fs laser flash at 610 nm. Filters needed to reject residual 610-nm light following continuum-probe light generation block the 560- to 620-nm wavelength range.



Transient absorption changes at 820 nm for PSII RCs after a 100- μ J, 500-fs laser flash at 610 nm.



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#3

Reaction Centers and Primary Photochemistry: in Photosynthetic Bacteria, in Cyanobacteria and in Plants

Outline

1. **Reaction Centers of Plants and Bacteria:** They are the world's most efficient solar battery where light energy is converted into chemical energy : extremely efficient (quantum efficiency = 0.90 to 0.95) and rapid (within a few picoseconds); they work efficiently and rapidly even at liquid helium temperature (4° Kelvin).
2. **A Major Breakthrough in 1982:** Crystallization of anoxygenic Bacterial Reaction Center (the first membrane protein to be ever crystallized), followed by X-ray diffraction measurements, and electron density map that led to the picture of chromophore arrangement, distances and angles between them; and finally the detailed 3-dimensional (3-D) structure of the four polypeptides of *Rhodospseudomonas viridis* reaction center : Nobel Prize in Chemistry was awarded to H. Michel, J. Deisenhofer, and R. Huber of W. Germany in 1988. The first crystallization paper was by H. Michel (1982) *J. Mol. Biol.* 158: 567-572.
3. **Composition of the anoxygenic Bacterial Reaction Center Complex**
 - 4 Bacteriochlorophyll molecules
(one special pair; dimer—primary electron donor) (P)
(two monomer molecules) (B)
 - 2 Bacteriopheophytin molecules (B Pheo; sometimes written as "H")
 - 2 Ubiquinone molecules; or 1 ubiquinone(UQ)+ 1 menaquinone(MQ)
(one electron acceptor Q_A)
(one two-electron acceptor Q_B)
 - 1 Non-heme Iron (Fe)
 - Cytochrome *c*-38 kilodaltons (bound in *R. viridis*; not in *Rhodobacter sphaeroides*)
 - Polypeptide "L" - 31 kilodaltons - 282 amino acids (281 in *R. sphaeroides*)
 - Polypeptide "M" - 34 kilodaltons - 307 amino acids (307 in *R. sphaeroides*)
 - Polypeptide "H" - 28 kilodaltons - 254 amino acids (260 in *R. sphaeroides*)
4. **Structure of the Reaction Center Complex of *R. viridis***

The reaction center complex (150 kilodaltons) from *R. viridis* has the size of 30x70x140Å (Å= Angstrom). Most of the "L" and "M" polypeptides are in the membrane (50Å) : each has 5 membrane spanning helices. The cytochrome is on top of the membrane on one side (periplasm) whereas most of the H polypeptide is on the opposite side of the membrane (cytoplasm) with one hydrophobic membrane spanning helix. The L and M polypeptides are to some extent intertwined.

In the "special pair" of bacteriochlorophylls (P), the acetyl group of one bacteriochlorophyll *b* is on the "Mg" of the other; they are at an angle of 15° and the center to center distance is 3Å (Å = Angstrom). The bacteriochlorophyll monomer (B) is at an angle of 70° to the special pair, with a center to center distance of 13Å. The bacteriopheophytin (BPheo) is at an angle of 64° to B, with a center to center distance of 11Å. Although Q_A and BPheo have quite a bit of overlap due to the tail of the quinone Q_A , they are within 11Å. The distance between Fe and Q_A is approximately 7Å and between the two quinones Q_A and Q_B about 14Å.

The location of all the above chromophores with respect to the specific amino acids in the L and M subunits is known and site-directed mutagenesis has begun to yield information on their role and importance.

Interesting points to note are:

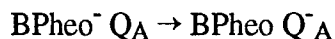
The reaction center complex is a symmetric molecule in a "gross sense," but only one side is functional, i.e., there is a *functional asymmetry*. Why?

5. *Function of the Bacterial (not cyanobacterial) Reaction Center Complex*

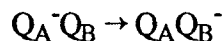
Within a few ps, excitonic energy is converted into redox energy, but the products are pretty unstable; the steps are suggested to be as follows:



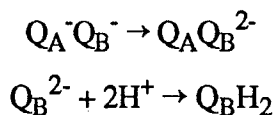
Here, P stands for the bacteriochlorophyll dimer (also called special pair), B for Bacteriochlorophyll monomer, and BPheo stands for bacteriopheophytin. This is the *primary charge separation*. The excitonic energy is consumed to produce the charge separated state. The exact nature of the involvement of the bacteriochlorophyll monomer (referred to as "B") is not yet settled, however. Within 200 ps, BPheo⁻ delivers its electron to Q_A:



Within 100-200 microseconds (μs), electrons are transferred to Q_B:

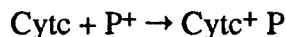


Q_B serves as a *two electron gate* and, thus, two turnovers of reaction center are needed to doubly reduce Q_B and complete the cycle:



Quinol Q_BH₂ exchanges with Quinone Q_B restoring the original state of the reaction center.

The oxidized P, P⁺, must also recover; this occurs by the transfer of electrons from, in about 300 nanoseconds, cytochrome *c* to P⁺:



6. *Types of Reaction Centers*

Anoxygenic photosynthetic bacteria have 1 type of reaction centers although the special pairs have slightly different spectral characteristics: P960 (in *Rhodospseudomonas viridis*; bacteriochlorophyll *b*); P860 (in *Rhodobacter sphaeroides*; bacteriochlorophyll *a*); P840 (in *Chlorobium* species, a green bacterium; also a bacteriochlorophyll *a*). The number after P indicates one of the absorption peaks, in nanometers, of these "special pairs".

On the other hand, oxygenic photosynthesizers (plants and cyanobacteria) have two types of reaction centers: P680 (having very high redox potentials, approximately +1.2 volts, capable of oxidizing water to oxygen; a chlorophyll *a* molecule) and P700 (having a redox potential of approximately +0.4 volts; also a chlorophyll *a* molecule). P680 belongs to photosystem II, and P700 to photosystem I.

[Note that P860 (or P870) was discovered by LNM Duysens et al. (Biochim. Biophys. Acta 19: 188-199, 1956) in *Rhodospirillum rubrum*. JHC Goedheer (Biochim. Biophys. Acta 38: 389-399, 1960) established that "bleaching" (absorption decrease at 870 nm) was due to oxidation of P870 to P870⁺.]

There is tremendous similarity in the amino acid sequence of polypeptides L and M of anoxygenic bacterial reaction centers with polypeptides D-1 and D-2 of plant and cyanobacterial photosystem II.

The Following text is taken from Whitmarsh and Govindjee (1996); it includes a short description of anoxygenic bacteria and of factors controlling electron transport, the two themes of this handout:

[Some of the material below repeats some of the material already presented; it should help understand the overall picture.]

A. ANOXYGENIC PHOTOSYNTHESIS

Anoxygenic photosynthetic bacteria differ from oxygenic organisms in that each species has only one type of reaction center (Blankenship et al., 1995). In some photosynthetic bacteria the reaction center is similar to photosystem II and in others it is similar to photosystem I. The latter two will be discussed at a later time. However, neither of these two types of bacterial reaction center is capable of extracting electrons from water, so they do not evolve O₂. Many species can only survive in environments that have a low concentration of O₂. To provide electrons for the reduction of CO₂, anoxygenic photosynthetic bacteria must oxidize inorganic or organic molecules available in their environment. For example, the purple bacterium *Rhodobacter sphaeroides* can use succinate to reduce NAD⁺ by a membrane-linked reverse electron transfer that is driven by a transmembrane electrochemical potential. Although many photosynthetic bacteria depend on Rubisco (Ribulose bis-phosphate carboxylase-oxygenase) and the Calvin cycle (to be discussed later) for the reduction of CO₂, some are able to fix atmospheric CO₂ by other biochemical pathways.

Despite these differences, the general principles of energy transduction are the same in anoxygenic and oxygenic photosynthesis. Anoxygenic photosynthetic bacteria depend on bacteriochlorophyll, a family of molecules that are similar to the chlorophyll, that absorb strongly in the infrared between 700 and 1000 nm. The antenna system consists of bacteriochlorophyll and carotenoids that serve a reaction center where primary charge separation occurs, as discussed in the outline earlier. The electron carriers include quinone (e.g., ubiquinone, menaquinone) and the cytochromes b and c and Rieske-iron sulfur center in the cytochrome bc complex, which is similar to the cytochrome bf complex of oxygenic photosynthetic apparatus. As in oxygenic photosynthesis, electron transfer is coupled to the generation of an electrochemical potential that drives phosphorylation by ATP synthase and the energy required for the reduction of CO₂ is provided by and ATP and NADH, a molecule similar to NADPH.

A.1 Purple Bacteria

There are two divisions of photosynthetic purple bacteria, the non-sulfur purple bacteria (e.g., *Rhodobacter sphaeroides* and *Rhodospseudomonas viridis*) and the sulfur purple bacteria (e.g., *Chromatium vinosum*) (Blankenship et al., 1995). Non-sulfur purple bacteria typically use an organic electron donor, such as succinate or malate, but they can also use hydrogen gas. The sulfur bacteria use an inorganic sulfur compound, such as hydrogen sulfide as the electron donor. The only pathway for carbon fixation by purple bacteria is the Calvin cycle. Sulfur purple bacteria must fix CO₂ to live, whereas non-sulfur purple bacteria can grow aerobically in the dark by respiration on an organic carbon source.

The determination of the three-dimensional structures of the reaction center of the non-sulfur purple bacteria, *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides*, has provided an unprecedented opportunity to understand the structure and function of photosynthetic reaction centers (Deisenhofer et al., 1984, 1985; Feher et al., 1989; Lancaster et al., 1995). The positions of the electron transfer components in the reaction center of *Rhodobacter sphaeroides* are shown in the Figure section (Norris and van Brakel, 1986), and those of the three protein subunits L, M,

and H, are also shown in the Figure section.. The reaction center contains four bacteriochlorophyll and two bacteriopheophytin molecules. Two of the bacteriochlorophyll molecules form the primary donor (P870). At present, there is controversy over whether a bacteriochlorophyll molecule is an intermediate in electron transfer from the P870 to bacteriopheophytin. However, there is agreement that the remaining steps involve two quinone molecules (Q_A and Q_B) and that two turnovers of the reaction center results in the release of reduced quinone (QH_2) into the photosynthetic membrane. Although there is a non-heme Fe between the two quinone molecules, there is convincing evidence that this Fe is not involved directly in transferring an electron from Q_A to Q_B . Because the primary donor (P870), bacteriopheophytin and quinone acceptors of the purple bacterial reaction center are similar to the photosystem II reaction center, the bacterial reaction center is used as guide to understand the structure and function of photosystem II.

Light driven electron transfer is cyclic in *Rhodobacter sphaeroides* and other purple bacteria (see Figure section). The reaction center produces reduced quinone, which is oxidized by the cytochrome bc complex. Electrons from the cytochrome bc complex are transferred to a soluble electron carrier, cytochrome c_2 , which reduces the oxidized primary donor $P870^+$. The product of the light driven electron transfer reactions is ATP. The electrons for the reduction of carbon are extracted from an organic donor, such as succinate or malate or from hydrogen gas, but not by the reaction center. The energy needed to reduce NAD^+ is provided by light driven cyclic electron transport in the form of ATP. The energy transformation pathway is complicated. Succinate is oxidized by a membrane bound enzyme (succinate dehydrogenase) that transfers the electrons to quinone, which is the source of electrons for the reduction of NAD^+ . However, electron transfer from reduced quinone to NAD^+ is energetically uphill. By a mechanism that is poorly understood, a membrane bound enzyme is able to use energy stored in the proton electrochemical potential to drive electrons from reduced quinone to NAD^+ .

A.2 Green Sulfur Bacteria

Green sulfur bacteria (e.g., *Chlorobium thiosulfatophilum* and *Chlorobium vibrioforme*) can use sulfur compounds as the electron donor as well as organic hydrogen donors (Blankenship et al., 1995). As shown in the figure section the reaction center of green sulfur bacteria is similar to the photosystem I reaction center of oxygenic organisms (Feiler and Hauska, 1995). The FeS centers in the reaction center can reduce NAD^+ (or NADP^+) by ferredoxin and the ferredoxin- NAD(P)^+ oxidoreductase enzyme, therefore green sulfur bacteria are not necessarily dependent on reverse electron flow for carbon reduction. The antenna system of the green sulfur bacteria is composed of bacteriochlorophyll and carotenoids and is contained in complexes known as a chlorosomes that are attached to the surface of the photosynthetic membrane. This antenna arrangement is similar to the phycobilisomes of cyanobacteria. Green sulfur bacteria can fix CO_2 without Rubisco. It has been proposed that they accomplish this by using the respiratory chain that normally oxidizes carbon (known as the Krebs cycle), resulting in the release of CO_2 . With the input of energy this process can be run in the reverse direction, resulting the uptake and reduction of CO_2 .

A.3 Green Gliding Bacteria

Green gliding bacteria (e.g., *Chloroflexus aurantiacus*), also known as green filamentous bacteria, can grow photosynthetically under anaerobic conditions or in the dark by respiration under aerobic conditions. Like the green sulfur bacteria, green gliding bacteria harvest light using chlorosomes. The green gliding bacteria appear to have reaction centers similar to those of the purple bacteria, but there are several notable differences. For example, instead of two monomer bacteriochlorophyll molecules, *C. aurantiacus* has one bacteriochlorophyll and one bacteriopheophytin and the metal between the two quinones is Mn rather than Fe (Feick et al., 1995). *C. aurantiacus* appears to fix CO_2 by a scheme that does not involve the Calvin cycle or the reverse Krebs cycle (Ivanovsky et al., 1993).

A.4 Heliobacteria

Heliobacteria (e.g., *Heliobacterium chlorum* and *Heliobacillus mobilis*) are in the phylum Gram Positive Bacteria that are strict anaerobes. Although the heliobacterial reaction center is similar to photosystem I in that it can reduce NAD^+ (or NADP^+), it contains a different type of chlorophyll known as bacteriochlorophyll g (Amesz, 1995).

B. CONTROL OF INTRAPROTEIN ELECTRON TRANSFER

The three-dimensional structure of the reaction center of *Rhodospseudomonas viridis* and *Rhodobacter sphaeroides* reveals the distances between the electron donors and acceptors (Deisenhofer et al. 1984, 1985; Norris and van Brakel, 1986; Feher et al. 1989) and has had an important influence on biophysical and molecular genetics studies designed to identify the factors that control the rate of electron transfer within proteins. There is currently a controversy concerning the importance of specific amino acid composition of the protein on the rate of intraprotein electron transfer. In part, the disagreement centers on whether the protein between the donor and acceptor molecules can be treated as a uniform material, or whether the specific amino acid composition of the protein significantly alters the rate. For example, it has been proposed that aromatic amino acids may provide a particular pathway that facilitates electron transfer between a donor and acceptor pair. [This is the case in the photosystem II reaction center, where a tyrosine residue on one of the reaction center core proteins (precisely, Tyr 161 on the D1 protein) donates an electron to the primary donor chlorophyll, P680+. However, in other cases, replacement of an aromatic by another non-aromatic residue has resulted in relatively minor changes in the rate of electron transfer.] L. Dutton and coworkers (Moser et al., 1992) have analyzed electron transfer reactions in biological and chemical systems in terms of electron tunneling theory developed by R. Marcus and others (DeVault, 1984). Dutton and coworkers argue that protein provides a uniform electronic barrier to electron tunneling and a uniform nuclear characteristic frequency. They suggest that the specific amino acid residues between an electron transfer pair is generally of less

importance than the distance in determining the rate of pairwise electron transfer. In their view, protein controls the rate of electron transfer mainly through the distance between the donor and acceptor molecules, the free energy, and the reorganization energy of the reaction. The importance of distance is demonstrated by electron transfer data from biological and synthetic systems showing that the dependence of the electron transport rate on the edge to edge distance is exponential over 12-orders of magnitude when the free energy is optimized (Moser et al., 1992). Increasing the distance between two carriers by 1.7 Å slows the rate of electron transfer 10-fold. The extent to which this view is generally applicable for intraprotein transfer remains to be established (Williams, 1992). One of the challenges in understanding pairwise electron transfer rates from first principles is illustrated by the reaction centers of *Rhodospseudobacter sphaeroides* in which the redox components are arranged along two-fold axis of symmetry that extends from the primary donor (P870) to the non heme Fe. Despite the fact that the reaction center presents two spatially similar pathways for electron transfer from P870 to quinone, nearly all electrons are transferred down the right-arm of the reaction center as shown in Fig. 17. The same is true for the reaction center of *Rhodopseudomonas viridis*, in which it is estimated that electron transfer down the left-arm is less than 1:100 (Kellogg et al., 1989). The challenge to theorists is to explain the surprisingly high probability that electron flow goes down the right-arm. Since the distances are similar, it has been suggested that electron transfer down the left-arm is less probable due to an endothermic free energy change (Parson et al., 1990) or to an unfavorable rearrangement energy for the reaction (Moser et al., 1992).

[References used in the above text (both A and B) will be shown below in larger size.]

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A. Basic reviews

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(3) G. Feher, J.P. Allen, M.Y. Okamura and D.C. Rees (1989) Structure and function of bacterial photosynthetic reaction centers. *Nature (London)* 339, 111-116.

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B. References

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C. Kirmaier, D. Gaul, R. deBey, D. Holten and C. Schenck (1991) Charge separation in a reaction center incorporating bacteriochlorophyll for photoactive bacteriopheophytin. *Science* 251: 922-926.

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Some Comments on Electron transport and Primary Photochemistry

1. The "uphill" charge separation becomes a "downhill" reaction with energy from light.
2. When an electron is suggested to flow from primary donor (D) of high mid-point redox potential (at pH 7) to an acceptor (A), ΔG is positive and thus no such reaction can occur. However, if we add light, D can be excited to D* and now ΔG is negative, and, charge separation can take place.

Remember: $\Delta G = nF \Delta E$, where G is free energy; F is Faraday's constant; n is number of electron transferred; and ΔE is difference in redox potential.

3. Review of Primary Photochemistry:

- (a) is very efficient, almost 95-98% quantum efficiency
- (b) occurs very rapidly: usually within a few picoseconds
- (c) is the first chemical reaction to take place
- (d) is equally efficient at liquid helium temperature (4 K)
- (e) leads to a charge separation between a "reluctant" donor and a "reluctant" acceptor
- (f) loss of an electron on "D" (or "P", as you may have called it) means that an unpaired electron now exists; this means you a "doublet" formed from a singlet, and, EPR can be used also to detect it.
- (g) clearly, energy is stored here; it is the only true "light reaction", the rest are "dark" reactions.

4. The concept of quantum mechanical tunneling in biology arose first when Don DeVault and Brit Chance (1966) discovered that the rate of cytochrome oxidation in a photosynthetic bacterium *Chromatium* was absolutely temperature independent from 100 to 4 K. Thus, no activation energy is involved. (Don DeVault spent most of his life at UIUC, Urbana, until his death. [I have published 2 papers with him giving the theory for "thermoluminescence in photosynthetic apparatus.]

In tunneling, a particle disappears from one side of a potential energy barrier and appears on the other side without having kinetic energy to mount the barrier! Perhaps, one can imagine that this is possible because of the wave-nature of the particles: they "ooze" through the barriers. Well, this is possible in quantum mechanics, not classical mechanics. Thus, I personally have difficulty in imagining it.

In Physics, Gamow (1928) described it as follows: If a particle with kinetic energy E hits a barrier (barrier height V in energy units, and barrier width b), then the probability P of finding it on the other side is given by the following equation:

$$P = 16 E V (E+V)^{-2} \exp [-2b (2 mV)^{1/2} 2\pi/\hbar]$$
, where m is the mass of the particle.

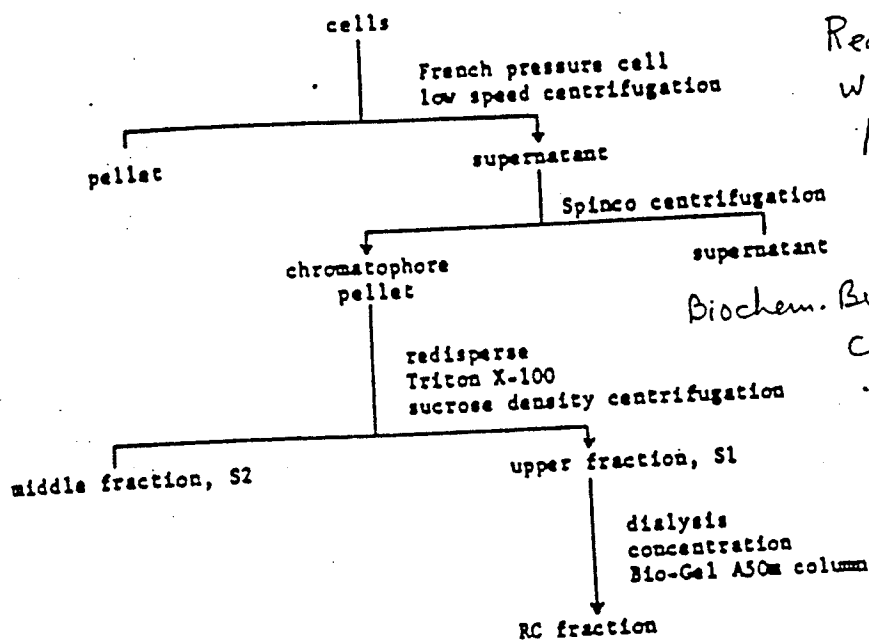
Some questions

- [1] If you were asked to plan an experiment to find out if a reaction center chlorophyll exists, what type of experiment would you propose to your advisor?
- [2] What is difference absorption spectroscopy? What is Electron paramagnetic spectroscopy? How do these techniques help you "finger print" P680, P700, P870, P960, etc.
- [3] We ask again: Why is light energy needed for photosynthesis?
- [4] What are the criteria of primary photochemical reactions in photosynthesis? Efficiency? Rates? Temperature dependence?
- [5] How are the electrons transferred? What on Earth is "quantum mechanical tunneling"? Why did the Nobel committee give Rudy Marcus a Nobel Prize recently? What is the dependence of rate of electron flow on distance? Les Dutton's ruler?
- [6] What is the structure of the bacterial reaction center? Why was a Nobel Prize given to Michel/Deisenhofer/Huber? How was this structure determined? How are the proteins arranged in the reaction center? What are the chromophores? How are they arranged in the reaction center? Angles? Distances? Why is there a functional asymmetry in the reaction centers when the centers look very symmetrical?
- [7] What is the biological function of bacterial reaction center? Is Bacteriochlorophyll monomer a voyeur or a real intermediate ?

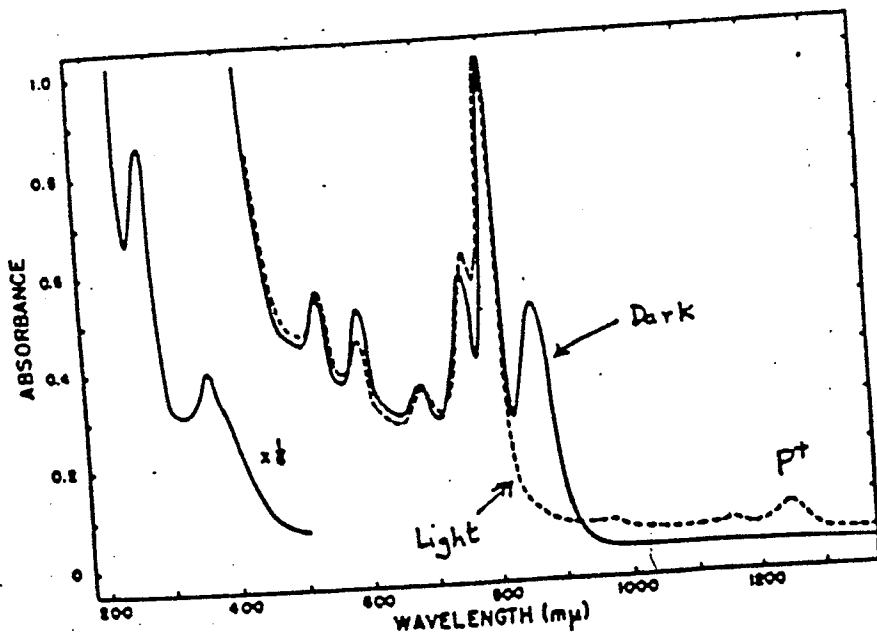
DIAGRAMS AND FIGURES FOLLOW: WE NEED TO STILL INTEGRATE THEM IN THE TEXT. THIS IS A NECESSARY EXERCISE BECAUSE OF MY LAZINESS!

Clayton, R. K.

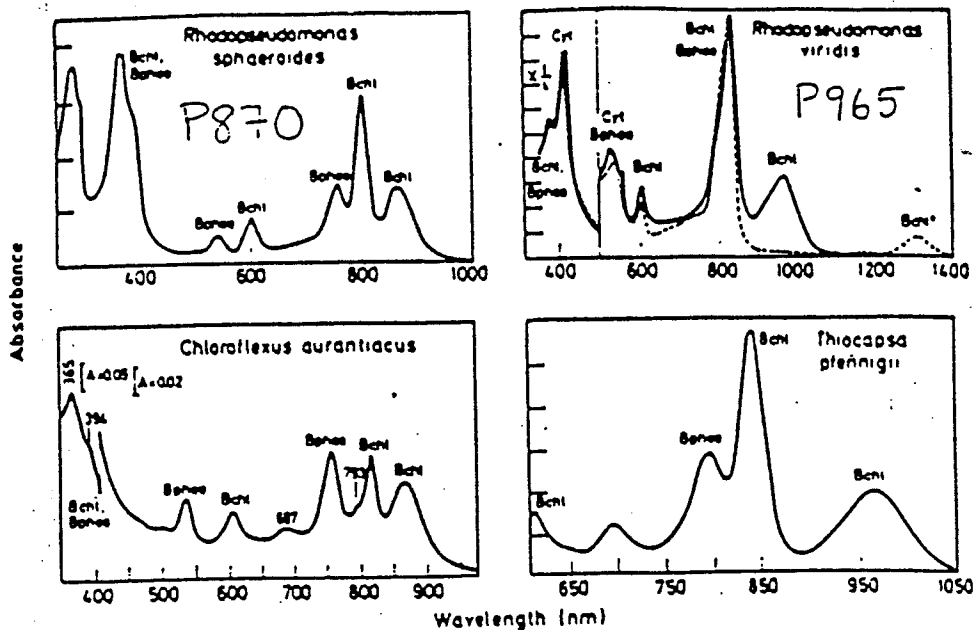
ISOLATION OF RC FRACTION



Reaction Center
was first
prepared by
Reed and
Clayton (1968)
Biochem. Biophys. Res.
Commun.
30, 471-475.

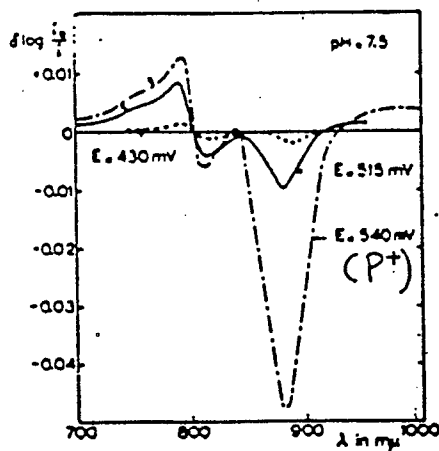


Absorbance spectra of Reaction Center fraction in 0.01 M Tris hydrochloride buffer, pH 7.5, containing 0.001 M dithiothreitol. Spectra were determined in the "IR-1", ———, and "IR-2", - - - - - , modes of the Cary model 14R recording spectrophotometer. In the "IR-2" mode the sample was exposed to undispersed white light.



Absorption spectra of some typical reaction center preparations from three purple and one green (*Chloroflexus aurantiacus*) bacteria. *Rhodospseudomonas sphaeroides* and *Chloroflexus aurantiacus* reaction centers contain bacteriochlorophyll a and bacteriopheophytin a, whereas the other two reaction centers contain the equivalent b forms, which absorb at longer wavelengths in vitro and in vivo.

J. C. GOEDHEER



Difference spectra of *Rhodospirillum rubrum* chromatophores obtained by the addition of ferri-ferrocyanide mixtures: ---- potential 430 mV, ——— 515 mV, ····· 540 mV (P^+).

A comparison of the composition of some isolated photochemical reaction centers

Species	<i>Rps. sphaeroides</i>	<i>Rps. viridis</i>	<i>Chloroflexus aurantiacus</i> ← green
Pigment content per P-870 or P-960 molecule	4 molecules BChl a 2 molecules BPheo a 2 molecules UQ ₁₀ 1 molecule carotenoid	4 molecules BChl b 2 molecules BPheo b 1 molecule UQ ₁₀ 1 molecule MQ ₇ 1 molecule carotenoid	3 molecules BChl a 3 molecules BPheo a No carotenoid
<i>Subunit composition</i>			
Number per P-870 or P-960 (and names)	3 (L, M and H)	4 (L, M, H and two c type cytochrome(s) ^a)	2 (L and M?)
Apparent size of subunits from SDS-PAGE (from amino acid analyses)	* H -28 (36) kDa * M -24 (32) kDa * L -21 (28) kDa	H -35 kDa M -28 kDa L -24 kDa Cytochrome - 38 kDa	M -30 kDa L -28 kDa

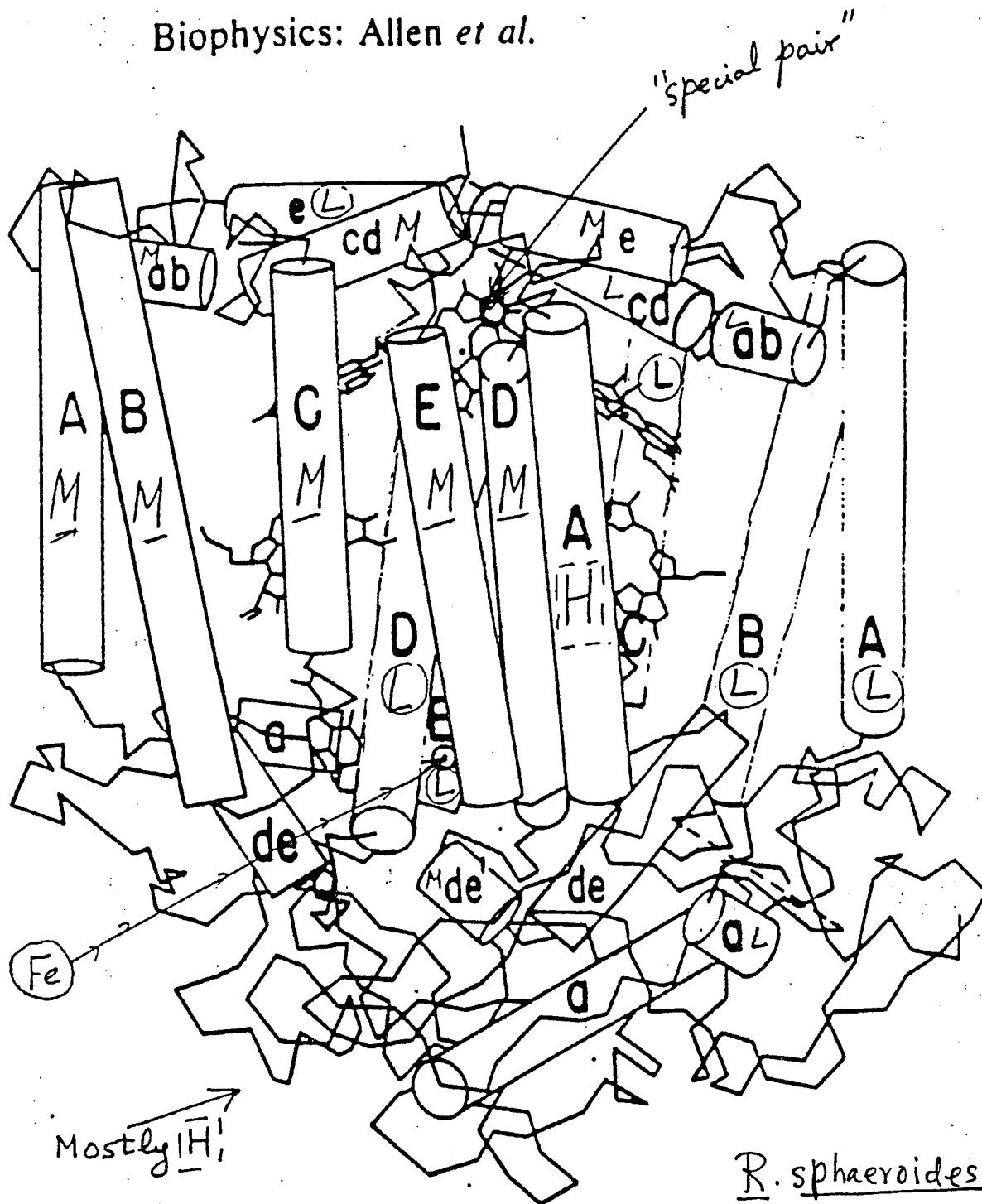
^a 4-5 hemes per P-960

Notes:

- 1-2% of total pigments
- Carotenoids protect against photooxidation
- 3 molecules of Bchl are necessary as 4th can be modified without loss of activity
- * d) correct molecular weights are:

L	31 kDa	old 21 (28)	AA 282
M	34 kDa	old 24 (32)	AA 307
H	28 kDa	old 28 (36)	AA 254

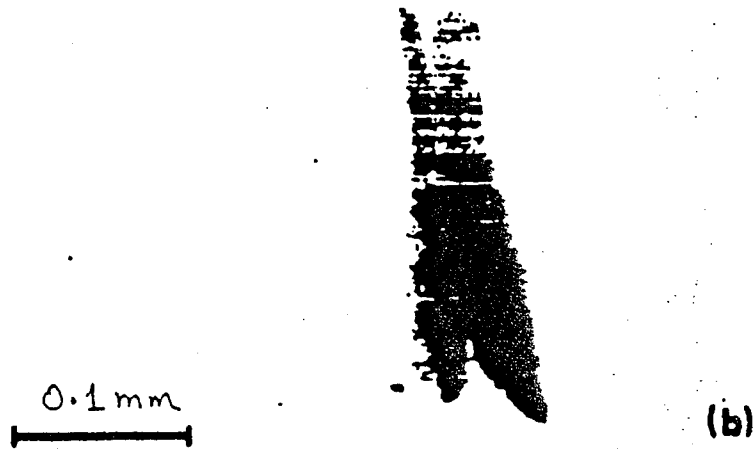
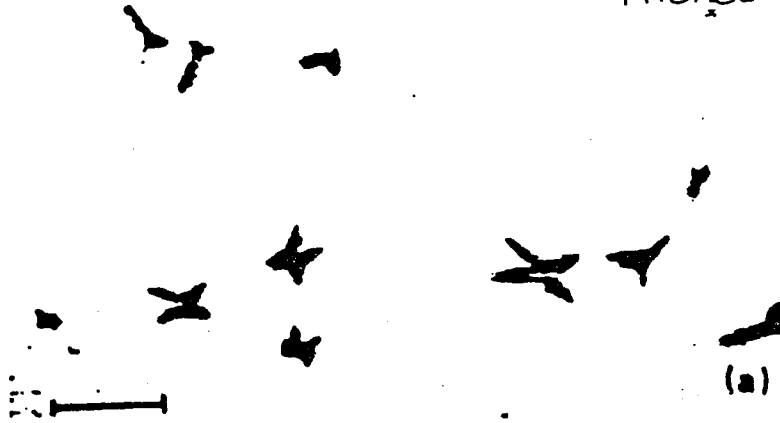
↑
SDS gels



The RC structure with L (yellow), M (blue), and H (green) subunits and the cofactors (red) with truncated phytol and isoprenoid chains. The α -helices have been approximated by straight cylinders (~~Table 1 lists their curvatures~~). The view is the same as that shown in Fig. 4a. The dotted line in the C α backbone of H corresponds to residues 48-53, for which only a weak electron density was obtained. The circle (red) near the bottom of the D and E helices is the position of the nonheme (iron).

(After: Allen *et al.*,)

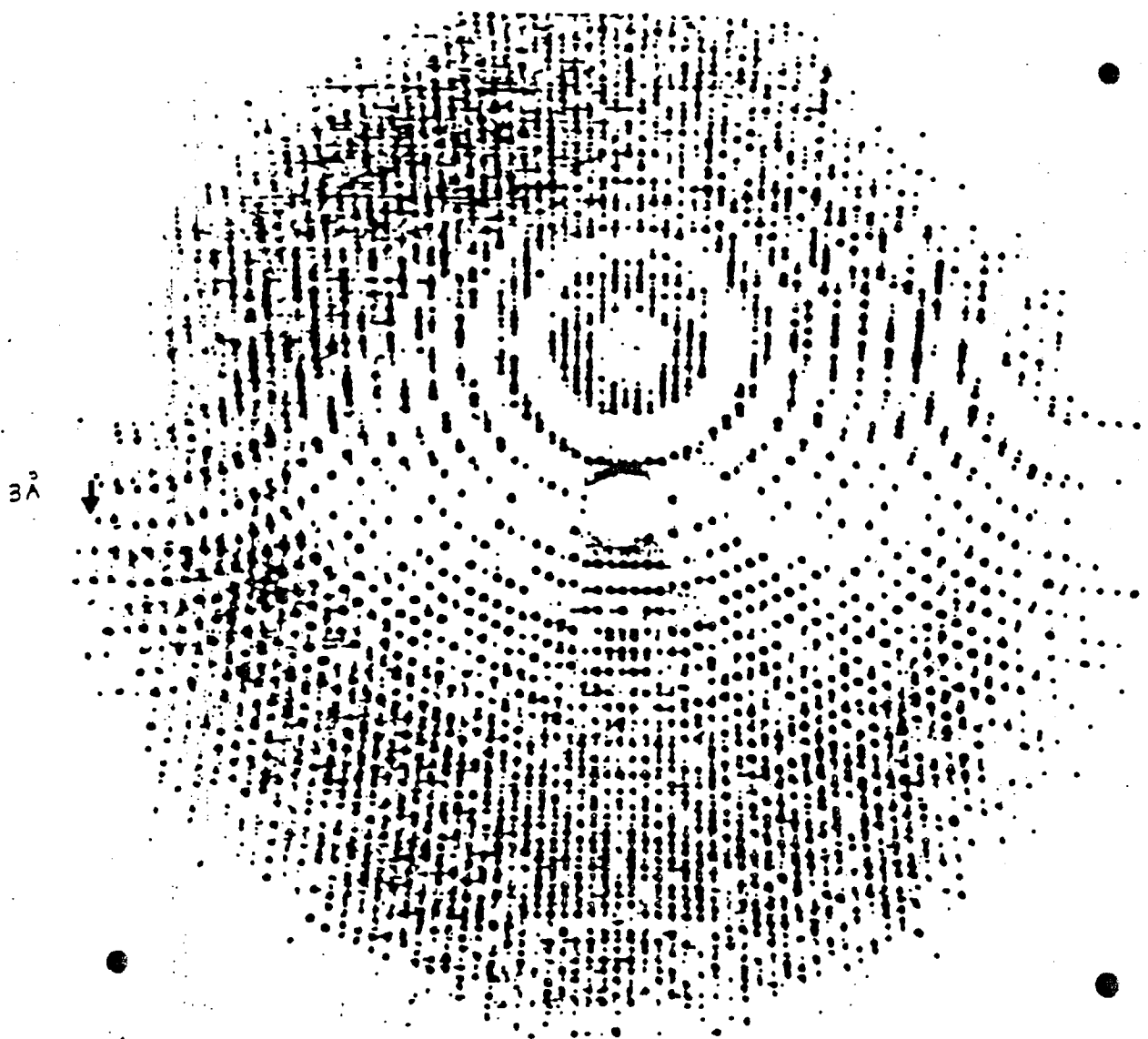
Michel (1982)



(Crystals obtained by vapour diffusion of a concentrated reaction centre solution (2 units of absorbance at 830 nm) in 1.5 M ammonium sulphate against: (a) 3 M, (b) 2.5 M and (c) 2.4 M ammonium sulphate at room temperature, pH 6.)

Various types of crystals of Reaction Centres of R. viridis (after Michel, 1982).

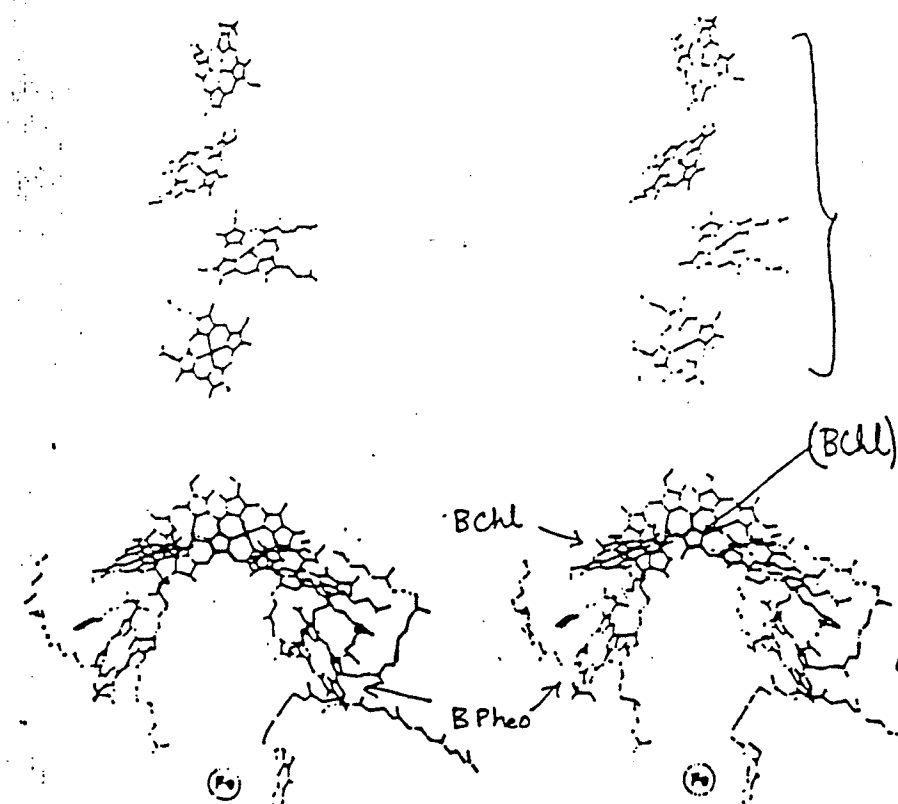
Michel (1982)



(X-ray diffraction pattern of a single reaction centre crystal (rotation photograph. 1 of rotation around the c-axis). The exposure time was 20 h using monochromatized X-rays from a Rotaflex rotating anode (Rigaku, Japan) operated at 45 kV and 100 mA, and a 0.4 mm collimator. The focus to crystal distance was 30 cm. The arrow indicates 3 Å resolution.)

X-ray diffraction pattern of a single reaction centre crystal. The arrow indicates 3 Å resolution (after Michel and coworkers)

Stereo drawing (scale 0.08 cm/Å) of 10 layers (distance between layers 1.2 Å) of the electron density distribution at 3 Å resolution. Contour lines start at 1.5 with increments of 1.8 (arbitrary units). The map was rotated so that the central local symmetry axis runs vertically in the middle layer. The section shows part of the central region with some chromophores: parts of the cytochrome (top), and of the H-subunit (bottom) can be seen



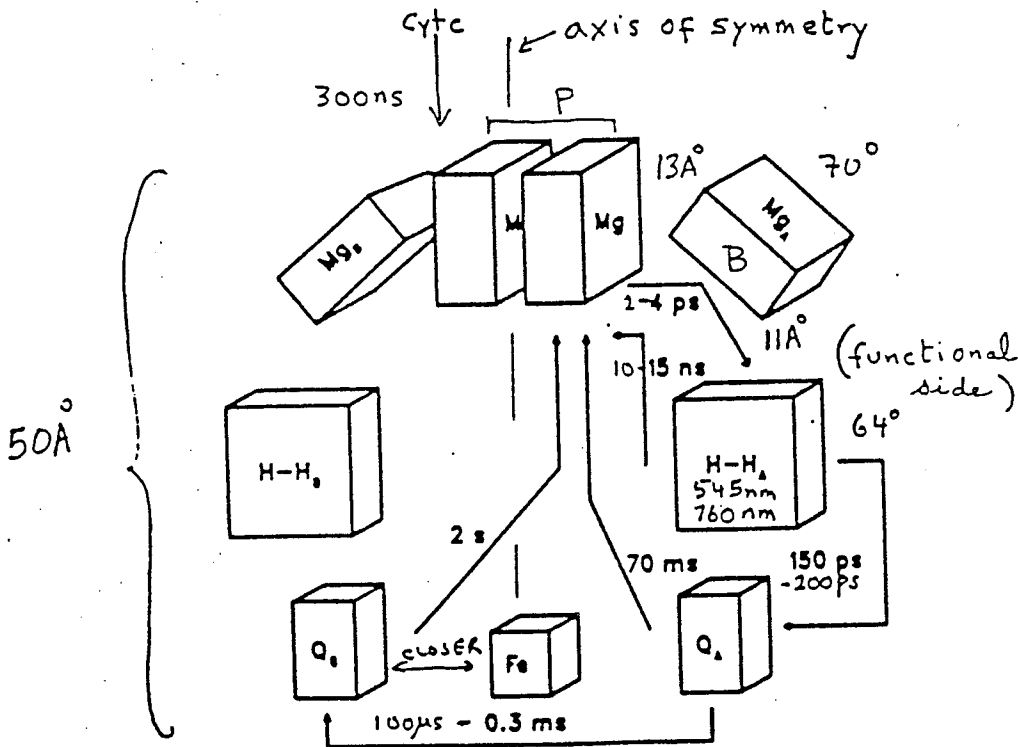
Stereo drawing of the prosthetic groups of the RC showing 4 BChl b, 2 BPh b, 1 nonheme iron (Fe), 1 menaquinone, and 4 heme groups. The central local symmetry axis runs vertically in the picture plane. The plane of the membrane is assumed to be oriented perpendicular to the central local symmetry axis, i.e., horizontal and perpendicular to the picture plane. The BChl b, BPh b, quinone, and iron are located within the cylindrical central part of the RC complex

- Cytochrome
- (Bchl)₂
- (1) Pyrrole ring I is stacked on top of each other
 - (2) ~ 3 Å apart;
 - (3) Acetyl group of one Bchl is over Mg of other
 - (4) angle between ring planes is few °
 - (5) Mg - Mg : 7 Å
 - (6) No H₂O !!
- ⊗ But Acetyl group H-bonded to His L-168 + Trp - M which π?

Biophysics: Yeates et al.

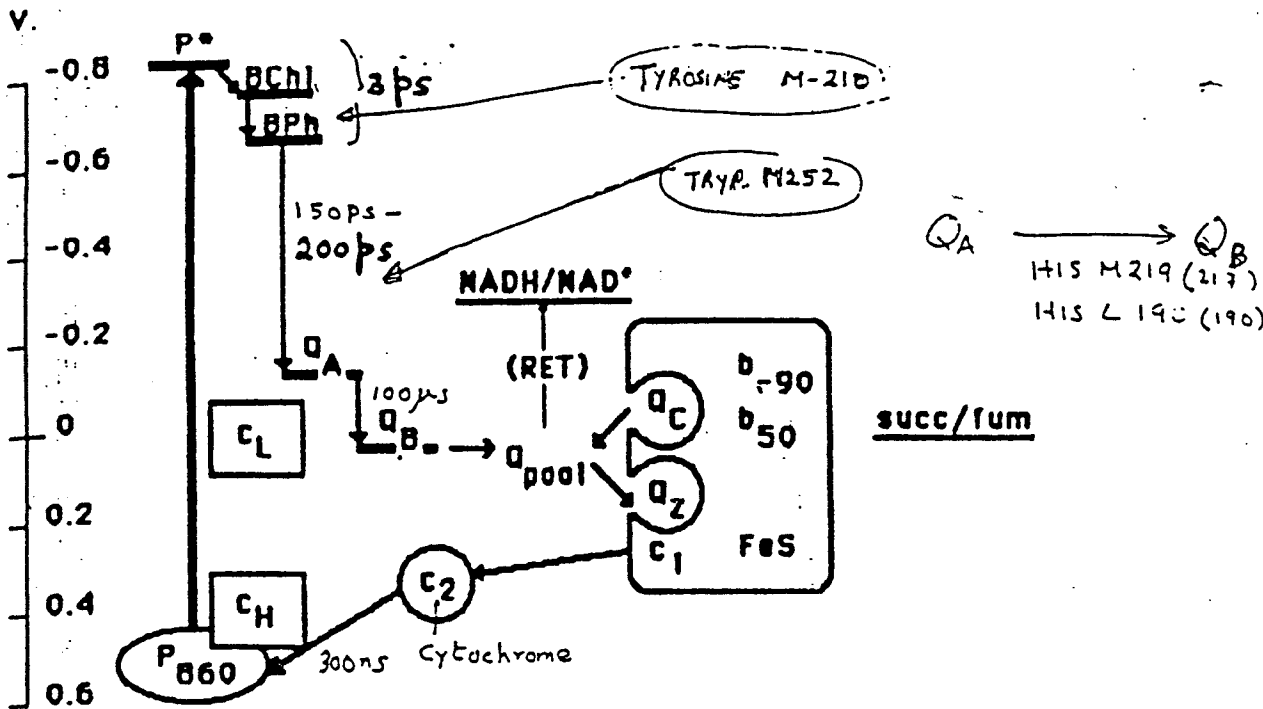
Membrane Position, Å	Fe Q	Bphe	Bchl (Bchl) ₂
0			
10			
20			
30			
40			
LA	32G	Q	T
LB	111L	K	C
LC	116H	I	P
LD	198A	A	S
LE	225G	L	G
MA			
MB	139A	A	R
MC			
MD	226V	A	L
ME	262M	G	I
HA	37R	M	E

Approximate positions of C* atoms in the 11 transmembrane helices of the RC. Labeling of the helices follows - p. 4. (First letter refers to subunit.) Locations of the cofactors (top) and all charged residues (bottom) of the RC are indicated. Residues in hatched circles have >50% of their surface area exposed to the membrane, while residues in plain circles have 20-50% of their surface area exposed to the membrane. The remaining residues are buried inside the protein. Histidine residues marked with an asterisk are ligands to Fe or Bchl. Of the major residues in the nonpolar region, alanine is preferentially buried; phenylalanine and isoleucine are preferentially exposed to the membrane; the remaining residues show no preference. Amino acids are designated by the single-letter code.



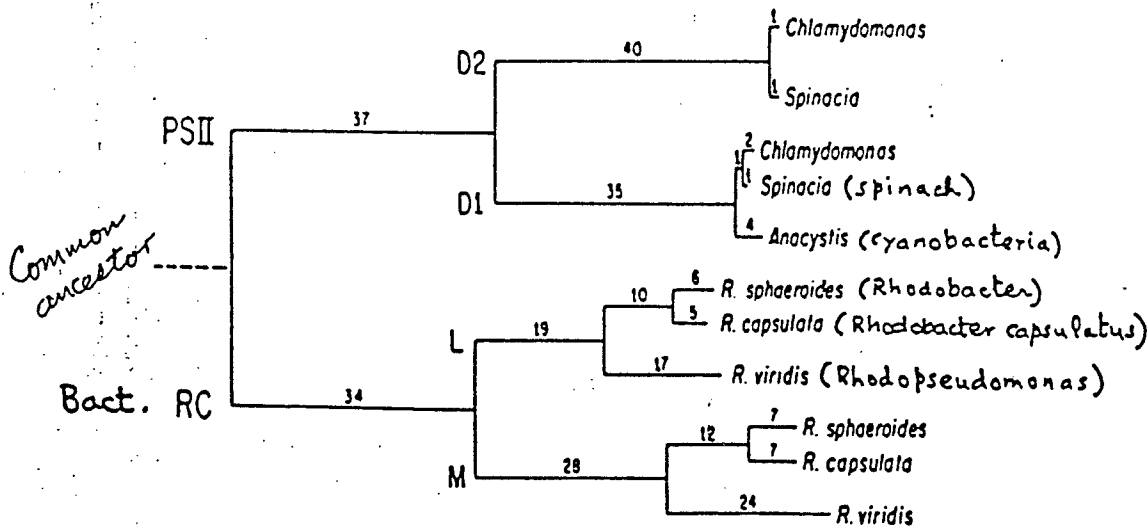
P* = excellent donor
P/p* 1.38 eV
P/p+ 0.45 V
P*/p+ = -0.9 V
B/B- -0.85 V
H/H- -0.55 V
Q_A/Q_A- ~ 0 V
P+BH- = P_F
Fe: High spin
; M GLU 23

Schematic approximation of the electron transfer components of the donor acceptor complex as found in bacterial reaction centers. The protein holding this assembly together is omitted. This drawing is based in part on X-ray structural information of *Rps. viridis* (DEISENHOFER et al. 1984; MICHEL and DEISENHOFER, Chap. 8.4, this Vol.) and on spectroscopic data on *Rps. sphaeroides*. Boxes represent aromatic ring systems associated with bacteriochlorophyll (M Mg) and (Mg), and bacteriopheophytin (H-H), and the quinones (Q) such as ubiquinone or menaquinone. One box does not represent a ring system, namely the Fe²⁺. The subscripts A and B represent two potential electron transfer pathways of which only the A pathway appears active



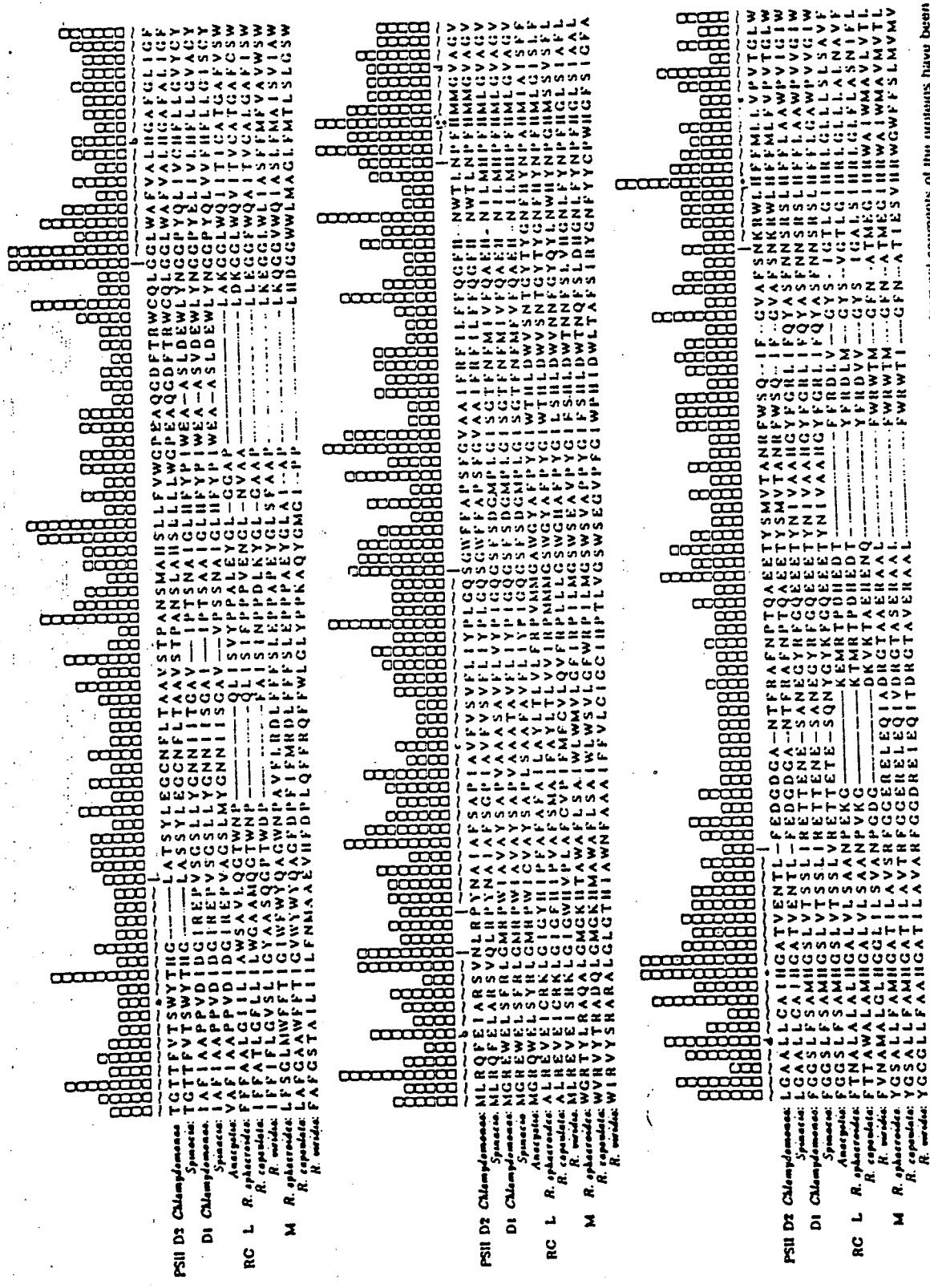
PURPLE SULFUR and NON-SULFUR BACTERIA
(Chromatiales and Rhodospirillales)

RET = Reverse Electron Transport
 C₁, C_L, C_H = Cytochrome c
 b = cytochrome b



Evolutionary tree of RC and PSII proteins. Vertical lines indicate divergencies of species or genes. The lengths of the horizontal branches (indicated by numbers, rounded to the nearest unit) show the relative evolutionary distance between proteins as determined from a least square fit of difference scores

(see text). The standard deviation between the difference scores calculated from the tree and the difference scores calculated from the original alignments (Table II) is 6%. The common ancestor represented by the root of the tree was arbitrarily placed so that it is approximately equidistant to each descendant.



Comparison of sequences of RC and PSII proteins. Gaps were introduced to maximize the homology. The amino- and carboxy-terminal segments of the proteins have been deleted. Shown are residues 50-280 (68% of the sequence) of the D2 protein from *Chlamydomonas reinhardtii*,¹⁸ residues 51-281 (65% of the sequence) of the D2 protein from *Spirulina oboracia*,¹⁹ residues 50-284 (65% of the sequence) of the D1 protein from *Chlamydomonas reinhardtii*,²¹ residues 50-284 (67% of the sequence) of the D1 protein from *Spirulina oboracia*,²⁰ residues 50-284 (65% of the sequence) of the D1 protein from *Anacystis nidulans*,²⁹ residues 39-242 (73% of the sequence) of the L subunit from *R. sphaeroides*,⁸ residues 40-243 (72% of the sequence) of the L subunit from *R. capsulata*,⁹ residues 39-242 (75% of the sequence) of the L subunit from *R. capsulata*,⁹ residues 59-276 (67% of the sequence) of the M subunit from *R. sphaeroides*,⁸ residues 59-276 (71% of the sequence) of the M subunit from *R. capsulata*,⁹ residues 59-276 (67% of the sequence) of the M subunit from *R. sphaeroides*,⁸ residues 59-276 (71% of the sequence) of the M subunit from *R. capsulata*,⁹ and residues 59-276 (67% of the sequence) of the M subunit from *R. sphaeroides*.⁸ The boxes indicate the largest number of identical residues at each position. Positions of putative transmembrane helices a-e ([-]) and ligands to the donor bacteriochlorophyll (C) and iron (I) in the L and M subunits are indicated.

$$P = 16 EV (E + V)^{-2} \exp [-2b (2mV)^{1/2}/\hbar],$$

where \hbar is Planck's constant (h) divided by 2π and m is the mass of the particle.

Fig. 2 Quantum-mechanical tunnel penetration of a barrier. A plot of potential energy v . distance for a symmetrical rectangular barrier. E is the kinetic energy of the approaching particle; V is the barrier height above the particle energy; b is the barrier width. (After Gamow, 1928.)

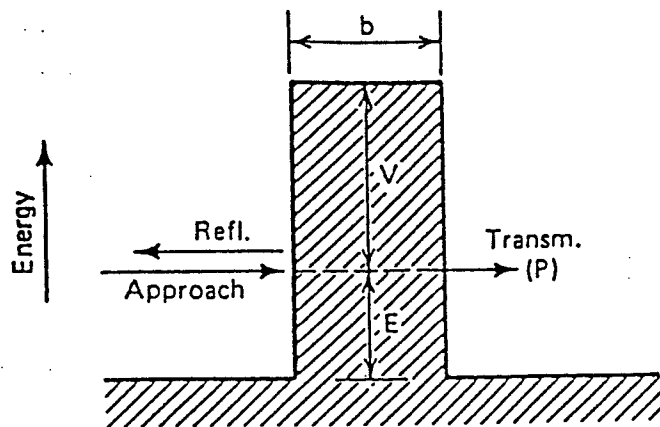
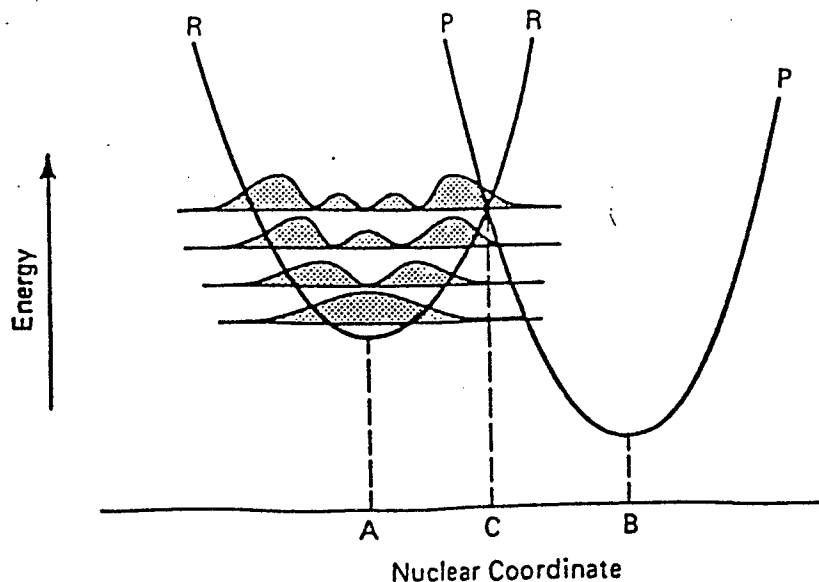


Figure 3 explains tunneling (nuclear, atomic or molecular): R and P are potential energy curves for, say, DA and D^+A^- states, respectively. The curve for R has the probability curves (wave-functions squared) for the system vibrating, but all below where R and P surfaces overlap. What we see is that the exponential tails of the wave function of R extend into the realm of P. At very low temperatures, molecules in the R state will be at the lowest vibrational state and there could be sufficient overlap with the wave-function of the P state and there would be electron or nuclear transfer (that we call tunneling here) and it would be the same at all the temperatures at which the bulk of the molecules are in their lowest vibrational state. This gives the temperature-independent region. (From: D. DeVault: Quantum-mechanical tunnelling in Biological Systems, 2nd edition, Cambridge University Press, 1984).

Fig. 3 Nuclear tunnelling. Nuclear vibrational energy states are pictured in electronic state R. Their wave-functions reach across configuration C even when the total energy is less than the potential energy at C.



5. Marcus theory

* Rudolf Marcus, who was at the UIUC for almost 10 years, received the 1992 Nobel Prize in Chemistry for his work on the mechanism of electron transfer in chemistry and biology.

Marcus defined a term λ , the reorganizational energy (Fig. 4A). (This is not the wavelength! We know that $E = h\nu$, where $E = \text{energy}$ and $h = \text{Planck's constant}$, and $\nu = \text{frequency}$. $\nu = E/h = \lambda/h$ since λ is energy.) This λ is the energy from the potential energy curve minimum of the reactants (R in section 4) to where the vertical hits the curve of the products.

Marcus was the first to calculate λ . He also realized that the leap of the electron only requires $\lambda/4$ via the crossing point of the R and P curves. The activation energy (E_a) = $\lambda/4$.

Thus, the reaction rate (or electron transfer rate), $k_{et} = \nu \exp(-E_a/kT)$, where $k = \text{Boltzmann constant}$ and $T = \text{temperature (absolute)}$. ν , the preexponential factor, is the vibration frequency of the atoms around their equilibrium positions. The reorganization energy, λ , and hence E_a , varies greatly from one reaction to another. Thus, large variations in electron transfer rates can be explained.

If in a reaction, energy E is liberated, the curve "P" is moved down by energy E (Fig. 4B). The activation energy is decreased and is, instead, $E_a = (E - \lambda)^2 / 4\lambda$. This is borne out by experimental results. When the liberated energy E is equal to λ , then $E_a = 0$ and the reaction is very fast. Since energy is released, E is negative and thus, ΔG is negative, the correct equation is $(\Delta G + \lambda)^2 / 4\lambda$.

If, on the other hand, the liberated energy E is large than λ (the so-called inverted region), we have an interesting situation (Fig. 4C): E_a increases with E . That is, the reaction is slower, greater the energy liberated. This remarkable result has also been experimentally confirmed.

Another point: λ depends also upon the solvent. If the dielectric constant is large (as in water) λ is large. Thus, water is a poor solvent for fast electron transport.

6. General Electron Transfer Theory

The appropriate equation for the electron transfer rate, k_{et} , is:

$$\frac{2\pi}{\hbar} V^2 FC$$

Here, V^2 is the quantum mechanical matrix element that couples the reactant and product electronic states and is dependent upon the extent of the overlap of the reactant and product wave functions. $\hbar = h/2\pi$. FC is the so-called Franck-Condon weighted density

* A personal note: Prof. Marcus attended all my lectures one semester when he was at Urbana! I feel great about it.

of states and reflects the integrated overlap of the reactant and product nuclear wavefunctions.

In biology, V^2 is the continuously decreasing overlap of electronic wavefunctions of the redox centers with increasing distance between them:

$$V^2 = V_0^2 e^{-\beta R}$$

where, R is the distance and β a constant. Les Dutton's research group has plotted for many systems log ket versus distance (see Figure 5) and shown that distance is the most significant parameter in a large number of cases. β was calculated to be 1.4 \AA^{-1} .

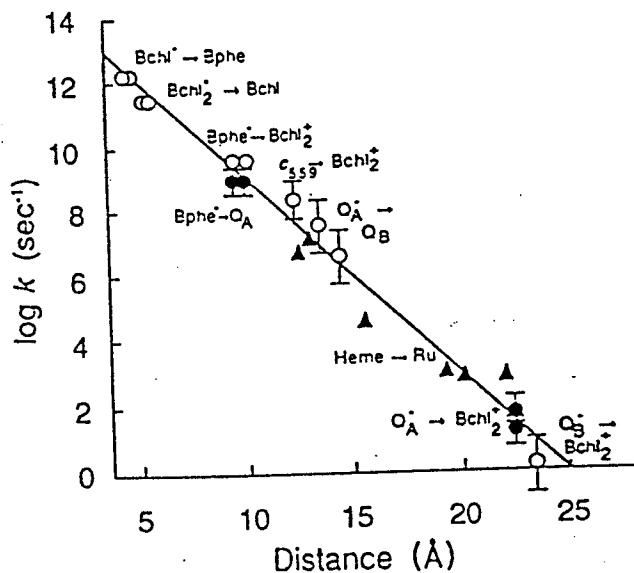


FIGURE 5 The free energy optimized rate vs. edge-to-edge distance relationship for intraprotein electron transfer. Photosynthetic reaction center rates are shown (○) and excited heme-ruthenium electron transfers in modified myoglobin and cytochromes *c* are shown (△). Filled symbols represent reactions with extensive free energy dependence, presented in Fig. 2, whereas open symbols represent reactions with a smaller experimental free energy dependence and more uncertain optimal rates, presented in Fig. 3. Error bars are associated with uncertainties in rate optima. For cytochrome *c* to $Bchl_2^+$, Q_A^- to Q_B^- , and Q_B^- to $Bchl_2^+$ electron transfer, this uncertainty represents the λ ranges shown in Fig. 3. The fit line represents a simple exponential decay of Eq. 1 with a β of 1.4 \AA^{-1} . Distance is defined as center of edge atom of donor to center of edge atom of acceptor; thus, the vertical line at 3.6 \AA represents van der Waals contact. Distances were derived from the crystal structures.

Oxygenic Photosynthesis: Membrane Organization and Components

Here I start with a text taken from Whitmarsh and Govindjee (1996):

Chloroplasts - Structure and Organization

In plants, the photosynthetic process occurs inside chloroplasts, which are organelles found in certain cells. Chloroplasts provide the energy and reduced carbon needed for plant growth and development, while the plant provides the chloroplast with CO_2 , water, nitrogen, organic molecules and minerals necessary for the chloroplast biogenesis. Most chloroplasts are located in specialized leaf cells, which often contain 50 or more chloroplasts per cell. Each chloroplast is defined by an inner and an outer envelope membrane and is shaped like a meniscus convex lens that is 5-10 microns in diameter (see **Figure section**), although many different shapes and sizes can be found in plants. For details of chloroplast structure, see Staehlin (1986). The inner envelope membrane acts as a barrier, controlling the flux of organic and charged molecules in and out of the chloroplast. Water passes freely through the envelope membranes, as do other small neutral molecules like CO_2 and O_2 . There is evidence that chloroplasts were once free living bacteria that invaded a non-photosynthetic cell long ago. They have retained some of the DNA necessary for their assembly, but much of the DNA necessary for their biosynthesis is located in the cell nucleus. This enables a cell to control the biosynthesis of chloroplasts within its domain.

Inside the chloroplast is a complicated membrane system, known as the photosynthetic membrane (or thylakoid membrane), that contains most of the proteins required for the light reactions. The proteins required for the fixation and reduction of CO_2 are located outside the photosynthetic membrane in the surrounding aqueous phase. The photosynthetic membrane is

composed mainly of glycerol lipids and protein. The glycerol lipids are a family of molecules characterized by a polar head group that is hydrophilic and two fatty acid side chains that are hydrophobic. In membranes, the lipid molecules arrange themselves in a bilayer, with the polar head toward the water phase and the fatty acid chains aligned inside the membrane forming a hydrophobic core (see **Figure section**). The photosynthetic membrane is vesicular, defining a closed space with an outer water space (stromal phase) and an inner water space (lumen). The organization of the photosynthetic membrane can be described as groups of stacked membranes (like stacks of *roti* or *chapati* with the inner pocket representing the inner aqueous space), interconnected by non-stacked membranes that protrude from the edges of the stacks . Experiments indicate that the inner aqueous space of the photosynthetic membrane is likely continuous inside of the chloroplast. It is not known why the photosynthetic membrane forms such a convoluted structure. To understand the energetics of photosynthesis the complicated structure can be ignored and the photosynthetic membrane can be viewed as a simple vesicle.

Reference

Staehelin, L.A. (1986) Chloroplast structure and supermolecular organization of photosynthetic membranes. *Encyclopedia of Plant Physiology* New Series 19:1-84.

Topic: Membrane Organization and Components

(1) Membrane Organization in Higher Plants

- (A) **Chloroplast:** (Figure 1) It has two envelope membranes, outer and inner, plus thylakoid membranes (thylakoid = "sac" like, in Greek); there are 20-100 chloroplasts/cell. A chloroplast has a diameter ranging from 3-30 μm . (*Peperomia* chloroplasts are "giant" in size.) One average chloroplast may occupy $3.3 \times 10^{-17} \text{ m}^3$. An average chloroplast may contain about 10^9 chlorophyll molecules.
- (B) **Thylakoid membrane:** (Figure 2) The total thylakoid area per average chloroplast is about $6 \times 10^{-10} \text{ m}^2$, *i. e.*, (roughly) about 10^{-18} m^2 per chlorophyll molecule, or about 10^6 m^2 per mole of chlorophyll.
- In higher plants, thylakoid membranes are organized into grana (appressed or stacked regions) and stroma lamellae (unappressed or unstacked regions).
 - Stroma lamellae are those that connect one granum with another (Figures 3 and 4)
- (C) **Particles in membranes:** (Figures 5 and 6) Freeze fracture electron microscopy has revealed tremendous details about the thylakoid membranes, particularly the presence of "particles" that must be ultimately related to the photosynthetic components. Nomenclature for surfaces (S) and fracture faces (F) in these studies are:

EFs: Endoplasmic (E, in contact with thylakoid lumen) fractured face (F) in the appressed (grana or stacked, s) region. These have large particles (15 and 11 nm) at a density of $1500 \mu\text{m}^{-2}$ (perhaps, they are photosystem II and light-harvesting complex-II); there are also small (10nm) particles, about $700 \text{ particles } \mu\text{m}^{-2}$.

EFu: Endoplasmic (E, in contact with lumen) fractured face (F) in the unappressed (stroma or unstacked, u) region. They contain small (10nm) particles, $450\text{-}600 \text{ particles } \mu\text{m}^{-2}$; these may be some type of photosystem II particles ($\text{PSII}\beta$).

PFs: Protoplasmic (P, in contact with the stromal side) fractured face (F) in the appressed (grana or stacked, s) region. These have deep pits to fit EFs particles. Also, they have $3,500\text{-}4,500 \text{ particles } \mu\text{m}^{-2}$, about 8nm in diameter.

PFu: Protoplasmic (P, in contact with the stromal side) fractured face (F) in the unappressed (stroma or unstacked, u) region. These have pits to fit the EFu particles and 8 and 11 nm particles, about $3,600 \text{ particles } \mu\text{m}^{-2}$. These could be photosystem I, coupling factor zero (CF₀) particles, etc.

PSu: Protoplasmic surface of unstacked region (by now, you know what it stands for). It contains ribulose-bis-phosphate carboxylase-oxygenase (RUBISCO) loosely attached to it and the CF₁ (the knobs of ATP synthase)

PSs: Protoplasmic surface of stacked region (you know what it means, right?). It also shows RUBISCO and CF₁.

ESs: Endoplasmic surface of stacked region (Figure 6). It contains water splitting complex particles (perhaps, the 17, 23, and 33 kDa polypeptides).

Note: Cytochrome b_6/f complexes are present in both unstacked and stacked regions and some of the particles must represent these complexes!

(2) Components (Figure 7)

(A) Four major protein complexes:

- 1) **Photosystem II Complex:** Photosystem II reaction center proteins D1, D2, cytochrome b_{559} , etc; CP-43 and CP-47; CP-24 and CP-29; light-harvesting complex-II (LHC-II), and several small molecular weight proteins
- 2) **Cytochrome b_6/f complex:** a Rieske Fe-S protein; a 17 kDa protein; cytochrome b_L ; cytochrome b_H ; and a small molecular weight protein
- 3) **Photosystem I complex:** to be discussed later -- contains several polypeptides for photosystem I reaction center and its environment and light-harvesting complex - I (LHC-I) proteins
- 4) **ATP synthase:** to be discussed later-- contains several polypeptides in its CF_1 part and in its CF_0 part.

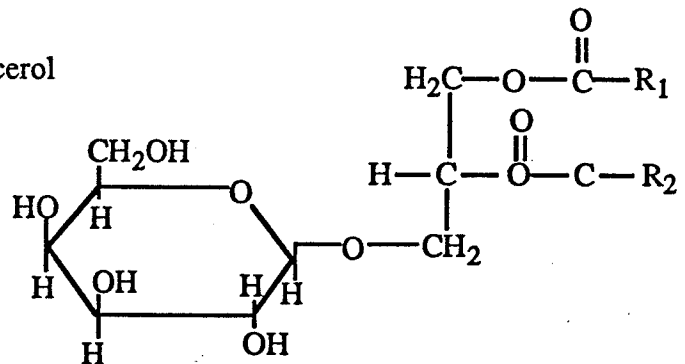
Photosystem II is also known as water-plastoquinone oxidoreductase. Cyt. b_6/f is also known as plastoquinol - plastocyanin oxidoreductase, and Photosystem I as plastocyanin ferredoxin oxidoreductase. All these names specify their functions just as ATP synthase specifies the function of $CF_0 - CF_1$ complex. Pigments (chlorophylls and carotenoids) are contained in several, not all, polypeptides of Photosystem I and II only.

Light absorbed in Photosystems I and II regulates energy distribution between the two systems (Figure 8).

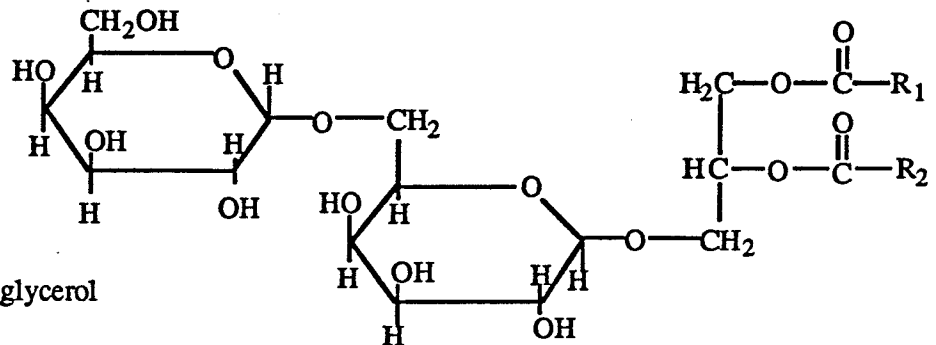
(B) Lipids:

- 1) **Galactolipid: Monogalactosyl diacylglycerol (MGDG)** - 40% of total acyl lipid. It is the most abundant lipid in the biosphere. It is highly unsaturated—provides low viscosity and high fluidity to thylakoid membranes; it is located preferentially in the inner "leaflet" of the membrane (endoplasmic side). It is neutral at neutral pH.

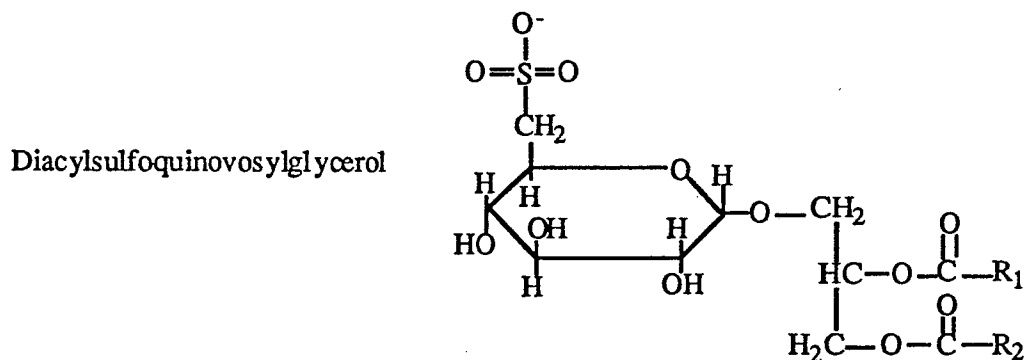
Monogalactosyldiacylglycerol



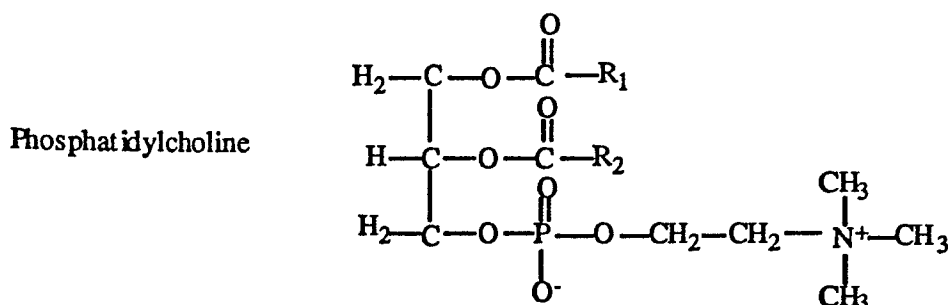
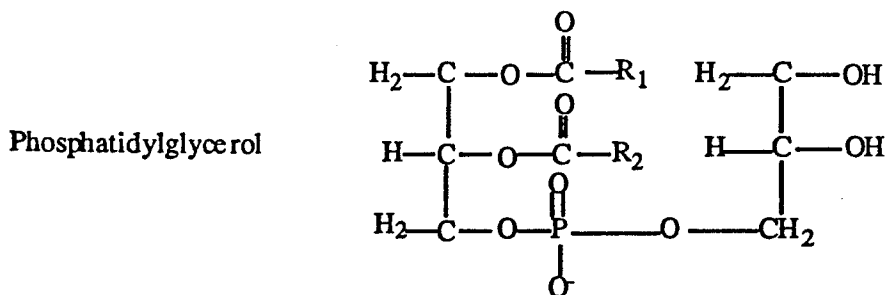
- 2) **Galactolipid: Digalactosyl acylglycerol (DGDG)** - 30% of total acyl lipid. It is preferentially located in the outer "leaflet" of the thylakoid membrane (protoplasmic side).



- 3) **Sulfolipid: Diacylsulfoquinovosyl glycerol** (galactose has sulphate on it):
10% of the total acyl lipids. It is anionic at neutral pH and is mostly in the inner "leaflet" of the thylakoid membrane.



- 4) **Phospholipids: phosphatidylglycerol** - 15% of total acyl lipids, is anionic at neutral pH; **phosphatidylcholine** - only 5% of total acyl lipids, is dipolar ionic at neutral pH. These are evenly distributed on the two leaflets of the thylakoid membranes.



(C) Mobile electron carriers:

Plastoquinone (lipidic/soluble in lipids) and plastocyanin (a copper protein). Asymmetry is "absolute" in terms of organization of components on the thylakoid membrane. Electron flow leads to oxidation of water to O_2 and reduction of $NADP^+$ to $NADPH$, but protons are translocated from outside to inside: protons are taken up where plastoquinone is reduced to plastoquinol (outside) and released where H_2O is oxidized (inside, photosystem II) and where plastoquinol is oxidized (inside, cyt b_6/f complex). This proton gradient, when dissipated through ATP synthase complex, produces ATP.

(3) Genetic Coding of the Various Polypeptides (Figure 7)

(A) Nuclear coded:

- LHC-II (28, 26, 24 kDa proteins);
- extrinsic (peripheral) 17, 23 and 33 kDa proteins of oxygen-evolving complex;
- 4 polypeptides (labeled as II, III, V and VI, about 25, 20, 16 and 8 kDa) of photosystem I complex;
- 4 polypeptides from 19 to 24 kDa of LHC-I;
- 20 kDa protein of Cyt b_6/f complex;
- γ (37 kDa in CF_1), δ (18 kDa in CF_1) and II (11 kDa in CF_0) polypeptides of ATP synthase;
- ferredoxin (11 kDa);
- plastocyanin (10.5 kDa); and
- ferredoxin - $NADP^+$ reductase (33 kDa).

(B) Chloroplast coded:

- CP-47, CP-43, D2, D1 and a 10 kDa protein of Photosystem II complex;
- subunit Ia and Ib and perhaps subunit IV (18 kDa) of Photosystem I complex;
- 33, 34 and 17 kDa proteins of Cyt b_6/f complex;
- α (59 kDa in CF_1), β (56 kDa in CF_1), ϵ (13 kDa in CF_1) and I (17 kDa in CF_0) and IV (9 kDa in CF_0) polypeptides of ATP synthase.

It is thus, clear that chloroplast structure and function would depend upon the DNA's of both the nucleus and the chloroplast. Furthermore, note that all the 4 protein complexes have one or more polypeptides coded by nuclear DNA.

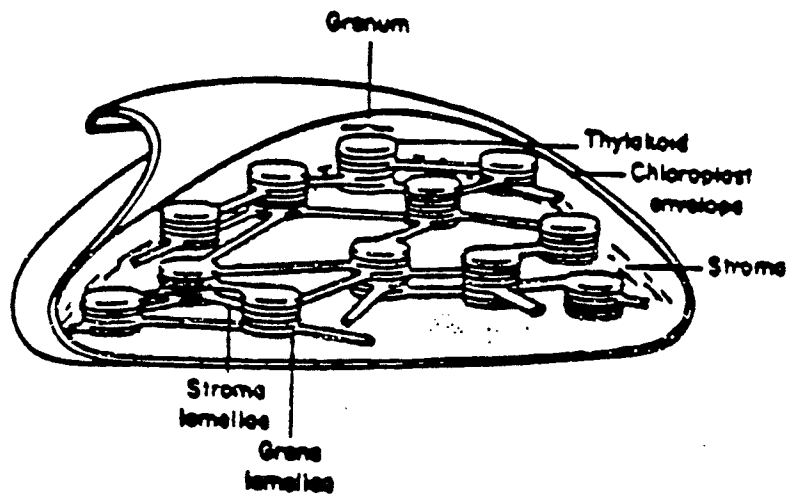


Fig. 1. A small portion of a lettuce (*Lactuca sativa*) leaf cell showing a mature chloroplast. A double membrane surrounds the chloroplast forming the plastid envelope (PE). Within the stroma (S) lie the unpaired stroma lamellae (SL), thylakoids stacked to form grana (G), and large, densely staining lipid droplets, plastoglobuli (P). The smaller, darkly staining droplets localized on the periphery of the grana stacks have been described by Sabnis *et al.* (1970); their chemical nature and physiological function, if any, remains unknown. This sample was prepared for electron microscopy by chemically fixing a portion of the intact leaf, embedding the leaf segment in plastic, and cutting ultrathin sections of the sample on a diamond knife.

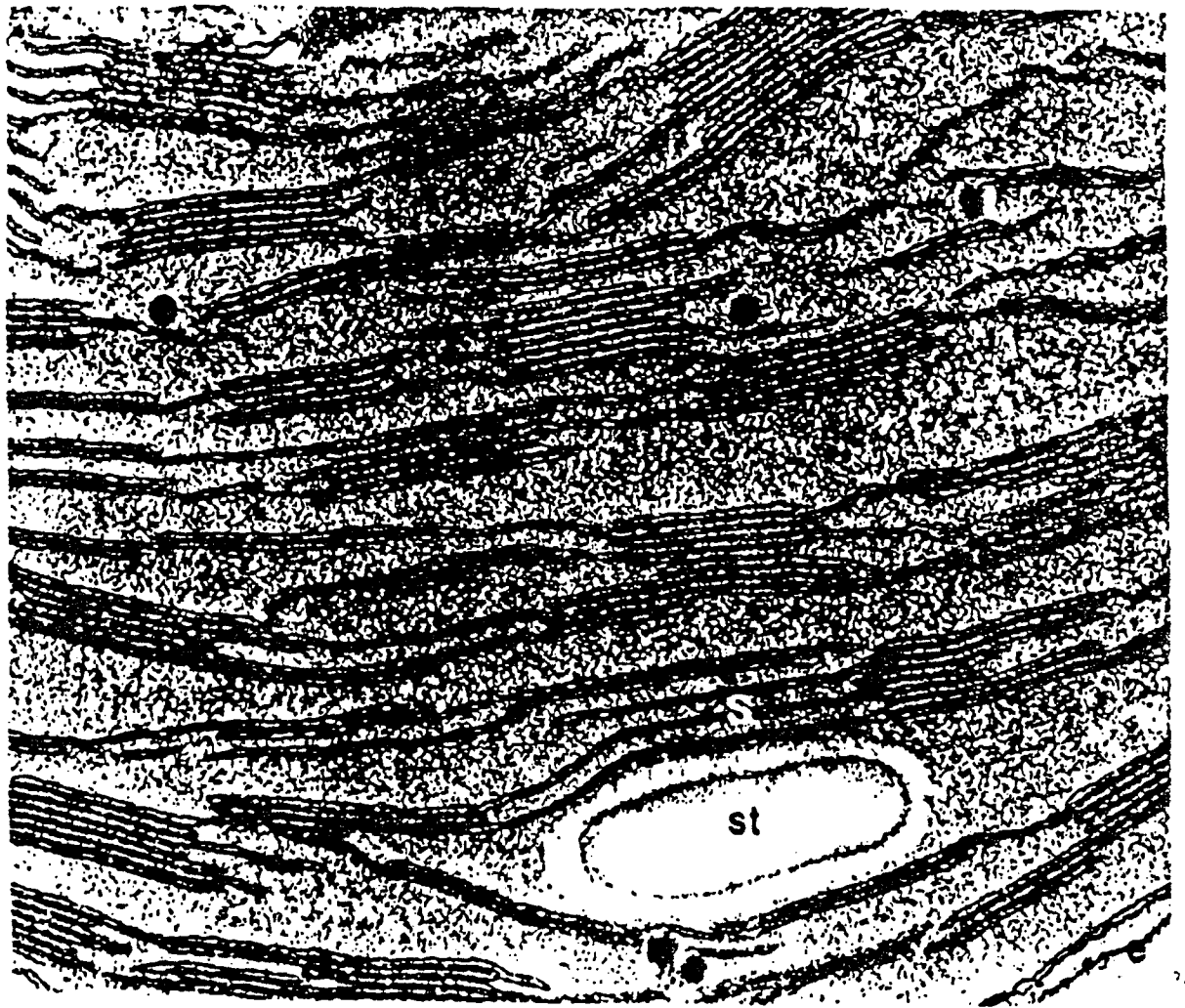
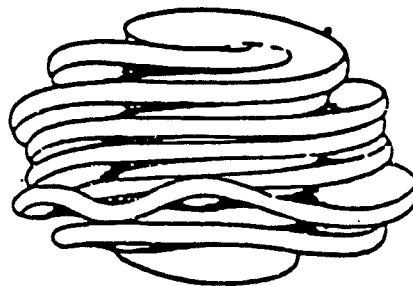
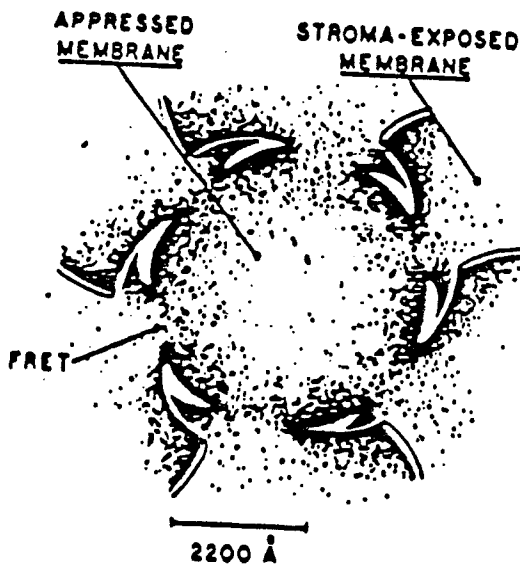


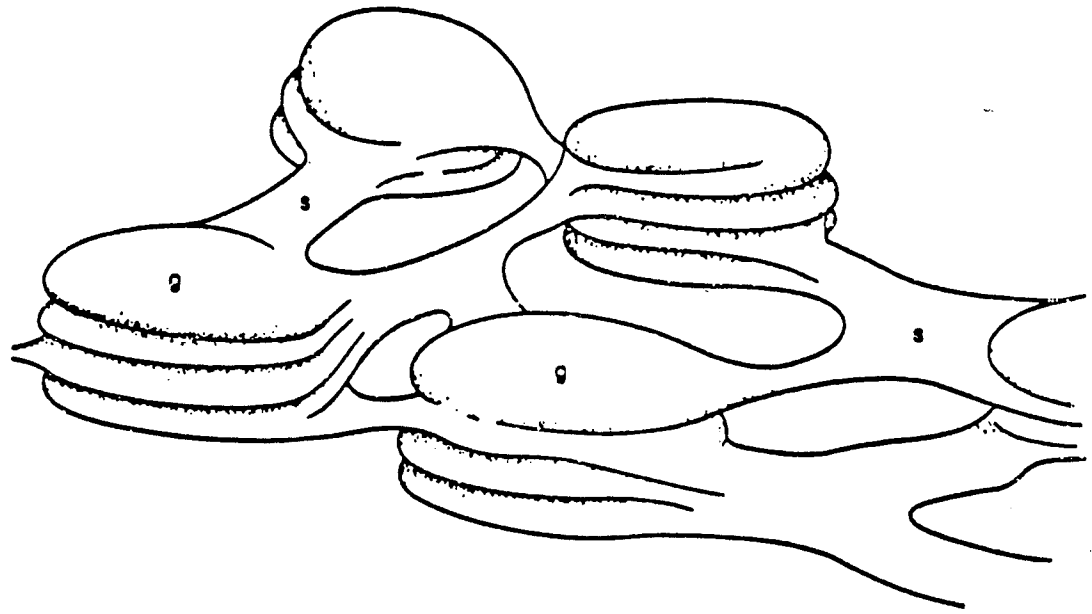
FIG. 2 Transverse section of spinach (*Spinacea oleracea*) chloroplast by electron microscopy ($\times 103\ 000$); thylakoid membranes are associated into grana (g) joined by the stromal thylakoids (S). Osmophilic lipid globules (l) are apparent and a starch granule (st) also. The envelope (e) appears as a single membrane at the lower right hand corner. Courtesy of Dr A.D. Greenwood, Imperial College, London.



Appearance of thylakoid membranes from scanning electron microscope studies.



Top view of cut through grana stack and associated stroma membrane



(a)



ES_s

(b)

FIG. 4.4(a) Diagram of the connections between granal (g) and stromal (s) thylakoids. (b) Spinach chloroplasts showing interconnections (—) between the granal thylakoids (ES_s) and a single stromal thylakoid (ES_u). Electron micrograph ($\times 185\,000$) courtesy of Professor L.A. Staehelin, University of Colorado, Boulder, Colorado, USA.

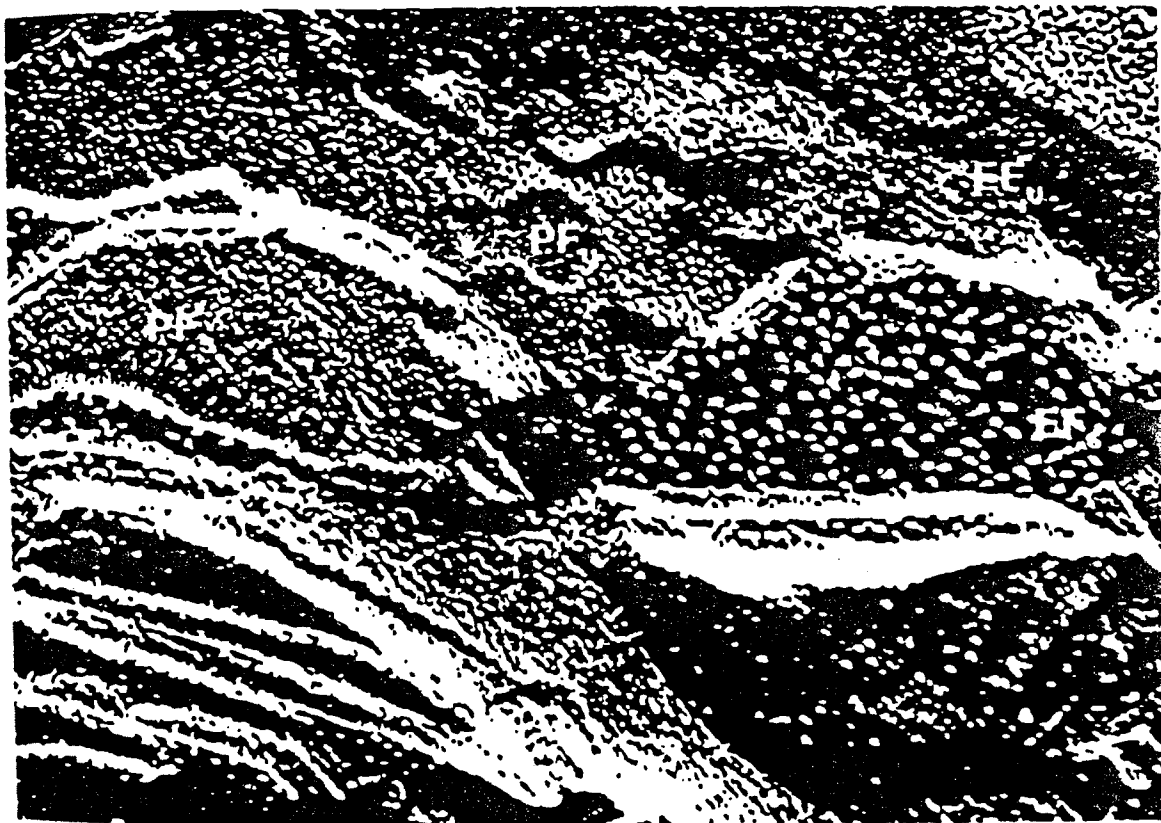
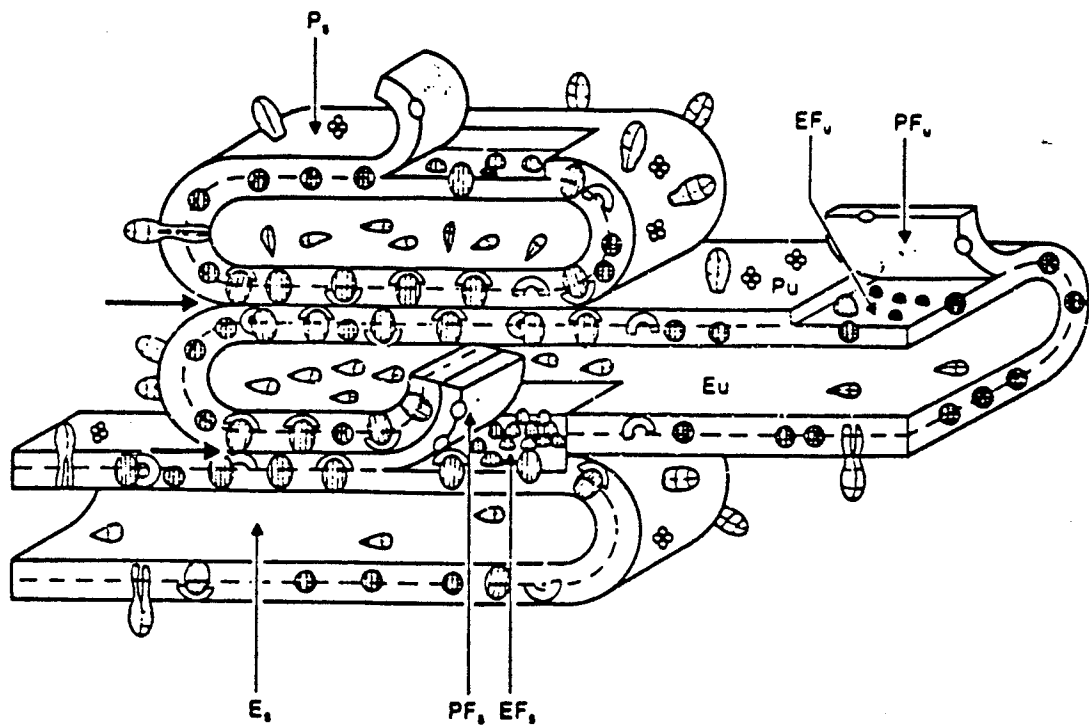


FIG. 5 Freeze-fractured granal stacks of isolated thylakoids of a spinach chloroplast, showing the EF and PF faces of stacked and unstacked areas. Note the characteristic large (15 nm) particles on EF_s. The stacks are linked by a sheet of unstacked membrane (l). Electron micrograph ($\times 185\ 000$) by courtesy of Professor A.L. Staehelin, University of Colorado, Boulder, Colorado, USA.

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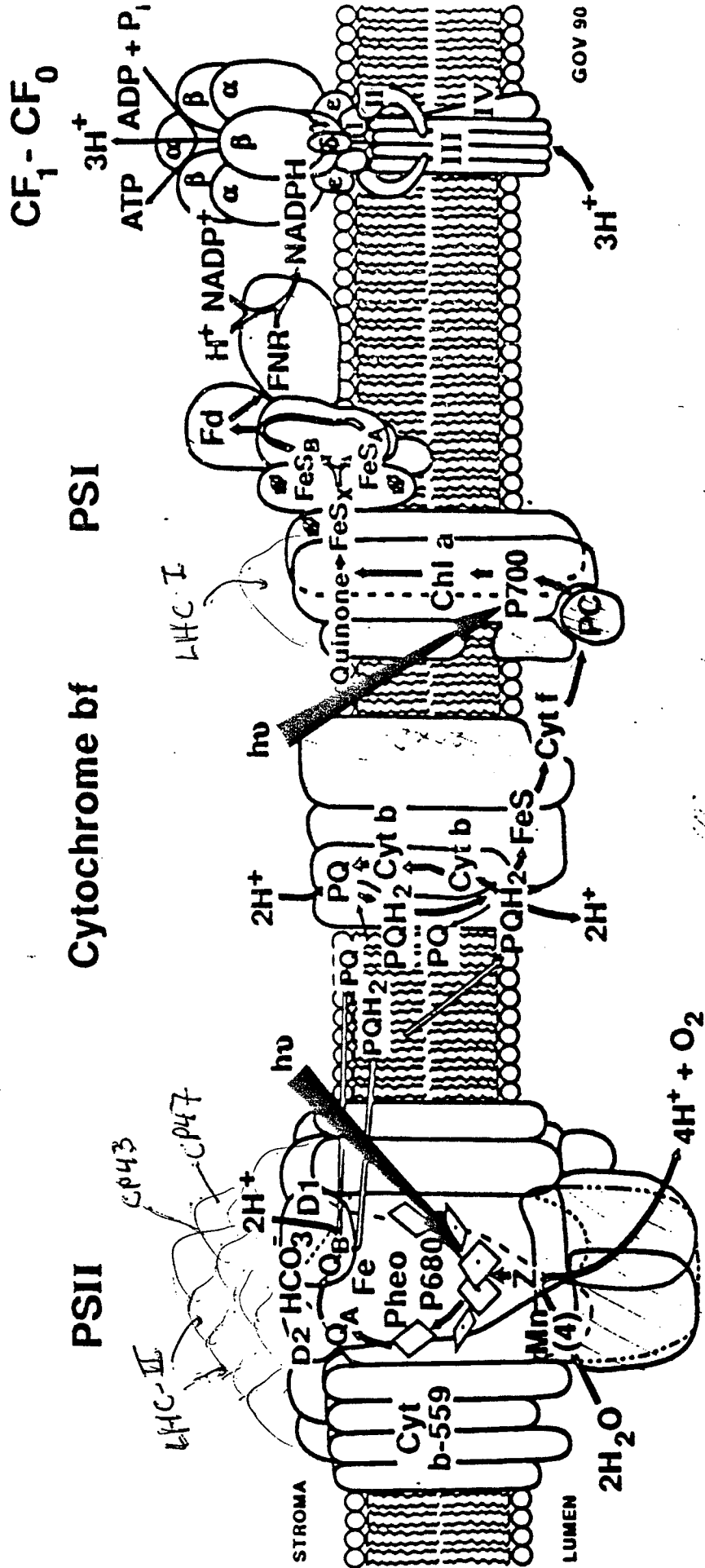
Symbol	Component		
	RuBP carboxylase		LHC entire or projecting
	Water-splitting complex		LHC plus PSII
	PSII entire or projecting		CF ₁ joined to CF ₀ in membrane
	PSI entire or projecting		
	Pits in membrane		

FIG. 6 Diagram of the thylakoids of a higher (C_3) plant chloroplast. Part of a granal stack and stromal thylakoid is shown. Coupling factor (CF_1) and ribulose biphosphate carboxylase (RuBPc) are attached but not in the partition region (\rightarrow). In the lumen of the granal thylakoids are particles of the photosystem II, water-splitting complex. The partition region contains particles on the endoplasmic fracture face (EF_s) identified with PSII and the light-harvesting protein complex. Smaller particles on other membranes may be PSI and the base of coupling factor. See text for explanation of membrane surfaces and particles.

Figure 7



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Govindjee
#5

Two Photosystems and Two light Reactions: in Plants and in Cyanobacteria, but not in Photosynthetic Bacteria

First, a description from Whitmarsh and Govindjee (1996) on the two-photosystems and two light reactions (slightly modified):

OXYGENIC PHOTOSYNTHESIS

It was Emerson's work on the Emerson enhancement effect that led to the concept of two-light reactions and two-pigment system concept, as enunciated by Duysens (see Emerson, 1958; and Duysens, 1989).

A. Light Absorption - The Antenna System

Plant photosynthesis is driven primarily by visible light (wavelengths from 400 to 700 nm) that is absorbed by pigment molecules (mainly chlorophyll *a* and *b* and carotenoids). The chemical structure of chlorophyll *a* molecule was shown earlier. In chlorophyll *b*, CH₃ in ring II is replaced by CHO group. Plants appear green because of chlorophyll, which is so plentiful that regions of the earth appear green from space. The absorption spectrum of chloroplast chlorophyll *a* and *b* and carotenoids along with the action spectrum of photosynthesis of a chloroplast is shown in the **Figure section**. Light is collected by 200-300 pigment molecules that are bound to light-harvesting protein complexes located in the photosynthetic membrane. And, there are two such systems: Pigment system I and Pigment system II. The light-harvesting complexes surround the reaction centers (reaction center I and reaction center II) that serve as an antenna. The three-dimensional structure of the light-harvesting complex, called LHCI**II**b (obtained by electron diffraction : see Kühlbrandt et al., 1994) shows that the protein determines the position and

orientation of the antenna pigments. Photosynthesis is initiated by the absorption of a photon by an antenna molecule, which occurs in about a femtosecond (10^{-15} s) (see Govindjee # 1) and causes a transition from the electronic ground state to an excited state. Within 10^{-13} s the excited state decays by vibrational relaxation to the first excited singlet state. The fate of the excited state energy is guided by the structure of the protein. Because of the proximity of other antenna molecules with the same or similar energy states, the excited state energy has a high probability of being transferred by resonance energy transfer to a near neighbor. Exciton energy transfer between antenna molecules is due to the interaction of the transition dipole moment of the molecules. The probability of transfer is dependent on the distance between the transition dipoles of the donor and acceptor molecules ($1/R^6$), the relative orientation of the transition dipoles, and the overlap of the emission spectrum of the donor molecule with the absorption spectrum of the acceptor molecule (see van Grondelle and Amesz, 1986; Govindjee # 2). Photosynthetic antenna systems are very efficient at this transfer process. Under optimum conditions over 90% of the absorbed quanta are transferred within a few hundred picoseconds from the two antenna systems to their reaction centers which acts as a trap for the exciton. [It seems that the reaction center complexes of Photosystem I include quite a bit of antenna chlorophyll a molecules, but that of Photosystem II are more like the bacterial reaction center (see Govindjee# 3).]

B. Primary Photochemistry - Photosystem II and Photosystem I Reaction Centers

Photosystem II uses light energy to drive two chemical reactions - the oxidation of water and the reduction of plastoquinone. Details will be presented elsewhere. A brief summary follows. The photosystem II complex is composed of more than fifteen polypeptides and at least nine different redox components (chlorophyll, pheophytin, plastoquinone, tyrosine, Mn, Fe, cytochrome b559, carotenoid and histidine) have been shown to undergo light-induced electron transfer (Debus, 1992). However, only five of these redox components are known to be involved in transferring electrons from H_2O to the plastoquinone pool - the water oxidizing manganese cluster $(Mn)_4$, the amino acid tyrosine, the reaction center chlorophyll (P680), pheophytin, and the plastoquinone molecules, Q_A and Q_B . Of these essential redox components, tyrosine, P680, pheophytin, Q_A and Q_B have been shown to be bound to two key polypeptides that form the

heterodimeric reaction center core of photosystem II (D1 and D2). Recent work indicates that the D1 and D2 polypeptides also provide ligands for the $(Mn)_4$ cluster. The three-dimensional structure of photosystem II is not known. Our knowledge of its structure is guided by the known structure of the reaction center in purple bacteria and biochemical and spectroscopic data.

Photochemistry in photosystem II is initiated by charge separation between P680 and pheophytin, creating $P680^+/Pheo^-$. The two major proteins of the Photosystem II reaction center are labeled as D1 and D2 proteins that are somewhat equivalent to L and M subunits of bacterial reaction centers. Primary charge separation takes about a few picoseconds. A controversy looms whether it takes 3 picoseconds or 20 picoseconds. Subsequent electron transfer steps have been designed through evolution to prevent the primary charge separation from recombining. This is accomplished by transferring the electron within 200 picoseconds from pheophytin to a plastoquinone molecule (Q_A) that is permanently bound to photosystem II. Although plastoquinone normally acts as a two-electron acceptor, it works as a one-electron acceptor at the Q_A -site. The electron on Q_A^- is then transferred to another plastoquinone molecule that is loosely bound at the Q_B -site. Plastoquinone at the Q_B -site differs from Q_A in that it works as a two-electron acceptor, becoming fully reduced and protonated after two photochemical turnovers of the reaction center. The full reduction of plastoquinone requires the addition of two electrons and two protons, *i.e.*, the addition of two hydrogen atoms (cf. Govindjee # 3). The reduced plastoquinone then debinds from the reaction center and diffuses into the hydrophobic core of the membrane. After which, an oxidized plastoquinone molecule finds its way to the Q_B -binding site and the process is repeated. Because the Q_B -site is near the outer aqueous phase, the protons added to plastoquinone during its reduction are taken from the outside of the membrane.

Photosystem II is the only known protein complex that can oxidize water, resulting in the release of O_2 into the atmosphere. Despite years of research, little is known about the molecular events that lead to water oxidation. Energetically, water is a poor electron donor. The oxidation-reduction midpoint potential ($E_{m,7}$) of water is +0.82 V (pH 7). In photosystem II this reaction is driven by the oxidized reaction center, $P680^+$ (the midpoint potential of $P680/P680^+$ is estimated to

be +1.2 V at pH 7). How electrons are transferred from water to P680⁺ remains a mystery (Govindjee and Coleman, 1990). It is known that P680⁺ oxidizes a specific tyrosine on the D1 protein and that Mn plays a key role in water oxidation. Four Mn ions are present in the water oxidizing complex. X-ray absorption spectroscopy shows that Mn undergoes light-induced oxidation. Water oxidation requires two molecules of water and involves four sequential turnovers of the reaction center. This was shown by an experiment demonstrating that oxygen release by photosystem II occurs with a four flash dependence (Joliot et al., 1969; Joliot and Kok, 1975). Each photochemical reaction creates an oxidant that removes one electron. The net reaction results in the release of one O₂ molecule, the deposition of four protons into the inner water phase, and the transfer of four electrons to the Q_B-site (producing two reduced plastoquinone molecules) (reviewed by Renger, 1993; Klein et al., 1993; and Lavergne and Junge, 1993).

Photosystem II reaction centers contain a number of redox components with no known function. An example is cytochrome b559, a heme protein, that is an essential component of all photosystem II reaction centers (discussed by Whitmarsh and Pakrasi, 1996). If the cytochrome is not present in the membrane, a stable PS II reaction center cannot be formed. Although the structure and function of Cyt b559 remain to be discovered, it is known that the cytochrome is not involved in the primary enzymatic activity of PS II, which is the transfer of electrons from water to plastoquinone. Why PS II reaction centers contain redox components that are not involved in the primary enzymatic reactions is a puzzling question. The answer may be found in the unusual chemical reactions occurring in PS II and the fact that the reaction center operates at a very high power level. Photosystem II is an energy transforming enzyme that must switch between various high energy states that involve the creation of the powerful oxidants required for removing electrons from water and the complex chemistry of plastoquinone reduction which is strongly influenced by protons. In saturating light a single reaction center can have an energy throughput of 600 eV/s (equivalent to 60,000 kW per mole of PS II). Operating at such a high power level results in damage to the reaction center. It may be that some of the "extra" redox components in photosystem II may serve to protect the reaction center.

Photosystem II has another perplexing feature. Many plants and algae have been shown to have a significant number of photosystem II reaction centers that do not contribute to photosynthetic electron transport. Why plants devote resources for the synthesis of reaction centers that apparently do not contribute to energy conversion is unknown (for reviews of photosystem II heterogeneity, see Melis, 1991; Lavergne and Briantais, 1996; Whitmarsh et al., 1996)

The **photosystem I** complex catalyzes the oxidation of plastocyanin, a small soluble Cu-protein, and the reduction of ferredoxin, a small FeS protein. Photosystem I is composed of a heterodimer of proteins that act as ligands for most of the electron carriers (Krauss et al., 1993). The reaction center is served by an antenna system that consists of about two hundred chlorophyll molecules (mainly chlorophyll *a*) and primary photochemistry is initiated by a chlorophyll *a* dimer, P700. As mentioned earlier, in contrast to photosystem II, many of the antenna chlorophyll molecules in photosystem I are bound to the reaction center proteins. Also, FeS centers serve as electron carriers in photosystem I and, so far as is known, photosystem I electron transfer is not coupled to proton translocation. Primary charge separation occurs between a primary donor, P700, a chlorophyll dimer, and a chlorophyll monomer (A_0). (see Golbeck, 1994).

C. Electron Transport

Electron transport from water to $NADP^+$ requires three membrane bound protein complexes operating in series - photosystem II, the cytochrome *bf* complex and photosystem I. Electrons are transferred between these large protein complexes by small mobile molecules (plastoquinone and plastocyanin in plants). Because these small molecules carry electrons (or hydrogen atoms) over relatively long distances, they play a unique role in photosynthetic energy conversion. This is illustrated by plastoquinone (PQ), which serves two key functions. Plastoquinone transfers electrons from the photosystem II reaction center to the cytochrome *bf* complex and carries protons across the photosynthetic membrane (see Kallas, 1994). It does this by shuttling hydrogen atoms across the membrane from photosystem II to the cytochrome *bf* complex. Because plastoquinone is hydrophobic its movement is restricted to the hydrophobic core of the photosynthetic membrane.

Plastoquinone operates by diffusing through the membrane until, due to random collisions, it becomes bound to a specific site on the photosystem II complex. The photosystem II reaction center reduces plastoquinone at the Q_B -site by adding two electrons and two protons creating PQH_2 . The reduced plastoquinone molecule debinds from photosystem II and diffuses randomly in the photosynthetic membrane until it encounters a specific binding site on the cytochrome bf complex. The cytochrome bf complex is a membrane bound protein complex that contains four electron carriers, three cytochromes and an FeS center. The crystal structure has been solved for cytochrome f from turnip (Martinez et al., 1994) and the FeS center from bovine heart mitochondria (Iwata et al., 1996). In a complicated reaction sequence that is not fully understood, the cytochrome bf complex removes the electrons from reduced plastoquinone and facilitates the release of the protons into the inner aqueous space. The electrons are eventually transferred to the photosystem I reaction center. The protons released into the inner aqueous space contribute to the proton chemical free energy across the membrane.

Electron transfer from the cytochrome bf complex to photosystem I is mediated by a small Cu-protein, plastocyanin (PC). Plastocyanin is water soluble and operates in the inner water space of the photosynthetic membrane. Electron transfer from photosystem I to $NADP^+$ requires ferredoxin, a small FeS protein, and ferredoxin-NADP oxidoreductase, a peripheral flavoprotein that operates on the outer surface of the photosynthetic membrane. Ferredoxin and $NADP^+$ are water soluble and are found in the outer aqueous phase.

The pathway of electrons is largely determined by the energetics of the reaction and the distance between the carriers (cf. Govindjee # 3). The electron affinity of the carriers is represented by their midpoint potentials, which show the free energy available for electron transfer reactions under equilibrium conditions. (It should be kept in mind that reaction conditions during photosynthesis are not in equilibrium.) Subsequent to primary charge separation, electron transport is energetically downhill (from a lower (more negative) to a higher (more positive) redox potential). It is the downhill flow of electrons that provides free energy for the creation of a proton chemical gradient. Photosynthetic membranes effectively limit electron transport to two

dimensions. For mobile electron carriers, limiting diffusion to two dimensions increases the number of random encounters (Whitmarsh, 1986). Furthermore, because plastocyanin is mobile, any one cytochrome *b₆f* complex can interact with a number of photosystem I complexes. The same is true for plastoquinone, which commonly operates at a stoichiometry of about six molecules per photosystem II complex.

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What follows is a discussion of the pioneering work of Robert Emerson that was the basis for the emerging concept of two-pigment systems and two light reactions in photosynthesis before 1960.

(1) Definitions and Notes:

Action spectrum:: Rate of photochemical activity per incident photon as a function of wavelength of light; at low absorbance, action spectrum follows absorption (absorbance or optical density) spectrum; it follows percent or fractional absorption spectrum at all absorbance values.

Maximum Quantum Yield (or Minimum Quantum Requirement) of O₂ evolution:

At low light intensities where rate of O₂ evolution increases linearly with light intensity, maximum quantum yield of O₂ evolution (# of molecules of O₂ evolved per quantum of light absorbed) was measured by Robert Emerson to be 0.12 (*i.e.*, the minimum quantum requirement was 8 photons/O₂). In contrast, Otto Warburg (a Noble Prize Winner) had

measured the minimum quantum requirement to be 4 or less. Emerson, not Warburg, was right.

Quantum Yield Action Spectrum: Rate of photochemical activity per absorbed photon (# of incident photons x fractional absorption); if all absorbing pigments are equally active, it should be a flat line throughout the spectrum.

Red Drop: Decline in the red end of the spectrum of the action spectrum *before* the decline in absorption spectrum. This is also manifested in a drop in the quantum yield action spectra; instead of a flat line up to 720 nm, Robert Emerson and co-workers discovered a drop in the quantum yield action spectrum around 680 nm in the photosynthesis (O₂ evolution) of green algae (*Chlorella*) and around 650 nm in red algae (*i.e.*, *Porphyridium*) (see Figure section).

Emerson Enhancement Effect: Emerson discovered, working in 155-157 Natural History Building, at the University of Illinois at Urbana, that the rate of photosynthesis (O₂ evolution) was enhanced when two beams of light [light I: far-red 710 nm etc. and light II: red 650 nm (in green algae *Chlorella*) or orange 620 nm (in cyanobacteria) or green 545 nm (in red alga *Porphyridium*)] were given together than when they were given separately. (see Figure section) For Example:

Rate of O₂ evolution, arbitrary units

- (1) In light I = 5
- (2) In light II = 20
- (3) Sum (when given separately) = 25
- (4) In light I + light II (given together) = 40
- (5) Enhancement = (4) - (3) = 15
- (6) Enhancement Ratio was calculated as:

$$\frac{(4)-(2)}{(1)} = \frac{40-20}{5} = 4$$

Discovery of Emerson Enhancement can be easily explained in the postulate that plant and cyanobacterial photosynthesis requires two pigment systems and two light reactions. Govindjee and coworkers showed in 1960 that both the pigment systems used chlorophyll *a*, but having different spectral characteristics (see Figure section).

In 1945, E.I. Rabinowitch had suggested a two-light reaction scheme for transfer of electrons from H₂O to CO₂ (see Figures section) to explain the minimum quantum requirement of 8/O₂. In 1956, Rabinowitch suggested that one light reaction oxidizes cytochrome *f* (as L.N.M. Duysens had found) and another reduces the oxidized cytochrome *f*. The real evidence was provided by Duysens and co-workers (see #3 below).

The above material is described in the updated version of "The Role of Chlorophyll *a* in Oxygenic Photosynthesis" by Rabinowitch and Govindjee (Scientific American, July, 1965).

(2) **The Current Series "Z" Scheme of Electron Flow in Oxygenic Photosynthesis** (see Figure section; and re-read the text from Whitmarsh and Govindjee, 1996).

(3) **Evidences for the Series Model or the "Z" Scheme**

*(A) Antagonistic effect of light I and light II on the redox state of the reaction center chlorophyll *a* P700 of photosystem I (Bessel Kok, 1959) and of the cytochrome *f* (Lou Duysens and coworkers, 1961) [see Figure section]

(B) Physical separation of the photosystems

(C) Chemical manipulations: partial reactions; use of inhibitors, artificial electron acceptors and donors

(D) Genetic/mutant approach

(E) Kinetic measurements of electron flow

*Most important

Some Questions

[1] What is the minimum quantum requirement of photosynthesis in normal oxygenic plants? for oxygen evolution? for carbon di-oxide uptake? How does this information relate to the current two light reaction scheme of photosynthesis?

[2] What are the unique properties of Photosystem II that are not found in any other photosystem in Nature?

[3] What is the crucial experiment that proves the existence of the Series "Z" scheme of photosynthesis?

[4] What were the beginnings of the concept of two-light reaction scheme of photosynthesis? How do you define the "Red drop" in photosynthesis? How do you define and explain the Emerson Enhancement Effect of photosynthesis?

[5] What are the basics of the "Z" scheme of photosynthesis? Where does it occur? Do the two photosystems begin functioning simultaneously, or, does Photosystem II start first?

[6] What are the similarities and differences between Photosystem II and bacterial reaction centers? between Photosystem I and bacterial reaction centers?

- If you are able to get on the World Wide Web and can see graphics, I recommend that you go to my page: <http://www.life.uiuc.edu/govindjee/>
- You will find , if you are able to download, slides that will help you understand some of the material I am presenting or will present. There are several other "packages" there that allow a fuller understanding of some of the concepts presented.

Figure 1

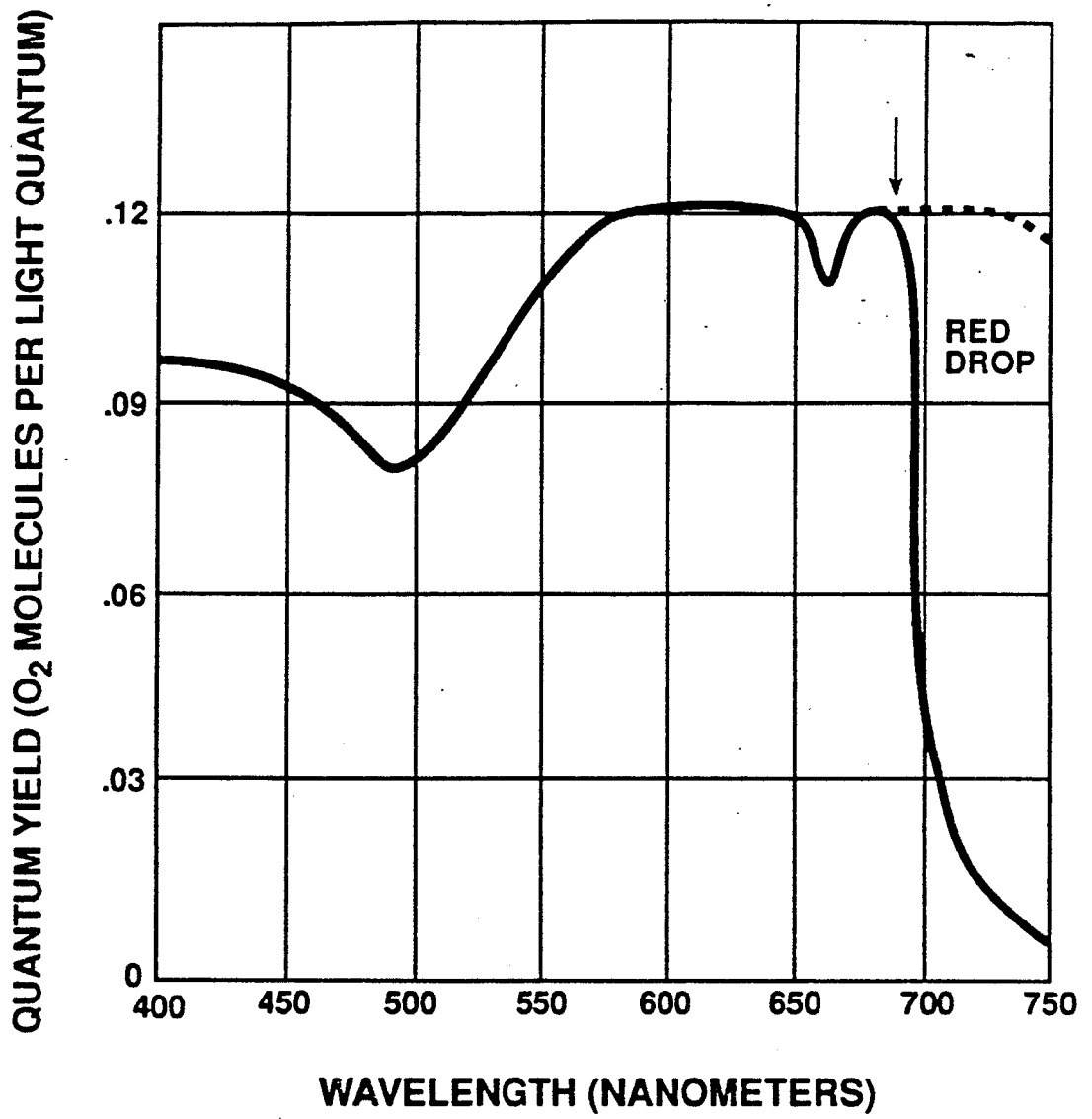


Figure 2

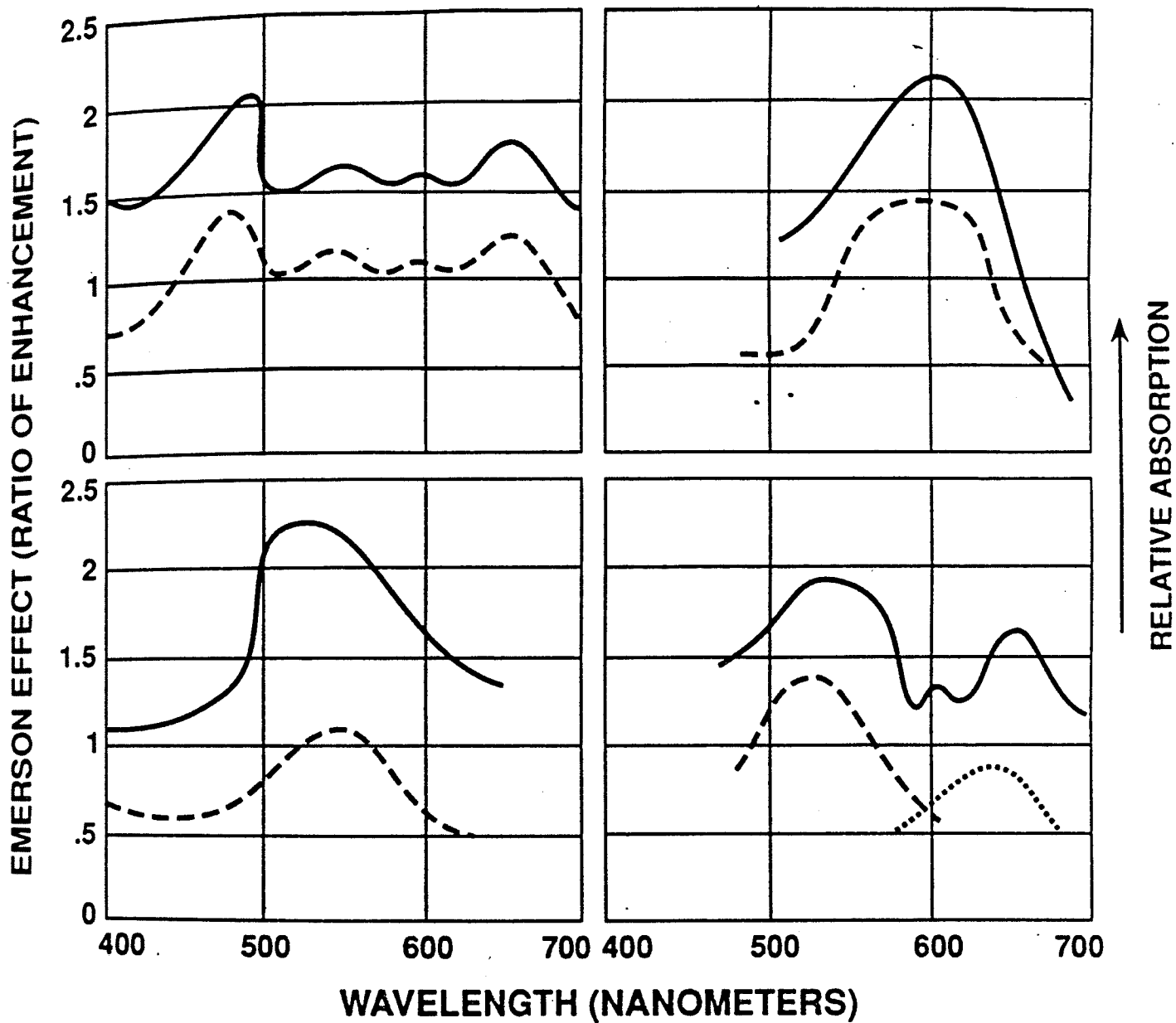


Figure 3

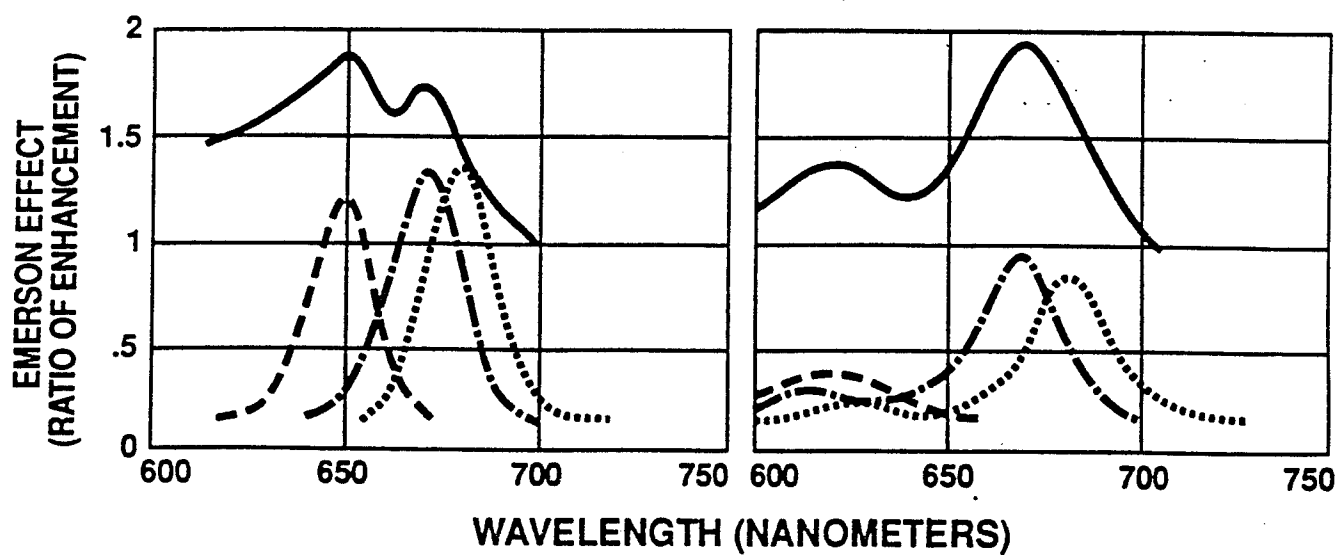


Figure 4

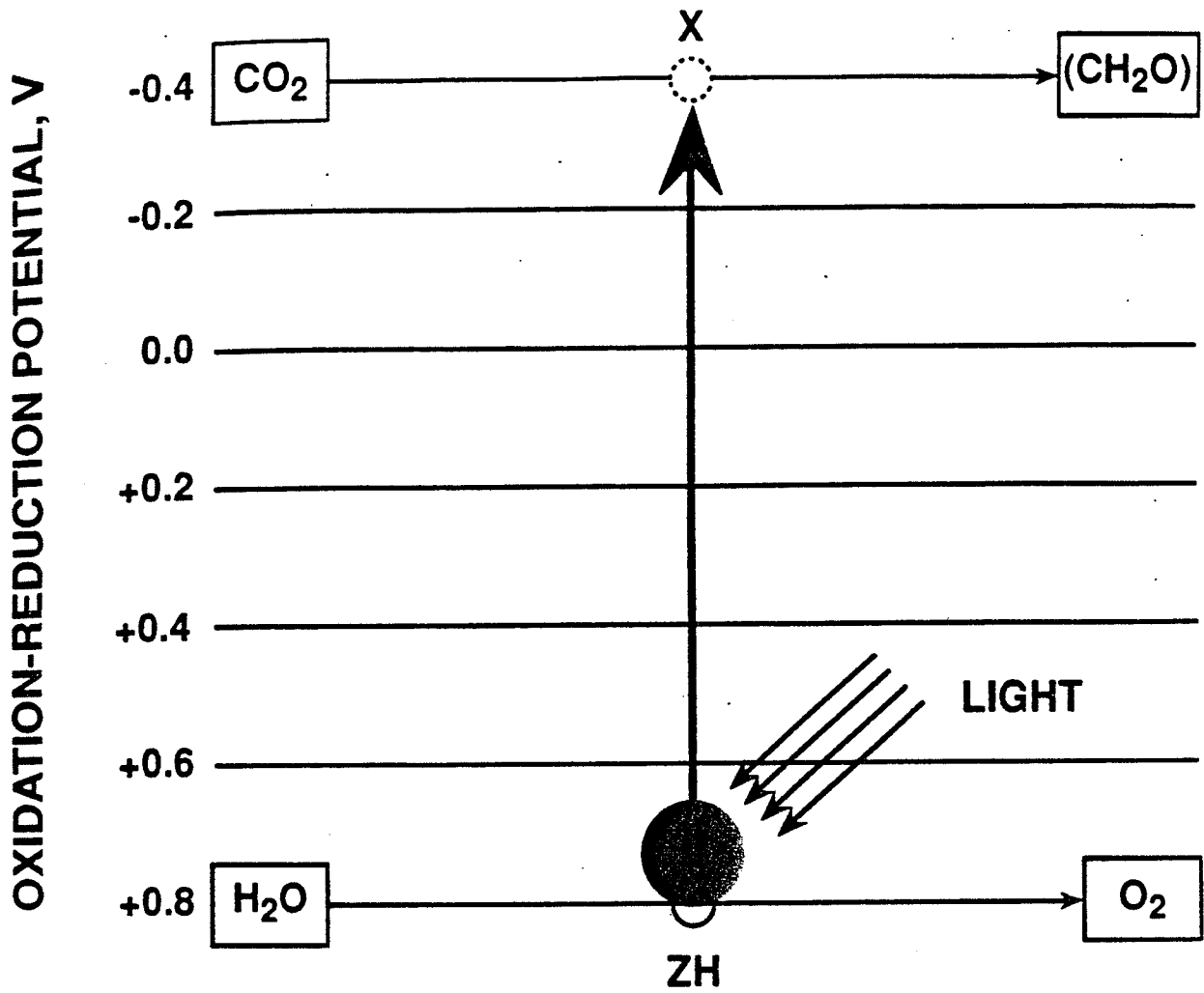


Figure 5

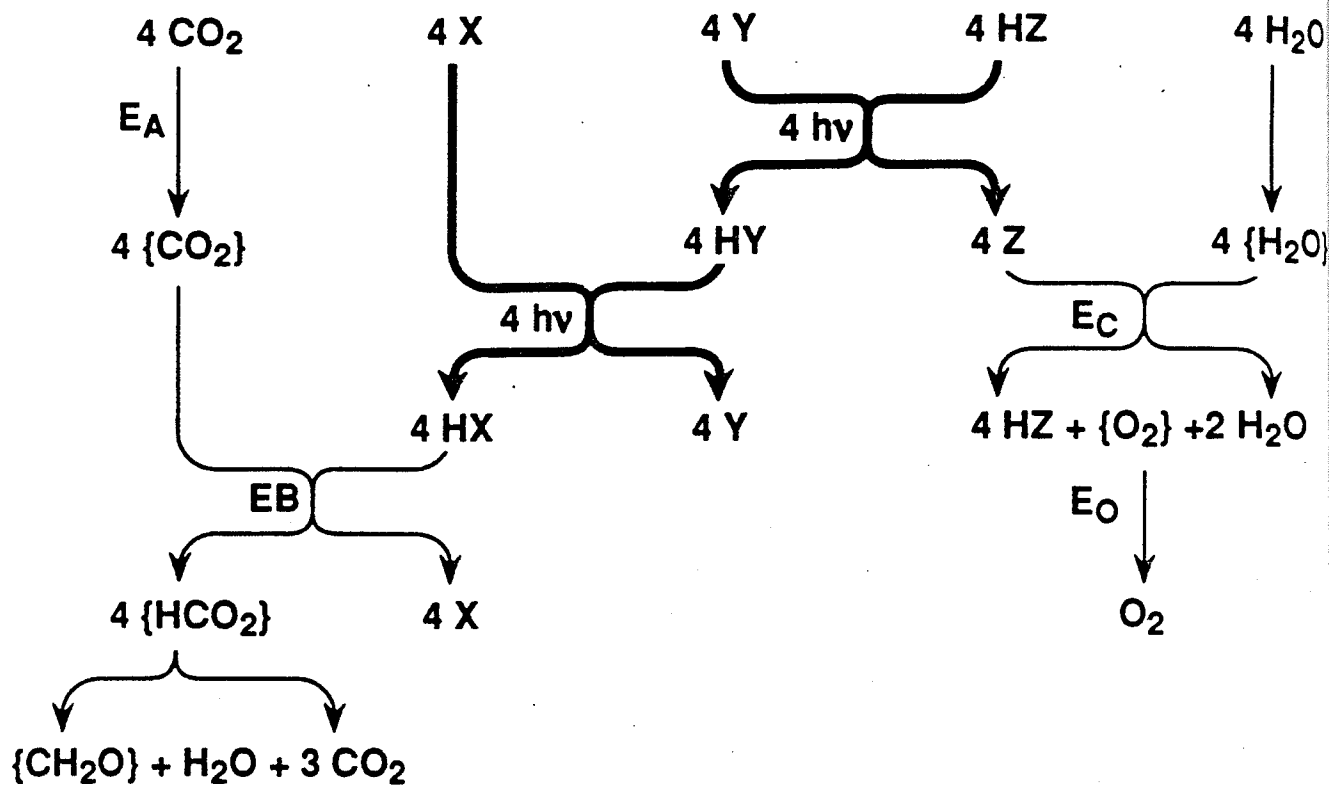
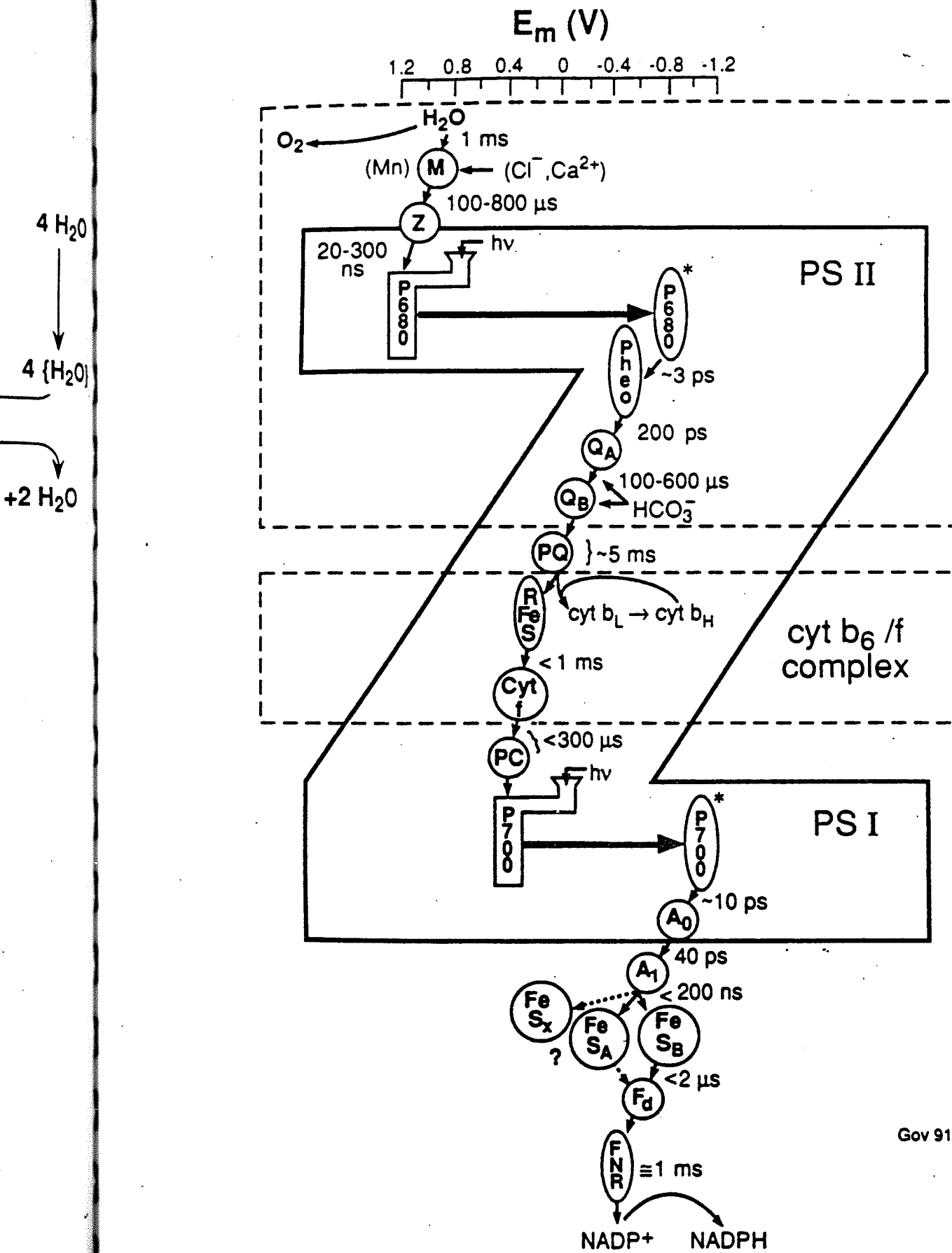


Figure 6



Gov 91

Symbols used in the "Z" scheme are (from top to bottom of the scheme):

Photosystem II (PSII)

PSII is also known as water-plastoquinone oxido-reductase as it oxidizes water to O₂ and reduces plastoquinone to plastoquinol.

"M": The charge accumulator; stores positive charges, up to 4; it includes 4 Mn atoms.

"Z": The electron donor to the reaction center chlorophyll *a* of photosystem II, the so-called P680; molecular genetic experiments show that it is an amino acid tyrosine (#161) on the 3rd helix of the polypeptide D1.

P680: This is the reaction center chlorophyll *a* of photosystem II; P stands for "Pigment" and 680 because one of its absorption maximum is at 680 nm; it is not certain whether it is a dimer as P870 or P960 is in anoxygenic photosynthetic bacteria. After 1 flash, electron flow from Z (to P680⁺) is fast (20 ns), but is slower after 2 or more flashes (100 ns).

P680*: first excited singlet state of P680

Pheo: Pheophytin, *i.e.*, chlorophyll without magnesium in the center-Mg is replaced by H. It appears, by analogy with anoxygenic photosynthetic bacteria, that one of the two pheophytin molecules in the reaction center are functional. Wasielewski et al. (1989) have shown that Pheo is reduced within a few ps. Electron flow from Pheo⁻ to Q_A occurs in 200 ps.

Q_A: This is a bound plastoquinone molecule; it can accept only one electron; it transfers its electron to Q_B, a "two electron acceptor" within 100-600 μs.

Q_B: This is also a bound plastoquinone molecule; its semiquinone form Q_B⁻ is tightly bound; its doubly reduced form QBH₂ is very weakly bound. Formate and bicarbonate affect Q_A and Q_B reactions in plants and cyanobacteria (but not in photosynthetic bacteria). In photosynthetic bacteria, Q_A and Q_B are ubiquinones; menaquinone can also replace ubiquinone.

PQ: This is also plastoquinone, but it is free; there are several molecules, *i.e.*, it is sort of a "pool" of molecules.

Reoxidation of Plastoquinol (PQH₂) by the Cytochrome b/f complex is the bottleneck reaction of electron flow (5 ms)

Cytochrome b/f Complex

R-FeS: Rieske iron sulphur center; it is bound in the Cytochrome b/f complex; the latter is also known as "Plastoquinol plastocyanin oxido-reductase" because its function is to oxidize plastoquinol and reduce plastocyanin.

Cyt f: Cytochrome f; f stands for "frond" for leaf; it is a c type cytochrome.

PC: Plastocyanin; it is a copper protein.

Cyt b_L or b_H: Low (L) or high (H) potential cytochrome b.

[These may be involved in a special cycle called the "Q-cycle".]

Photosystem I (PSI) Complex

This is also known as the plastocyanin - NADP⁺ oxido-reductase as it oxidizes plastocyanin and reduces NADP⁺.

P700: reaction center chlorophyll *a* of photosystem I, with one of its absorption maxima at 700nm; in all likelihood, it is a dimer of two chlorophyll *a* molecules.

A₀: This is a chlorophyll molecule located on the reaction center I complex.

A₁: In all likelihood, it is a vitamin K₁ type molecule, a phylloquinone.

F_X(FeS_X), F_A(FeS_A), F_B(FeS_B):
Iron sulfur centers "X", "A", "B"; they have unpaired spins in their reduced states and can be measured by the "EPR" method just as several other intermediates are. (EPR = electron paramagnetic resonance).

F_d: Ferredoxin; it is an iron-sulfur protein

FNR: Ferredoxin-NADP⁺Reductase.

NADP⁺: Nicotinamide Adenine Dinucleotide Phosphate.

Figure 6B: A Simplified Diagram of Figure 6.

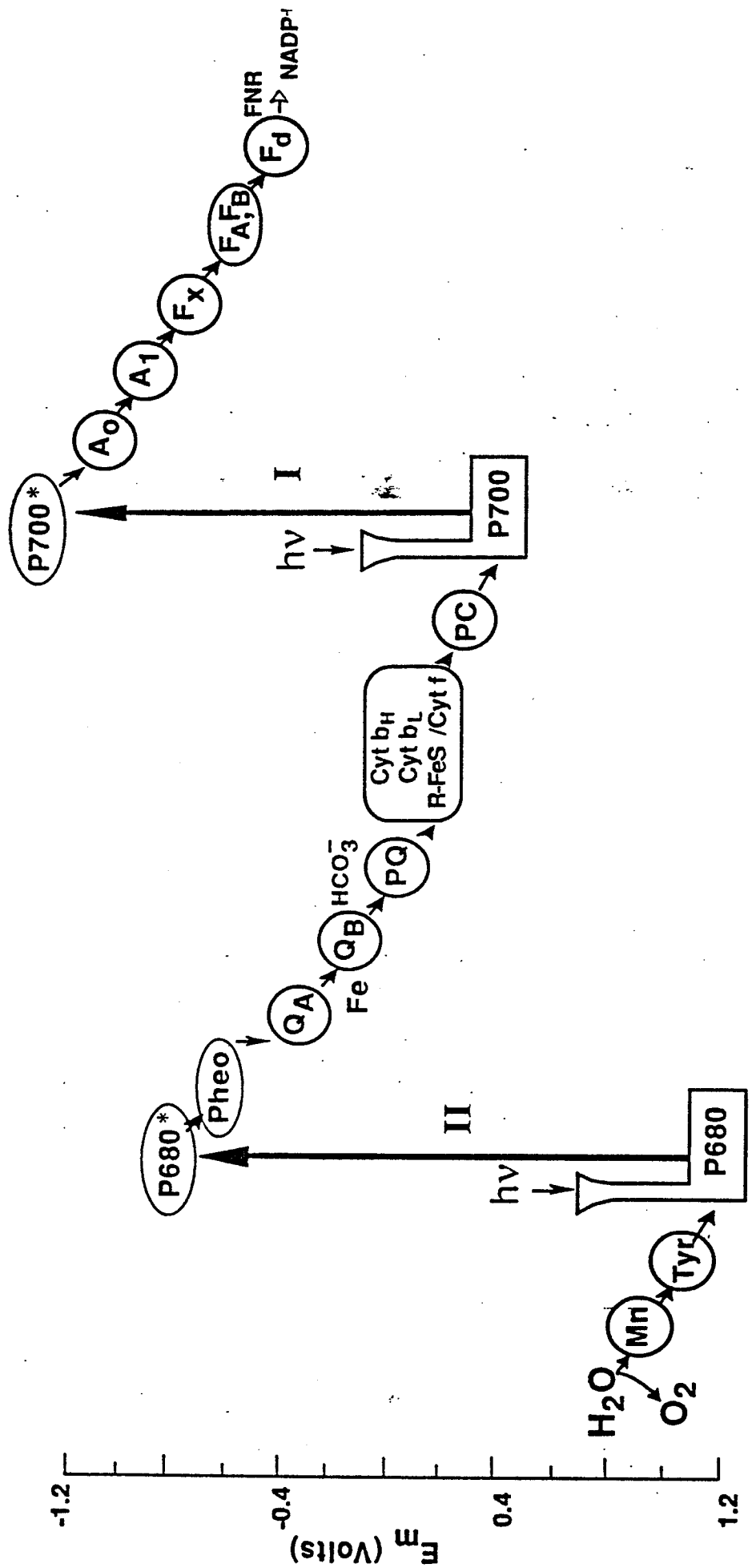


Figure 6C: A comparison of electron flow schemes in various organisms.

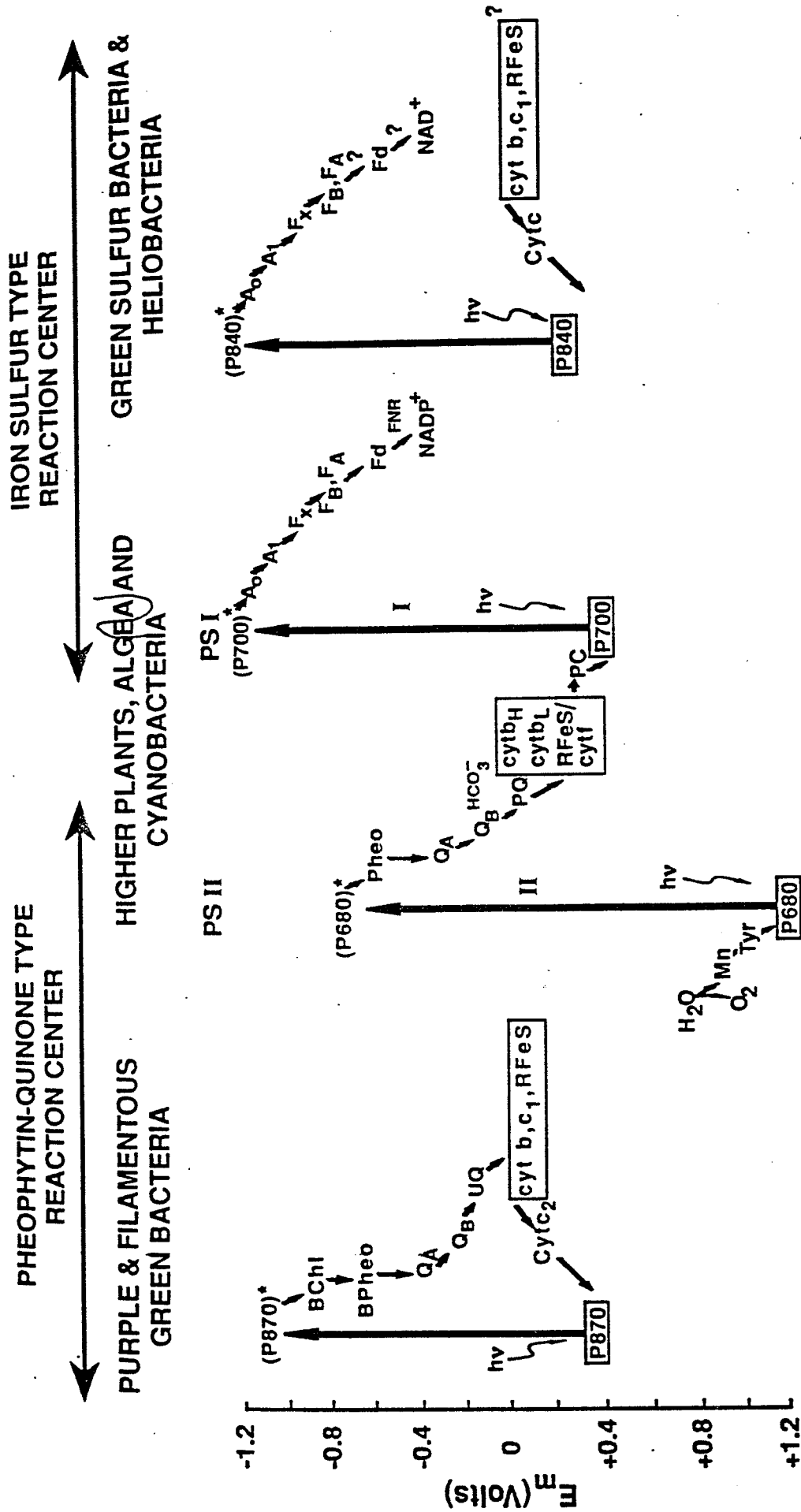
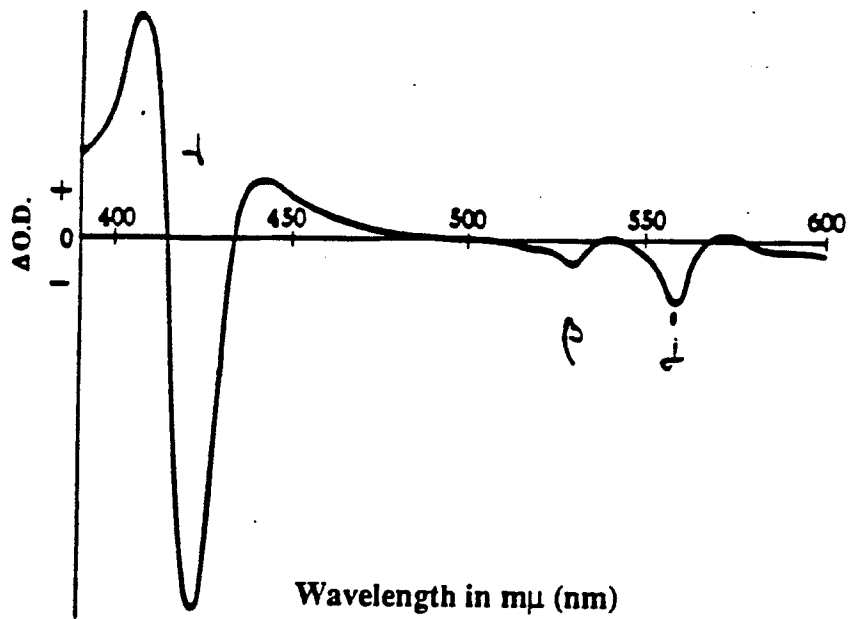


Figure 7

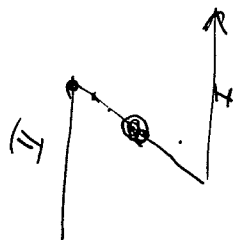
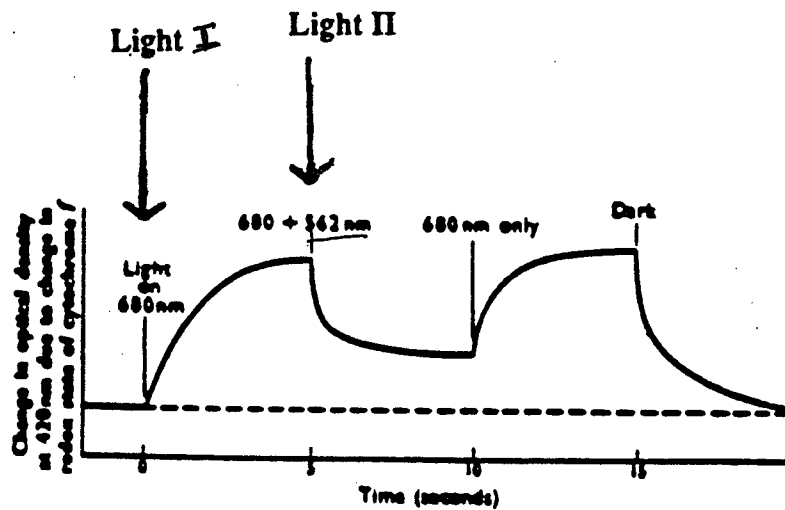
Oxidized Minus Reduced Spectrum of a Cytochrome



Antagonistic Effect of Light I and II on Cytochrome f in a Red Alga Porphyridium (after Duysens et al., 1961)

Porphyridium cruentum
Red Alga

Cyt f
Oxidation ↑



Govindjee

#6

Water-plastoquinone-oxido-reductase (Photosystem II): Oxygen Evolution in Plants and Cyanobacteria, but not in Photosynthetic Bacteria

Prelude:

- *"A living organism is like a running clock. If it is not wound up it will sooner or later run out of free energy and stop. If the clock of life on earth would be left to run down without rewinding, it would take less than one hundred years for all life on the planet to approach its end. First, green plants would die from starvation. Man and other animals who feed on plants would follow. And finally, bacteria and fungi feeding on dead animal and plant tissues would exhaust their food and die too What is that winds up the machinery of life on earth? The answer.... is photosynthesis"*

(E. Rabinowitch and Govindjee, 1969, Photosynthesis, John Wiley & Sons, New York, see p. 28)

- In Govindjee# 5, we provided a general picture of "Oxygenic photosynthesis"; to emphasize that this process occurs not only in higher plants, but also in algae, cyanobacteria and prochlorophytes, I reproduce some statements made by Whitmarsh and Govindjee (1996):

A. Oxygenic Photosynthesis in Algae

Algae are photosynthetic eukaryotic organisms that, like plants, evolve O_2 and reduce CO_2 . They represent a diverse group that include the dinoflagellates, the euglenoids, yellow-green algae, golden-brown algae, diatoms, red algae, brown algae, and green algae. The photosynthetic apparatus and biochemical pathways of carbon reduction of algae are similar to plants. Photosynthesis occurs in chloroplasts that contain photosystems II and I, the cytochrome *b_f* complex, the Calvin cycle enzymes and pigment-protein complexes containing chlorophyll a, and other antenna pigments (*e.g.*, chlorophyll b in green algae, chlorophyll c and fucoxanthol in brown algae and diatoms, and phycobilins in red algae). Green algae are thought to be the ancestral group from which land plants evolved (see Douglas, 1994). Algae are abundant and widespread on the earth, living mainly in fresh and sea water. Some algae live as single celled organisms, while

others form multicellular organisms some of which can grow quite large, like kelp and seaweed. Phytoplankton in the ocean is made up of algae and oxygenic photosynthetic bacteria. Most photosynthesis in the ocean is due to phytoplankton, which is an important source of food for marine life. [These algae have been often used to measure the maximum quantum yield of oxygen evolution in oxygenic photosynthesis and for oxygen evolution in a series of single-turnover flashes that provided information on the existence of the "Oxygen clock" discussed below.]

B. Oxygenic Photosynthesis in Bacteria

Cyanobacteria are photosynthetic prokaryotic organisms that evolve O₂ (Bryant, 1994). Fossil evidence indicates that cyanobacteria existed over 3 billion years ago and it is thought that they were the first oxygen evolving organisms on earth (Wilmotte, 1994). Cyanobacteria are presumed to have evolved in water in an atmosphere that lacked O₂. Initially, the O₂ released by cyanobacteria reacted with ferrous iron in the oceans and was not released into the atmosphere. Geological evidence indicates that the ferrous Fe was depleted around 2 billion years ago, and earth's atmosphere became aerobic. The release of O₂ into the atmosphere by cyanobacteria has had a profound affect on the evolution of life.

The photosynthetic apparatus of cyanobacteria is similar to that of chloroplasts. The main difference is in the antenna system. Cyanobacteria depend on chlorophyll *a* and specialized protein complexes (phycobilisomes) to gather light energy (Sidler, 1994). They do not contain chlorophyll *b*. As in chloroplasts, the chlorophyll *a* is located in membrane bound proteins. The phycobilisomes are bound to the outer side of the photosynthetic membrane and act to funnel exciton energy to the photosystem II reaction center. They are composed of phycobiliproteins, protein subunits that contain covalently attached open ring structures known as bilins that are the light absorbing pigments. Primary photochemistry, electron transport, phosphorylation and carbon reduction occur much as they do in chloroplasts. Cyanobacteria have a simpler genetic system than plants and algae that enable them to be easily modified genetically. Because of this cyanobacteria have been used as a model to understand photosynthesis in plants. By genetically

altering photosynthetic proteins, researchers can investigate the relationship between molecular structure and mechanism (Barry et al., 1994).

Over the past three decades several types of oxygenic bacteria known as prochlorophytes (or oxychlorobacteria) have been discovered that have light harvesting protein complexes that contain chlorophyll *a* and *b*, but do not contain phycobilisomes (Palenik and Haselkorn 1992, Urbach et al., 1992; Matthijs et al., 1994). Because prochlorophytes have Chlorophyll *a/b* light harvesting proteins like chloroplasts, they are being investigated as models for plant photosynthesis.

References for the above section:

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Outline

(1) Photosystems are located on **thylakoid membranes** (see **Figure section**).

(2) **Components** (see **Figure section**)

Four major protein complexes:

- 1) **Photosystem II Complex:** Photosystem II reaction center proteins D1, D2, two subunits of cytochrome b559, a psbI and a psbW gene products ; inner antenna CP-43, CP-47, CP-24 and CP-29 complexes; light-harvesting complex-II (LHC-IIb), and several small molecular weight proteins. (CP = chlorophyll-protein)
- 2) **Cytochrome b₆/f complex:** a Rieske Fe-S protein; a 17 kDa protein; cytochrome b_L; cytochrome b_H; and a small molecular weight protein (see Govindjee # 7)
- 3) **Photosystem I complex:** contains several polypeptides for photosystem I reaction center and light-harvesting complex - I (LHC-I) proteins (see Govindjee # 8).
- 4) **ATP synthase:** also contains several polypeptides in its CF₁ part and in its CF_o part (see Govindjee# 9)

•Photosystem II is also known as *water-plastoquinone oxidoreductase*. Cyt. b₆/f is also known as *plastoquinol - plastocyanin oxidoreductase*, and Photosystem I as *plastocyanin ferredoxin oxidoreductase*. All these names specify their functions just as *ATP synthase* specifies the function of CF_o - CF₁ complex. Pigments (chlorophylls and carotenoids) are contained in several, not all, polypeptides of Photosystem I and II only.

Mobile electron carriers:

•Plastoquinone (lipidic/soluble in lipids) and plastocyanin (a copper protein).

•Asymmetry is “absolute” in terms of organization of components on the thylakoid membrane. Electron flow leads to oxidation of water to O₂ and reduction of NADP⁺ to NADPH, **but** protons are translocated from outside to inside: protons are taken up where plastoquinone is reduced to plastoquinol (outside) and released where H₂O is oxidized (inside, photosystem II) and where plastoquinol is oxidized (inside, cyt b₆/f complex). This proton gradient, when dissipated through ATP synthase complex, produces ATP.

(3) Genetic Coding of the Various Polypeptides (see Figure section))

(A) Nuclear coded:

- LHC-II (light-harvesting complex II) (28, 26, 24 kDa proteins);
- extrinsic (peripheral) 17, 23 and 33 kDa proteins of oxygen-evolving complex;
- 4 polypeptides (labeled as II, III, V and VI, about 25, 20, 16 and 8 kDa) of photosystem I complex;
- 4 polypeptides from 19 to 24 kDa of LHC-I (light-harvesting complex I);
- 20 kDa protein of Cyt b₆/f complex;
- γ (37 kDa in CF₁), δ (18 kDa in CF₁) and II (11 kDa in CF₀) polypeptides of ATP synthase;
- ferredoxin (11 kDa);
- plastocyanin (10.5 kDa); and
- ferredoxin - NADP⁺ reductase (33 kDa).

(B) Chloroplast coded:

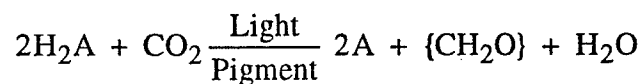
- CP-47, CP-43, D2, D1 and a 10 kDa protein of Photosystem II complex;
- subunit Ia and Ib and perhaps subunit IV (18 kDa) of Photosystem I complex;
- 33, 34 and 17 kDa proteins of Cyt b₆/f complex;
- α (59 kDa in CF₁), β (56 kDa in CF₁), ε (13 kDa in CF₁) and I (17 kDa in CF₀) and IV (9 kDa in CF₀) polypeptides of ATP synthase.

It is, thus, clear that chloroplast structure and function would depend upon the DNA's of both the nucleus and the chloroplast. Furthermore, note that all the 4 protein complexes have one or more polypeptides coded by nuclear DNA.

(4) The Source of Oxygen in Photosynthesis

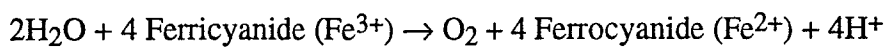
Water versus CO₂; almost everybody else versus Otto Warburg: H₂O

(a) Cornelius van Niel and Rene Wurmser's basic equations:



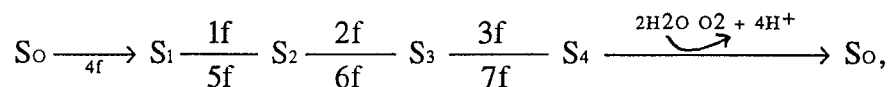
(b) Sam Ruben and Martin Kamen's 0-18 experiment: O₂ released has mass 36 when H₂¹⁸O is used, but not when C¹⁸O₂ is used.

(c) The Hill reaction, named after Robin Hill



(5) The Oxygen Clock: Period 4 Oscillation (see Figure section)

- (a) *Experiments by Pierre Joliot:* Oxygen per flash as a function of flash number shows a periodicity of 4, i.e., O₂/flash is maximum at flash 3, 7, 11, 15, etc.
- (b) *Theory by Bessel Kok:* The redox state of oxygen evolution complex is described by the symbol "S". (Please do not confuse these S-states with the excited states of absorbing molecules; S-states here represent the redox state of the oxygen evolving complex that may be a manganese complex or more generally "M" in the Z-scheme.) The "S" state theory for the oxygen clock: oxygen evolving centers are independent of each other; they are in the "S₁" state in darkness. Each light reaction, at the reaction center P680 of Photosystem II, leads ultimately to the oxidation of the S-state. Thus first flash takes the system to S₂, second to S₃ and third to S₄ (S₄ means 4 positive charges have accumulated); S₄ reacts with water yielding O₂ and S₀. then the cycle starts again and after the 7th flash, there is again a "gush" of O₂:



where f stands for a flash of light; and S₁ = dark state.

NOTE: These S-state changes of the oxygen evolving complex are not direct light reactions.

We can write S₁ to S₂ reaction as follows:

1. P680 Pheo + 1 flash $\xrightarrow{1f}$ P680* Pheo (P680 = reaction center chlorophyll; Pheo = pheophytin)
2. P680* Pheo $\xrightarrow{3ps}$ P680⁺ Pheo⁻
3. P680⁺ Pheo⁻ + Q_A $\xrightarrow{200ps}$ P680⁺ Pheo + Q_A⁻ (Q_A = one-electron acceptor plastoquinone)
4. P680⁺ Pheo⁻ + Z $\xrightarrow{20ns}$ P680 Pheo + Z⁺ (Z = electron donor tyrosine entity)
5. Z⁺ + S₁ $\xrightarrow{50;100\mu s}$ Z + S₂
6. Q_A⁻ + Q_B $\xrightarrow{200\mu s}$ Q_A + Q_B⁻ (Q_B = a two-electron acceptor plastoquinone)

Thus, the oxygen evolving complex has been oxidized and Q_B has been reduced.

(fs = femtosecond; ps = picosecond; ns = nanosecond; μ s - microsecond)

Other complications are:

Misses

Double hits

These complications lead to damping of the flash pattern with increasing flash number.

(c) *Proton Release Patterns*

The extent of measured proton release often differs between the four steps (S_0 to S_1 ; S_1 to S_2 ; S_2 to S_3 ; and S_3 to S_0 via S_4) Protons are often released in the following ratio: 1, 0.5, 1, 1.5 during S_0 to S_1 , S_1 to S_2 , S_2 to S_3 and $S_3 \rightarrow S_4 \rightarrow S_0$ transitions.

However, under other experimental conditions, this pattern is 1, 1, 1,1, or 1, 0, 1, 2.

The pattern varies as a function of pH in thylakoids and PSII membranes. In contrast, it is constant in PSII core particles and it is 1:1:1:1 at all pHs. It is now generally accepted that these patterns do not directly reflect the water protons (i.e., chemical deprotonation), but, instead protons from aminoacids close to the Mn atoms; these protons are then replaced by water during S_4 to S_0 transition.

Lavergne and Junge (1993) commented that the process of release of protons at the oxygen evolving complex may have many sources and it is because of this that the proton release, observed after a series of single-turnover flashes, does not represent protons originating in water (see **Figure section**).

(d) *Comparison of period 4 flash patterns:*

S_4 ; O_2 ; maxima after 3rd and 7th flashes (see **Figure section**)

S_3 ; recombination of S_3 and Q_B^- ; thermoluminescence: maxima after 2nd and 4th flashes (see **Figure section**)

S_2 ; Mn multiline ESR (electron spin resonance) signal at 4K: maxima after 1st and 5th flashes (see **Figure section**)

The S-states, themselves, are suggested to be composed of 4Mn atoms and undergo transitions as shown in the Figure section.

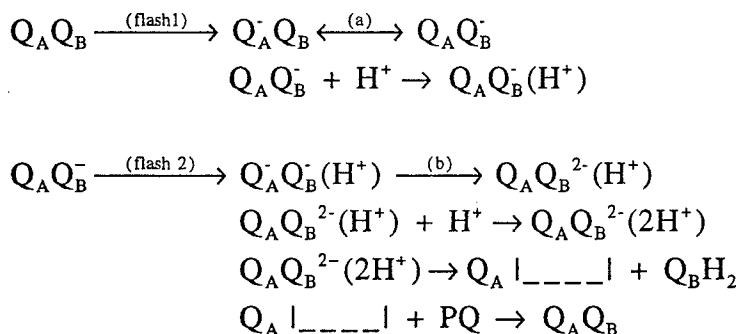
X-ray absorption studies (XAS; and EXAFS= Extended X-ray Absorption Fine Structure) show that during S-state transitions, it is Manganese that undergoes oxidation reaction. Manganese is oxidized by loss of one electron, and one can observe a 1 eV shift in the X-ray Absorption Edge of Mn. This, however, is not observed during S_2 to S_3 transition that led to suggestions that an aminoacid, instead of Mn, may be oxidized during that transition, a suggestion that had come first from T. Kambara and later S. Padhye (working with me).

Please remember that although the assignment of "Mn" to the "S-states" is based on several experiments, the multiline EPR signal fingerprints S2 as Mn(III)/Mn(IV) complex, the X-ray absorption spectroscopy prove that during S-state transitions, Mn undergoes redox changes (in certain steps, charges are shared with other nearby aminoacids) and EXAFS data allow the construction of a model as to how Mn atoms are arranged and what other ligands may be involved in the "Oxygen Evolving Complex".

(6) Components for Oxygen Evolution (see Figure section)

- (a) Polypeptides: D-1; D-2; two subunits of cytochrome *b*-559; a *psbI* and a *psbW* gene product ; extrinsic 33kDa, 24kDa, and 17kDa
(cyanobacteria lack 24 + 17 kDa polypeptides but, instead have a 10 kDa polypeptide and a cytochrome *c*)
- (b) Manganese: at least 4Mn—direct measurement by X-ray absorption spectroscopy
- (c) Chloride ions
- (d) Calcium ions

(7) The Two Electron Gate



- Since (a) is faster than (b), Chlorophyll *a* fluorescence yield at 100-200 μs after an actinic flash is lower after flash 1 than after flash 2. Thus, one observes a periodicity of 2 in the flash-number dependence of Chl *a* fluorescence yield 100-200 μs after a strong flash.

- The binary flash-number dependent Chl *a* fluorescence yield can also be observed by the following experiment: Illuminate with flash 1 (see reactions after flash 1, above), then add the herbicide diuron (DCMU); the latter cannot displace Q_B^- which has a long lifetime, but equilibrium reaction (a) is shifted towards $[Q_A^-]$. Chl *a* fluorescence yield is

high when $[Q_A^-]$ is high. Now, illuminate with two flashes (spaced 1s apart), then add the herbicide diuron (DCMU) (see reactions after flash 2, above). There is \hat{Q}_B at the site and diuron displaces it, but the system is in Q_A state, and Chl *a* fluorescence yield is low as Q_A is a quencher of fluorescence. Thus, Chl *a* fluorescence in this experiment is high after flash 1 and low after flash 2 (see figure section).

(8.) **Effects of Herbicide:** Herbicides work by displacing Q_B from its binding site.

- (a) Herbicides slow down the rate of decay of Q_A^- to Q_A since Q_B is displaced by herbicides. Chlorophyll *a* fluorescence yield is high when $[Q_A^-]$ is high and is low when $[Q_A^-]$ is low.
- (b) Herbicides allow a faster rise of chlorophyll *a* fluorescence yield in continuous illumination as Q_A^- is unable to transfer electrons to Q_B .

(9.) **Use of Site-Directed Mutagenesis:**

Site-directed mutagenesis of a cyanobacterium *Synechocystis* sp. PCC 6803 have established, among other things, that:

- (a) "Z" is a tyrosine residue (Y-161) on the D1 protein.
- (b) Even the 33 kD extrinsic polypeptide may not be an absolute requirement for O_2 evolution.
- (c) Certain specific amino acids (*e.g.*, Aspartic 170 on the D1 protein) may be involved in Mn binding.
- (d) Certain arginine residues on the D2 protein may be involved in a unique role of bicarbonate on the two electron gate.

Sources

A. Basic Material

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Structure of Photosystem II has been modeled:

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Water Oxidation: Oxygen Evolution

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Proton Release

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Use of Molecular Biology in Photosystem II

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Protection of Photosystem II against Light: Mechanism involving Xanthophyll-cycle

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Questions

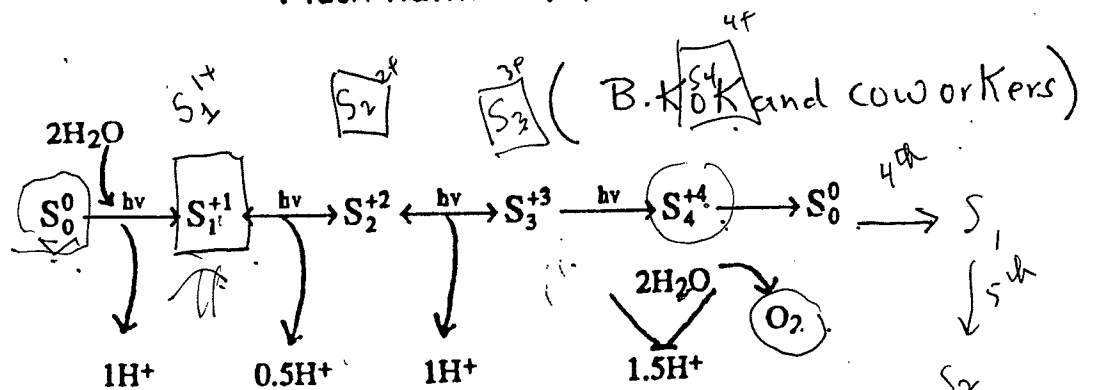
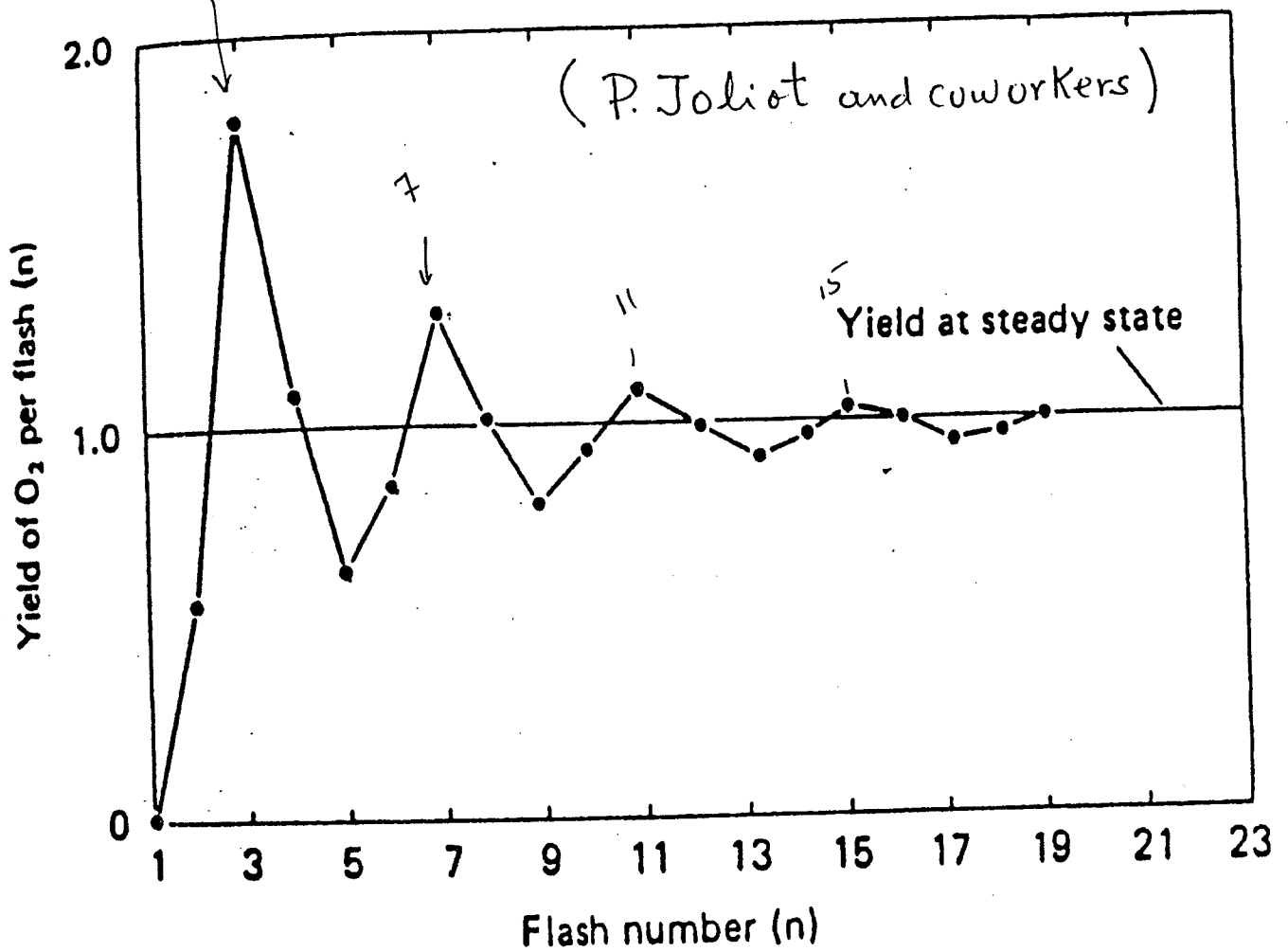
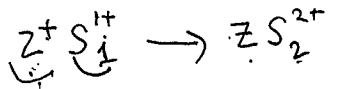
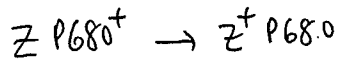
- [1] First, please answer some basic questions that you should have figured out by now: What is really electron transport? What does oxidation-reduction mean? Would you have an spontaneous electron flow from an intermediate having a midpoint redox potential (at pH 7) of +0.4 eV to one with 0.0 eV ?
- [2] What is the evidence that the oxygen evolving complex starts in the dark in the so-called S1 , not So, state? What would be the pattern of oxygen evolution/ flash as a function of flash number if the system started in 100% So state in the dark, and there were no "misses" and no "double-hits"?
- [3] What is the best evidence that manganese is oxidized sequentially prior to water oxidation? What information has been provided by techniques like XAE (X-ray Absorption Edge), EXAFS (Extended X-ray Absorption Fine Structure), NMR (nuclear magnetic resonance, not mentioned thus far), EPR (Electron Paramagnetic Resonance), and UV (Ultra-violet) absorption spectroscopies regarding the role of manganese in photosynthesis ? { J. Amesz and A.J. Hoff (eds.) (1996) *Biophysical Techniques in Photosynthesis, Advances in Photosynthesis, Vol. 3, Kluwer Academic, Dordrecht* }
- [4] Does the observed flash-number dependent proton release patterns inform us about water chemistry? If not, why not? Do they contain any important information regarding the functioning of Photosystem II?

- [5] Although a phenomenon, called thermoluminescence, may not have been discussed yet, an example of results on flash-number dependent thermoluminescence from leaves may have been presented. What do you think thermoluminescence can tell us about photosystem II?
- [6] What is the key experimental evidence for the existence of a two-electron acceptor, QB, in photosystem II?
- [7] What is the molecular mechanism of inhibition of photosynthesis (that leads to death of the treated plants) by herbicides such as DIURON or ATRAZINE?
- [8] What is the experimental basis of the suggestion that one of the functions of bicarbonate is to stimulate the functioning of the two electron gate of photosystem II, but not the photosynthetic bacteria? [An answer to this question may require discussions with Govindjee]
- [9] Write a short essay on the structure and function of Photosystem II emphasizing the uniqueness of this system. In what major fashion, this system is similar and different from reaction centers of anoxygenic bacteria?
- [10] How does photosystem II protect itself against light, especially with the help of changes in xanthophyll cycle?

Pheo⁻

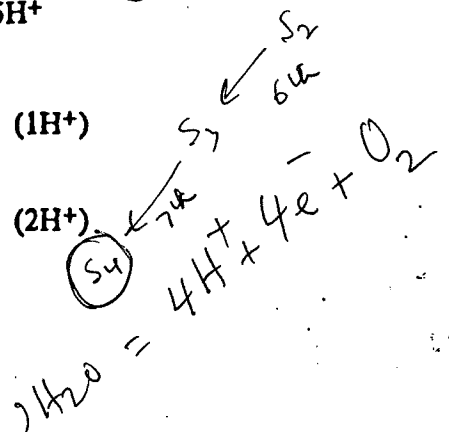
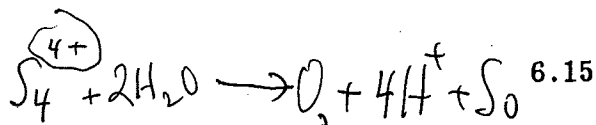
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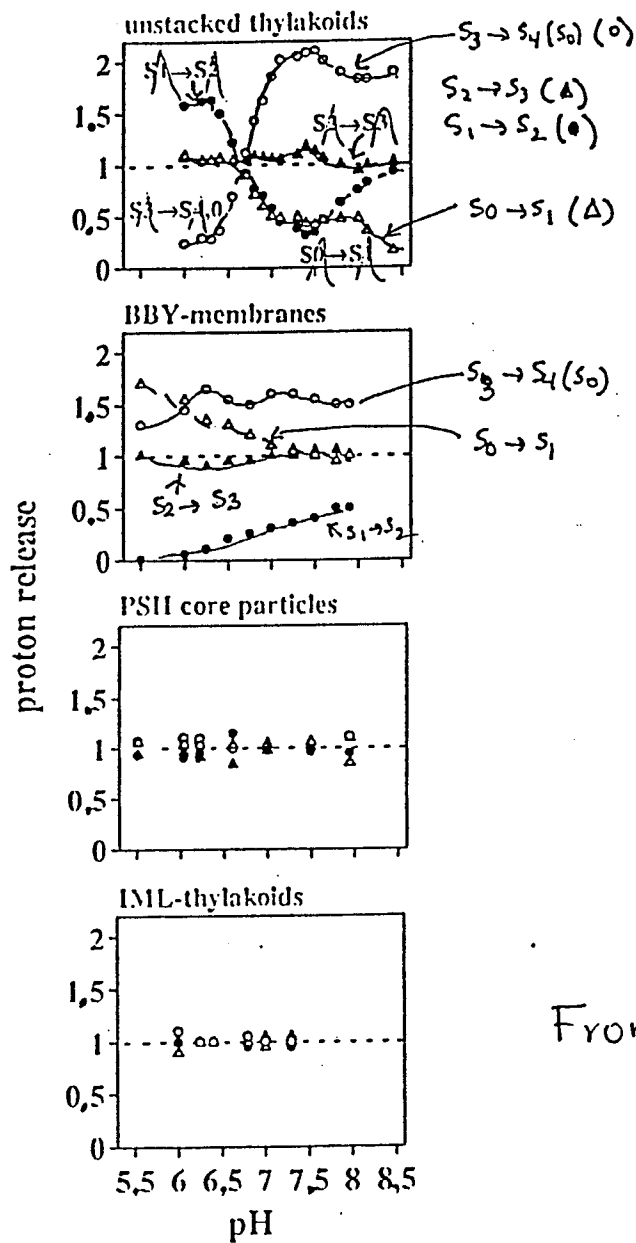
Figure 1



or (1H⁺) (1H⁺) (1H⁺) (1H⁺)

or (1H⁺) (OH⁺) (1H⁺) (2H⁺)



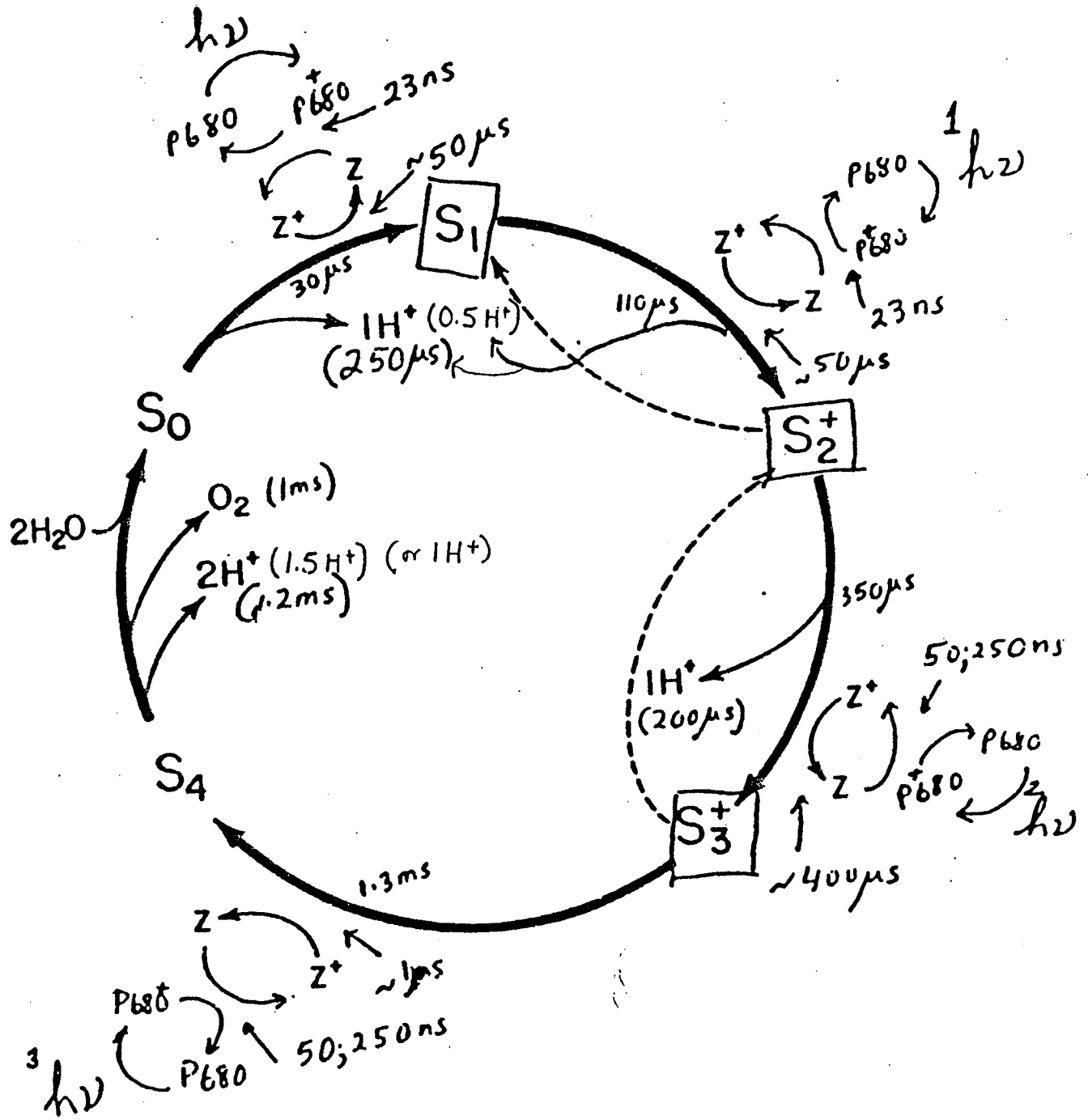


From Lavergne and Junge (1993)

Fig. 1 Patterns of proton release by water oxidation as a function of pH. Original patterns as function of flash number were deconvoluted to yield the extent at any of the four transitions, namely $S_0 \Rightarrow S_1$ (open triangles), $S_1 \Rightarrow S_2$ (closed circles), $S_2 \Rightarrow S_3$ (closed triangles), and $S_3 \Rightarrow S_4 \rightarrow S_0$ (open circles). Four different types of material, all capable of oxygen evolution, were used; from top to bottom: unstacked pea thylakoids (M. Haumann and W. Junge, unpublished); BBY-membranes (Rappaport and Lavergne 1991); PS II core particles (Lübbbers et al. 1993); and IML-thylakoids (Jahns and Junge 1992b).

IML = plants grown in intermittent light

Figure 2



and
993)

Fig. 2A

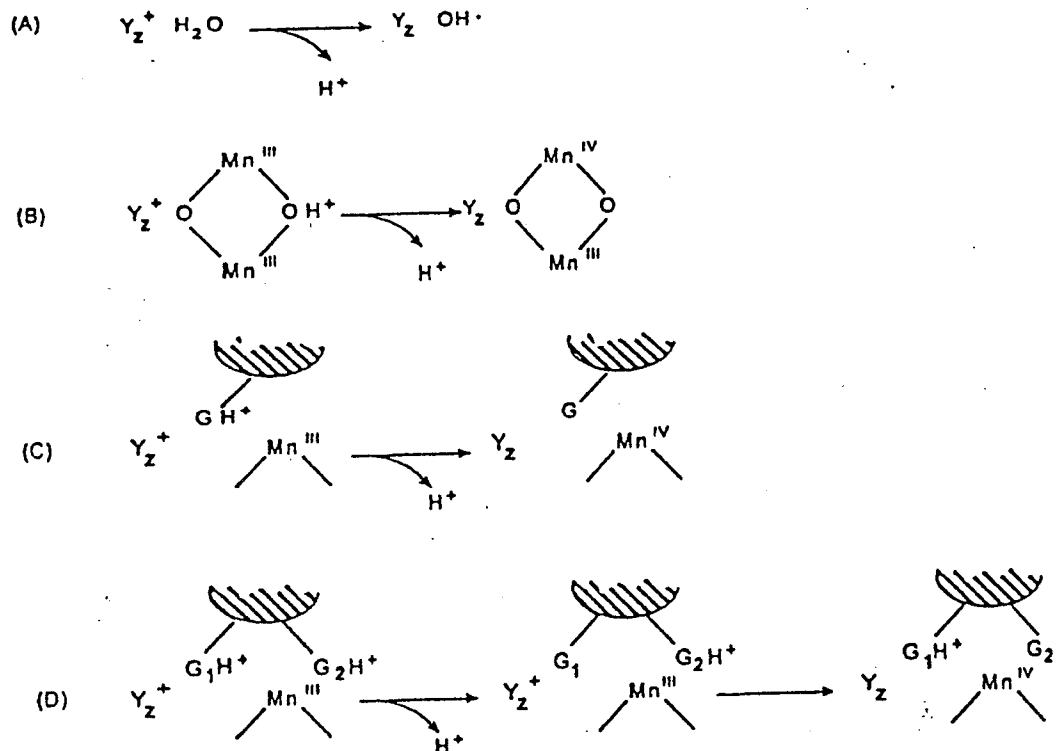
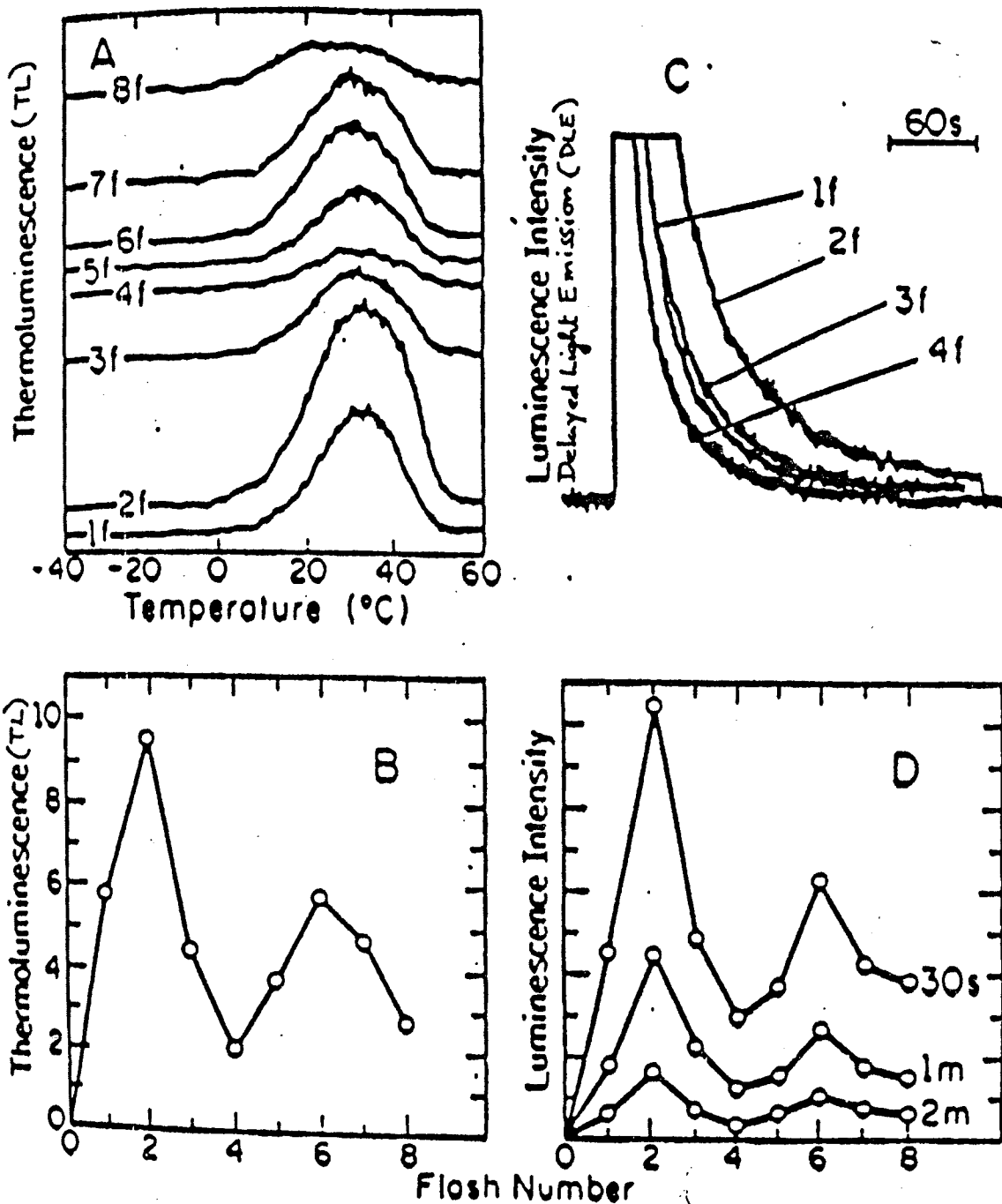


Fig. 2A scheme illustrating various possibilities for proton release on the donor side of Photosystem II. A, Chemical deprotonation. The particular example shown (formation of an OH· radical) was chosen for its simplicity rather than likelihood, since the redox potential of the OH/H₂O couple (in aquo) (E₀ = 2.85 V) is much higher than that of Y₂[•]/Y₂. B, Deprotonation of a Mn ligand. C, Electrostatic deprotonation of a group close (but not liganded) to a Mn atom. Depending on geometry and rate factors, the deprotonation may be initiated before the electron transfer from Mn to Y₂[•]. D, Push-pull process involving two groups. The deprotonation is initiated by Y₂[•] acting on G₁ and stabilized, after the electron transfer step, by proton transfer from G₂ to G₁.

Lavergne and Junge (1993)

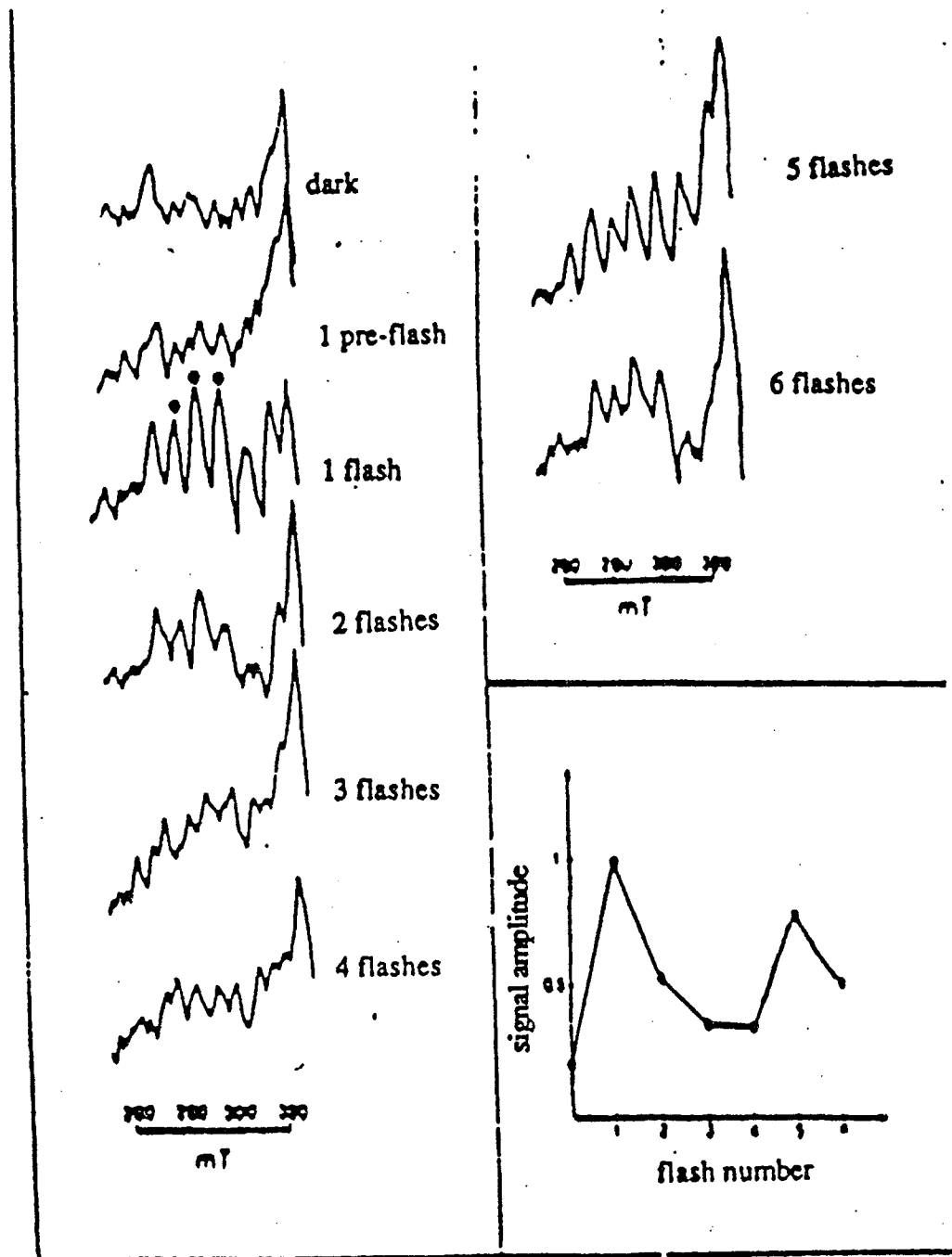
Figure 3



(A) TL recorded from spinach leaf discs after a series of flashes (f). The traces show luminescence intensity (arbitrary units) as a function of temperature. (B) TL intensity at 35°C plotted as a function of flash number. (C) DLE recorded from spinach leaf discs after a series of flashes. (D) DLE intensity at 30 s, 1 min, and 2 min after a flash, plotted as a function of flash number. All flashes were given at room temperature.

Rutherford, Govindjee and Inoue (1984)

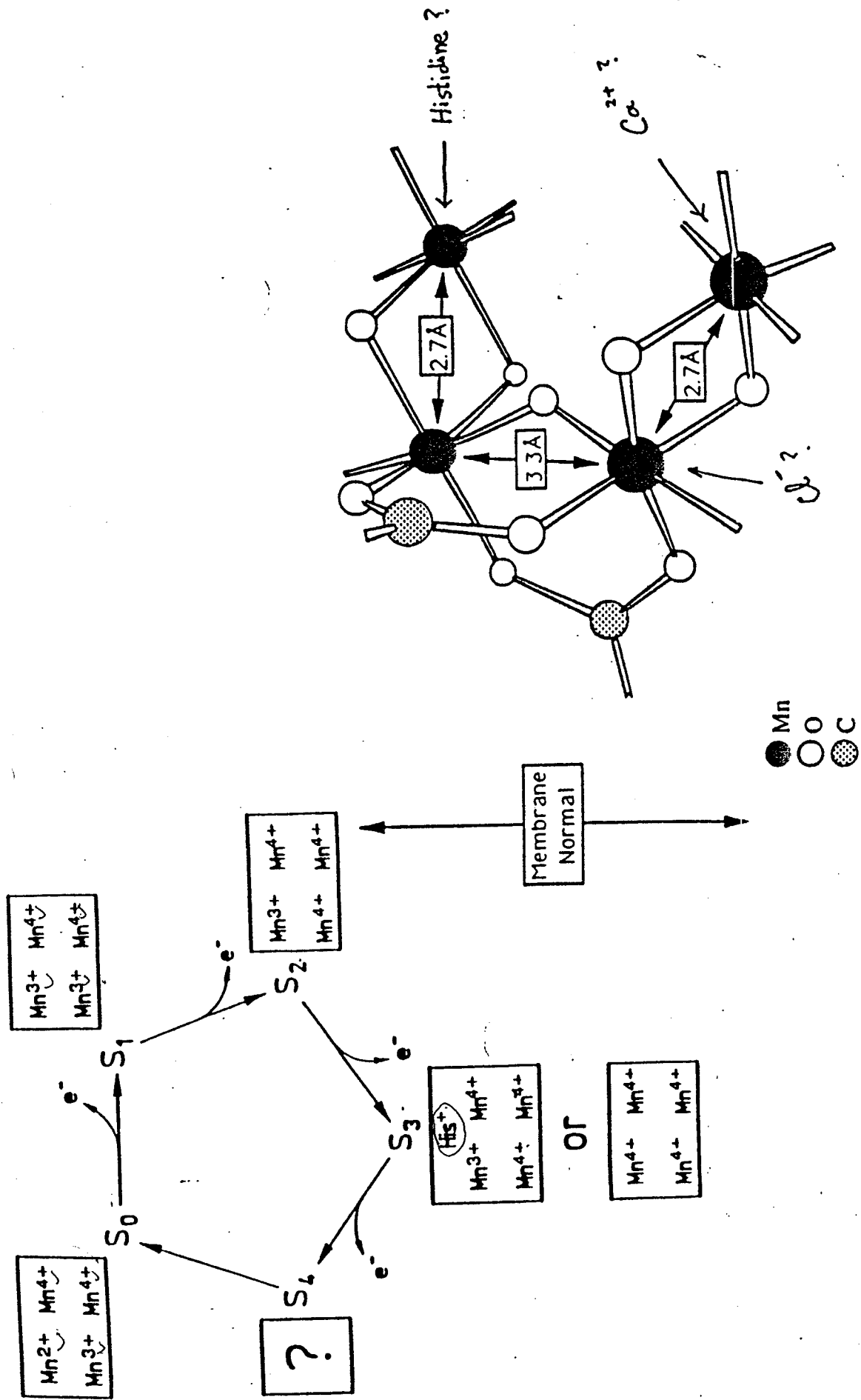
Figure 4



Multiline ESR signals produced by a series of light flashes. Samples of Photosystem II membranes were given one preflash at 20°C, dark-adapted for 15 min. and then illuminated by 1-6 flashes at 0°C. Temperature of measurement, 8 K; frequency, 9.44 GHz; microwave power, 31.5 mW; modulation amplitude, 25G. The amplitudes of the signal, measured as the heights of the three downfield peaks marked by *, as a function of flash number are shown in the lower right-hand panel. (After J.L. Zimmerman and A.W. Rutherford, 1984)

(First shown by Dismukes + Siderer)

Figure 5



(After K. Sauer and co-workers)

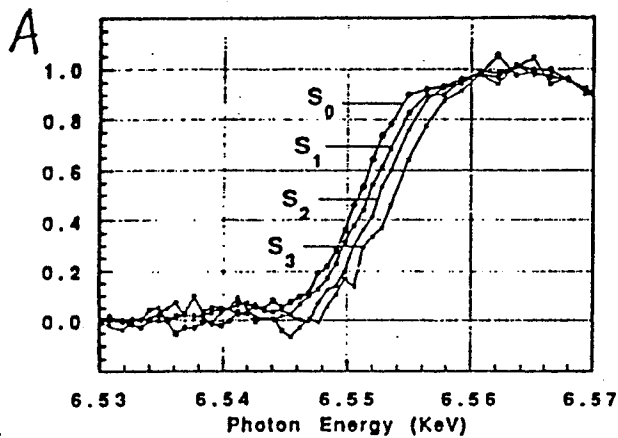


Fig. 5A Mn K-edge XANES spectra of Kok's true S_0 -, S_1 -, S_2 - and S_3 -states, whose K-edge half-height energies are found to be 6550.9 ± 0.2 eV, 6551.7 ± 0.2 eV, 6552.5 ± 0.2 eV and 6553.6 ± 0.2 eV, respectively.

Ono et al. (1992)

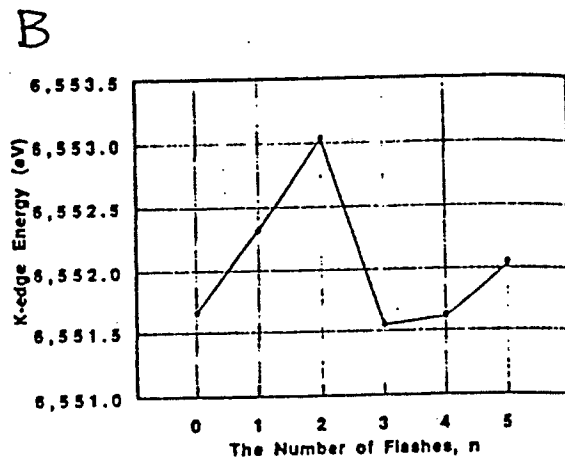


Fig. 5B Oscillatory pattern of the Mn K-edge energy in the XANES spectra of PS II membranes as a function of flash number n : The experimental data (circles) is compared with a theoretical one

drawn line
Ono et al. (1992)

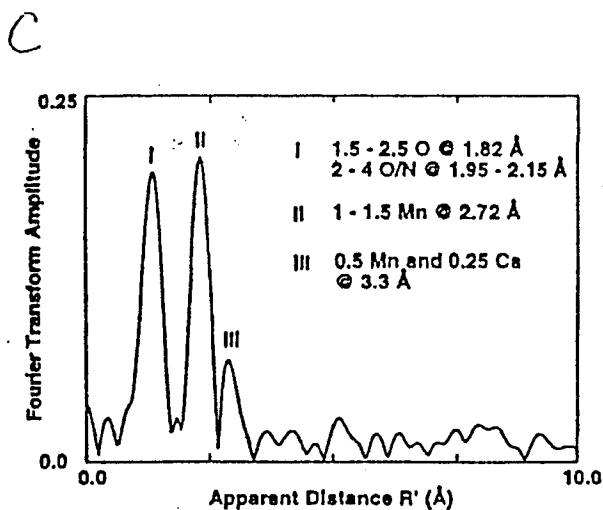
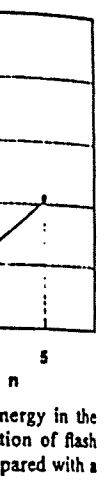


Fig. 5C Fourier-transform of Mn EXAFS data from *Synechococcus* in the S_1 state collected at 10 K using a 13-element Ge detector. The Fourier transform shows three well resolved peaks. In this radial distribution about Mn, each of the Fourier peaks represents X-ray photoelectron backscattering from the coordinating or neighboring atoms, and the assignments of each peak determined by simulations is shown on the figure (from DeRose 1990).

For Figure 5 model:

Yachandra, V.K., V.J. de Rose, M.J. Latimer, I. Mukerji, K. Sauer and Klein, M.P. (1993) Where Plants Make Oxygen: A structural model for the Photosynthetic Oxygen-Evolving Manganese Cluster. *Science* 260: 675-679.



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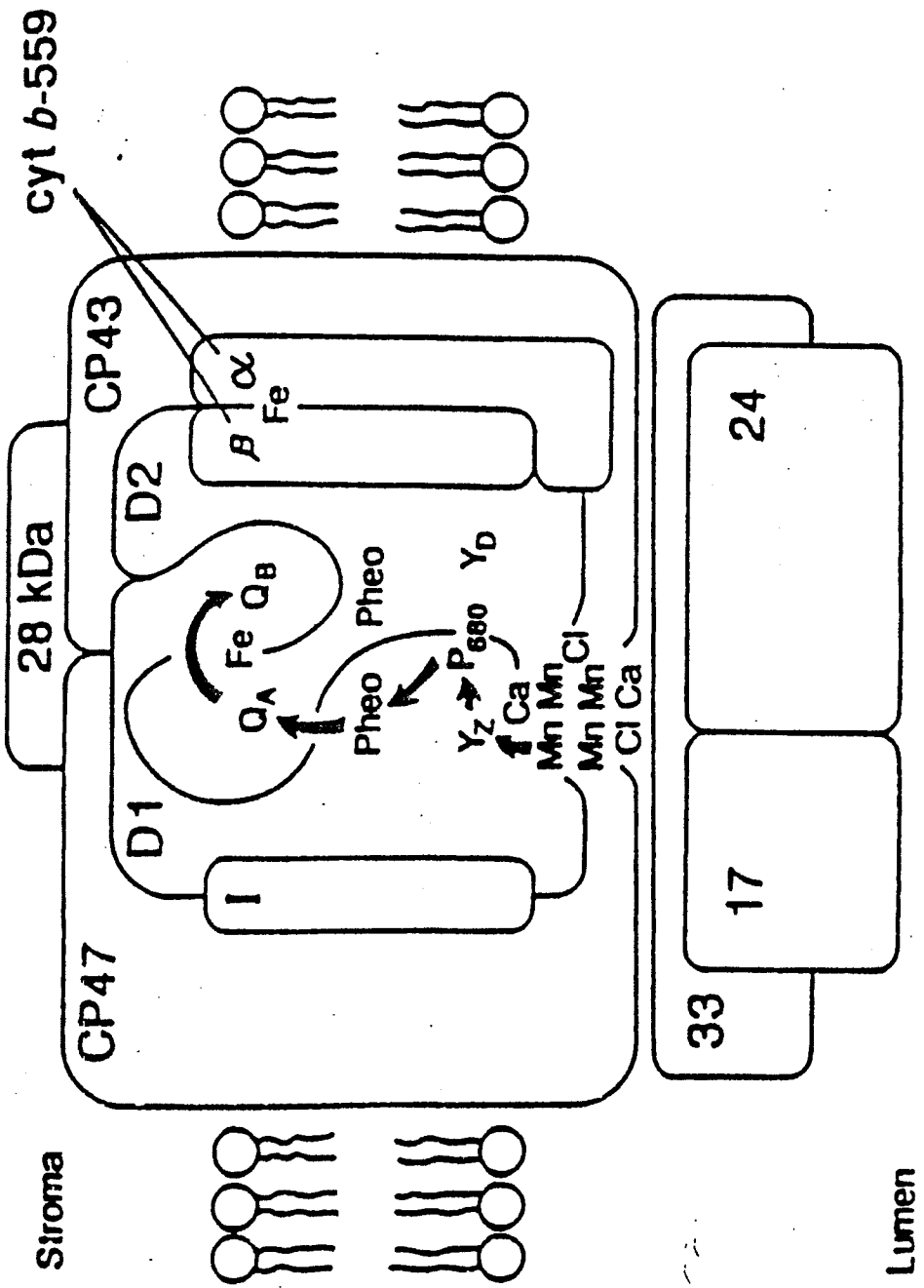


Figure 6

D1 PROTEIN

A

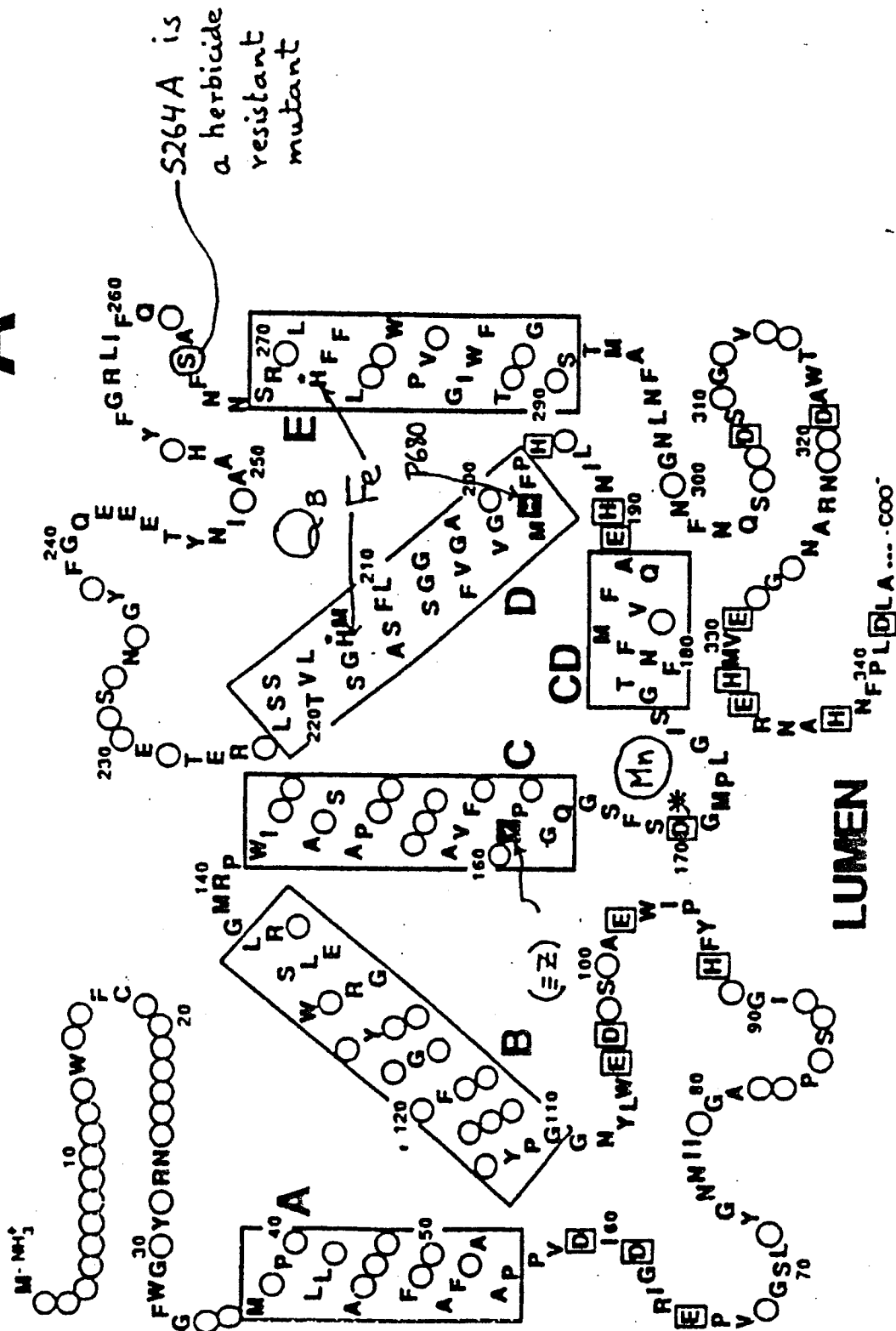


Figure 7

D2 PROTEIN

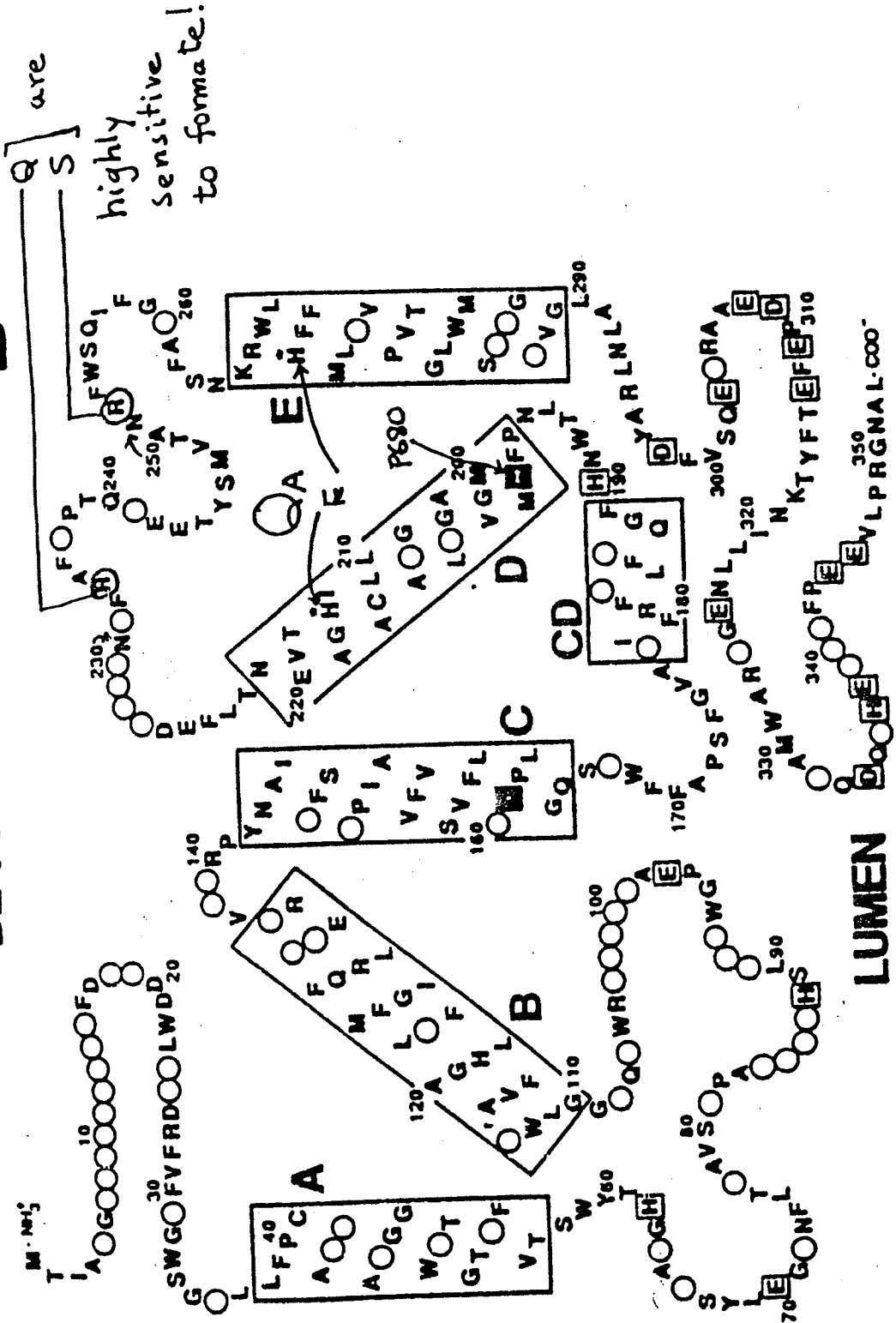
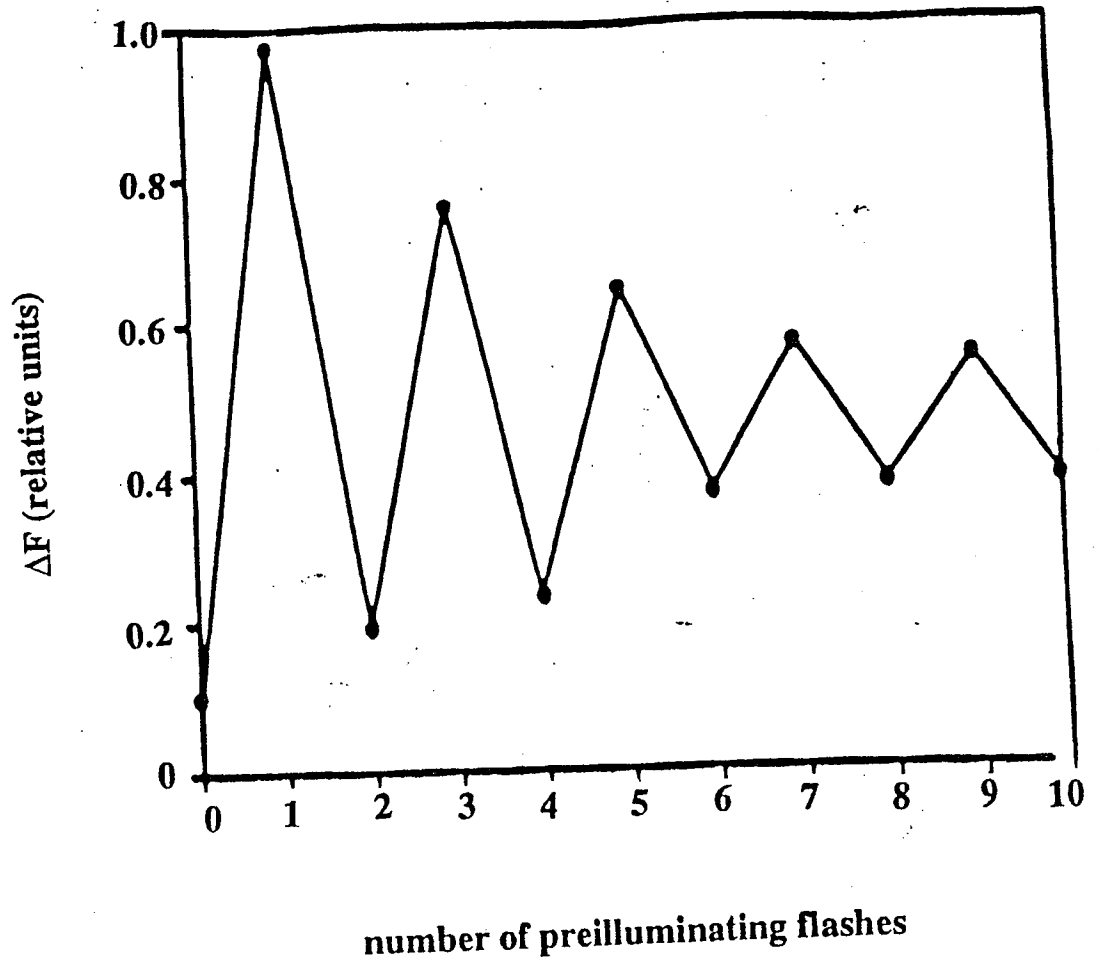


Figure 8

Figure 9



Chlorophyll Fluorescence (ΔF) observed after illumination of dark-adapted chloroplasts by a series of single-turnover flashes and subsequent DCMU addition. Units correspond to $(\Phi - \Phi_0) / \Phi_0$. Oxygen evolution was inactivated by Tris treatment and an artificial electron donor was added to replace H_2O . (From Velthuys and Amesz, 1974.)

Govindjee
#7

**Plastoquinone-plastocyanin oxido-reductase
(cytb6/f complex): Intersystem Electron
Transport; the "Q" cycle**

The major function of Cyt b6/f complex is to not only to provide the organisms with a proton gradient needed for ATP synthesis, but to act as an intermediate in the electron transfer chain between Photosystem II and Photosystem I; the "Q" cycle is great because it allows twice the protons/ electron transferred than available without it. Below, I have reproduced a portion of text from Whitmarsh and Govindjee (1996) that deals with the question of creation of a proton electrochemical gradient.

Creation of a Proton Electrochemical Potential

Electron transport creates the proton electrochemical potential of the photosynthetic membrane by two types of reactions. (1) The release of protons during the oxidation of water by photosystem II (see Govindjee # 6) and the translocation of protons from the outer aqueous phase to the inner aqueous phase by the coupled reactions of photosystem II and the cytochrome b6/f complex in reducing and oxidizing plastoquinone on opposite sides of the membrane. This creates a concentration difference of protons across the membranes ($\Delta\text{pH} = \text{pH}_{\text{in}} - \text{pH}_{\text{out}}$). (2) Primary charge separations at both the reaction centers (I and II) drive electrons across the photosynthetic membrane, which create electric potential across the membrane ($\Delta\Psi = \Psi_{\text{in}} - \Psi_{\text{out}}$). Together, these two forms of energy make up the proton electrochemical potential across the photosynthetic membrane ($\Delta\mu_{\text{H}^+}$) which is related to the pH difference across the membrane and the electrical potential difference across the membrane by the following equation:

$$\Delta\mu_{\text{H}^+} = F \Delta\Psi - 2.3 RT \Delta\text{pH},$$

where F is the Faraday constant, R is the gas constant, and T the temperature in Kelvin. Although the value of $\Delta\Psi$ across the photosynthetic membrane in chloroplasts can be as large as 100 mV, under normal conditions the proton gradient dominates. For example, during photosynthesis the outer pH is typically near 8 and the inner pH is typically near 6, giving a pH difference of 2 across the membrane that is equivalent to 120 mV. Under these conditions the free energy for proton transfer from the inner to the outer aqueous phase is -12 kJ/mol of protons.

Outline

- O. Instructor should provide a background on the meaning of electrochromism; redox potential and difference absorption spectroscopy before proceeding with a discussion of how this Cyt b₆/f complex functions..

From Plastoquinol to Plastocyanin

By now, you are into the "thick of things". I have provided here much more details than ever before! So, read on!

1. Components and their Function (see Figure section)

Protein Complex: Cytochrome b₆/f complex; another name for it is plastoquinol-plastocyanin oxido-reductase

Components; molecular weights; redox potentials; gene coding; arrangement; absorption and other properties are given below:

(1.) *One Copy of Cytochrome f*

Molecular Mass: 33 kilodaltons (285 amino acids)

Midpoint Redox potential (E_m,7): +360 millivolts

Gene is Located in: **Chloroplast** (gene name: pet A)

Arrangement: Most of the protein including the heme group is in the lumen; this part has been crystallized and its structure is now available; one helix spans the membrane, with the carboxylic group facing the N-side.

Absorption: Alpha band is at 554 nm; measured as absorbance at 554 nm minus that at 540 nm

Function: It transfers electrons from Rieske Iron Sulfur Center to plastocyanin which is a mobile protein; light reaction I oxidizes it and light reaction II reduces it.

(2.) *One Copy of Cytochrome b₆*: 2 hemes (low potential, b_L and high potential, b_H)

Molecular Mass: 24 kilodaltons (214 amino acids)

Midpoint Redox Potentials: Two values: -50 millivolts (b_H); -150 millivolts (b_L) the former being the high(H) potential form and the latter the low(L) potential form

Gene is Located in: **Chloroplast** (gene name: pet B)

Arrangement: This protein spans the membrane several times; there are two hemes located about 70 Ångstrom apart, almost vectorially and connected to histidines; the COOH end looks out into the **N-side**.

Absorption: The alpha band of b_H is at 563.3 nm; absorbance is measured as that at 563.3 nm minus that at 572 nm; that of b_L is at 564.8 nm.

Function: In the modified "Q" cycle model, b_L receives an electron from the PQH₂ (plastoquinol) and transfers it to plastoquinone via b_H; details are:

Plastoquinol (PQH₂) delivers an electron (near the Q_Z site) to the low potential form of the heme (Cyt b_L); this transfers its electron to the high potential form of the heme (Cyt b_H) sitting almost across on the other side of the membrane (~50 Ångstrom); this step is electrogenic and produces electrochromism on carotenoids and the membrane potential produced can be measured; reduced b_H (b_H⁻) delivers its electron to a plastoquinone molecule at the so-called Q_C site; a H⁺ is picked up from outside (**N side**) and PQH is made after one reaction and PQH₂ after two reactions.

(3.) *One Copy of Rieske Iron Sulfur Center*

Molecular Mass: 20 kilodaltons (179 amino acids)

Midpoint Redox Potential: +280 millivolts

Gene is Located in: **Nucleus** (gene name: pet C)

Arrangement: There is one membrane spanning helix; the amino terminal end looks to the **N-side** in this protein; the Fe-S and the rest of the protein are on the **P-side**.

Function: It receives one electron from the plastoquinol and delivers its electron to the oxidized cytochrome *f*.

(4.) *One Copy of Subunit IV*

Molecular Mass: 17 kilodaltons (160 amino acids)

Redox Potential: ?

Gene is Located in: **Chloroplast** (gene name: pet D)

Arrangement: Spans the membrane several times and its amino terminal end looks to the **N-side**.

Function: This and the cytochrome b_6 along with a missing loop are equivalent to the cytochrome b in mitochondria. What do you think its evolutionary significance may be?

- (5.) Recently discovered low molecular weight (4-6 KDa) polypeptides of unknown function. (gene name: pet G; coded in the nucleus)

2. Electron Flow Schemes

(Watch for superscript minus and plus signs, minus means electron, and plus means positive charge.)

(1.) Linear:

Electron flow may follow the following sequence:

- PQH_2 (made by PSII reactions) + RieskeFe-S Center (R-Fe-S) \rightarrow R-Fe-S⁻ + PQH with a H⁺ released on the **P-side**
- R-Fe-S⁻ + cytochrome f (Cyt f)⁺ (produced by PSI reaction) \rightarrow R-Fe-S + Cyt f
- $PQH + R-Fe-S \rightarrow PQ + R-Fe-S^-$ with the release of another H⁺ to the **P-side**
- $R-Fe-S^- + Cytf^+ \rightarrow R-Fe-S + Cytf$.

In the above scheme, PQH₂ is the plastoquinol that was made by the reactions in photosystem II (discussed in Govindjee #5). The net result is the transfer of **two** electrons in two one-step reactions and the release of two H⁺s to the **P-side** (lumen, inside).

Thus, the ratio of H⁺/e⁻ is **one** here.

- (2.) **The "Q" Cycle** [This can be best shown on an overhead using coins to mimick PQ, electrons and protons; also see "Photosynthesis and Time" on Govindjee's home page on WWW: <http://www.life.uiuc.edu/govindjee/>; click on the Cyt b₆/f complex; and then on the word "Q-cycle" in blue]

There are two turnovers of the cytb₆/f complex. The first one is: One possible sequence of events is: At the Q_Z site (quinol oxidase site) of the Cytb₆/f complex (see **Figure section**): [looks too complicated, but it is not!]

- (1) $\text{PQH}_2 + \text{R-Fe-S} \rightarrow \text{R-Fe-S}^- + \text{PQH}$, with a H^+ released inside into the **P-side** (this is like the linear scheme so far). But, see below:
- (2) $\text{PQH} + \text{Cytb}_L \rightarrow \text{Cytb}_L^- + \text{PQ}$, with the other H^+ released into the **P-side**
- (3) $\text{R-Fe-S}^- + \text{Cyt}f^+$ (produced by PSI reaction) $\rightarrow \text{R-Fe-S} + \text{Cyt}f$.
- (4) In the middle of the b₆/f complex, $\text{Cytb}_L^- + \text{Cytb}_H \rightarrow \text{Cytb}_L$ and Cytb_H^- (the electron flows vectorially across the membrane, creating membrane potential; see the above discussion on the function of cytochrome b₆).
- (5) At the Q_C site (quinone reductase site) of the Cytb₆/f complex, $\text{Cytb}_H^- + \text{PQ} + \text{H}^+$ (from the **N-side**) $\rightarrow \text{Cytb}_H + \text{PQH}$.

This ends the reactions of the first turnover of the complex.

A second turnover leads to the following (see **Figure section**):

- (6, 7, 8) At Q_Z Site: Same reactions (1,2 and 3) as above
- (9) In the Middle of the Complex: same reaction (4) as above
- (10) At Q_C Site: $\text{Cytb}_H^- + \text{PQH} + \text{another } \text{H}^+$ from the **N-side** $\rightarrow \text{Cytb}_H + \text{PQH}_2$.

The **NET RESULT** of the modified "Q" cycle, discussed here, is the net transfer of 4 H^+ s (delivered inside or the **P-side**) for the transfer of **only 2 electrons** to cytochrome *f*.

Thus, the H^+/e^- ratio is 2, not 1.

This is an ingenious devise to get twice as many H^+ s to the P-side.

(Always remember that to have made PQH₂, photosystem II had taken 2 H⁺s from the outside.)

We can write these as: 2 PQH₂ (at the Q_Z site) + 2 Cyt^f + 1 PQ (at the Q_C site) + 2 more H⁺s from the outside (N-side) → 2 PQ + 2 Cyt^f + 1 PQH₂, with 4 H⁺s delivered inside (P side).

This leads to: PQH₂ + 2 Cyt^f → PQ + 2 Cyt^f + 4 H⁺s released inside (NOTE: to make PHQ₂, 2 H⁺s were taken from outside, and then during the "Q" cycle 2 more H⁺s were picked up from outside).

Some more notes:

- (a.) Cytochrome *f* delivers its electrons to plastocyanin (coded in the nucleus; gene name: *pet E*), which, in turn, delivers its electron to P700⁺ (formed by light I)
- (b.) There are specific inhibitors of the Q_C and the Q_Z sites allowing one to study the "Q" cycle:

Inhibitors of Q_C Site (also known as the Quinone Reductase Site):
NQNO=2-n-nonyl-4-hydroxy-quinoline N-oxide; MOA - stilbene; mucidin.

Inhibitor of Q_Z Site (also known as Quinone Oxidase Site):
DBMIB=2,5-dibromo-3-methyl-6-isopropyl p-benzoquinone.

- (c.) The original "Q" cycle, proposed by the nobel laureate Peter Mitchel, was quite different than the above. Thus, what we describe here is called the modified "Q cycle".
- (d.) This cycle **should never** be confused with the two electron gate discussed earlier which involved electron flow from Q_A to Q_B to the PQ pool to produce PQH₂ used here. There, we dealt with the problem of producing PQH₂, that is reducing plastoquinone to plastoquinol. Here, we are dealing at the Q_Z site with the opposite process: oxidation of plastoquinol to plastoquinone; at the Q_C site, however, PQ is being reduced to PQH₂.

(e.) Both mitochondria and the photosynthetic machinery of photosynthetic bacteria have similar complexes and they are called *Cytb/c* complexes and they oxidize ubiquinol instead of plastoquinol.

(f.) Recent work shows, in agreement with the modified Q cycle scheme: (i) There are indeed two *cytb*: midpoint potentials (E_m , 7) of -50 mv (for b_H) and -150 mv (for b_L): b_L^- is energetically able to transfer electrons to b_H . Right? Do you understand it? (ii) Cytochrome *f* reduction is always coupled with cytochrome *b* reduction as predicted by the scheme; cytochrome *f* and cytochrome *b* are reduced simultaneously, and (iii) Kinetics of cytochrome *f* and cytochrome *b* reduction and oxidation, under conditions of inhibition of Q_C and Q_Z sites, are observed as predicted by the model. There are, however, questions as to whether this cycle really operates *in vivo* and there are many discrepancies in the literature. Conceptually, the "Q" cycle is a beautiful thing.

3. What Transports Electrons from one Complex to Another and How?

I will not have time to discuss this question at all, but you may wonder how electrons move from photosystem II to photosystem I in higher plants when the two are in different physical locations. Photosystem II is located in the appressed membranes in the "grana" and photosystem I in the end membranes or the unappressed membranes, mainly in stroma lamellae. Of course, photosystem II transfers electrons in the form of **plastoquinol** to the cytochrome b_6/f complex. And, the latter complex transfers electrons to photosystem I via reduced **plastocyanin** molecules. Both PQH_2 and PC (you know the abbreviations, right) are mobile carriers. Plastocyanin is a small protein (10 kilodaltons, for short, kDa).

The **diffusion** is certainly possible and can be very fast. The equation given by Einstein is:

$\langle r^2 \rangle = 4 D t$, where r^2 is the square of the distance of movement, D is the diffusion coefficient, and t is the diffusion time. (This equation is for a 2-dimensional case.)

If we imagine that a electron carrier has to move 1,000 Ångstroms, then, any entity having a D value of $\sim 1.2 \times 10^{-8}$ will reach its destination in only 2 milliseconds which is pretty good as the bottle-neck reaction of electron flow in photosynthesis is of the order of 5 milliseconds.

- * **Home Work:** Please check the above calculation. **How long will it take to reach 1,580 Ångstroms?**

(4). **News:**

- The hydrophilic portion of cytochrome f from turnip has now been crystallized at Purdue University. Thus, its detailed structure is now available:

Martinez, S.E., D. Huang, A. Szczepaniak, W.A. Cramer and J.L. Smith (1994) Crystal structure of chloroplast cytochrome f reveals a novel cytochrome fold and unexpected heme ligation. *Structure* (Current Biology Ltd.) 2:95-105.

- Cyt b/c complex from a totally different organism has been crystallized and its crystal structure is now available: Hopefully, I will provide the reference to you by the time we get to this section.
- Further, the structure of the hydrophilic portion of the Rieske iron-sulfur protein has been solved. Although it is from bovine heart mitochondria, it is expected that chloroplast component will have similarities to it:

Iwata, S., Saynovits, M., Link, T.A. and H. Michel (1996) Structure of a water soluble fragment of the 'Rieske' iron-sulfur protein of the bovine heart mitochondrial cytochrome bc₁ complex determined by MAD phasing at 1.5 Å resolution. *Structure* 4: 567-579.

(5) **References**

I will provide here only one reference to an excellent and very detailed review (other references may be found in that chapter):

Kallas, T. (1994) The Cytochrome b₆f complex. In: D. Bryant (ed.) *The Molecular Biology of Cyanobacteria*, pp. 259-317. Kluwer Academic, Netherlands.

Questions

- [1] Describe exactly how you will explain to a first year B.Sc. student and/or your younger brother or sister as to how cytochrome b/c in his/her mitochondria may be transferring two protons per electron going for the reduction of oxygen to water.
- [2] Would you be able to demonstrate the "Q" cycle at a party using differently colored balloons representing electrons and protons using your friends as intermediates? If so, please make a detailed map as to how many intermediates, electrons and protons you will need, and lay down the steps to be followed. Good luck.

Table 1. Protein subunits and redox centers common to cytochrome b_6f and bc_1 complexes^a

Cytochrome b_6f complexes				Cytochrome bc_1 complexes		
Gene (coding)	Protein	kDa ^b (amino acids)	Prosthetic group or substrate binding site	Gene ^c	Protein	kDa ^d
<i>petA</i> (ct)	cytochrome f	31.2 (285)	heme c (Cyt f) and a bind site for Cyt c_{553} or PC ^e	<i>fbcC</i> (<i>petC</i>)	cytochrome c_1	30.3
<i>petC</i> (nuc)	Rieske Fe-S	19.2 (179)	2Fe-2S center	<i>fbcF</i> (<i>petA</i>)	Rieske	20.4
<i>petB</i> (ct)	cytochrome b_6	24.3 (214)	heme b_H heme b_L	<i>fbcB</i> (<i>petB</i>)	cytochrome b (N-terminal)	49.4
<i>petD</i> (ct)	subunit IV	17.5 (160)	Q_{ox} and Q_{red} sites ^f		cytochrome b (C-terminal)	
<i>petG</i> (nuc)	unnamed	4.1	?	?	?	

^aThe *petG* gene product is as yet a possible and not an established component of the cytochrome b_6f complex.

^bMolecular masses are those for the *Nostoc* sp. strain PCC 7906 complex based on sequence data (Kallas et al., 1998b). The *petG* molecular weight is based on the sequence of the *Cyanophora paradoxa* cyanelle gene (Stirewalt and Bryant, 1989).

^cThese genes from purple photosynthetic bacteria have been designated either *fbc* (Gabellini and Sebald, 1986) or *pet* (Davidson and Daldal, 1987). Mitochondrial bc_1 complexes contain several additional protein to which there are no corresponding subunits in Cyt b_6f complexes (Trumpower, 1990)

^dMolecular weights are those for the *Rhodobacter capsulatus* complex based on sequence data (Davidson and Daldal, 1987).

^eKnown electron acceptors for the Cyt b_6f complex are one or both of the soluble proteins, cytochrome c_{553} or plastocyanin.

^fCurrent evidence points to involvement of both the cytochrome b_6 and subunit IV proteins (equivalent to cytochrome b in bc_1 complexes) in formation of two discrete quinone-binding sites.

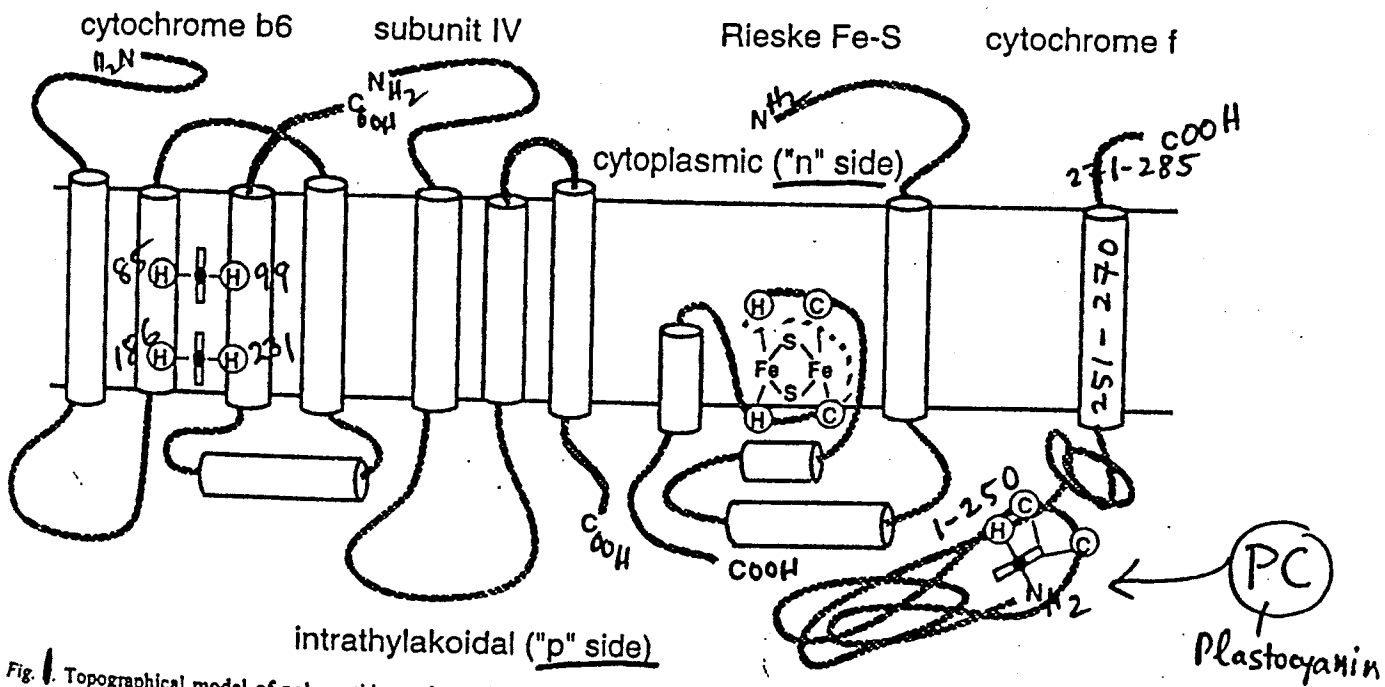
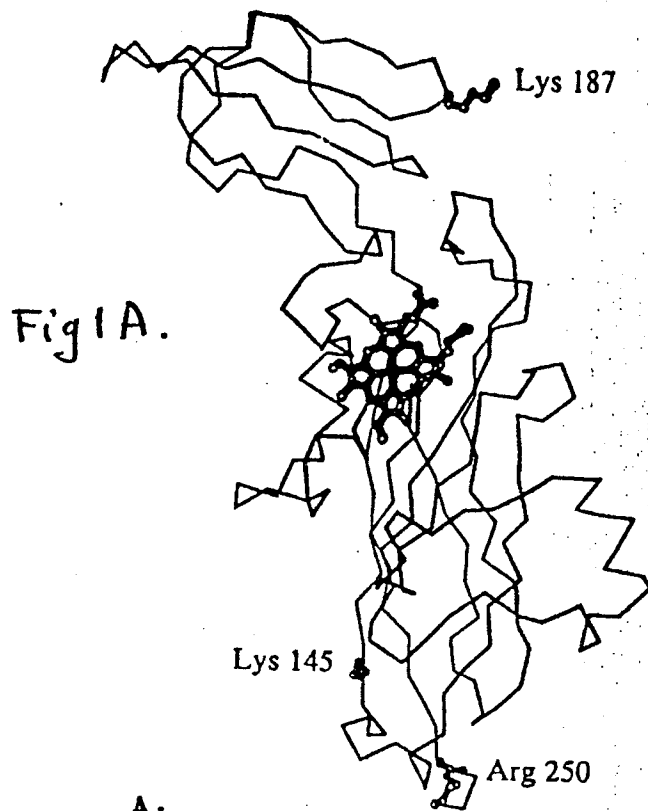


Fig. 1. Topographical model of polypeptides and prosthetic groups (two protohemes of Cyt b_6 , the 2Fe-2S Rieske center, and the covalently bound c-type heme of Cyt f) in the Cyt b_6f complex. Horizontal lines depict the membrane bilayer. Amino (N) and carboxyl (C) termini of polypeptides are shown as are probable ligands for the prosthetic groups. Cylinders represent likely membrane-spanning and other possible alpha helical regions.

(Kallas, T., 1994, in press)



A.
 Fig. 1. α -carbon backbone model of the lumen side extrinsic polypeptide of turnip chloroplast Cyt *f* showing residues Lys145 and Lys187 in the large and small domains, and Arg250 at the carboxyl-terminus near the membrane surface, that are separated by 33, 28, and 45 Å from the iron of the heme that is also shown. As discussed in the text, Lys187 forms a contact with plastocyanin and Arg250 may interact with the Rieske protein (note that Lys187 is not conserved in cyanobacteria). Lys145 was formerly thought to serve as an axial ligand for the heme. This figure is courtesy of Dr. W. A. Cramer and is reprinted with permission from Plenum Press.

FIGURE 2

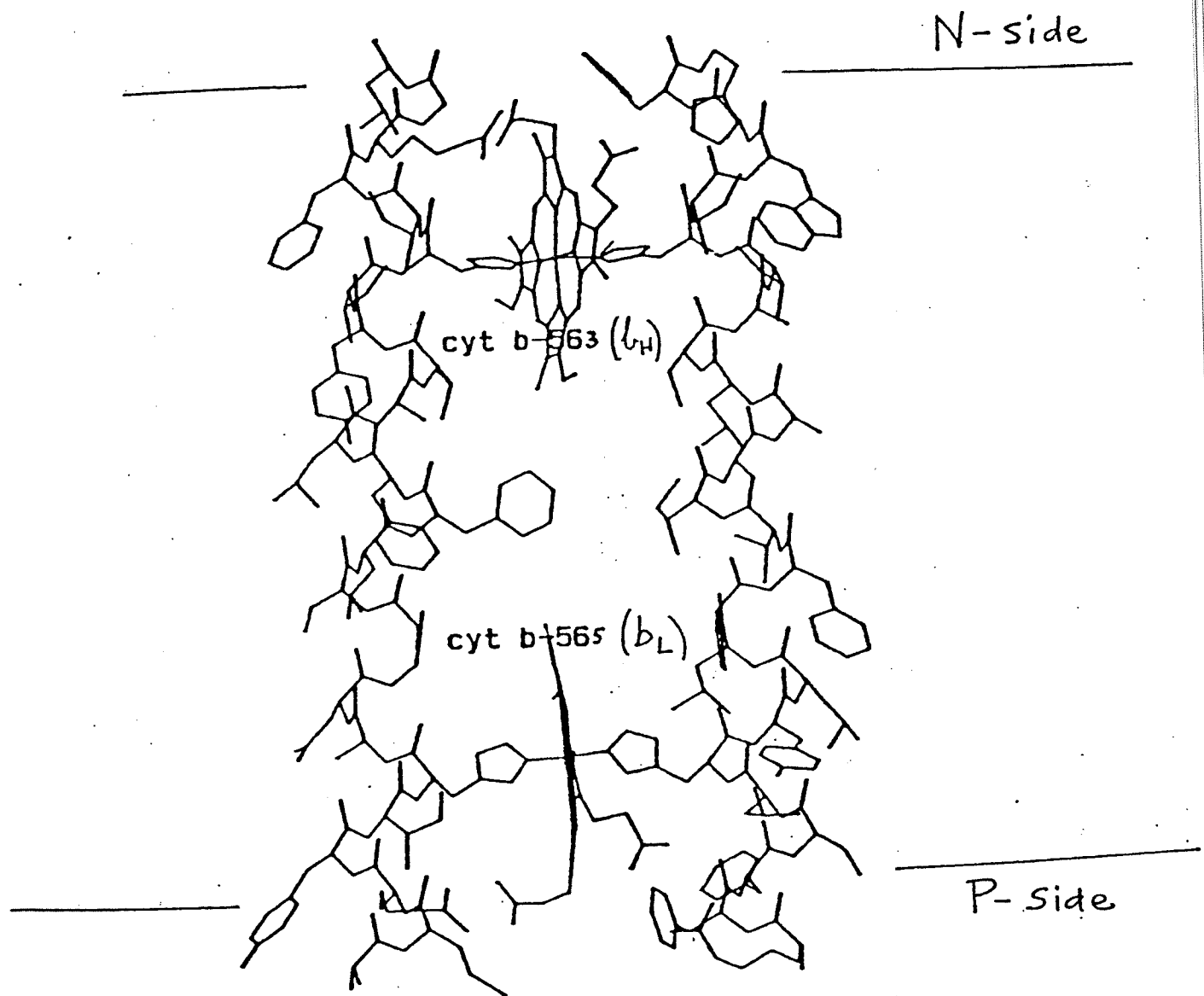
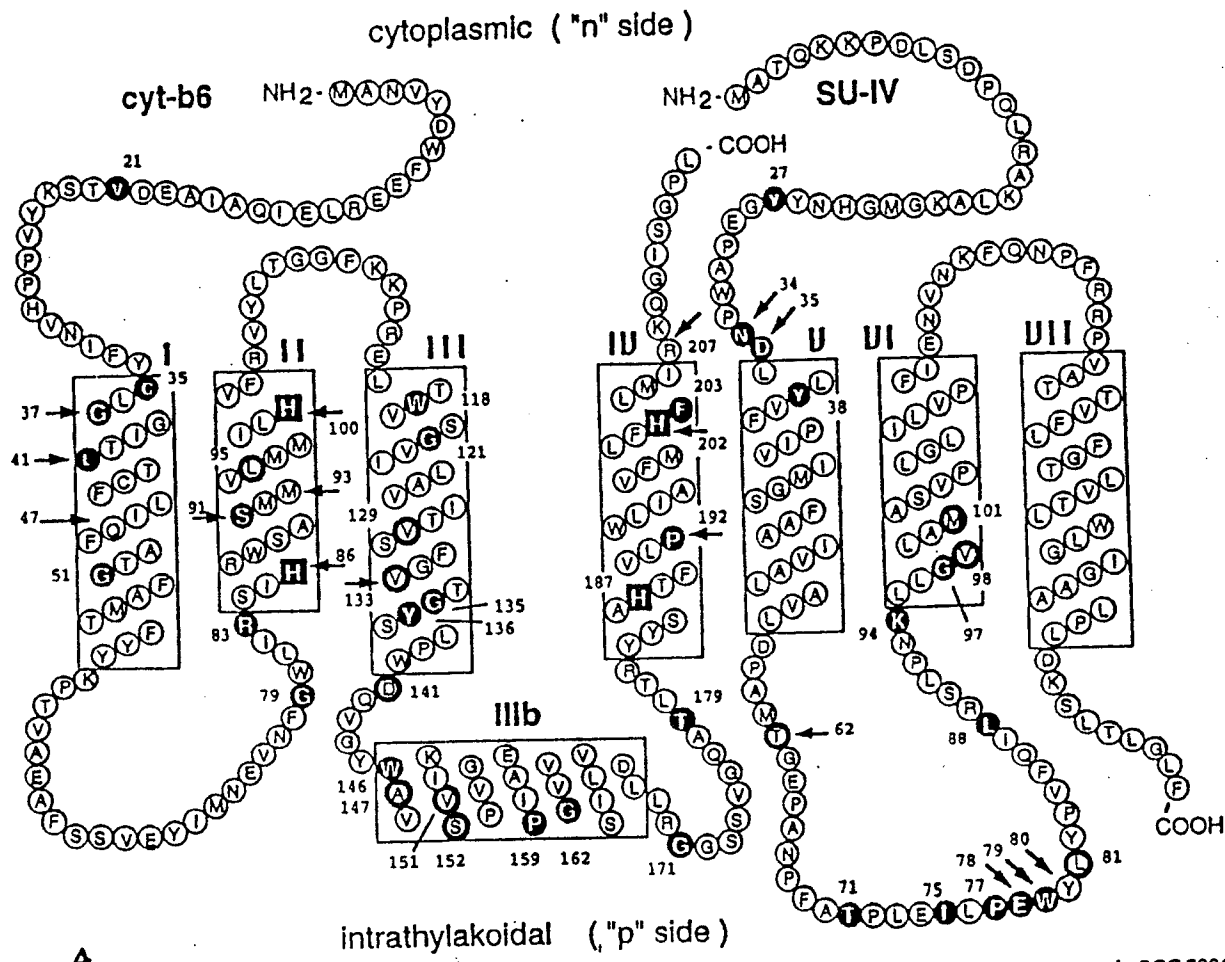
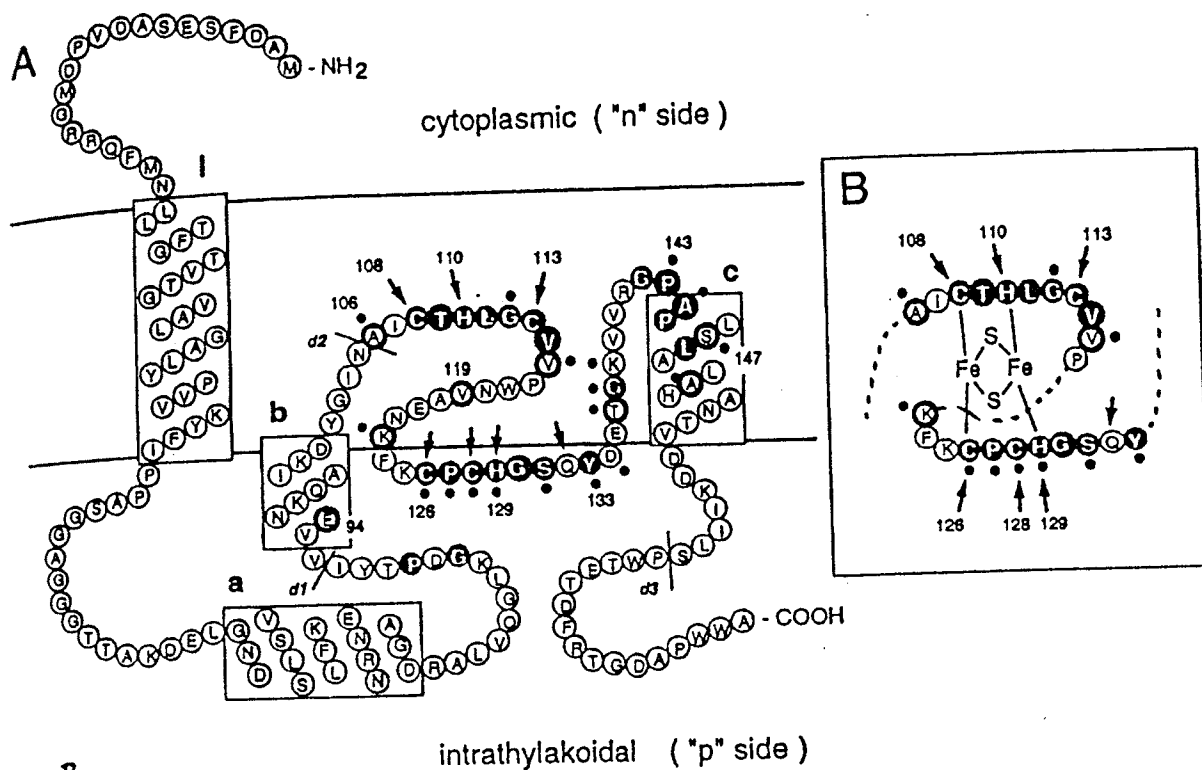


Diagram shows the location of two hemes (Cytb 563: b_H Cytb565: b_L) in Cyt b/c complex of an anoxygenic bacterium



A
 Fig. 2 Membrane-spanning model for the Cyt *b*₆ and subunit IV polypeptides based on the sequences from *Nostoc* sp. strain PCC 7906 (Kallas et al. 1988b). Large rectangles indicate membrane-spanning helices and one membrane-parallel (IIIb) helix. Shaded residues are those strictly conserved in all known Cyt *b*₆/SU IV (or Cyt *b*) sequences (Hauska et al. 1988, Widger and Cramer, 1991). The boxed His residues represent the conserved, probable ligands for hemes *b*_L (H86 and H187) and *b*_H (H100 and H202). Residues corresponding to sites of mutations in Cyt *b* proteins (*bc*₁ complex) conferring resistance to quinol-oxidation (bold circles) and quinone-reduction (darkened circles) inhibitors are shown in the Figure and described in Tables 5 and 10. Arrows show residues corresponding to site-directed mutations in Cyt *b* as described in Tables 8 and 11. The residue corresponding to Asp(D)35 in subunit IV is both conserved and the site for a mutation conferring resistance to the quinol-reduction inhibitor, antimycin-A (Park and Daldal, 1992).

Fig. 2A (after T. Kallas, 1994, in press)

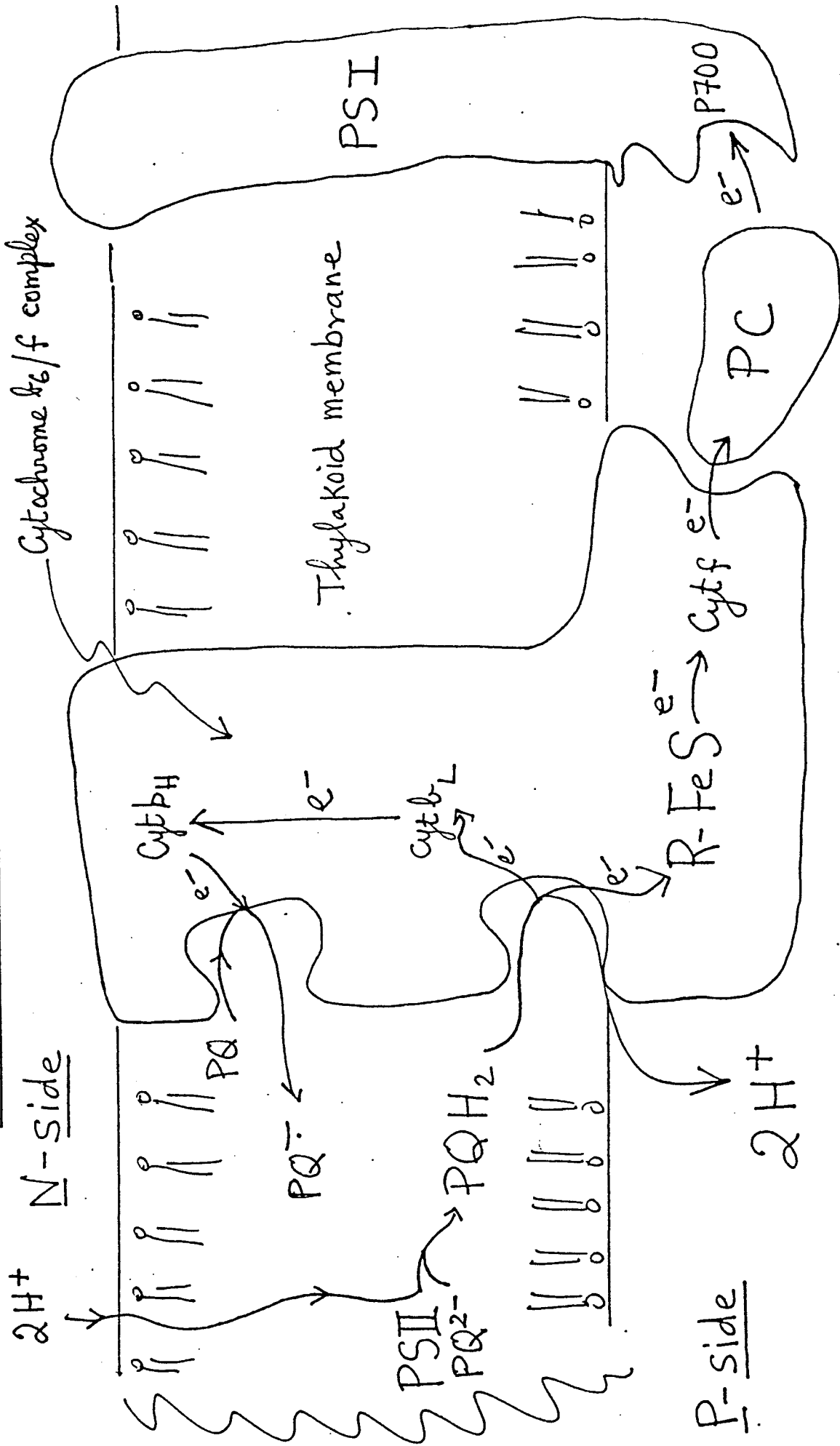


B
 Fig. 2 Panel A: Membrane-spanning model for the Rieske Fe-S protein based on the sequence from *Nostoc* sp. strain PCC 7906 (Kallas et al. 1988a). Inset B shows the region from A106 to Y133 which contains conserved Cys and His ligands for the 2Fe-2S center (details and references in the text). Shaded or darkened residues are those that have been strictly conserved in all known Rieske sequences (Graham et al. 1993). Residues corresponding to sites of respiratory-defective, petite (marked with dots) and temperature-sensitive mutations (bolded) in the yeast Rieske protein are described in Table 12. Arrows show residues corresponding to site-directed mutations in Rieske proteins from Cyt bc_1 complexes as described in Table 13. Darkened residues correspond to those at which site-directed mutations result in nonfunctional Cyt bc_1 complexes (Table 13). The junctions $d1$, $d2$, and $d3$ mark discontinuities in the Cyt b_6f -type Rieske relative to the Cyt bc_1 -type Rieske sequences. The alignment of the Cyt b_6f - and bc_1 -type Rieske sequences is open to interpretation (discussed in the text) but the residues beyond $d3$ are clearly absent in Rieske proteins from Cyt bc_1 complexes.

Fig 2B (after T. Kallas, 1994, in press).

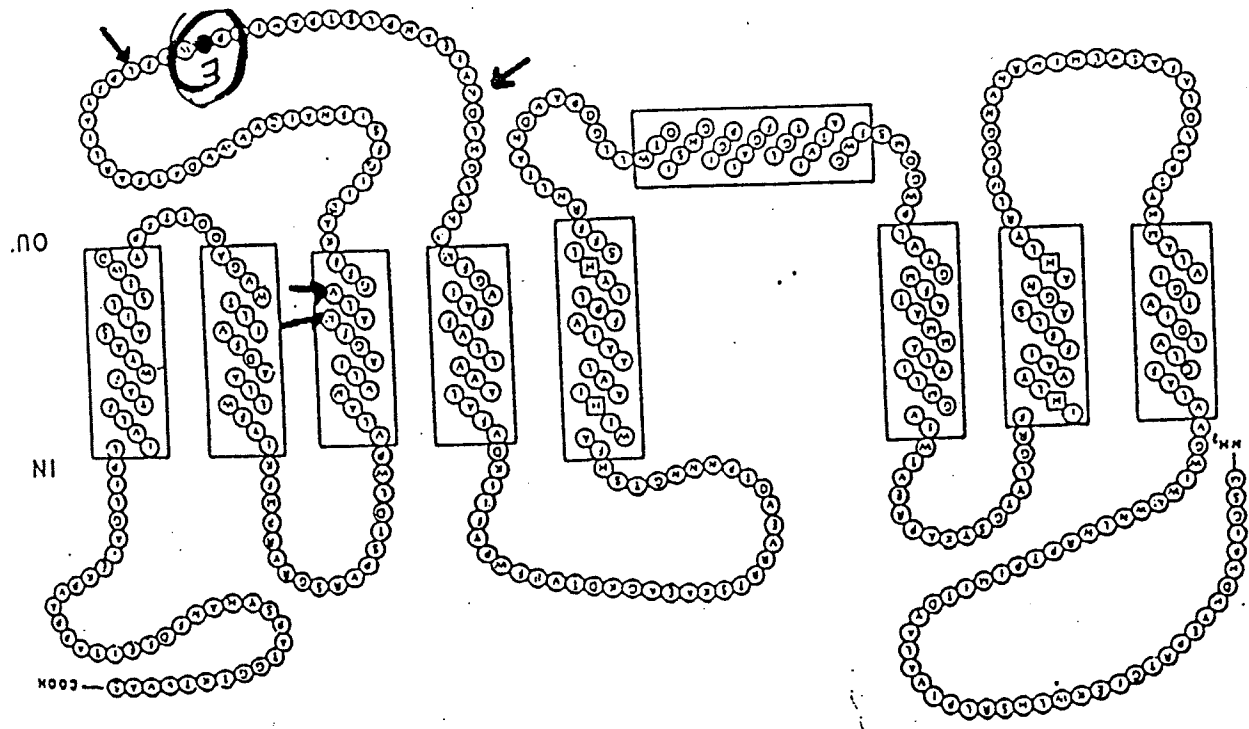
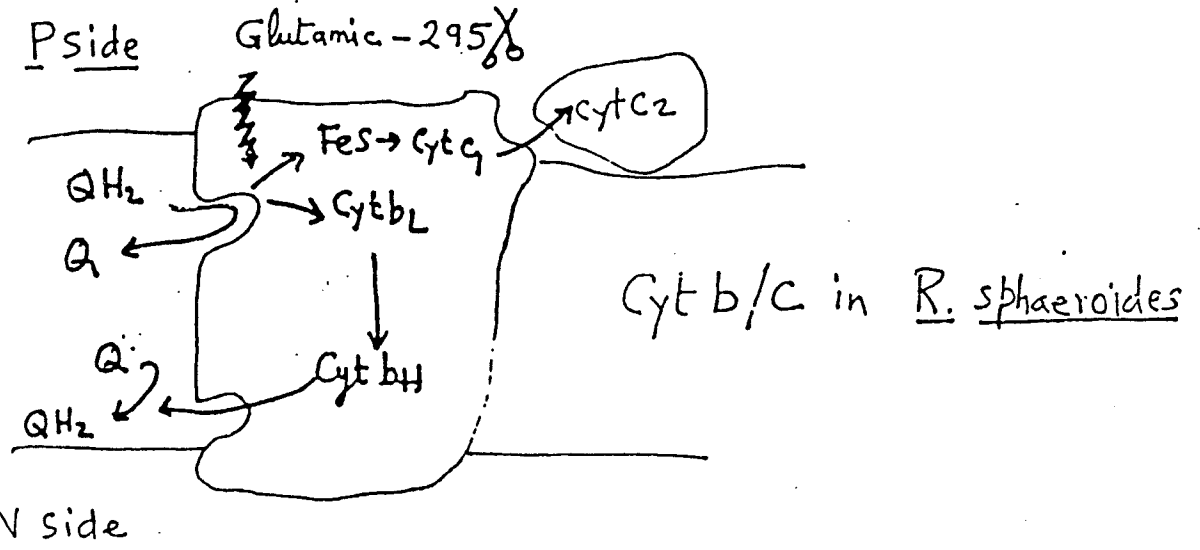
FIGURE 3

First PQH₂ Oxidized



The "Q" Cycle - step 1.

FIGURE 5

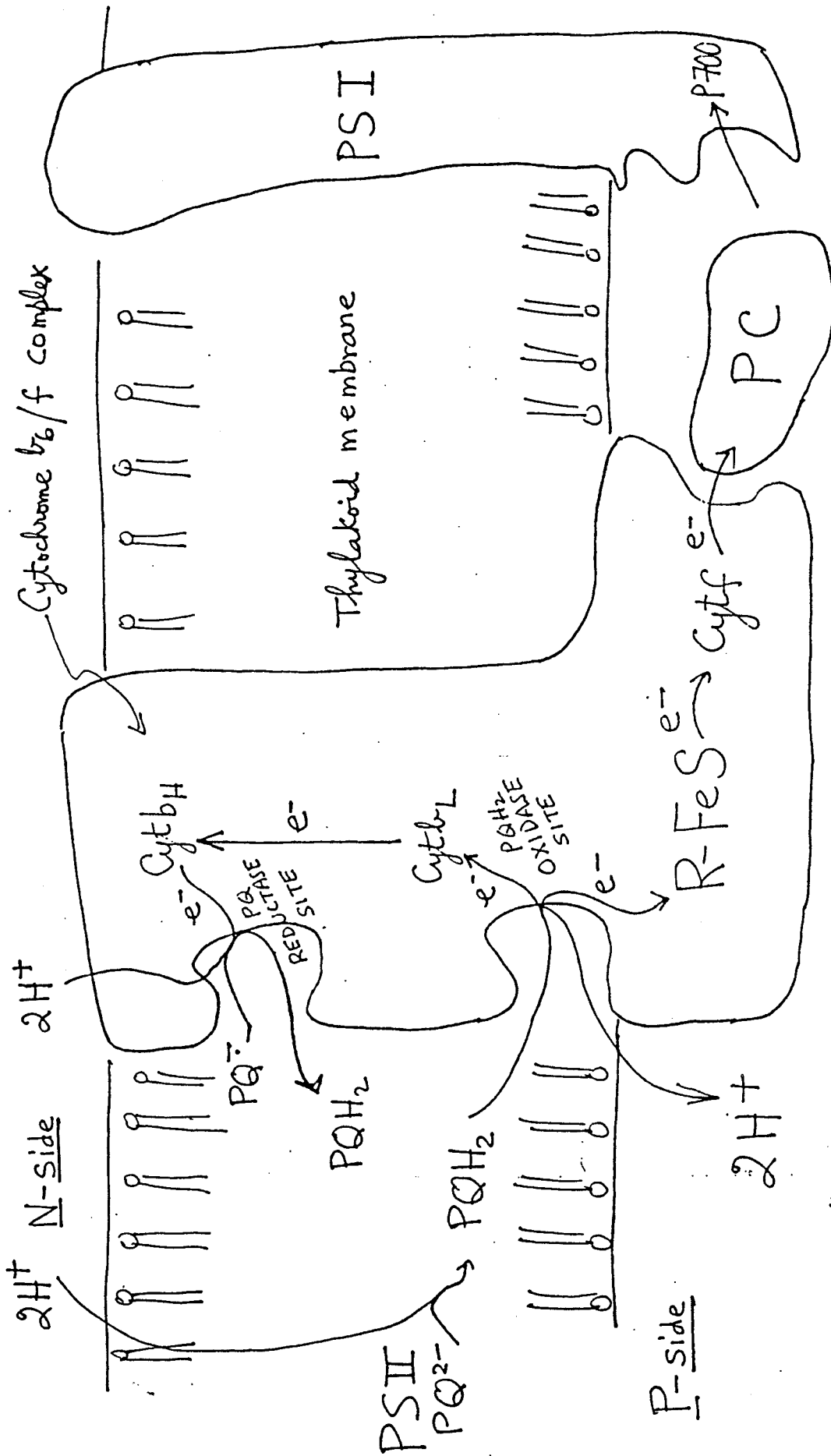


The "Q" Cycle - Step 1.

When glutamic-295 is changed by mutagenesis to glutamine, reduction of Cyt b_L and of Cyt b_H does not occur (Gennis and co-workers).

FIGURE 4

Second PQH₂ oxidized

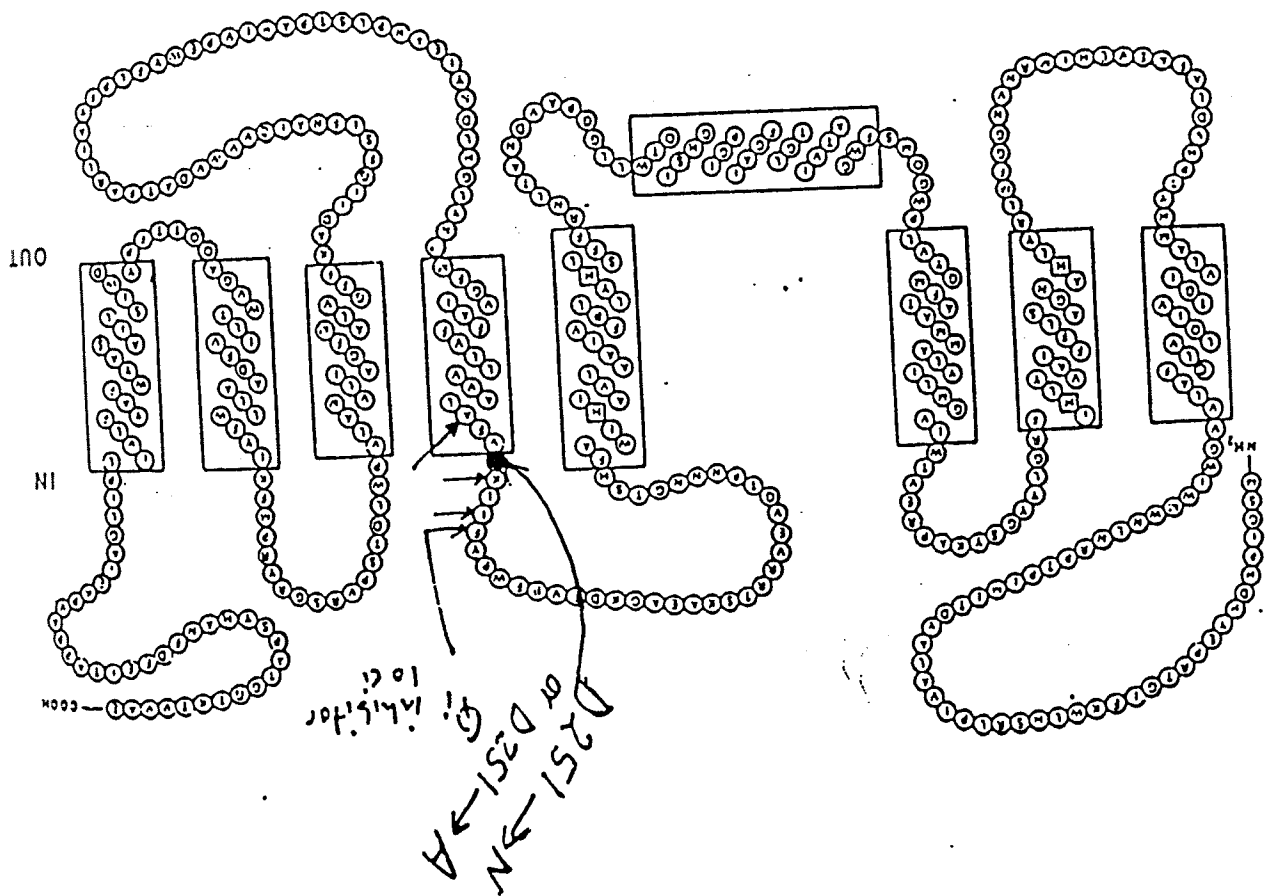
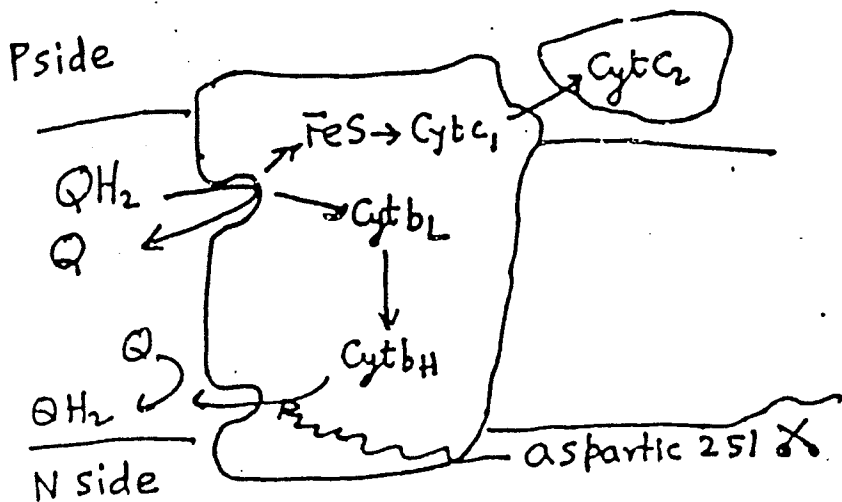


The "Q" cycle - step 2.

Result: 4H⁺ were released on the P side for 2 electrons passed to PSI. Thus, we have 2H⁺/e⁻ transferred! Two PQH₂ were used but one was produced. Only one PQH₂ was used.

Result: $4H^+$'s were released on the Pside for 2 electrons passed to PSI. Thus, we have $2H^+$'s/ e^- transferred! Two PQH₂ were used but one was produced. ∴ only one PQH₂ was used.

FIGURE 6



When aspartic acid 251 is changed by mutagenesis to asparagine or to alanine, reoxidation of Cyt bH is blocked (Gennis & coworkers).

Govindjee

8

Plastocyanin-ferredoxin oxido-reductase (Photosystem I): Components and Electron transfer pathways

"Controversies in scientific concepts usually are conditioned by the overemphasis of a limited number of observations. These controversies tend to disappear after extensive observations have made possible the formulation of broader concepts which include the apparent contradictions as special cases definitely governed by different, although equally definite conditions" (van Niel and Mueller, 1931)

Outline

From Plastocyanin to NADP⁺ (Photosystem I)

Please see a brief description of Photosystem I in Govindjee # 5.

(1) Function of PSI

The function of PSI is to reduce NADP⁺ and oxidize the copper protein plastocyanin that is, in turn, reduced by photosystem II via cytochrome b₆/f complex. Reduced NADP⁺ (NADPH) is used in carbon fixation cycle in stroma. The process starts with charge separation in which P700 (special pair chlorophyll *a* of photosystem I) is oxidized and A₀ (a special chlorophyll *a* molecule) is reduced. Measurements by Jim Fenton (in my laboratory) indicate that it occurs within 14 picoseconds (ps). The real time is now estimated to be 3 ps. Measured time includes excitation energy time. Charge separation means production of P700⁺ A₀⁻.

Spectral characteristics of P700 and of A₀ include specific, but different, difference (light-dark) absorption peaks (negative or positive); the dimer character of "P700" is inferred from the double peak due to excitonic interactions between the 2 molecules of the dimer. (see **Figure section**).

The figure section further shows a possible detailed scheme involving the following intermediates: A₀ (chlorophyll *a*); A₁ (a phylloquinone); F_X, F_B and F_A (all iron-sulfur centers); and F_d (ferredoxin). With the help of FNR (ferredoxin-NADP⁺ reductase), reduced ferredoxin reduces NADP⁺. And, oxidized P700 (P700⁺) recovers an electron from plastocyanin, the copper protein.

Approximate times of forward and back reactions are also shown in the figure section. Do you notice something interesting when you compare the times for the forward and the back reactions? Yes, the forward rates are many orders faster than the backward rates making photosynthesis possible.

The iron sulfur centers (F_X, F_A, F_B) can all be monitored, just as all components with "unpaired electrons" are, by **electron spin resonance spectroscopy (ESR, for short; some call it EPR or electron paramagnetic resonance)**. This technique has a lot of medical applications too. Do you know any?

The figure section also shows ESR spectrum of F_X; it is usually plotted as the first derivative. A brief explanation will be given in the lecture (note diagrams in the figure section, H stands for magnetic field). It may be a bit too complicated, but please try to get the **basics** from the description by the instructor.

Photosystem I has an extremely low fluorescence at room temperature and its fluorescence does not monitor its chemistry unlike that of photosystem II. However, at liquid nitrogen temperature, 77 degrees Kelvin, one observes fluorescence bands at 720 nm and at 735 nm, the former arises from the reaction center complex and the latter from the light harvesting complex of photosystem I.

(2) **Structure and Composition (see Figure section):**

Subunit Function	MW, kilodaltons	Gene	Gene Coding
IA (A) IB (B)	55-70 55-70	psaA psaB	chloroplast } chloroplast } P700, A ₀ , A ₁ , F _X and core antenna Chl <i>a</i>
II (D)	19-25	psaD	Nuclear Ferredoxin docking protein
III (F)	16-20	psaF	Nuclear Plastocyanin docking protein
VII (C)	8-9	psaC	Chloroplast F _A , F _B carrying protein

Function of other proteins that are products of genes *psaE*, *psaG*, *psaH*, *psaK* (all 4 nuclear) and two low molecular weight (4.1 and 1.5 kDa) proteins coded by chloroplast genes *psaJ* and *psaI* are unknown. (Names in parentheses refer to names used by some researchers.)

In addition to these components, there are light-harvesting chlorophyll *a* / chlorophyll *b* complexes, called LHC*a* and LHC*b* (20-24 kilodalton range); these function as antenna pigments.

The figure section also shows a schematic arrangement of subunit Ia in the membrane labeled as PSI-A. H stands for histidines where chlorophyll *a* molecules may bind; the rectangles represent the helices going through the membranes. For comparison, we show you the D1 protein from photosystem II—this has only 5 transmembrane helices, but subunit Ia (that harbors P700, we think) has a lot of antenna chlorophyll *a* molecules in addition to P700 that maybe shared, most likely, with the subunit Ib that is very similar to Ia.

News

It is interesting to note that several research groups have crystallized photosystem I (PS I) complexes, and the x-ray diffractions of PS I crystals have begun to yield a detailed picture in the laboratory of Professor H.T. Witt in Berlin. We will mention these results, but the handout may not include this work.

References

For a current description of PSI structure, SEE: Schubert et al. (1996)

Photosynthesis: From light to Biosphere. Vol. II, pp. 3-10. (editor P. Mathis), Kluwer, Dordrecht, the Netherlands.

Also, SEE: several chapters in the 1996 book: "Oxygenic Photosynthesis: Light Reactions", edited by D.Ort and C.F.Yocum, Kluwer Academic, Dordrecht (Advances in Photosynthesis Series, Vol. 4, Series editor, Govindjee)

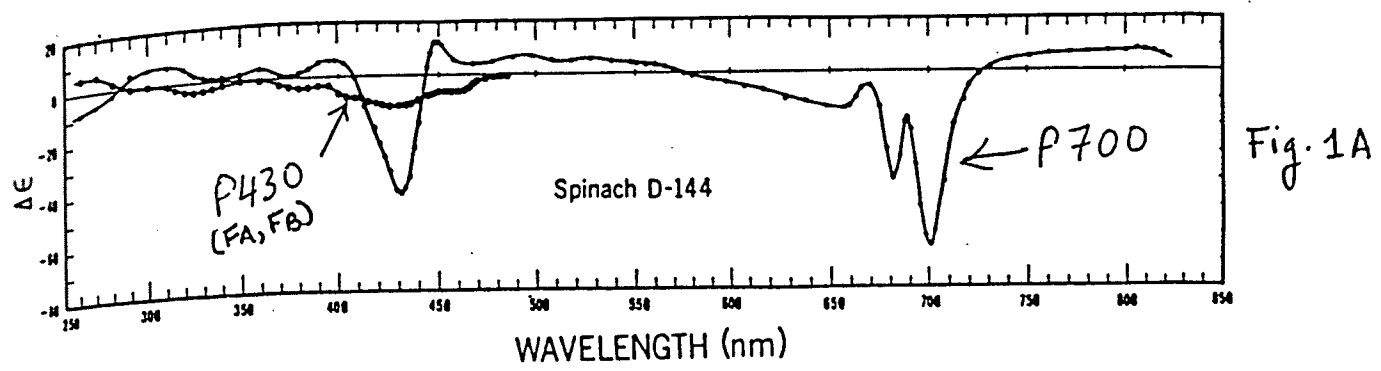
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Questions

- [1] It is now time to write an essay on similarities and differences between the two photosystems (I and II) of plant, algal and cyanobacterial photosynthesis. Wherever possible, a comparison must be made with the anoxygenic bacterial reaction center. The essay should be divided under the following headings: (1) Introduction; (2) Light capturing system and light capture; (3) Reaction center composition; (4) Genetic coding of the proteins; (5) Nature of electron carriers and their function; and (6) Overall energetics.
- [2] In 1995 and 1996, Elias Greenbaum, at Oak Ridge National laboratory, has published papers in NATURE* as well as SCIENCE # showing that a Photosystem I- less mutant of *Chlamydomonas reinhardtii* can live autotrophically suggesting that Photosystem I may not be necessary for the life of a plant. Please find these papers, read them, and, then write an essay on its possible implications on normal photosynthesis of wild type plants.
- * E.Greenbaum, J.W. Lee, C.V. Tevault, S.L. Blankenship and L.J. Mets (1995) CO₂ fixation and photoevolution of H₂ and O₂ in a mutant of *Chlamydomonas* lacking Photosystem I. *Nature*, **376**: 438-441.
- # J.W. Lee, C.V. Tevault, T.G. Owens and E. Greenbaum (1996) Oxygenic photoautotrophic growth without Photosystem I. *Science* **273**: 364-367.

Fingerprinting of intermediates by difference absorption spectroscopy.



Light-minus-dark difference spectra for (P700⁺-P700) and (P430⁻-P430) in digitonin-fractionated PSI particles. (From Ke.

1973.)

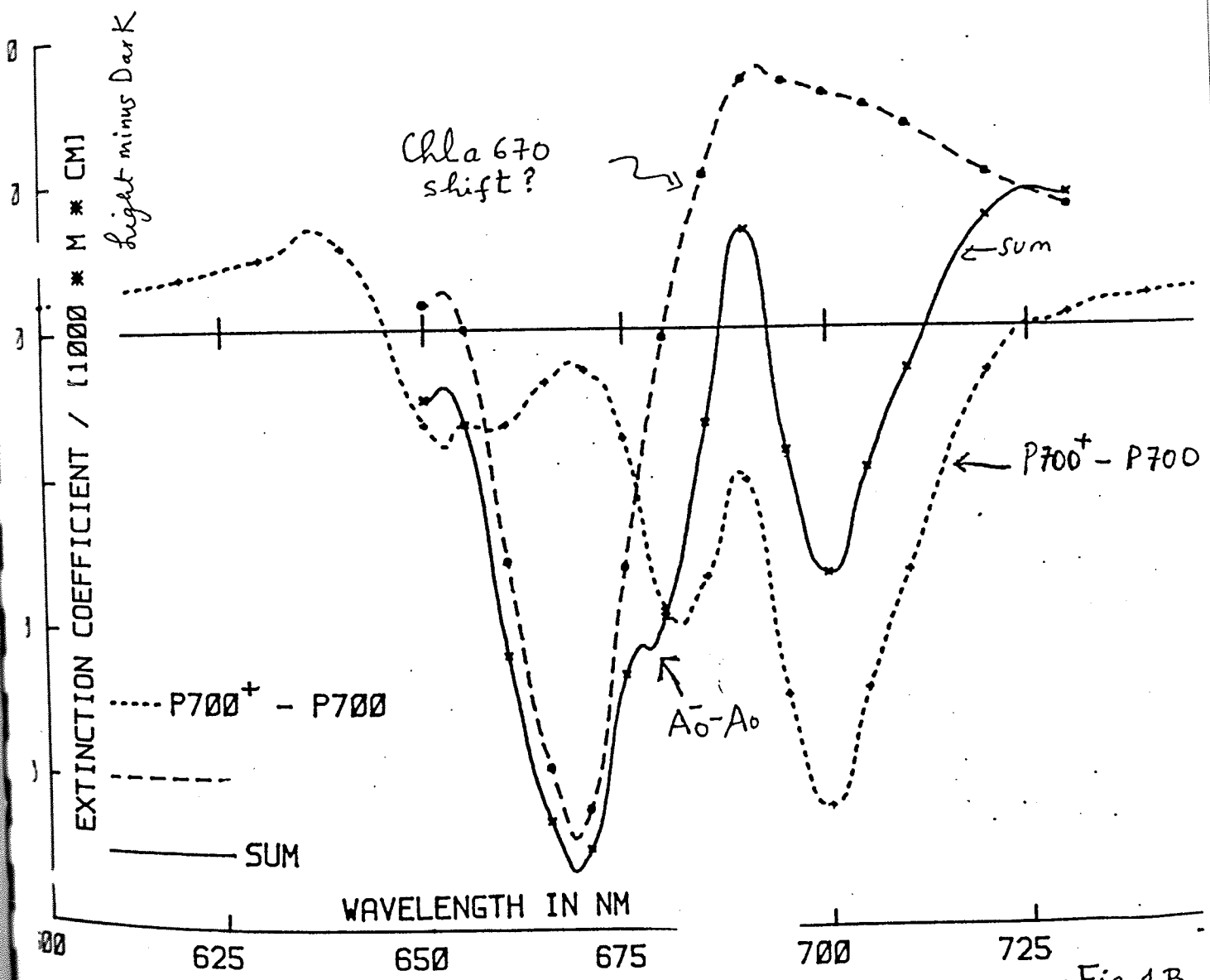


Fig. 1B

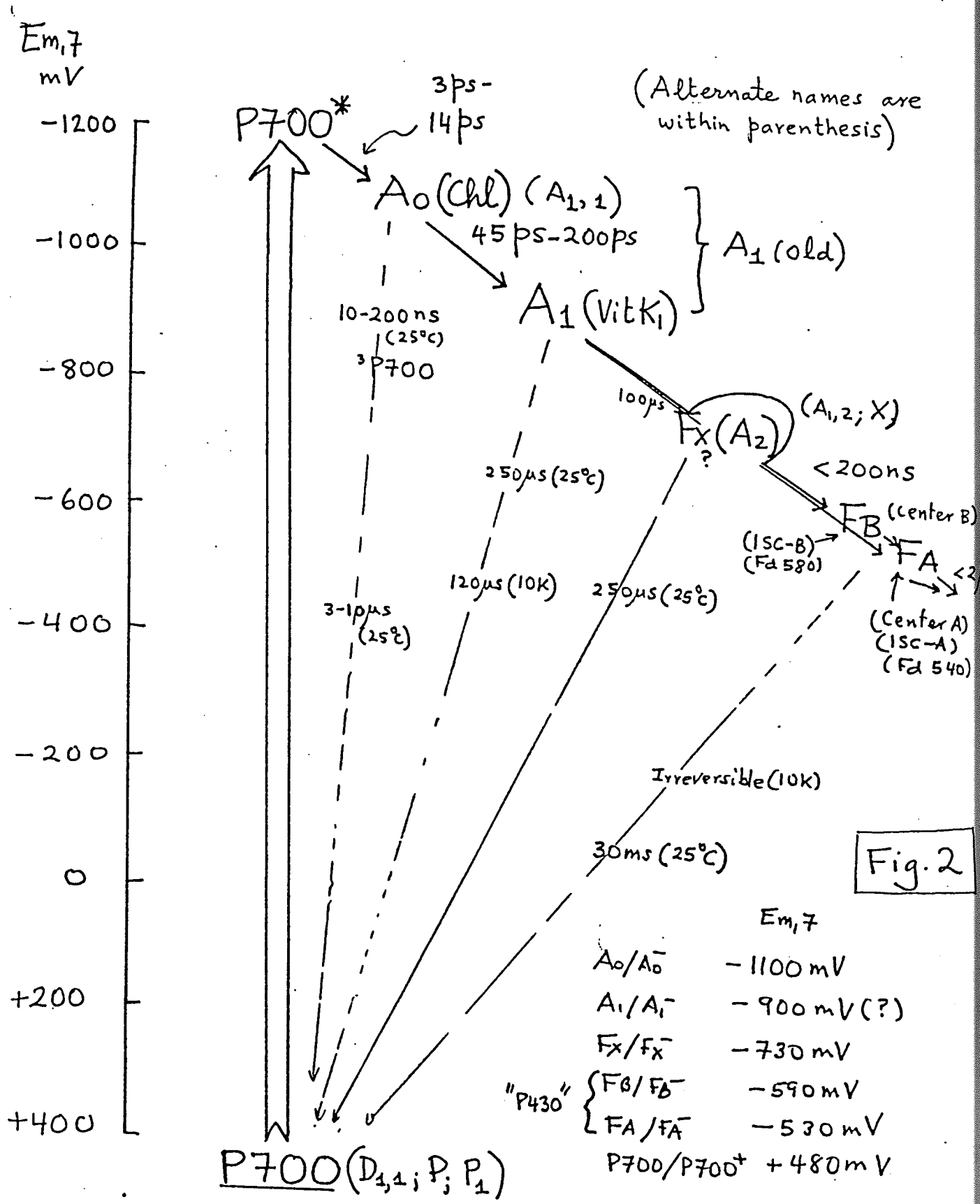


Fig. 2

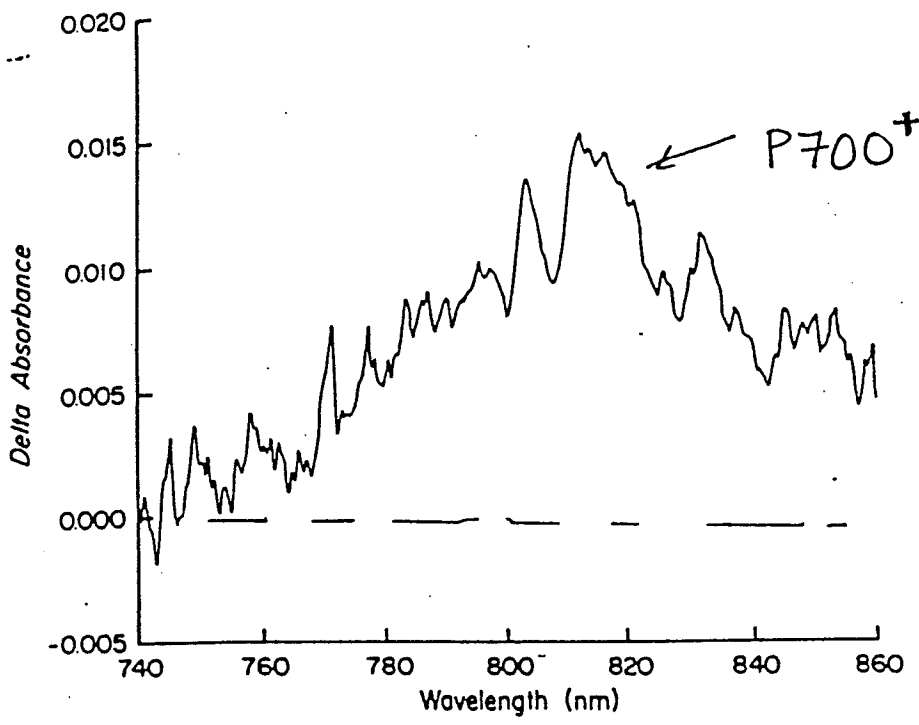


Fig. 3

Wavelength dependence of transient absorbance change of PS-I particles (with antenna changes subtracted) at 0 ps following a 1.5 ps, 610 nm laser pulse. (1987)

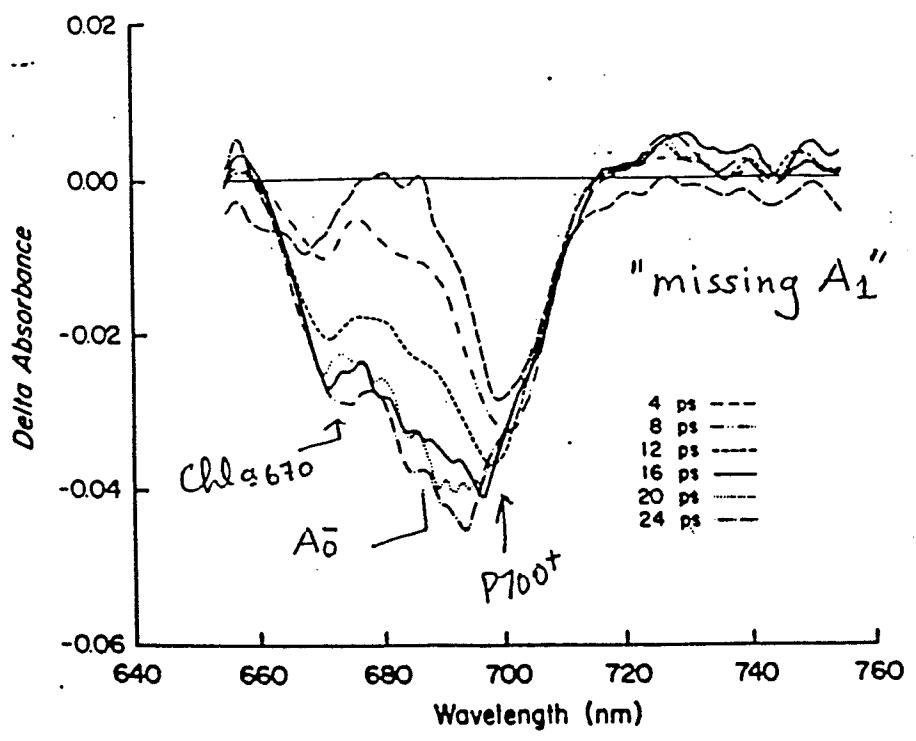
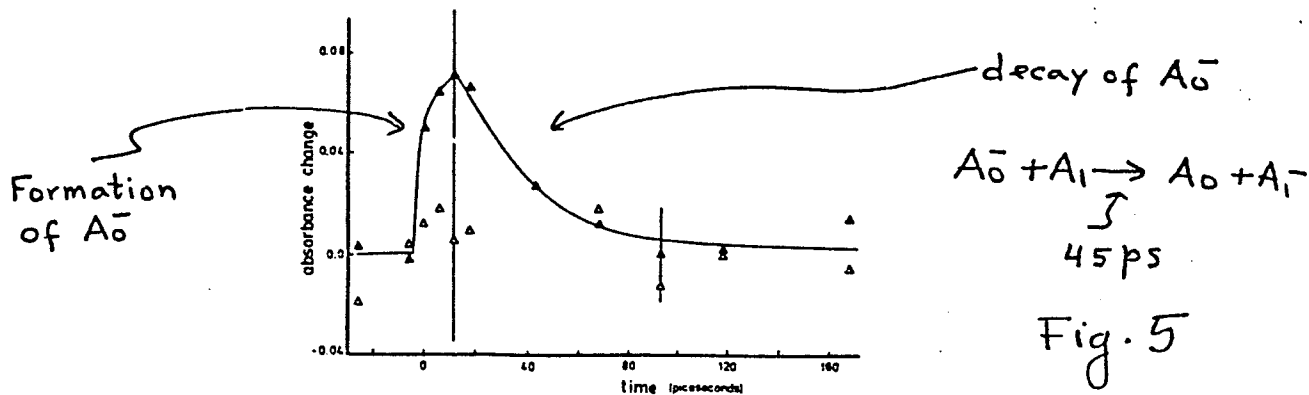


Fig. 4

Wavelength and time dependences of transient absorbance changes of PS-I particles (with antenna changes subtracted) following excitation with 1.5 ps, 610 nm laser pulse. (1987) Wasielewski et al.

Newer results are now going to be available from the laboratory of R. Blankenship (1994)



Kinetics at 730 nm, which is the isobestic point for the (P700⁺-P700) difference spectrum, in dark-adapted samples (\blacktriangle) and samples illuminated

Fenton et al. (1979)

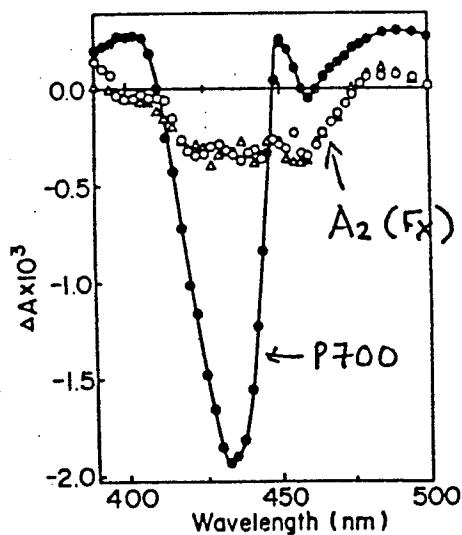
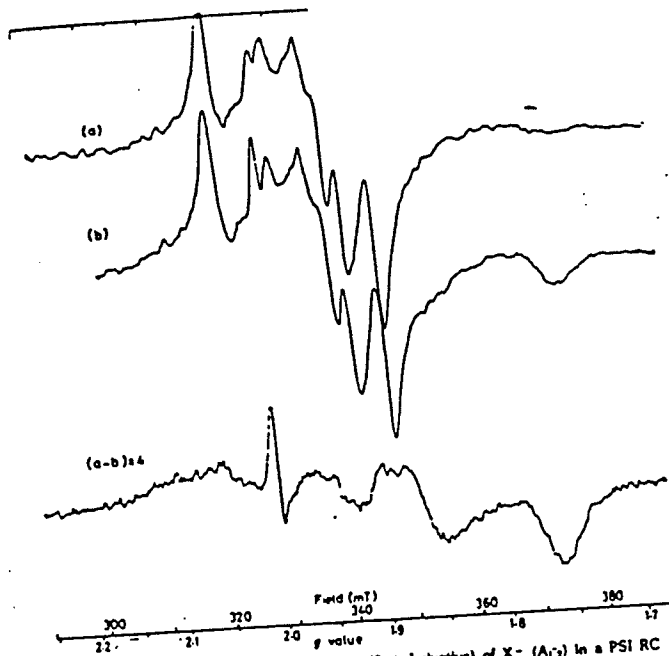


Fig. 6

The difference spectrum of A_2 :
 Solid circles, the difference in total absorption changes invoked by flashes. Open symbols, the difference between the difference spectrum of total absorption changes which indicate oxidation of P700 plus reduction of A_2 and that of the P700 photooxidation. Results from two sets of experiments are presented with (O) and (Δ).

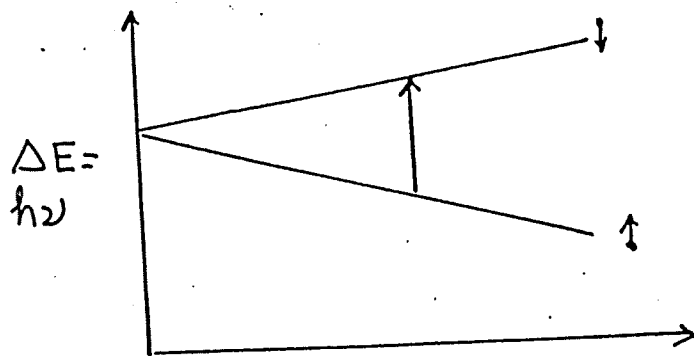
Fingerprinting of intermediates by ESR

Fig. 7

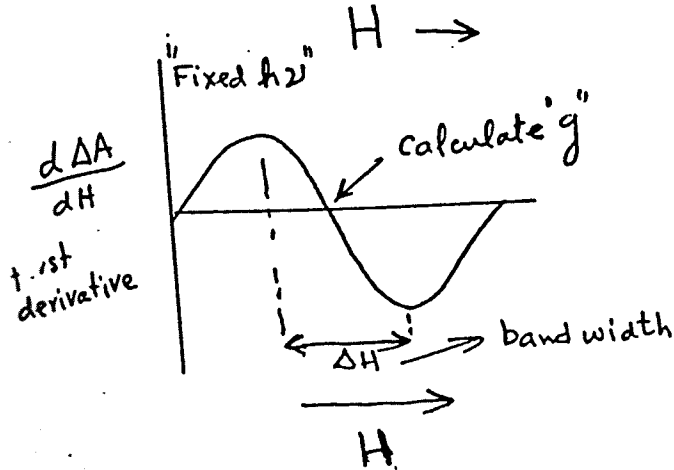


ESR of "Fx"

The light induced ESR spectrum (first derivative) of X- (A₁)₂ in a PSI RC preparation made with Triton X-100 (bottom). The dark adapted sample (top) was illuminated in the ESR spectrometer (middle). T. 10 K, ν. 9.25 GHz. (From M. C. W. Evans et al., 1976b.)

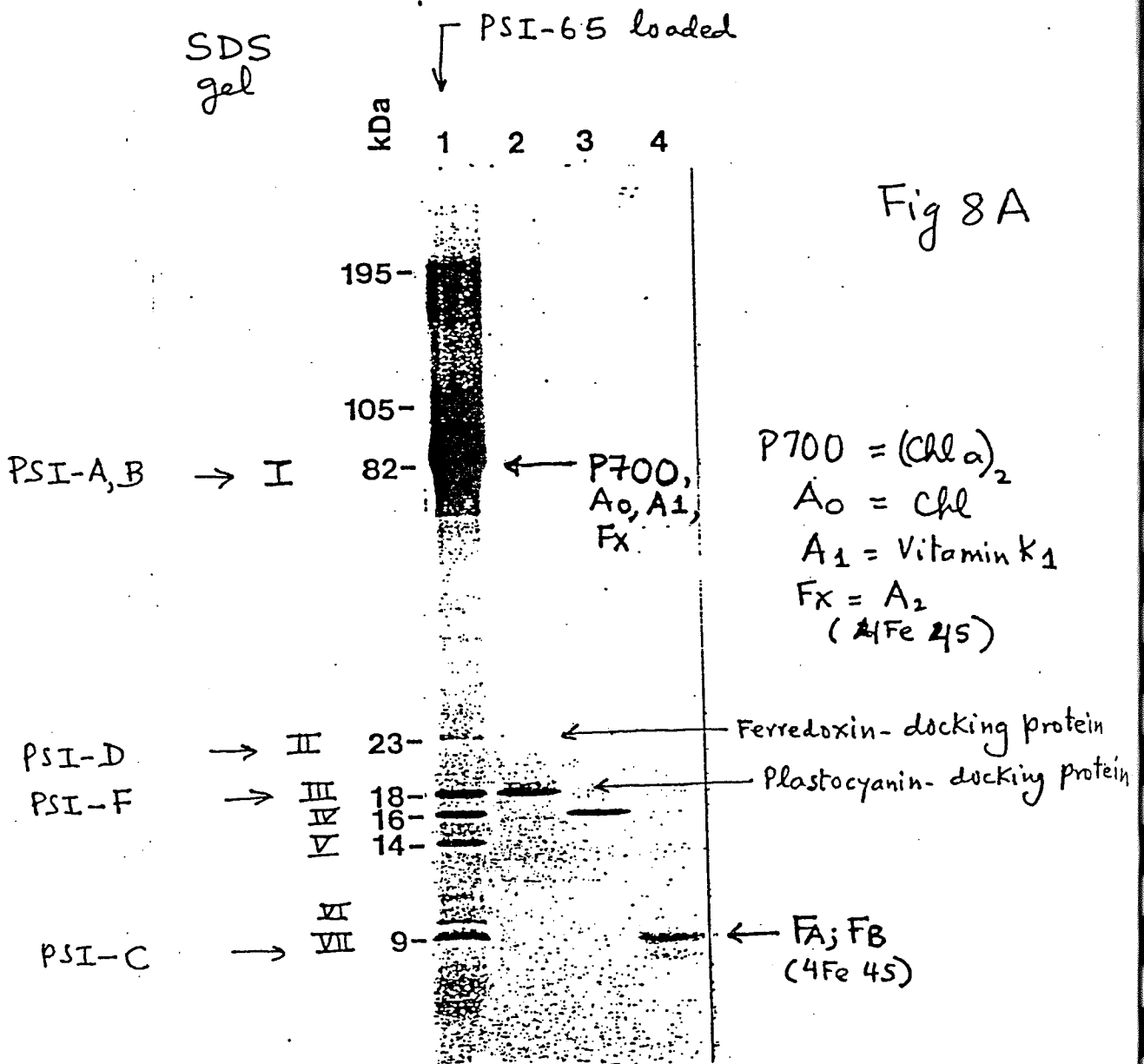


Bohr magneton
 $h\nu = g \beta H$ ← magnetic field
 condition for resonance - maximum absorbance



$$g = \frac{h\nu}{\beta H}$$

characteristic number characterizing the unpaired electron and its environment



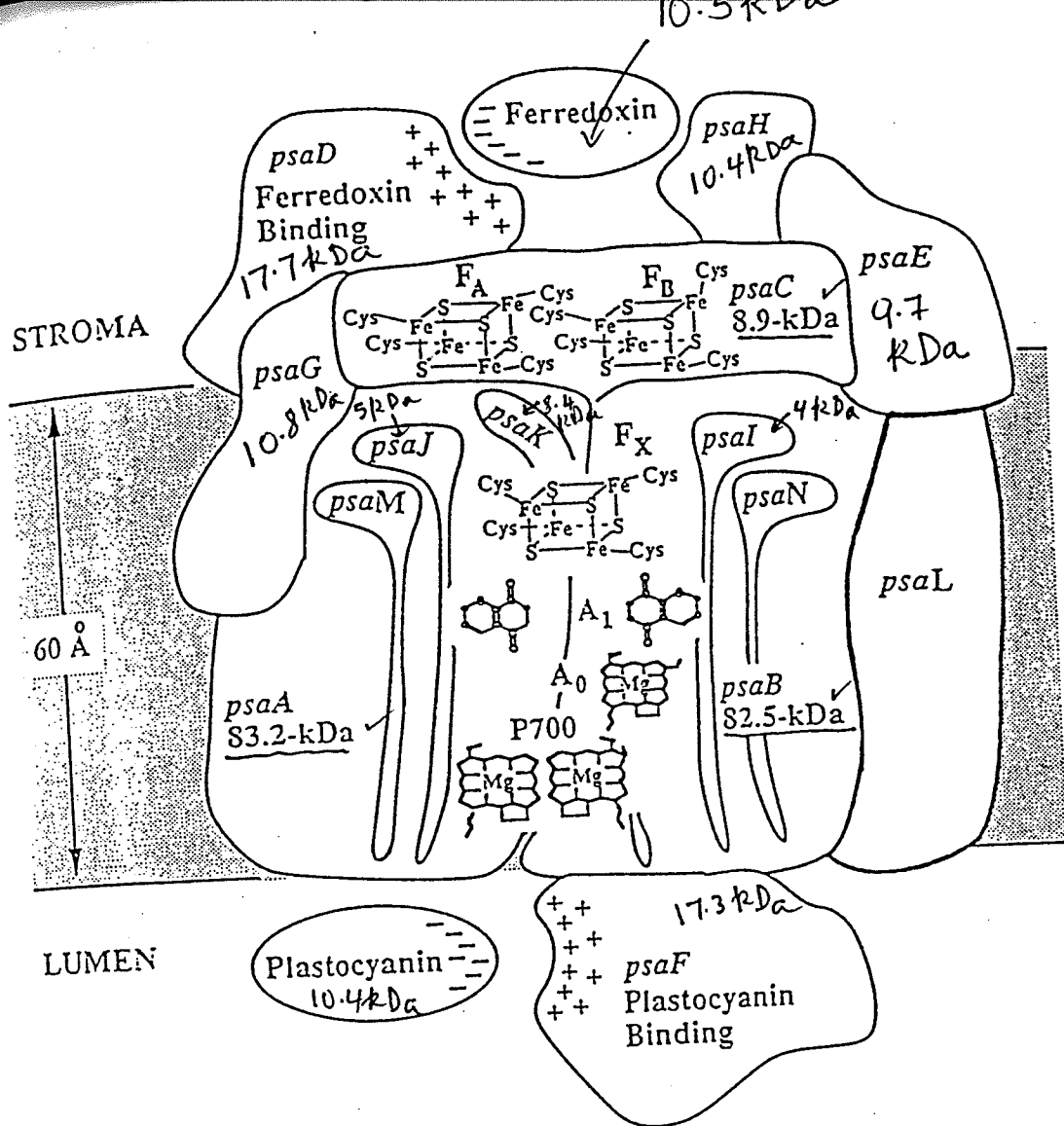
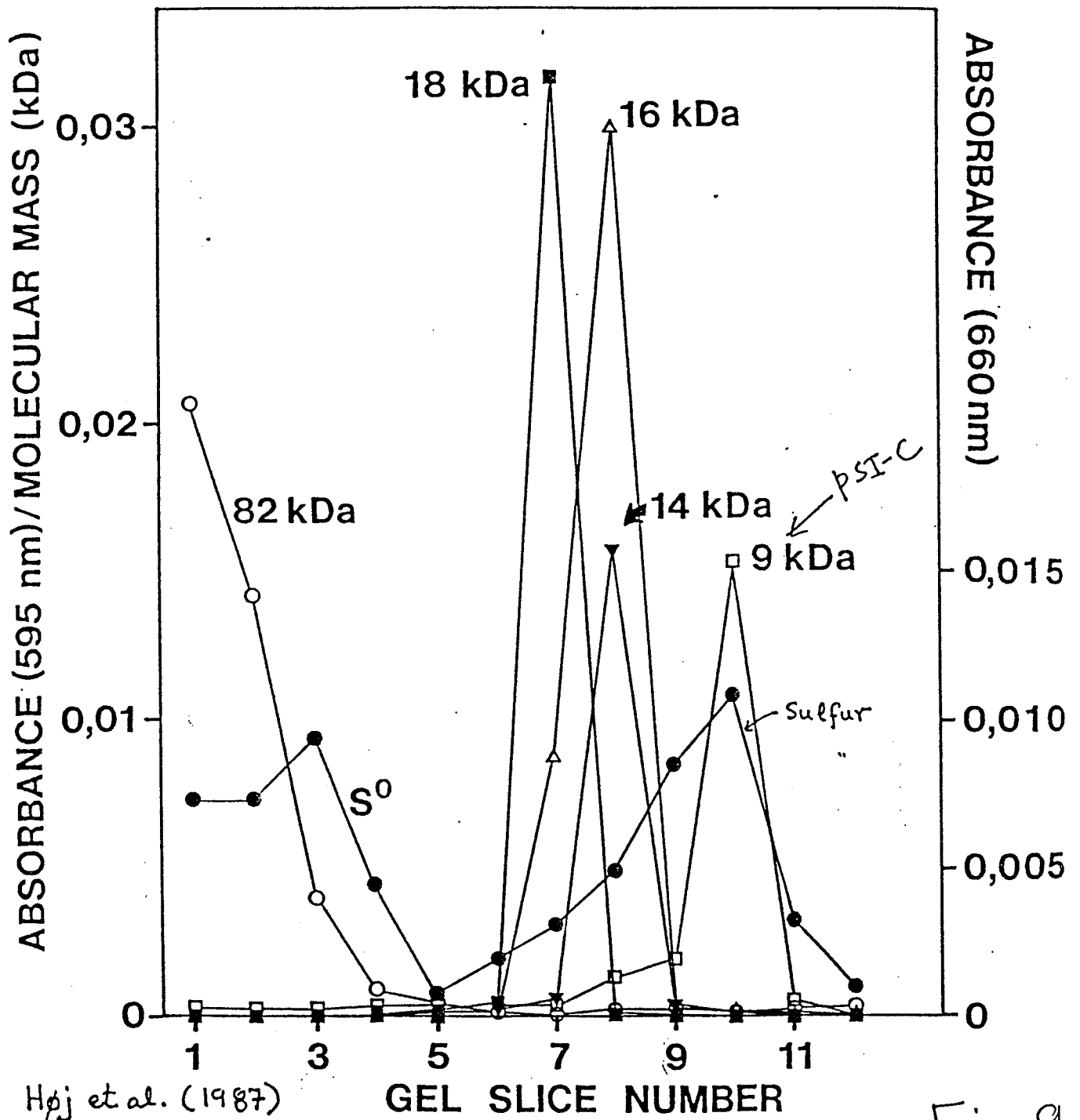
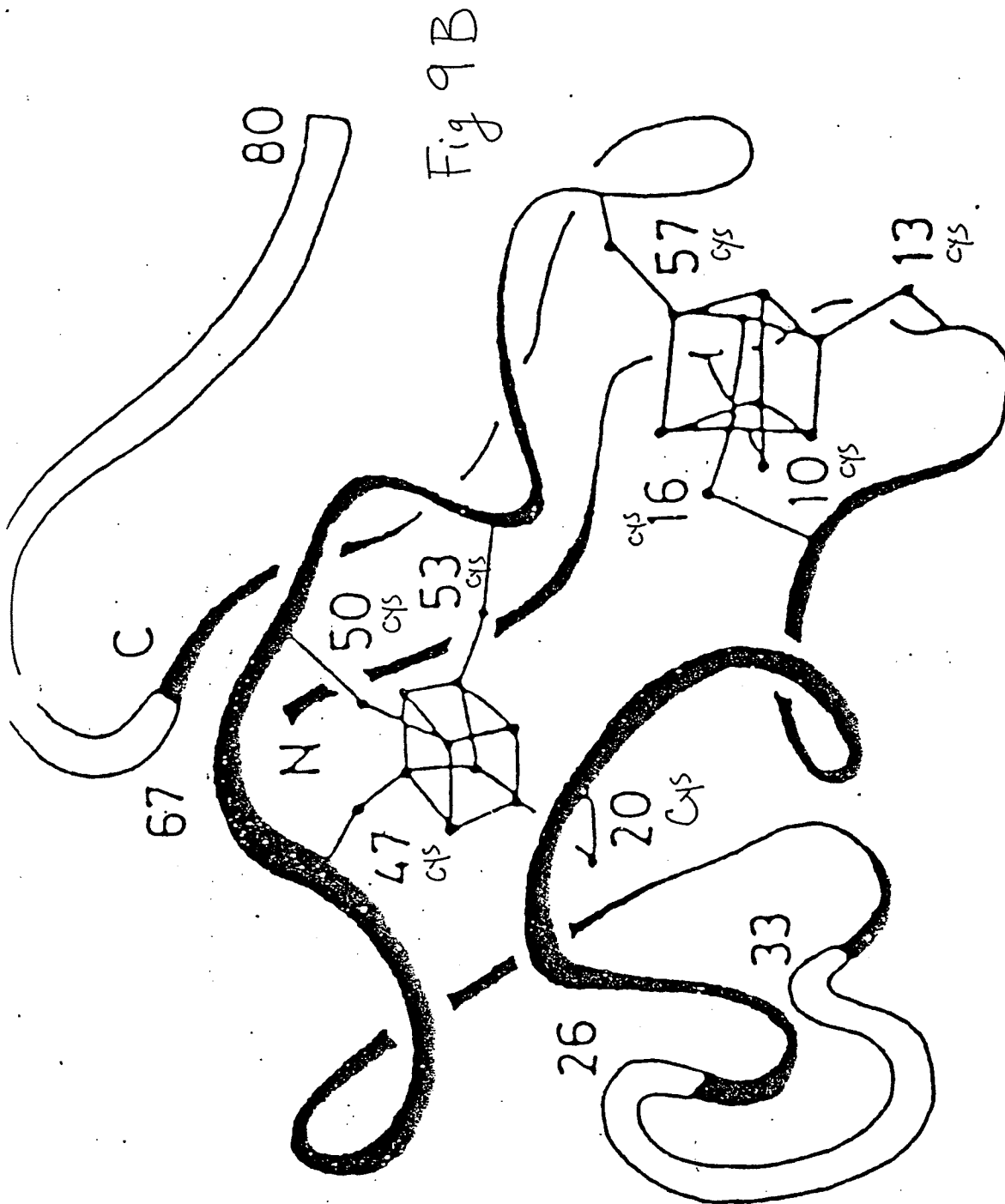


Figure 8B

Working model for the photosystem I complex of higher plants and cyanobacteria. The light-harvesting CAB proteins of the LHCl complex of higher plants and the possibly related 9-kDa 'PsaO' polypeptide are not shown. The PsaG and PsaH polypeptides have thus far only been demonstrated to occur in the PSI complexes of higher plants, and the PsaM and PsaN are provisionally named membrane-intrinsic polypeptides only observed in some cyanobacterial PSI complexes thus far (but see text). The locations of the low-molecular mass polypeptides PsaI, PsaJ, PsaK, PsaM, and PsaN are arbitrary. The hydrophilic PsaC, PsaD, PsaE, and PsaH polypeptides are known to be located on the stromal side of the membrane; the PsaF polypeptide is located on the luminal side of the membrane as shown. Polypeptides PsaF, PsaG, and PsaL are believed to be membrane-intrinsic and are probably membrane-spanning, as are PsaI, PsaJ, PsaK, PsaM, and PsaN. The two molecules of phylloquinone are arbitrarily shown as being equally distributed on the membrane-spanning PsaA and PsaB polypeptides. The primary donor, P700, is shown close to the luminal side of the thylakoid membrane, and the F_x interpolypeptide [4Fe-4S] cluster is shown close to the stromal surface of the thylakoid and close to the terminal electron-accepting F_A and F_B [4Fe-4S] clusters. The model is a revision of that of Parrett (1990).



g. 9A
015
010
005



Simulated three-dimensional structure of PSI-C 9 kD polypeptide. The black ribbon (■) shows the main chain folding of *Peptococcus aerogenes* ferredoxin. The white (□) shows the insertion and the extended C-terminal region of this protein (reprinted, with permission, from Oh-oka *et al.*, 1988a).

Plastocyanin

N of His 37
 S of Cys 84
 N of His 87
 S of Met 92

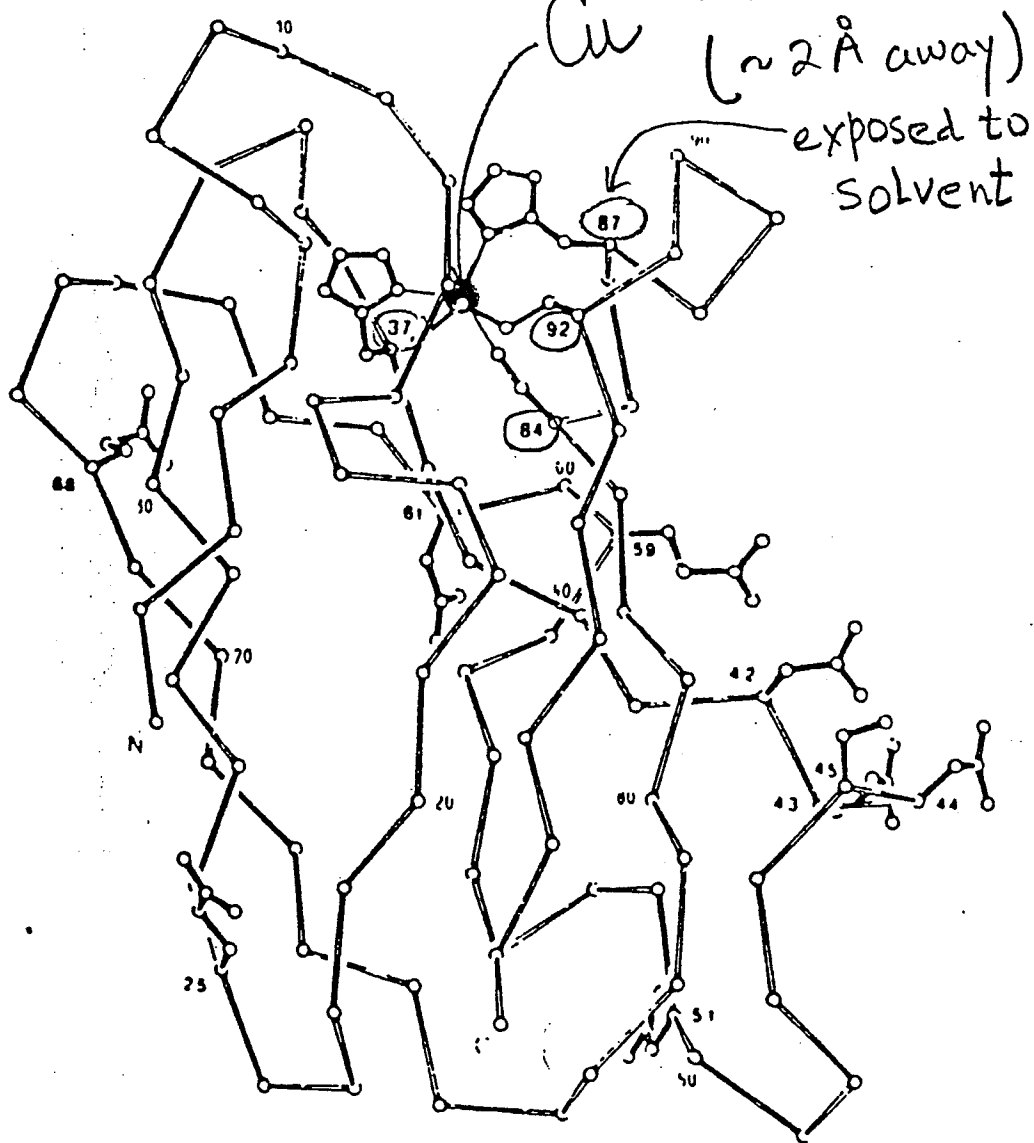


Fig. 9C

Fig. Molecular structure of poplar plastocyanin (FREEMAN 1981). The polypeptide backbone is represented by the C_α atoms with every tenth numbered. The four Cu-binding side-chains and the conserved acidic side-chains of plant plastocyanins are shown. (Residue 45 is Ser in polar but Glu in other plant plastocyanins)

(P)

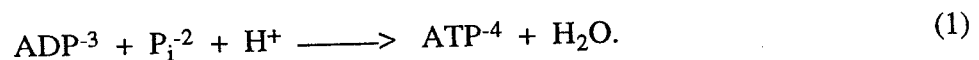
ATP Synthesis: Proton Gradient and Membrane Potential; Mitchell's Chemiosmotic Hypothesis; ATP Synthase and Boyer's binding change hypothesis

"ATP synthesis is a common activity between halobacteria (Archaea), anoxygenic photosynthetic bacteria, cyanobacteria, algae, spinach, wheat, rice, cows, myself and my dear friend Manmohan, among many other forms of life. Thus, its discussion is rather important." (Govindjee, Indore, 1996)

What follows is a brief discussion taken from Whitmarsh and Govindjee (1996):

Synthesis of ATP by the ATP Synthase Enzyme

The conversion of proton electrochemical energy into chemical free energy is accomplished by a single protein complex known as ATP synthase. P. Mitchell (1961) has discussed his theory whereby it is the proton motive force that is responsible for providing energy for ATP formation (see Govindjee # 7) This enzyme catalyzes a phosphorylation reaction, which is the formation of ATP by the addition of inorganic phosphate (P_i) to ADP



The reaction is energetically uphill ($\Delta G = +32 \text{ kJ/mol}$) and is driven by proton transfer through the ATP synthase protein. The ATP Synthase complex is composed of two major subunits, CF_0 and CF_1 (see Figure section). The CF_0 subunit spans the photosynthetic membrane and forms a proton channel through the membrane. The CF_1 subunit is attached to the top of the CF_0 on the outside of the membrane and is located in the aqueous space. CF_1 is composed of several different protein subunits, referred to as α , β , γ , δ and ϵ . The top portion of

the CF_1 subunit is composed of three $\alpha\beta$ -dimers that contain the catalytic sites for ATP synthesis. A recent major breakthrough has been the elucidation of the structure of ATPase of *bovine heart mitochondria* by Abrahams et al. (1994). The molecular processes that couple proton transfer through the protein to the chemical addition of phosphate to ADP are poorly understood. It is known that phosphorylation can be driven by a pH gradient, a transmembrane electric field, or a combination of the two. Experiments indicate that three protons must pass through the ATP synthase complex for the synthesis of one molecule of ATP. However, the protons are not involved in the chemistry of adding phosphate to ADP. Paul Boyer and coworkers have proposed an alternating binding site mechanism for ATP synthesis (Boyer, 1993). One model based on their proposal is that there are three catalytic sites on each CF_1 that cycle among three different states (see **Figure section**). The states differ in their affinity for ADP, P_i and ATP. At any one time, each site is in a different state. This model is supported by the structure of ATPase elucidated by Abrahams et al. (1994). Initially, one catalytic site on CF_1 binds one ADP and one inorganic phosphate molecule relatively loosely. Due to a conformational change of the protein, the site becomes a tight binding site, that stabilizes ATP. Next, proton transfer induces an alteration in protein conformation that causes the site to release the ATP molecule into the aqueous phase. In this model, the energy from the proton electrochemical gradient is used to lower the affinity of the site for ATP, allowing its release to the water phase. The three sites on CF_1 act cooperatively, i.e., the conformational states of the sites are linked. It has been proposed that protons affect the conformational change by driving the rotation of the top part (the three $\alpha\beta$ -dimers) of CF_1 . Such a rotating model has recently been supported by recording of a rotation of the gamma subunit relative to the alpha-beta subunits by Sabbert et al. (1996). This revolving site mechanism would require rates as high as 100 revolutions per second. It is worth noting that flagella that propel some bacteria are driven by a proton pump and can rotate at 60 revolutions per second.

References for the above section:

Abrahams, J.P., A.G.W. Leslie, R. Lutter and J.E. Walker (1994) Structure at 2.8 Å resolution of F_1 -ATPase from bovine heart mitochondria. *Nature* **370**: 621- 628.

- Boyer, P.D. (1993) The binding change mechanism of ATP synthase-- some probabilities and possibilities. *Biochim. Biophys. Acta* **1140**:215-250.
- Mitchell, P. (1961) Coupling of phosphorylation to electron and proton transfer by a chemiosmotic type of mechanism. *Nature* **191**:144-148.
- Sabbert, D., S. Engelbrecht and W. Junge (1996) Intersubunit rotation in active F-ATPase. *Nature*. **381**:623-625.

END OF TEXT FROM WHITMARSH AND GOVINDJEE (1996)

Outline

1. Various Systems

Note: The side to which protons are pumped is labeled as **P (positive) side** and the side from which they are pumped as the **N (negative) side**.

- A. Photosynthetic bacterial plasma membrane; halobacteria; bacterial plasma membrane; bacterial membrane ghosts (Kabakosomes); mitochondrial inner membrane; inside out thylakoid vesicles:

H⁺ gradient is directed outside; ATP is synthesized inside as H⁺s go through the enzyme.

- B. Chloroplast thylakoid membranes; purple membrane vesicles of halobacteria; submitochondrial particles; French-pressed spheroplasts of bacterial plasma membranes; chromatophores of purple bacteria:

H⁺ gradient is directed inside; and ATP is synthesized outside as H⁺s go through the enzyme.

(The lumen is labeled as P-phase (the side that acquires positive charges, and stroma matrix as N-phase in thylakoid membranes.)

2. Mitchell Theory: Delocalized Theory (Peter Mitchell received the Nobel Prize for this work)

- A. Membranes are impermeable to H⁺s
- B. Arrangement of electron and H-atom carriers is such that H⁺s are brought in or out naturally.
- C. ATP synthase is a H⁺ translocating machine

- D. Δp_{mf} (proton motive force) = $\Delta\Psi$ (membrane potential) + $(-60mV\Delta pH,$
or pH gradient) can be referred to as Δp .
- E. Δp_{mf} (also written as $\Delta\mu H^+$) is responsible for ATP synthesis

3. Key Experiments for Chloroplasts

- A. ΔpH is indeed created
- B. $\Delta\Psi$ is indeed created (ΔA_{515} : electrochromism or Stark effect)
- C. Acid-base bath experiments (André Jagendorf's experiments)
- D. Equivalence of ΔpH and $\Delta\Psi$: experiments in Peter Gräber's and Norman Good's laboratories

4. The ATP Synthase and Mechanism of ATP Synthesis

- A. The enzyme and its subunits:

Stoichiometry is: 3 α , 3 β , 1 δ , 1 γ , 1(or 2) ϵ

Molecular mass is: 522 kilodaltons

Active units are: α (nucleotide binding) and β (active site)

γ : gate for proton passage

δ : required for binding of CF_1 to CF_0

ϵ : inhibitor of ATPase activity

- B. Genes for α , β , ϵ subunits of CF_1 and I, III and IV subunits of CF_0 are coded in the chloroplast, whereas, the genes for γ and δ of CF_1 and II of CF_0 are coded in the nucleus.

There is sequence homology in selected regions of the α and β subunits of F_1 -ATPases and other ATP-requiring enzymes.

- C. Theory of "binding change" or "alternate sites" (Paul Boyer):

1. Three catalytic sites "rotate" sequentially through 3 distinct sites;
2. Each site is in a different state from the other two: one is binding ADP and P_i ; the other is making bound ATP from tightly bound ADP and P_i ; and the third is releasing ATP;

3. Release of ATP is coincident with tight binding of ADP and Pi at another site; and

4. Energy is needed for the release of ATP, not formation of ATP!

D. 3H^+ are needed to make one ATP.

5. Sources of H^+ s in the lumen

— Water oxidation

— Plastoquinol oxidation by Cytb₆/f complex (ubiquinol oxidation by Cytb/c complex in photosynthetic bacteria)

— Extra H^+ release by Cytb/c complex: by the so-called "Q" cycle

6. Equations

A. $\mu^i_{\text{H}^+} - \mu^o_{\text{H}^+} = -2.303 RT (\text{pH}^i - \text{pH}^o) + ZF (\Psi^i - \Psi^o)$

i = inside; o = outside; μ = chemical potential;

R = gas constant; T = temperature (in Kelvin); Z = charge;

F = Faraday constant; Ψ = membrane potential.

B. $\Delta p = \Delta\mu_{\text{H}^+} = -2.303 RT (\Delta\text{pH}) + F(\Delta\Psi)$ for Z = 1

C. $\Delta G_{\text{ATP}} < n\Delta\mu_{\text{H}^+}$ where n = number of protons translated

$$\Delta G_{\text{ATP}} = \Delta G^{\circ}_{\text{ATP}} + RT \ln \frac{[\text{ADP}] [\text{Pi}]}{[\text{ATP}]}$$

7. News

ATP synthase (F_1 portion) of beef heart mitochondria has been crystallized and the structure of the F_1 portion is, thus, available. Available pictures support the Boyer's model, and, they will be discussed (handout does not contain these details).

See: Abrahams et al. (1994) *Nature* 370:621-628 (Full citation appears above in this handout).

8. References

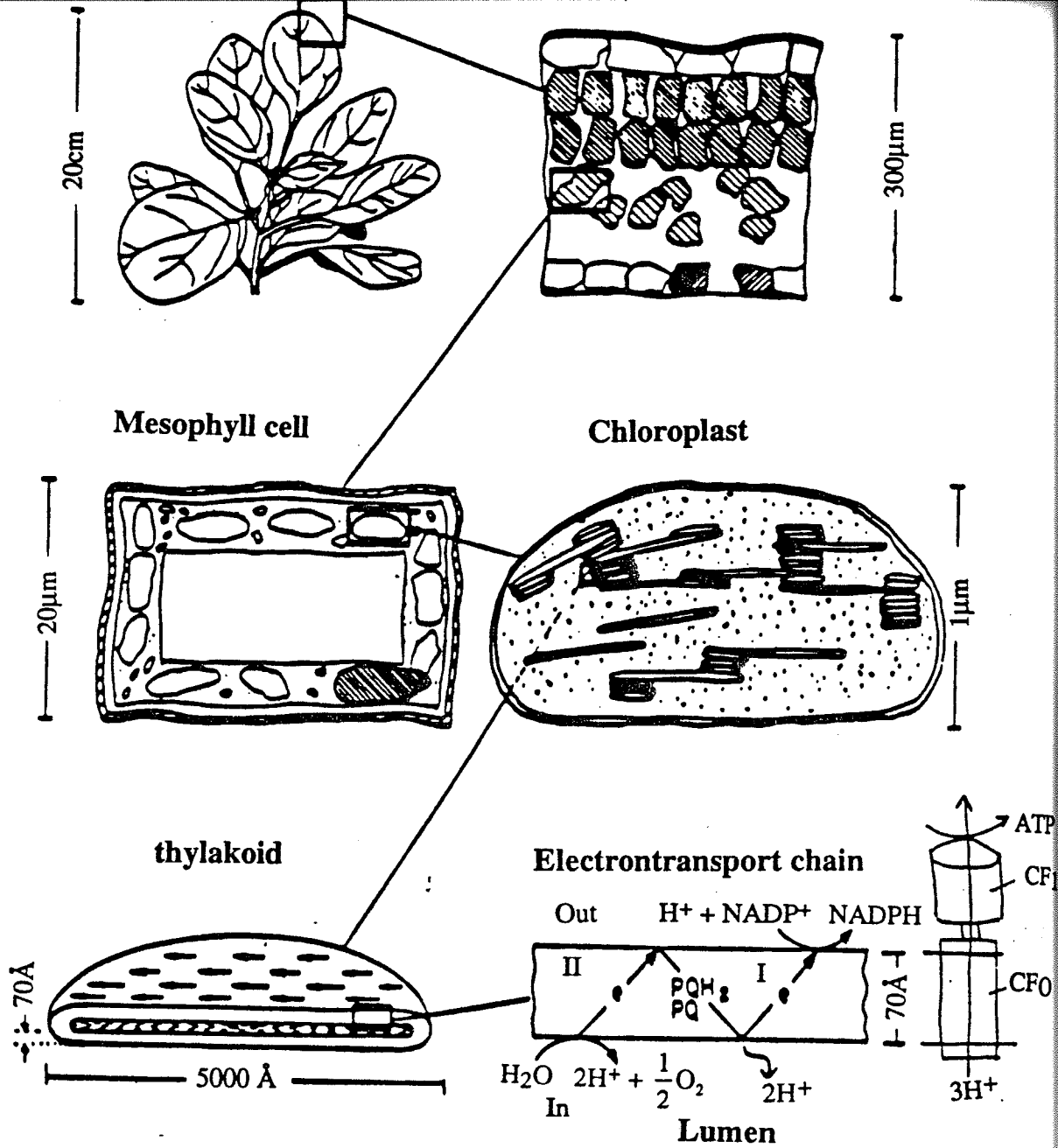
- [1] W.D. Frasch (1994) The F-type ATPase in cyanobacteria: pivotal point in the evolution of a universal enzyme. In: D. Bryant (ed.) *The Molecular Biology of Cyanobacteria*, pp. 361-380. Kluwer Academic, Dordrecht. (Advances in Photosynthesis, vol. 1, series editor: Govindjee)

- [2] **SEE various chapters on phosphorylation in D. Ort and C.F. Yocum (1996) Oxygenic photosynthesis: the light reactions, Kluwer Academic, Dordrecht (Advances in Photosynthesis, Vol. 4, Series editor: Govindjee).**
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- [13] R.P. Hangarter and N.E. Good (1982) Energy thresholds for ATP synthesis in chloroplasts. *Biochim. Biophys. Acta* **681**: 397-404.
- [14] D.O. Ort and B.A. Melandri (1982) Mechanism of ATP synthesis (pp. 537-587); W. Junge and J.B. Jackson (1982) The development of electrochemical potential gradient across photosynthetic membranes (pp. 589-646); R.E. McCarty and C. Carmeli (1982) Proton translocating ATPases of photosynthetic membranes (pp. 647-698). IN: Govindjee (editor) *Photosynthesis, Volume 1*, Academic Press, New York.

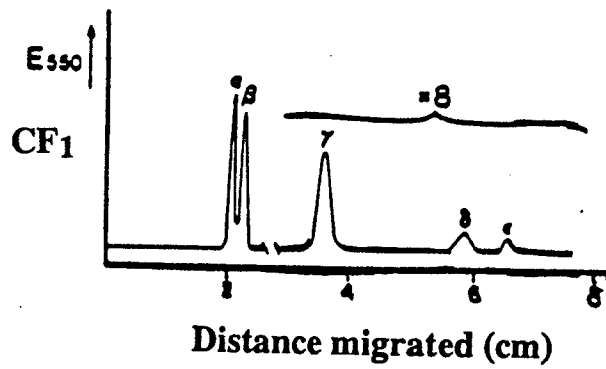
- [15] N. Nelson (1982) Structure and function of higher plant coupling factor (pp. 81-104); E. Schlodder, P. Graeber and H.T. Witt (1982) Mechanism of phosphorylation in chloroplasts SEE . J. Barber (ed.) Topics in Photosynthesis, volume 4. Elsevier.
- [16] A.T. Jagendorf (1975) Mechanisms of photophosphorylation. In: Govindjee (ed.) Bioenergetics of Photosynthesis, pp. 413-492. Academic Press, New York.

Questions

- [1] Discuss the Mitchell's theory for phosphorylation with emphasis on the key assumptions and the experimental evidence that support these assumptions. What are the major challenges to this theory and how have they been accomodated. [My presentation is not enough to answer this question.]
- [2] Discuss the Boyer's alternate binding change model for ATP synthesis. How do the current papers by Abrahams et al. (1994) and Sabbert et al. (1996) support this model. How do you reconcile to the fact that in this model energy is needed for the release of ATP whereas we learned in text books that it is needed for the synthesis of ATP?



Scheme of the localization of the photosynthetic electron transport chain in the plant.



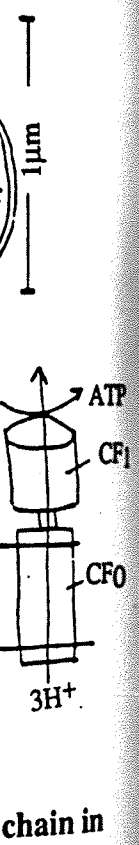
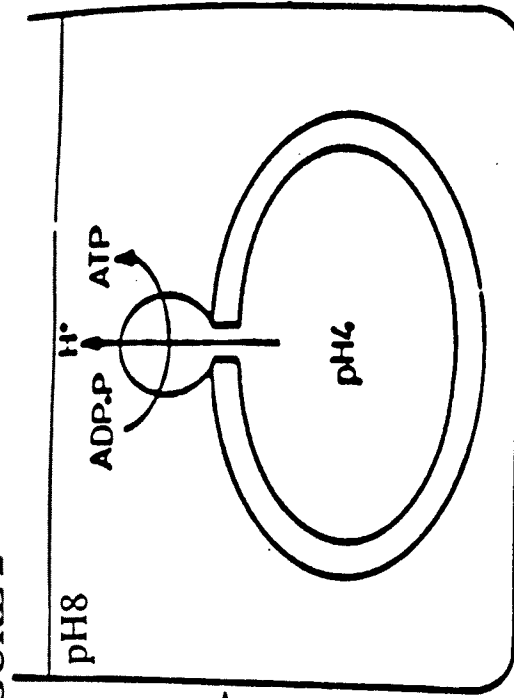
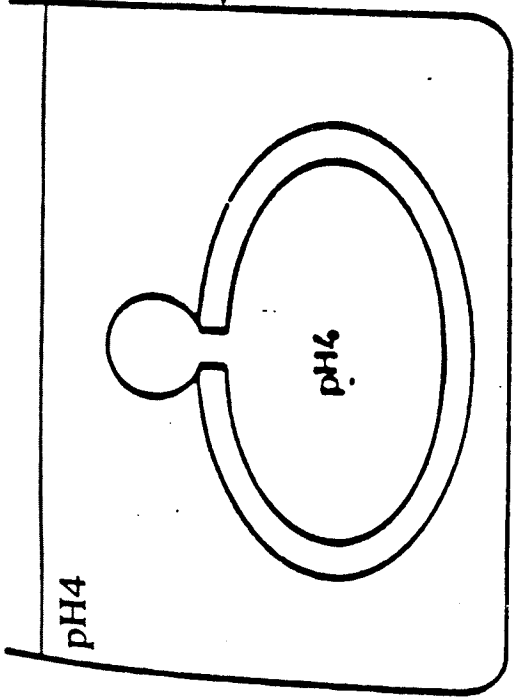


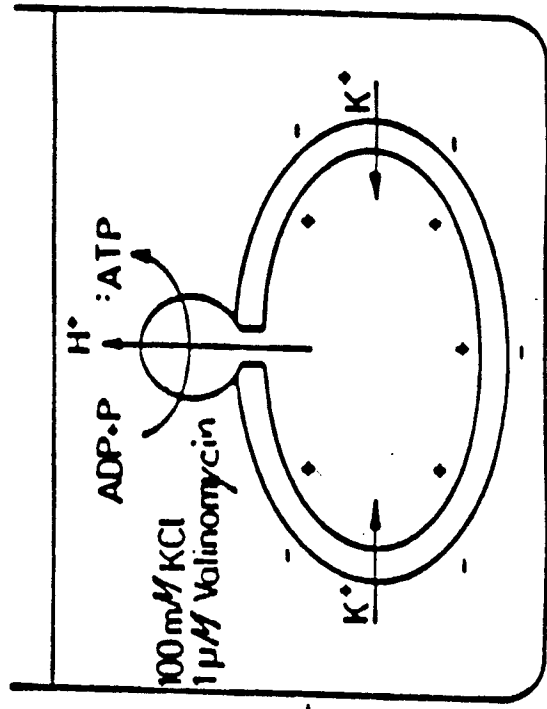
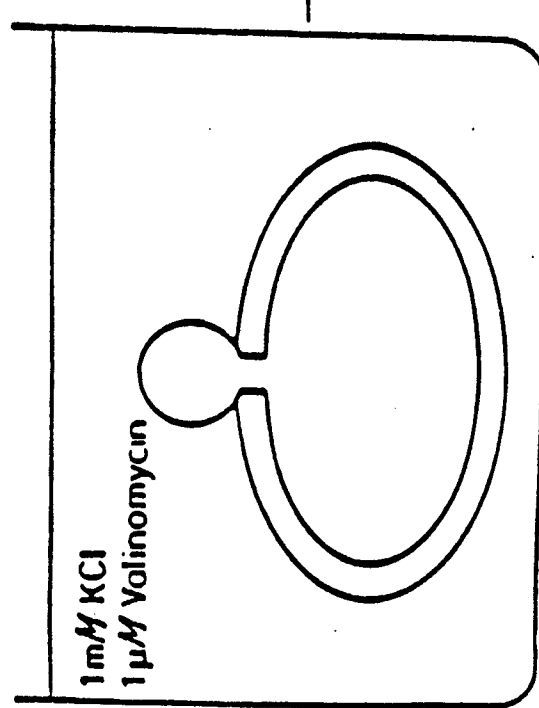
FIGURE 2



Acid-base phosphorylation proving ΔpH can drive ATP synthesis

(André Jagendorf)

$\Delta pH = 4$



Experiment proving $\Delta \psi$ can drive ATP synthesis

$\Delta \psi \approx 85 \text{ mV}$

Scheme of the procedure for artificial generation of a transmembrane proton concentration difference, ΔpH (top) and of transmembrane electric potential difference $\Delta \psi$ (bottom) in a chloroplast membrane

FIGURE 3

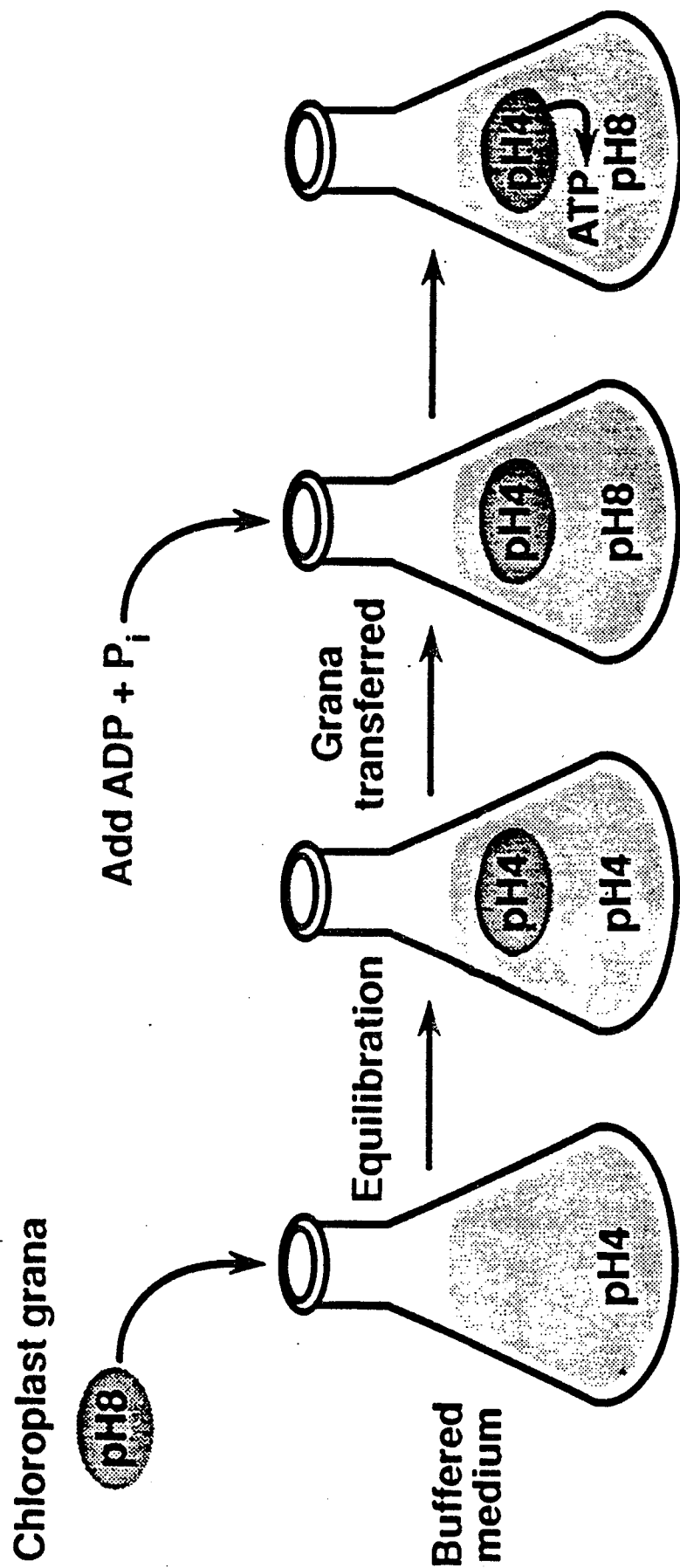
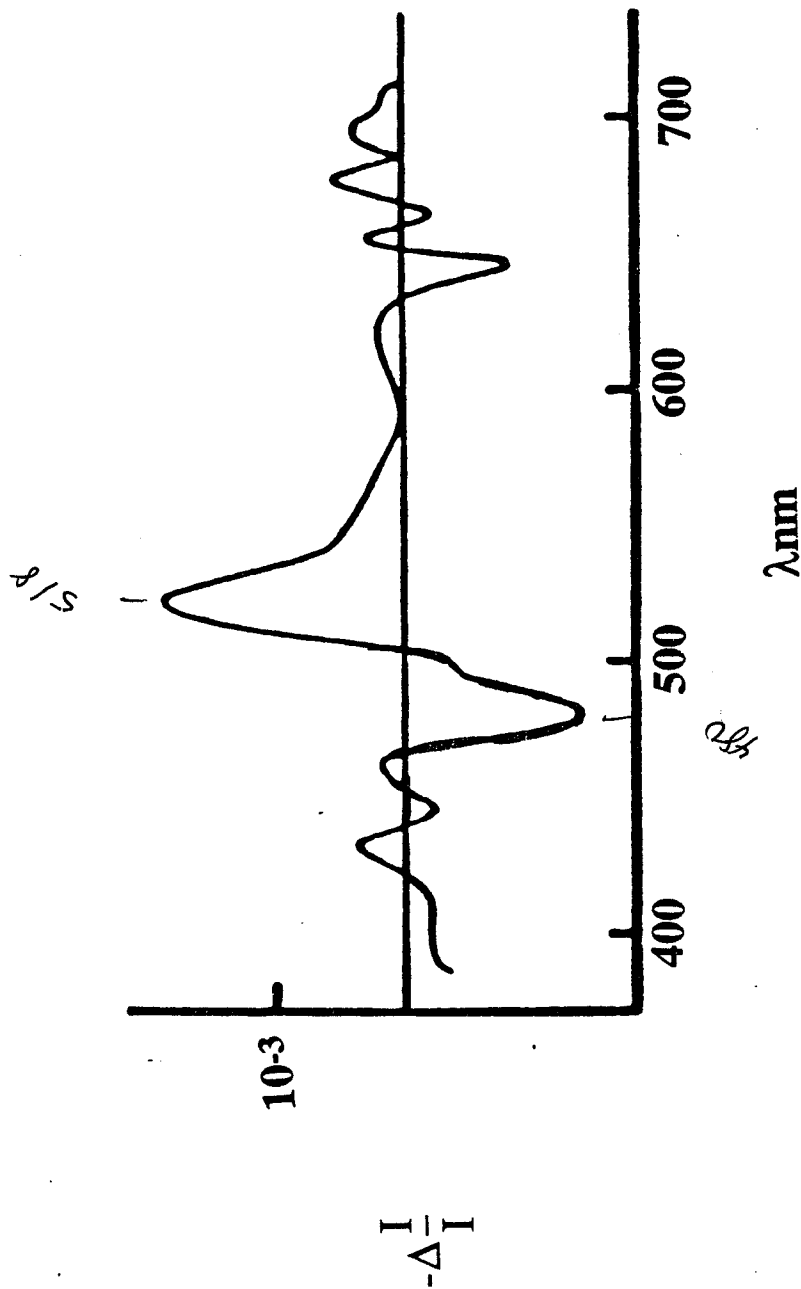
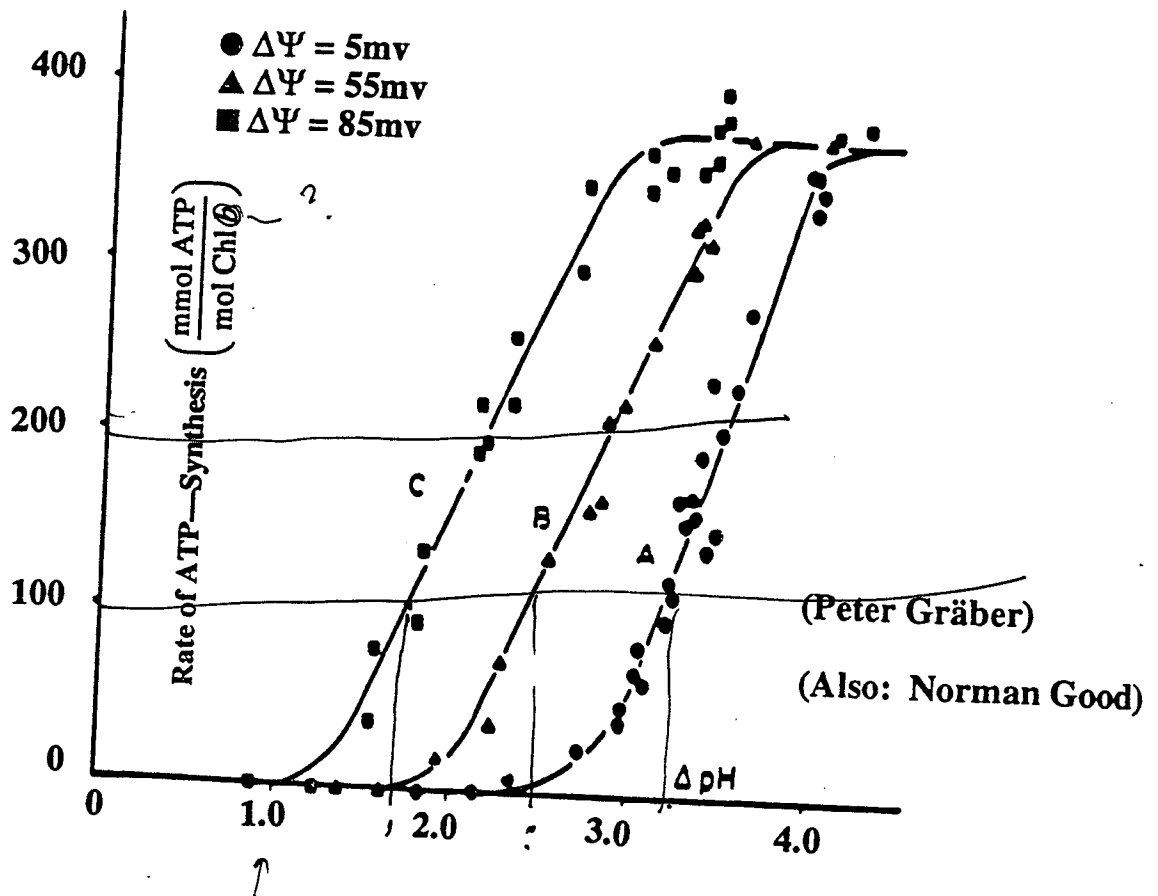


FIGURE 4



Light-induced electrochromic absorption changes in chloroplasts. (After Junge 1977.) The difference spectrum ($-\Delta I / I$) created by the light-induced red shift in the absorption spectra of the pigments is plotted. The large peak at 518 nm is mainly due to carotenoids, hence the "carotenoid band shift".

FIGURE 5



Rate of ATP synthesis as a function of ΔpH with three $\Delta \Psi$ potentials: A: $\Delta \Psi \approx 5$ mV, B: $\Delta \Psi \approx 55$ mV and C: $\Delta \Psi$

This experiment shows equivalence of ΔpH and $\Delta \Psi$ in making ATP
 $\Delta pH + \Delta \Psi \equiv \Delta pmf$ where, $\Delta pH = pH$ gradient and $\Delta \Psi =$ membrane potential
 and pmf stands for proton motive force.

$$\Delta pmf \text{ (or } \Delta \tilde{\mu}H^+) = \Delta \Psi - \frac{2.3RT}{F} \Delta pH$$

$$\text{or } \Delta \Psi - 60\Delta pH$$

The two are additive because ΔpH is negative!

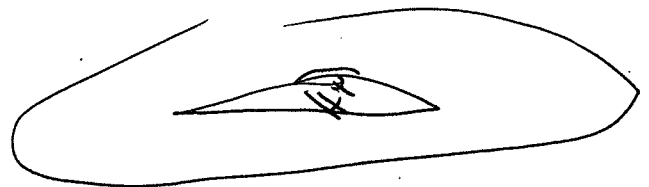


FIGURE 6

BBA 41146

ENERGY THRESHOLDS FOR ATP SYNTHESIS IN CHLOROPLASTS *

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(Received May 27th, 1982)

Key words: ATP synthesis; Chemiosmosis; Membrane potential; pH gradient; Proton-motive force; (Spinach chloroplast)

We have investigated the ATP synthesis associated with acid-base transitions in chloroplast lamellae under conditions which allow simultaneous control of the thermodynamic variables, ΔpH , membrane potential and ΔG_{ATP} . These variables have been directly imposed rather than simply inferred. Since the initiation of labeled P_i incorporation seems to measure accurately the initiation of net ATP synthesis, the following conclusions can be drawn: (1) The proton-motive force which is just sufficient for ATP synthesis provides almost exactly the required energy for ΔG_{ATP} if the efflux of three H^+ is required for each ATP molecule formed. (2) The membrane potential and the ΔpH contribute to the proton-motive force in a precisely additive way. Thus, the threshold can be reached or exceeded by a ΔpH in the absence of a membrane potential, by a membrane potential in the absence of a ΔpH , or by any combination of membrane potential and ΔpH . With a large enough membrane potential, ATP synthesis occurs even against a small inverse ΔpH . In each instance the combined ΔpH and membrane potential necessary for initiation of ATP synthesis represent the same threshold proton-motive force.

401

400

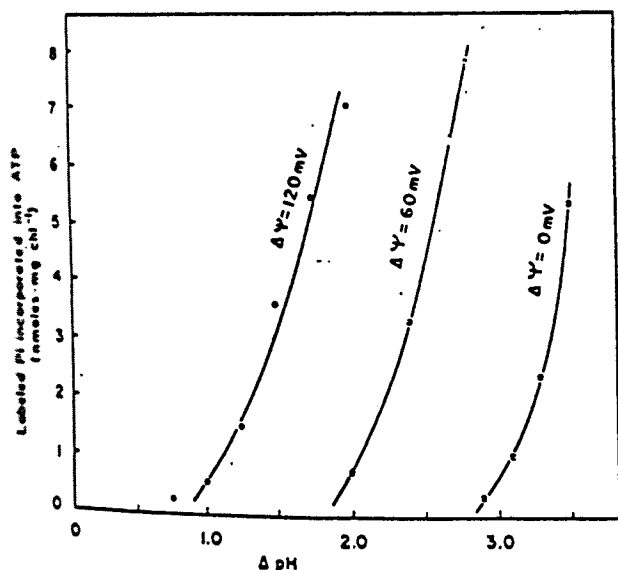


Fig. 2. Incorporation of labeled P_i into ATP by various combinations of ΔpH and membrane potentials.

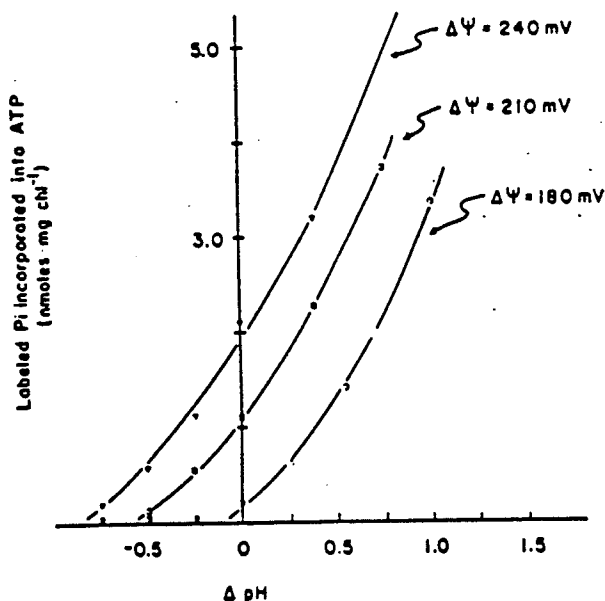
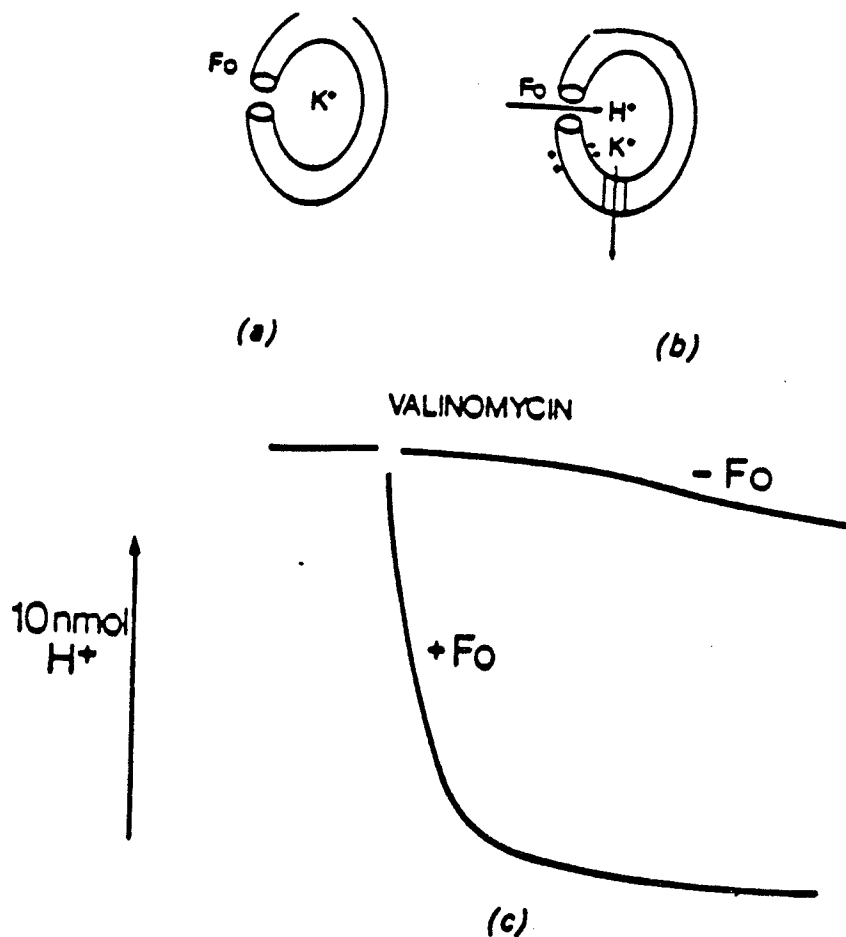


Fig. 5. Incorporation of labeled P_i into ATP in the absence of an H^+ concentration gradient.

FIGURE 7

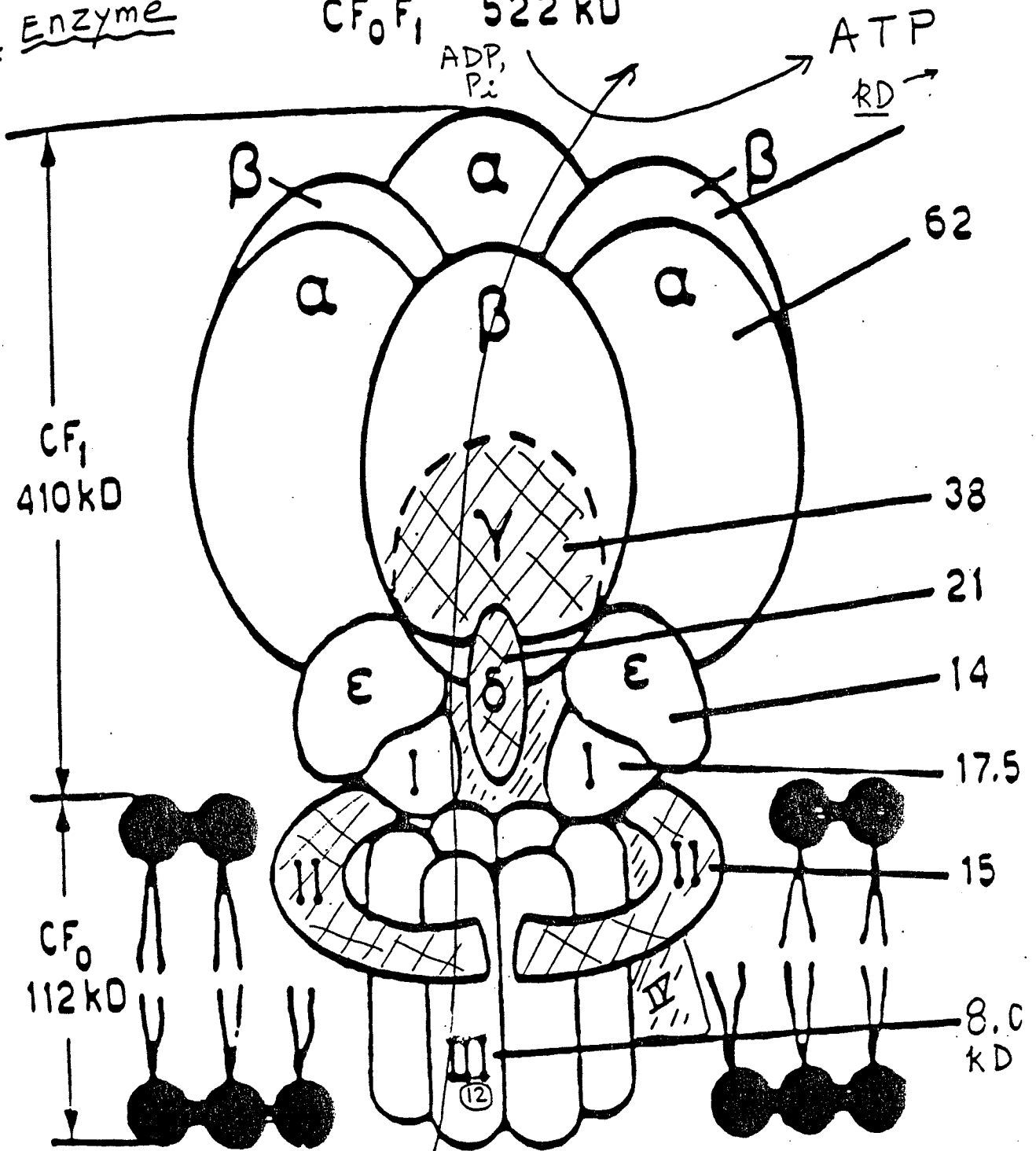


Purified F_0 conducts protons across artificial phospholipid bilayers. Highly purified F_0 from the thermophilic bacterium PS3 was suspended together with soybean phospholipids in a cholate solution containing tricine buffer. The cholate was dialysed away for 16 hours so that vesicles formed with F_0 incorporated into the bilayer. The vesicles were first loaded with KCl by incubating them at 55°C in a KCl medium. They were then washed and resuspended in a K^+ -free medium in a chamber with a pH-electrode (a). Valinomycin was then added, and a diffusion potential was generated by K^+ efflux down its concentration gradient H^+ -uptake through F_0 in response to this potential was monitored (b). The phospholipid bilayer itself was highly impermeable to protons, since little H^+ -uptake occurred in the absence of F_0 (c). (Data from Okamoto *et al.* 1977.)

FIGURE 8

CF_0F_1 522 kD

ATP ENZYME



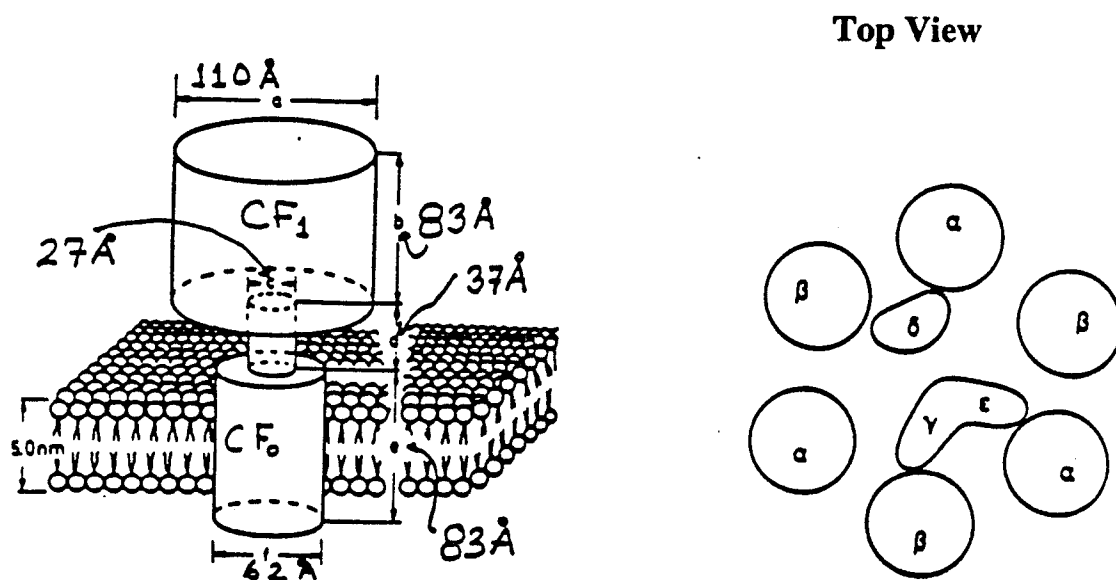
The ATP synthase
(ATPase;
The Coupling factor

3 H⁺

⊗ = nuclear-encoded

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FIGURE 9



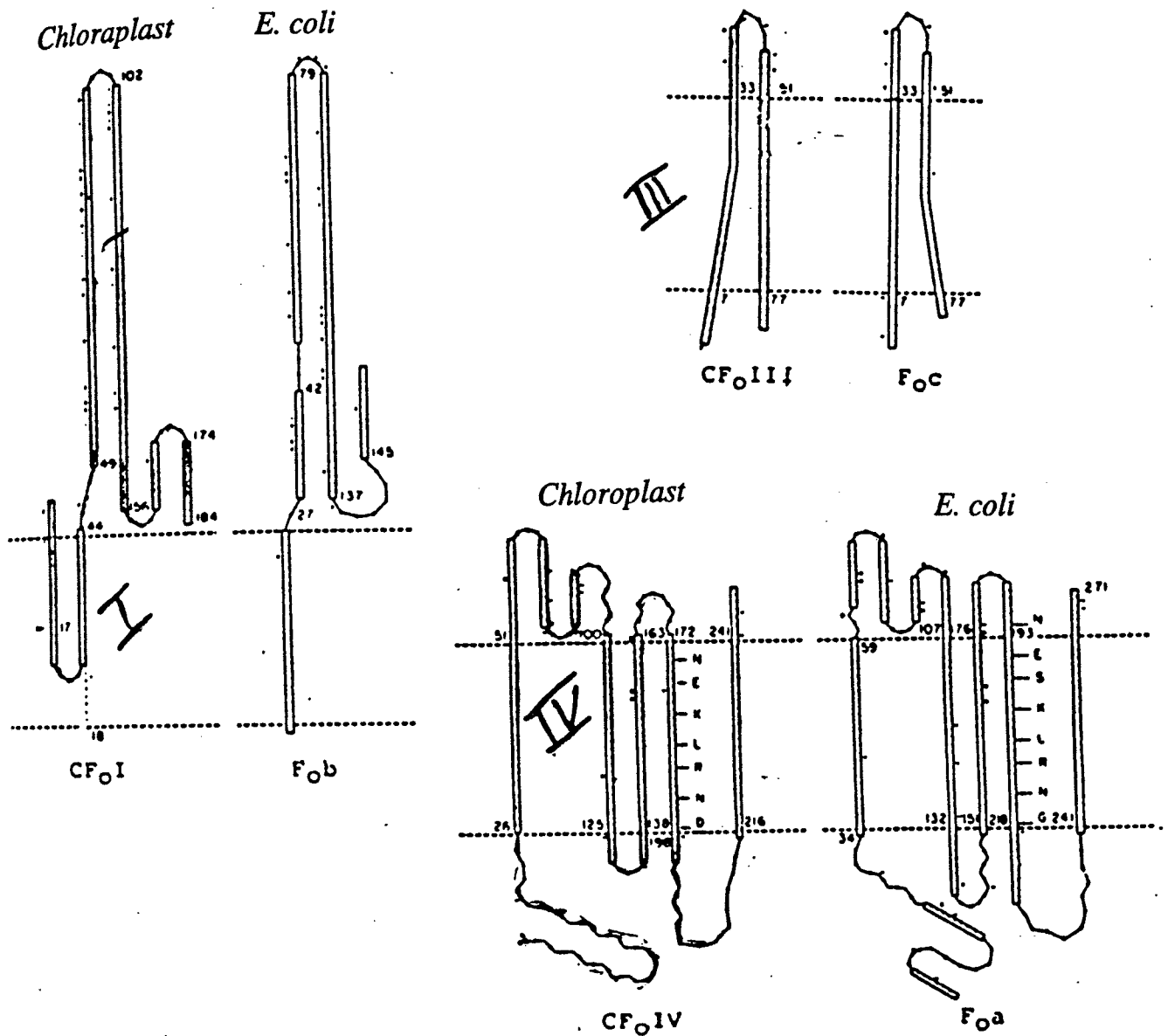
A model for the shape of the ATP-synthase in a lipid bilayer. The different measured parameters are defined in this figure (Boekema, *et al.*, 1988)

Scheme of the subunit arrangement in the hexagonal view of chloroplast F₁ ATP-synthase. (Boekma *et al.*, 1988)

SUBUNIT STRUCTURE AND FUNCTION OF THE CHLOROPLAST ATP-SYNTASE

Subunit	Molecular weight	Subunit's stoichiometry	Site of synthesis (coding)	Function	Name of the Gene
CF ₁					
α	62,000	3	Chloroplast	Nucleotide binding and regulation	atp A
β	57,000	3	Chloroplasts (C)	Active site	atp B
γ	38,000	1	Cytoplasm (nucleus) (or N)	Energy transduction from pmf to ATP. <u>Template for assembly of CF₁ to the membrane</u>	atp C
δ	21,000	1	Cytoplasm (nucleus)	<u>Necessary for proper binding and photophosphorylation</u>	atp D
ε	14,000	1 or 2	Chloroplast (C)	<u>ATPase inhibitor. Necessary for photophosphorylation</u>	atp E
CF ₀					
I	17,500	?(1)	Chloroplast (C)	Binding of CF ₁ ?	atp F
II	15,000	?(2)	Cytoplasm (N)	Assembly of CF ₀ ?	atp G
III	8,000	6(12)	Chloroplast (C)	<u>Proton conduction</u>	atp H
IV	25,000	1	?	?(newly discovered)	atp I

FIGURE 10

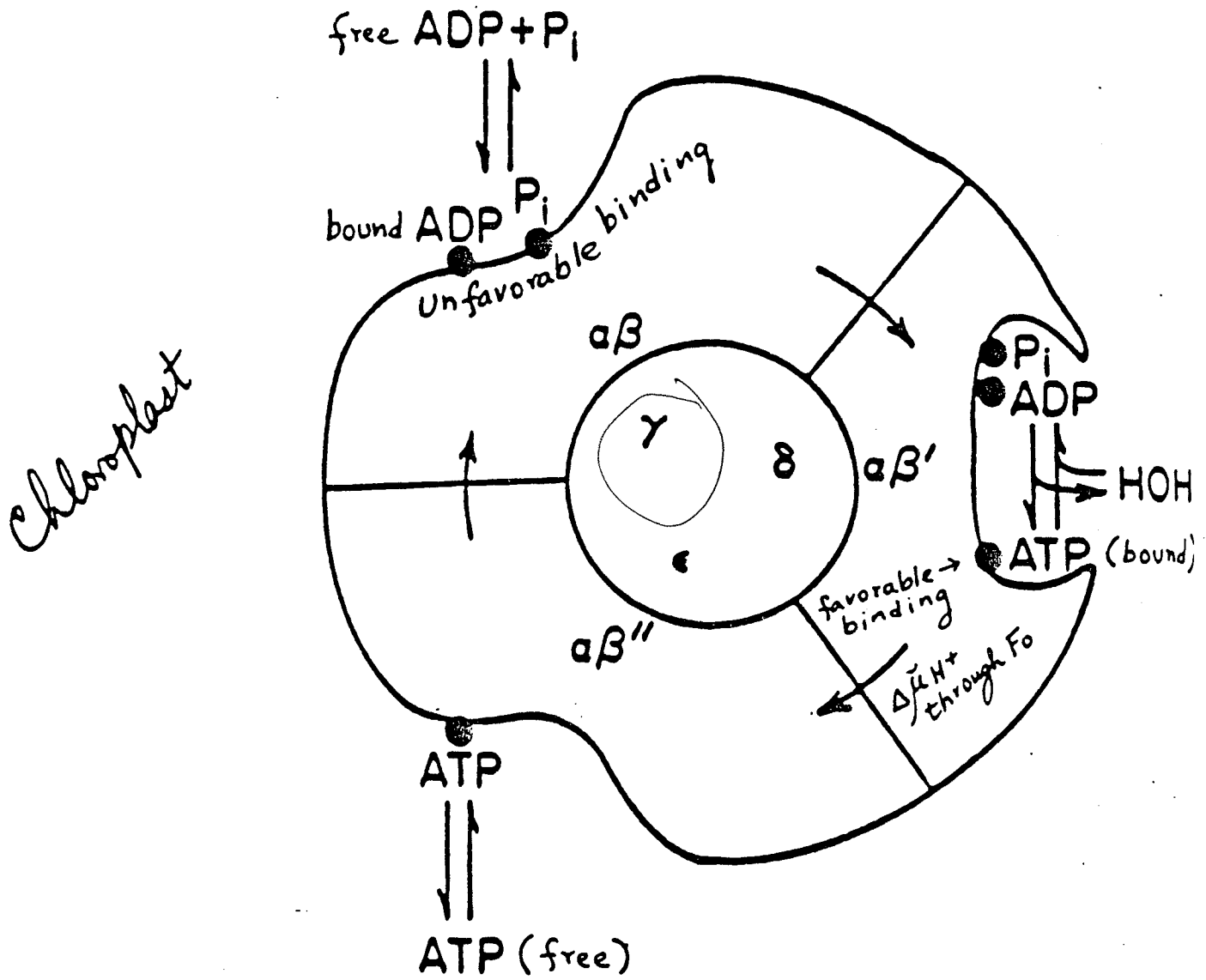


Predicted secondary structural arrangements within the membrane of subunits CF₀-I, CF₀-III, and CF₀-IV of spinach chloroplasts compared with those of subunits F₀-b, F₀-c and F₀-a, respectively, of *E. coli* [From Hudson *et al.* (1987)] Subunit I + II acts as a stalk: May bind CF₁; subunit III (many copies) is most conserved; atp F, atp H and atp I are genes for subunits I, III and IV respectively. Subunit III, perhaps, conduct protons. With $\Delta\mu_{H^+} = 30 \text{ mV}$, $2 \times 10^5 \text{ H}^+$ go through/CF₀/second.

ement
roplast
et al., 1988)

Name of the Gene
atp A
atp B
atp C
atp D
atp E
atp F
atp G
atp H
atp I

FIGURE 11



ALTERNATE SITE MODEL—(BOYER & OTHERS.)

HOH
 (bound)

Pyridine is a permeable buffer

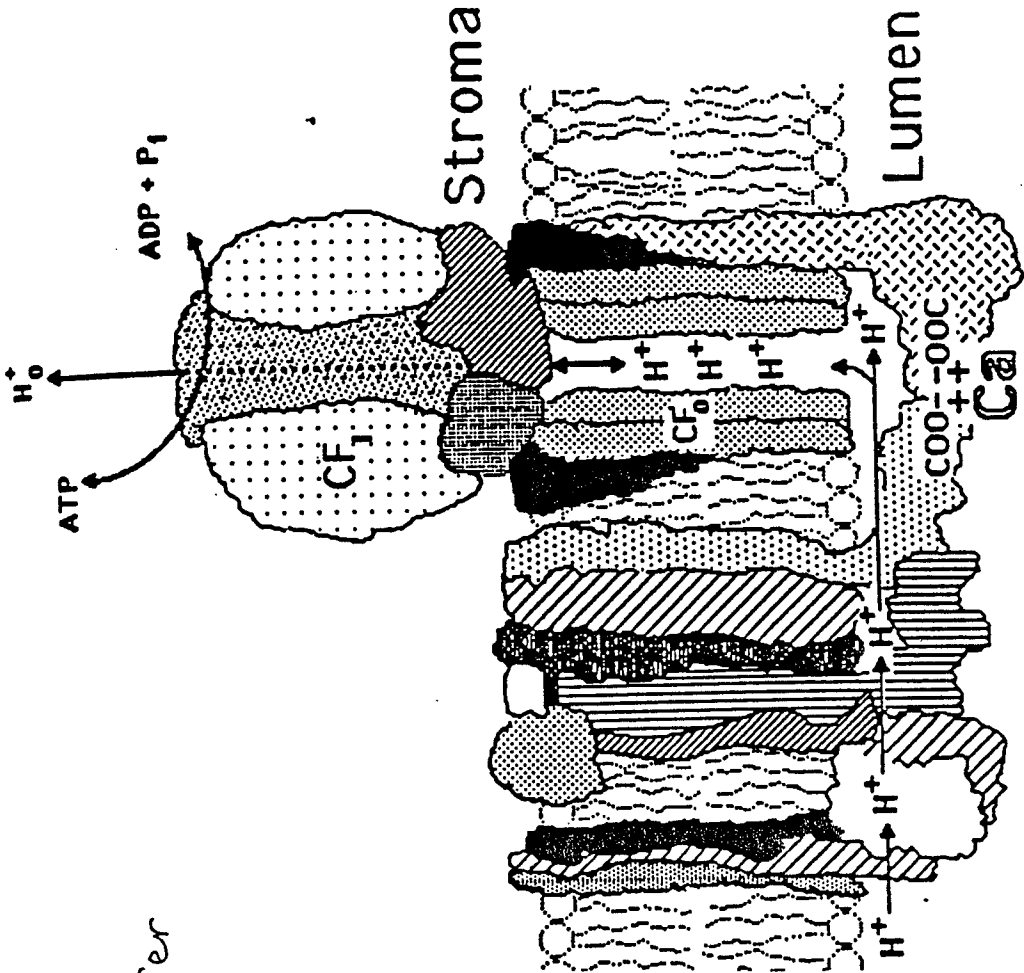
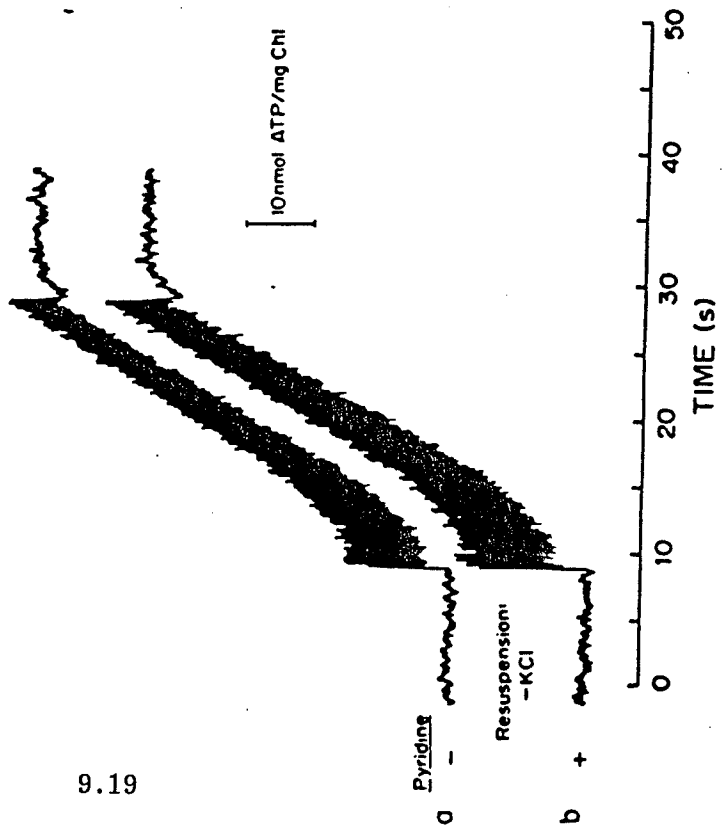


Fig. 12 (from R.A. Dilley)

Localized coupling

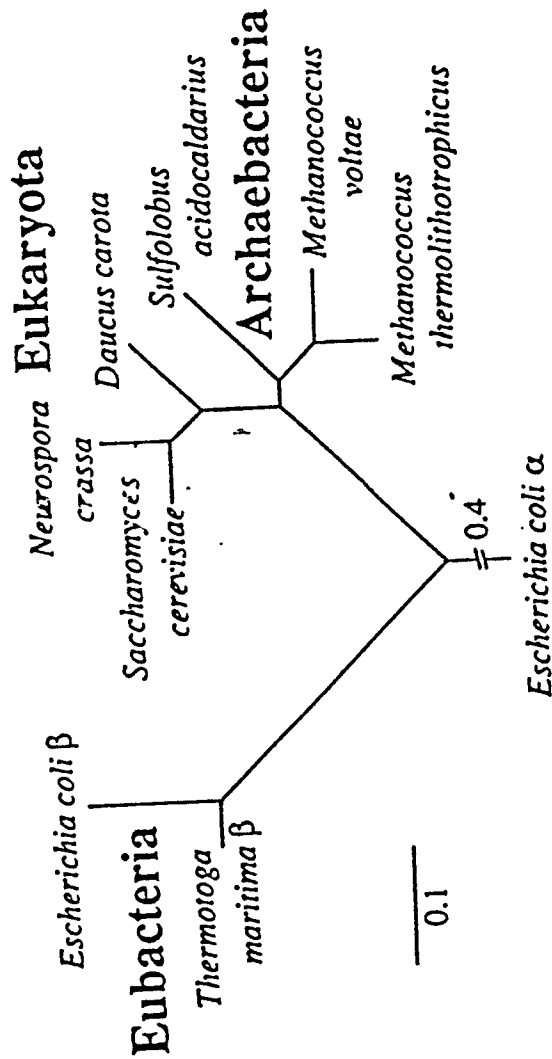


Fig. 13 Phylogenetic tree based on the catalytic subunits of V- and F-ATPases. This tree was calculated using a global alignment of the sufficiently conserved regions from DNA sequences encoding the catalytic subunits from the indicated organisms. In order to locate the root of the tree, the non-catalytic subunit of the *E. coli* F-ATPase is used as an outgroup. The topology and the branch lengths were calculated using Felsenstein's maximum likelihood method (1981). Parameters for the algorithm, sequences and their alignment were as described in Gogarten et al. (1989b); branches are scaled in terms of the probability for change of the first base of the codon. All branches were positive at the 1% significance level. In 100% of bootstrapped samples analyzed with parsimony the *M. thermolithotrophicus* and the *Sulfolobus acidocaldarius* sequences grouped together with the eukaryotic sequences. In 96% of the bootstrapped samples *Methanococcus thermolithotrophicus* and the *Sulfolobus acidocaldarius* formed a monophyletic group. When the sequence encoding the *Methanosarcina barkeri* plasmalemma ATPase (Inatomi et al. 1989) is included in the alignment, in 98 out of 100 bootstrapped samples the *Methanosarcina* sequence constitutes a separate branch that separates from the branch leading to the eukaryotes after the bifurcation leading to the thermophilic archaeobacteria (*Sulfolobus acidocaldarius* and *Methanococcus thermolithotrophicus*). Thus, not all archaeobacteria but only the mesophilic appear to be the sister group of the eukaryotes.

Govindjee
#10

Carbon fixation: The Calvin Cycle; RUBISCO; C-4 Pathway, and CAM

In this presentation, we provide a discussion of what your books call "dark reactions". Calvin cycle is also called Calvin-Benson-Bassham cycle or Reductive Pentose Phosphate cycle. RUBISCO stands for Ribulose bis phosphate carboxylase-oxygenase; It is the most abundant enzyme on Earth. C-4 pathway is also called Hatch-Slack-Kortschak pathway. C-4 plants are those that have this pathway, whereas the name arises from the fact that the pathway includes 4-C compounds (such as oxalo-acetate) as the product of CO₂ fixation. In contrast C-3 plants are those that have only 3-C compounds (phosphoglyceric acid) as the end product of CO₂ fixation. C-4 plants include C-3 intermediates. Thus, the C-4 pathway is an "add-on" mechanism. CAM refers to Crasulacean Acid Metabolism; here C-4 pathways occur. In C-4 plants, CO₂ fixation occurs in one type of cells; 4-C compounds are transferred to another type of cells where Calvin cycle takes place. In CAM plants, the entire process occurs in the same cell, but at different times.

An excerpt from Whitmarsh and Govindjee (1996) follows:

Synthesis of Carbohydrates

All plants and algae remove CO₂ from the environment and reduce it to carbohydrate by the Calvin cycle. The process is a sequence of biochemical reactions that reduce carbon and rearrange bonds to produce carbohydrate from CO₂ molecules. The first step is the addition of CO₂ to a five-carbon compound (ribulose 1,5-bisphosphate) (see **Figure section**). The six-carbon compound is split, giving two molecules of a **three-carbon** compound (3-phosphoglycerate). [Thus, these plants are called C-3 plants.] This key reaction is catalyzed by Rubisco, a large water

soluble protein complex. The 3-dimensional structure has been determined by X-ray analysis for Rubisco isolated from tobacco (Schreuder et al., 1993) from a cyanobacterium (*Synechococcus*) (Newman and Gutteridge, 1993) and from a purple bacterium (*Rhodospirillum rubrum*) (Schneider et al. 1990). *The carboxylation reaction is energetically downhill.* The main energy input in the Calvin cycle is the **phosphorylation** by ATP and subsequent **reduction** by NADPH of the initial three-carbon compound forming a three-carbon sugar, triosephosphate. Some of the triosephosphate is exported from the chloroplast and provides the building block for synthesizing more complex molecules. In a process known as **regeneration**, the Calvin cycle uses some of the triosephosphate molecules to synthesize the energy rich ribulose 1,5-bisphosphate needed for the initial carboxylation reaction. This reaction requires the input of energy in the form of one ATP. Overall, thirteen enzymes are required to catalyze the reactions in the Calvin cycle. The energy conversion efficiency of the Calvin cycle is approximately 90%. The reactions do not involve energy transduction, but rather the rearrangement of chemical energy. Each molecule of CO₂ reduced to a sugar [CH₂O]_n requires 2 molecules of NADPH and 3 molecules of ATP.

Rubisco is a bifunctional enzyme that, in addition to binding CO₂ to ribulose bisphosphate, can also bind O₂. This oxygenation reaction produces the 3-phosphoglycerate that is used in the Calvin cycle and a two-carbon compound (2-phosphoglycolate) that is not useful for the plant. In response, a complicated set of reactions (known as photorespiration) are initiated that serve to recover reduced carbon and to remove phosphoglycolate. The Rubisco oxygenation reaction appears to serve no useful purpose for the plant. Some plants have evolved specialized structures and biochemical pathways that concentrate CO₂ near Rubisco. These pathways (C₄ and CAM), serve to decrease the fraction of oxygenation reactions.[We shall discuss these pathways also.]

References cited in the above text:

- Newman, J. and S. Gutteridge (1993) The X-ray structure of *Synechococcus* ribulose-bisphosphate carboxylase/oxygenase-activated quaternary complex at 2.2 Å resolution. *J. Biol. Chem.* **268**:25876-25886.
- Schneider, G., Lindqvist, Y. and T. Lundqvist (1990) Crystallographic refinement and structure of ribulose-1,5-bisphosphate carboxylase from *Rhodospirillum rubrum* at 1.7 Å resolution. *J. Mol. Biol.* **211**:989-1008.
- Schreuder, H.A., Knight, S., Curmi, P.M.G., Andersson, I., Cascio, D., Sweet, R.M., Brändén, C.I. and D. Eisenberg (1993) Crystal structure of activated tobacco rubisco complexes with the reaction-intermediate analogue 2-carboxy-arabinitol 1.5-bisphosphate. *Protein Structure* **2**:1136-1146.

Outline

- ◆ 1. Discussion of the three major steps (carboxylation, reduction and phosphorylation, and regeneration of the receptor molecule for CO₂) in the formation of sugars from CO₂.
- ◆ 2. Discussion of the contrasting carboxylase and oxygenase function of the enzyme **RUBISCO** (Ribulose biphosphate carboxylase and oxygenase): *Calvin cycle versus photorespiration*.
- ◆ 3. Discussion of the three different pathways for CO₂ fixation in plants labeled as *C-3*, *C-4* and *CAM*.
- ◆ 4. Discussion of how these different plants function in different environment (light intensity, temperature, CO₂ and O₂ concentrations, etc).

"Fear no more the heat o' the sun,
Nor the furious winter's rages;
Thou thy worldly task hast done,
Home art gone, and ta' en thy wages:
Golden lads and girls all must,
As chimney-sweepers come to dust."

William Shakespeare

Table 1. Magnitude and Source of Energy for incorporation of CO₂ into Sugars

- 200 billion tons of CO₂ is fixed into biomass /year on Earth
- The source of energy is the solar energy converted by the light reactions into the reducing power (NADPH) and ATP; and it is specifically used in two key steps (see figure section)

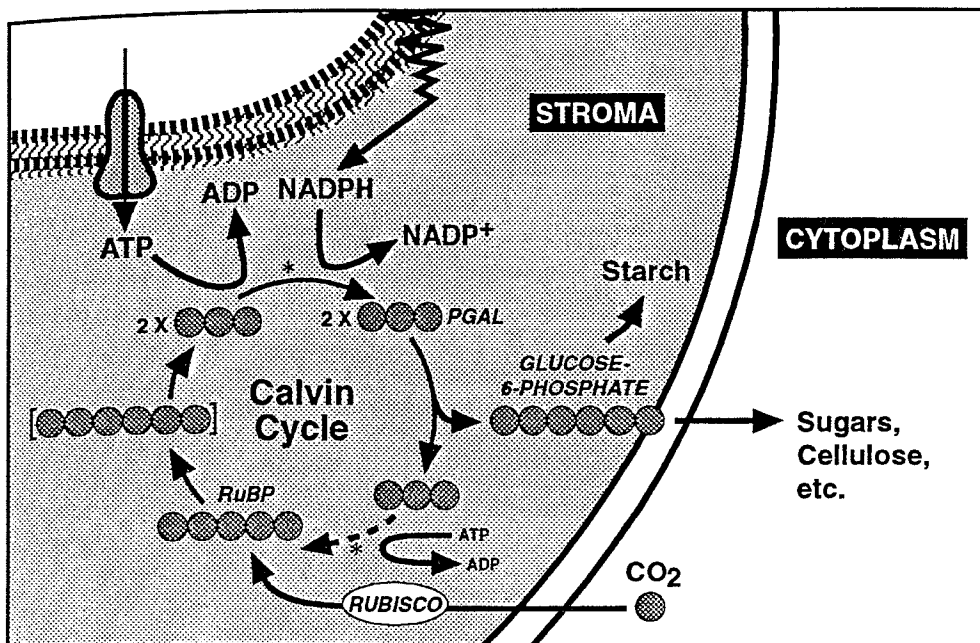


Fig. 1. "Light reactions" produce ATP and NADPH (on thylakoid membranes, see top left of diagram). These are used in the Calvin cycle for CO₂ fixation at two steps, marked with *. The Calvin cycle is shown here in its bare minimum. A 5-carbon receptor sugar molecule ribulose-bisphosphate (RuBP) is converted into a transient 6-carbon intermediate by the addition of CO₂ to it, catalyzed by the enzyme RUBISCO (ribulose bisphosphate carboxylase/oxygenase). The 6-carbon intermediate splits into 2 molecules of 3-carbon intermediate phosphoglyceric acid, and thus, the name C-3 for plants that have only this cycle. It is the conversion of phosphoglycerate to phosphoglyceraldehyde (P GAL in diagram, but also called triose-phosphate, a C-3 sugar) where most of the energy from "light reaction" (in the form of NADPH and ATP) is used. These triose phosphate molecules are the source of both sugars and starch (the food our life depends upon) as well as of regeneration of the receptor 5 carbon RUBP.

The Calvin Cycle: The Three Basic Steps

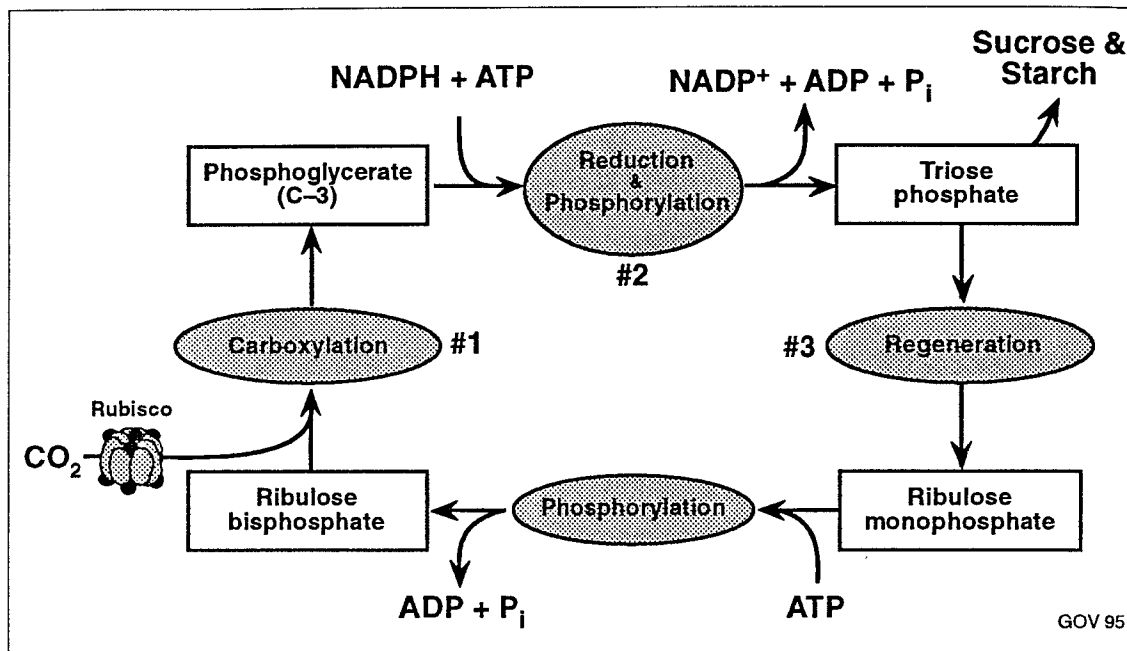
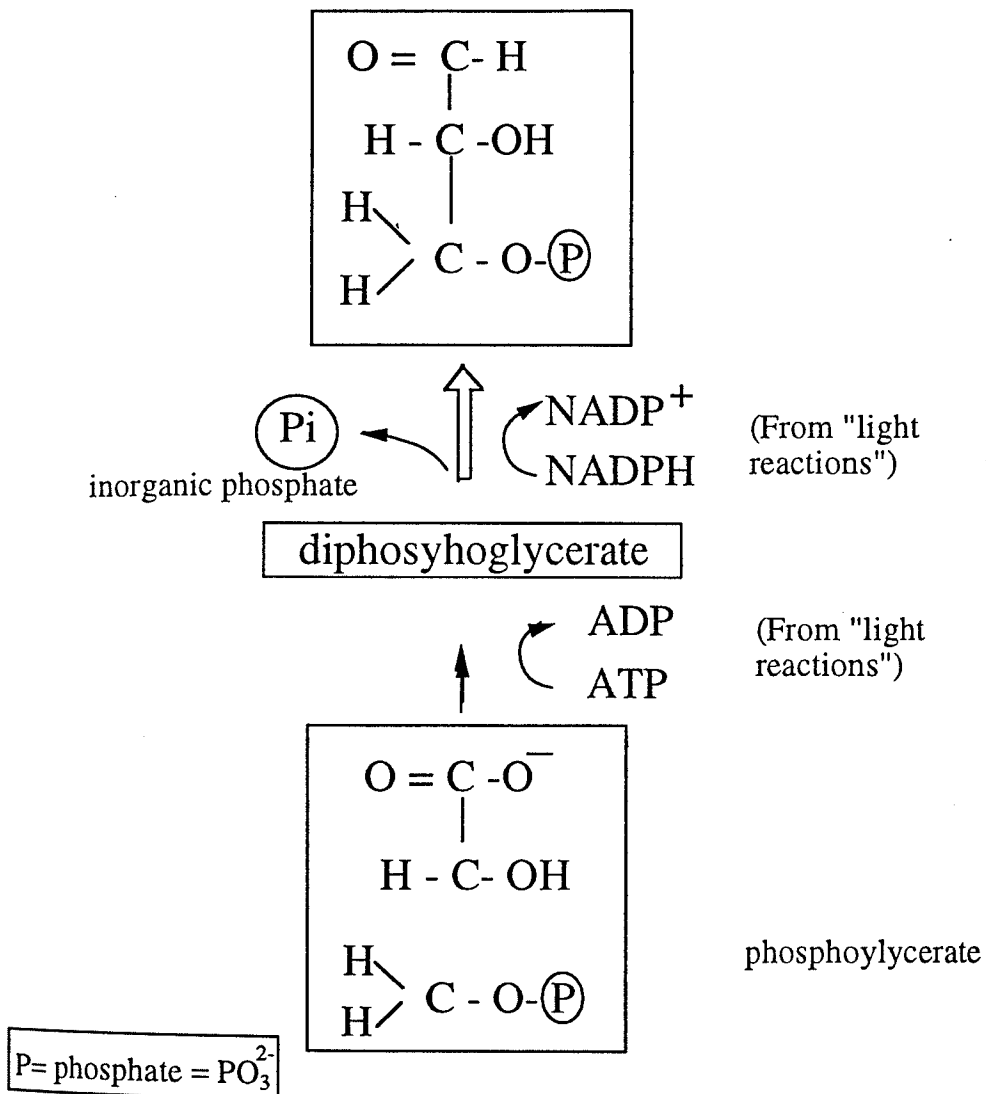


Fig. 2. An abbreviated scheme showing reduction of CO_2 by the Calvin cycle. Melvin Calvin received a Nobel Prize for this work. The first step (#1) is **carboxylation**, i.e., addition of CO_2 , to a receptor molecule a 5-carbon sugar Ribulose biphosphate using an enzyme RUBISCO (ribulose biphosphate carboxylase/oxygenase). **An estimated 10^7 tons of this enzyme (20kg/person) may be present on this Earth.** This step (#1) leads to the formation of 2 molecules of 3-C compound phosphoglycerate. There is then **reduction** (step #2) of phosphoglycerate to phosphoglyceraldehyde (PGAL in Fig. 1), also called triose phosphate. This is the step which requires energy as it is an "uphill" reaction. First phosphoglycerate is **phosphorylated** to diphosphoglycerate and only then it can be reduced by NADPH to triose phosphate. Two molecules of triose phosphate (C-3 sugar) can make one molecule of C-6 sugar. It is also used for **regeneration** of the receptor ribulose biphosphate with an additional phosphate step, as shown at the bottom of the diagram. **For fixation of 1 CO_2 molecule, 3 ATP and 2 NADPH are needed.**

Source of Energy for CO_2 Fixation is : ATP and NADPH

Table 2. The Energy Requiring Reaction



Calvin
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Experimental Evidence for Calvin Cycle

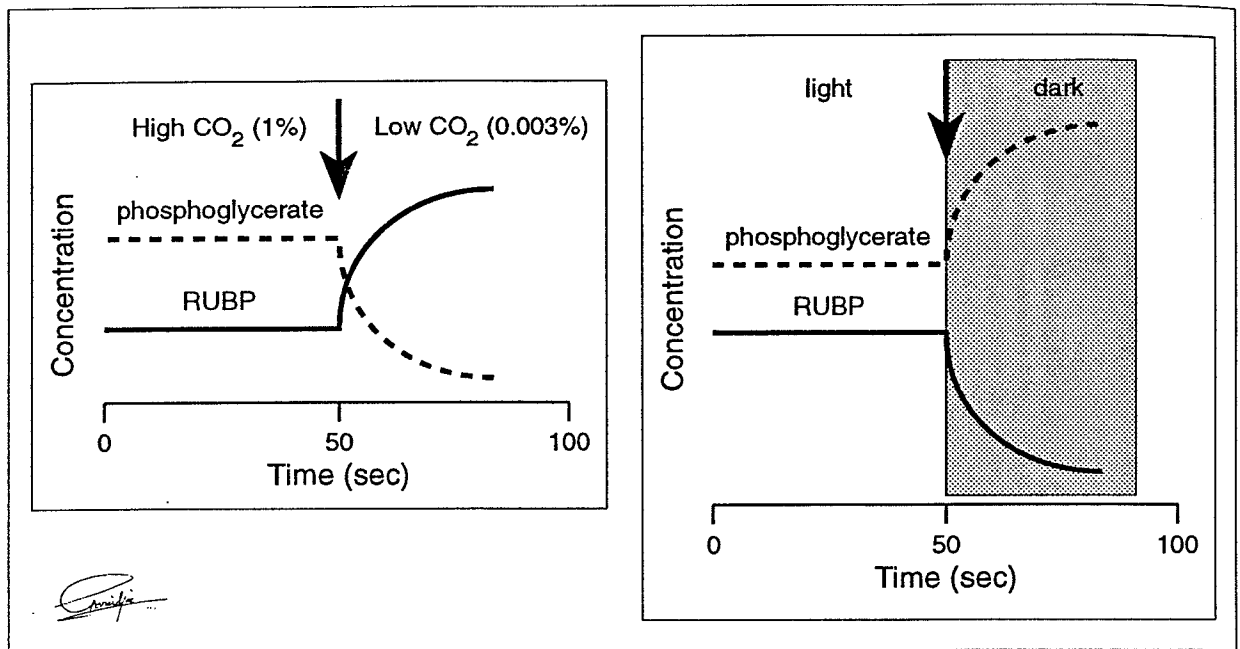
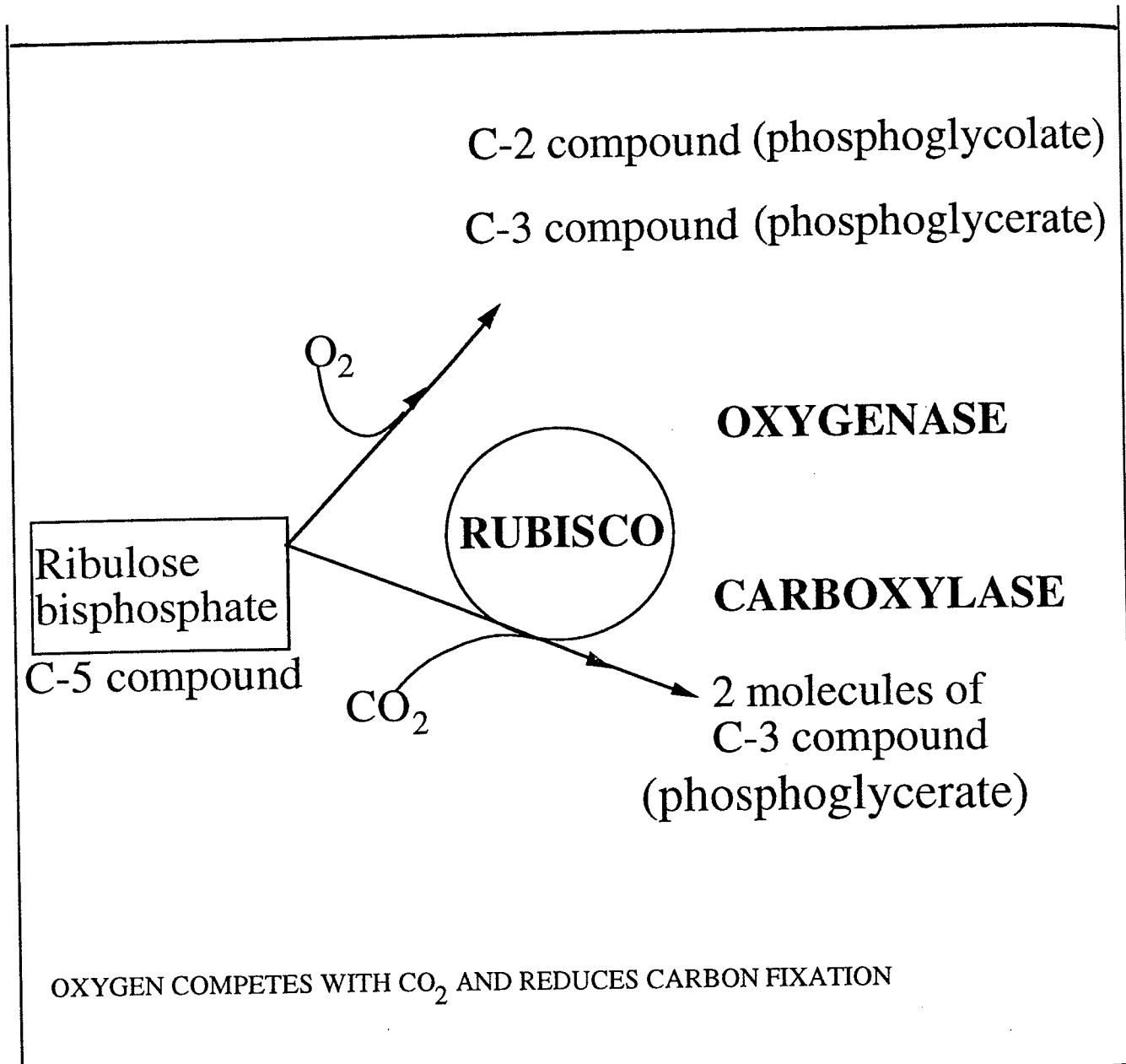


Fig. 3. Diagram showing the effect of lowering the CO₂ level (left) and turning off of the light (right) on the concentration of phosphoglycerate and ribulose bisphosphate (RUBP). If you see this graph together with Fig. 2, you will see that these results support the scheme there. Lowering CO₂ decreases the #1 reaction and thus phosphoglycerate decreases, but #2 and #3 steps continue for awhile temporarily increasing RuBP. On the other hand, turning off the light shuts off #2, as well as the phosphorylation step (at the bottom of Fig. 2) and, thus, Ribulose bisphosphate decreases, whereas phosphoglycerate increases as #1 reaction (in Fig. 2), the CO₂ fixation, continues with the pool of ribulose bisphosphate already there. These experiments were crucial in the acceptance of the Calvin cycle.

Table 3: THE RUBISCO

- The enzyme RUBISCO in higher plants is made up of 8 large (53 kilodaltons) and 8 small (13 kilodaltons) subunits.
- The active site, *i.e.*, where CO₂ and O₂ are added, is in the large subunit; it is formed at the interface between two large subunits.
- About 10⁷ tons of this enzyme is present on Earth; this amounts to 20 kilograms of this enzyme per person on Earth!
- It is a notoriously inefficient enzyme.

Table 4. Carboxylation versus Oxygenation at RUBISCO



* William Ogren, of UIUC, Urbana, was the one responsible for first recognizing the bifunctional nature of this enzyme, and the name RUBISCO arose from this understanding.

Photorespiration

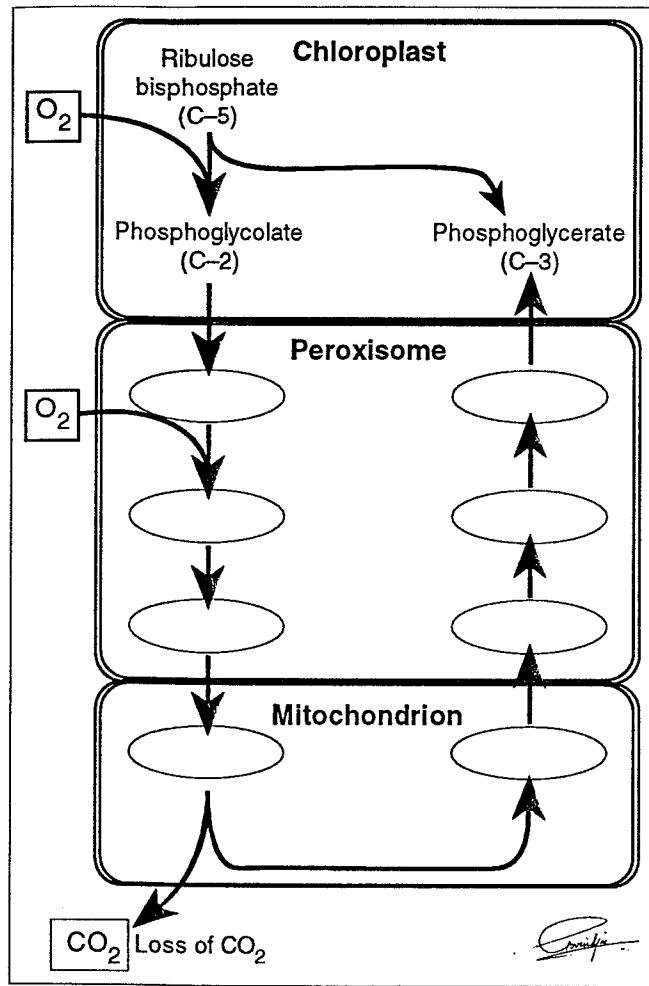


Fig. 4 Photorespiration occurs when RUBISCO reacts with O_2 in chloroplasts. The 2-carbon glycolate enters another organelle *peroxisome* and goes through a series of intermediates there and then in *mitochondrion*. During this process O_2 is taken up in peroxisome and CO_2 is lost in mitochondrion; and a part of carbon returns to chloroplast as a 3-carbon glycerate. Sounds mysterious. There is a lot of trafficking taking place. For every 2 molecules of 2-carbon glycolate, 1 molecule of CO_2 is lost, and one molecule of 3-carbon glycerate returns to chloroplast. So, plants try to make the best of the bad situation. However, the process of photorespiration *undoes* photosynthesis to some extent.

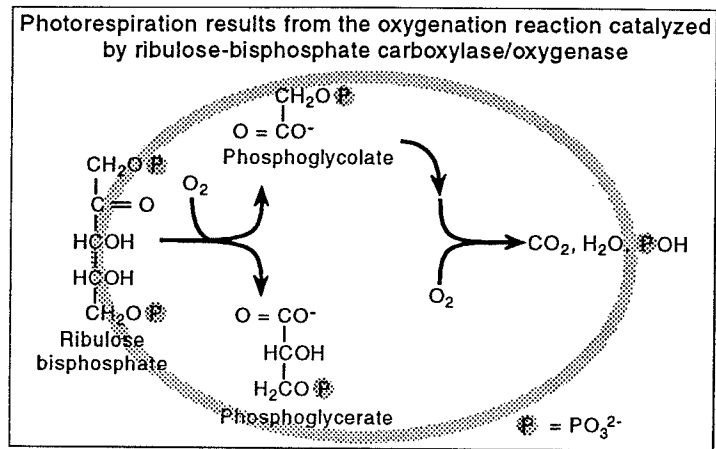


Fig.5 Shows the chemical structures of 5-C compound, the ribulose biphosphate, of 3-C phosphoglycerate and 2-C phosphoglycolate.

C-4 Pathway

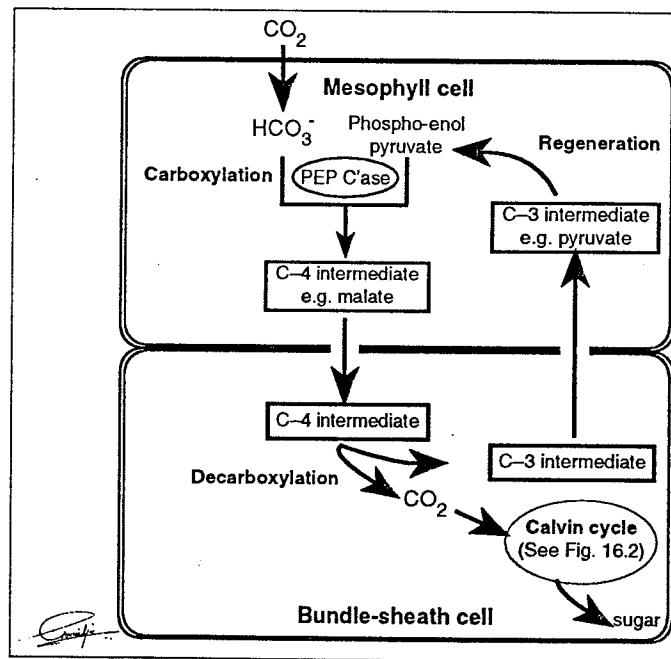


Fig. 6. The C-4 Pathway in its bare minimum. The purpose of this pathway is to concentrate CO_2 ; it is an "add-on" pathway to the Calvin cycle (see Fig. 2) is still used in these plants. Here CO_2 (in the form of bicarbonate ion, HCO_3^-) is added to a C-3 compound phosphoenol pyruvate

(PEP, for short) producing C-4 intermediates (e.g. oxaloacetate). The key enzyme is *phosphoenolpyruvate carboxylase*. It is an efficient enzyme and *does not* react with O_2 ! Oxaloacetate is converted into other C-4 compounds such as malate or aspartate. This efficient carboxylation uses HCO_3^- and occurs in *mesophyll cells* of C-4 plants. The C-4 intermediates are transported to *bundle sheath cells* where the *Calvin cycle* utilizes one of its carbons (released as CO_2) and the 3-carbon intermediate returns to the mesophyll cell to *regenerate* phosphoenolpyruvate. I would like to tell you that there is an additional *cost* involved here because 2 extra ATP's are used in the mesophyll cells.

Since phosphoenolpyruvate carboxylase (PEPC'ase) has a very high affinity of HCO_3^- , it can function at lower CO_2 levels, and the plant can afford to close their stomates. CO_2 is transported as part of C-4 intermediates (e.g. malate), CO_2 is concentrated in bundle sheath cells where RUBISCO is located. This CO_2 -concentrating mechanism decreases *photorespiration*.
Diagram by the author.

CAM Pathway

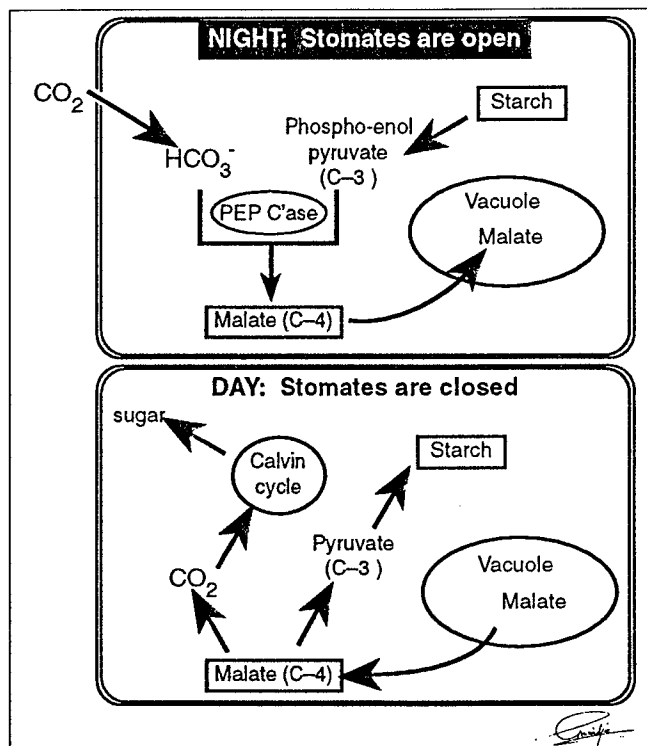


Fig.7. Crassulacean Acid Metabolism (CAM) pathway. Several plants are able to live in hot weather by keeping stomates closed during the day when it is hot and opening them at night when it is cold to avoid water loss.

In the dark (night), CAM plants open their stomates, and fix CO_2 by Phosphoenolpyruvate (PEP) carboxylase, accumulating the C-4 intermediate (malate), just as C-4 plants do in light in mesophyll cells. This malate, however, is stored in the vacuole. (The PEP is formed from the starch stored at earlier times.)

In the light (day), CAM plants close their stomata, malate comes back out of vacuole, and decarboxylates to release CO_2 that is then fixed by the Calvin cycle, and the 3-C intermediate pyruvate is converted into starch, to be used at night again.

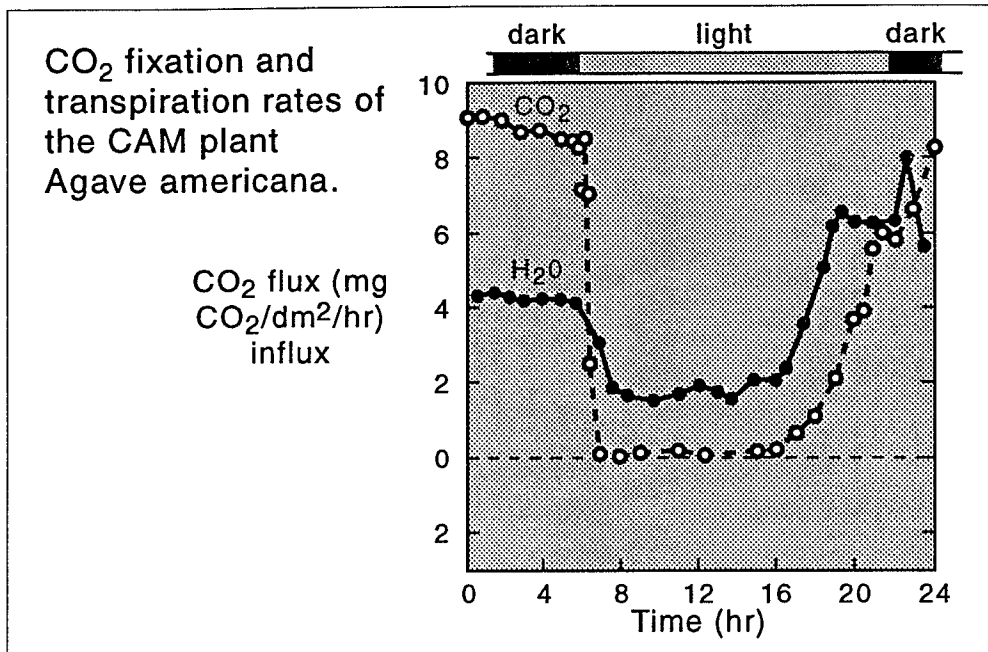


Fig. 8. In a CAM plant, CO₂ fixation occurs in dark. Due to lowered temperatures at night, not too much water is lost. During day, stomates are closed and no CO₂ uptake occurs. This condition, however, is good for these plants as very little water is lost, and these plants can thus survive in the hot temperatures where they grow. (Data of T.F. Neales, A.A. Patterson and V.J. Hartney, *Nature*, 219, 469-472, 1968) Diagram drawn by the author.

not
This
thus
V.J.

Table 5 A comparison of C-3, C-4 and CAM plants.

Characteristics	C-3	C-4	CAM
Examples	Soybean	Maize	Pineapple;
Anatomy	spongy and palisade cells	Mesophyll and Bundle sheath cells	Cells with large vacuoles
Growth Rates ($\text{gdm}^{-2} \text{day}^{-1}$)	1	4	0.02
Stomates	open in day closed at night	open in day closed at night	closed in day open at night

Water Use Efficiency gCO ₂ kg ⁻¹ H ₂ O	1-3	2-5	10-40
Maximum Photosynthetic Rate (+5 range)	30 mgCO ₂ dm ⁻² hr ⁻¹	60 mgCO ₂ dm ⁻² hr ⁻¹	3 mgCO ₂ dm ⁻² hr ⁻¹
Optimum temperature	20-30°C range	30-45°C range	30-45°C range
Compensation point: CO ₂ concentration when there is no net photosynthesis	50 parts per million (ppm)	5 ppm	2ppm (in dark)
Photorespiration	High	Low	Low
Key carboxylating Enzyme	RUBISCO only	PEP'Case RUBISCO	PEP'Case RUBISCO

C-3 versus C-4 Plants

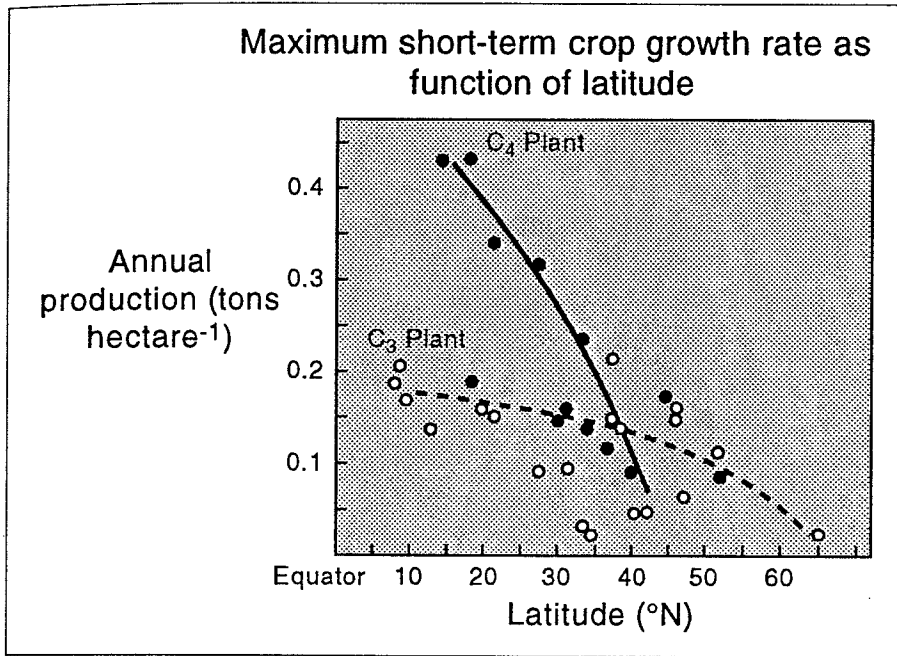


Fig. 9 Production (tons hectare⁻¹) of C-3 and C-4 plants at different latitudes. The C-4 plants predominate in warmer climate closer to the Equator, whereas C-3 plants predominate in temperate climate. Data compiled by Loomis and Gerakis, 1975.

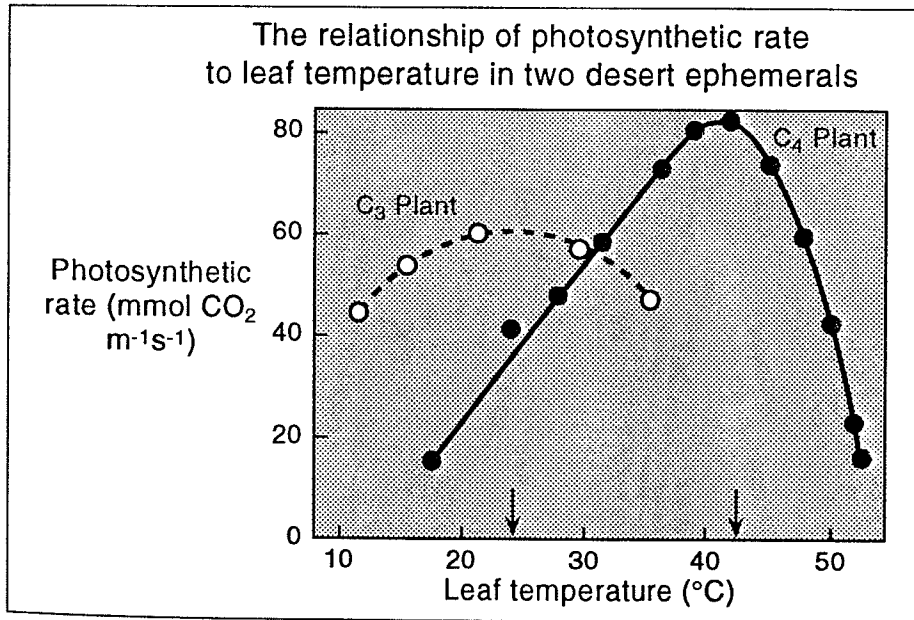


Fig. 10 A cool season C-3 plant has higher photosynthesis rates at lower temperature range, whereas a hot season C-4 plant has higher photosynthesis rates at higher temperatures.

C-3 versus C-4 Plants

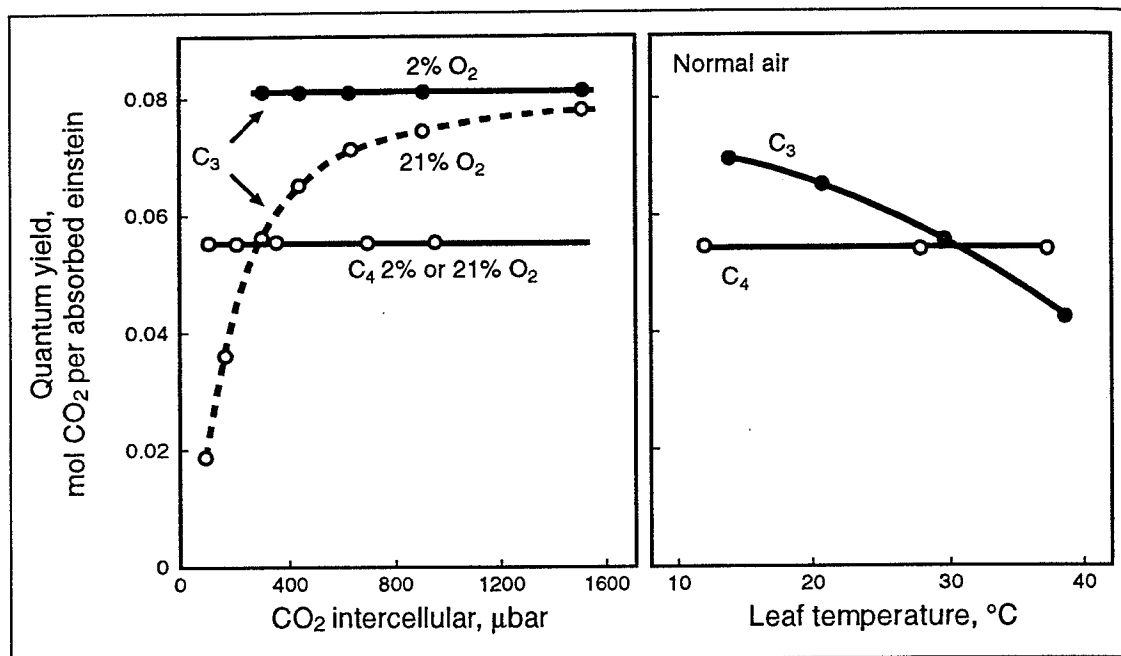


Fig. 11. Quantum yield of photosynthesis (molecules of CO₂ uptake or O₂ evolved, per absorbed photon; or moles of CO₂ uptake or O₂ evolved per mole of photon, also called Einstein) is calculated from the slope of the rate of photosynthesis versus number of photons absorbed at low light intensities. Its maximum value is 0.12 (or 1 CO₂/8 photons). **Remember Emerson.**

The graph on the left shows the following:

- (1) At 2% O₂ (when photorespiration is absent), quantum yield of CO₂ uptake for C-3 plants is maximal at all CO₂ concentrations, whereas that at 21% (when photorespiration is high) this quantum yield of C-3 plants is very low at low CO₂ concentration, increasing with increasing CO₂ concentration.
- (2) In C-4 plants (where photorespiration is always low due to the CO₂-concentrating mechanism (see Fig. 16.5)), quantum yield of photosynthesis is independent of O₂ concentration.

The graph on the right shows that the quantum yield of photosynthesis, always measured at low light intensities, is higher at lower than at higher temperatures for C-3 plants, whereas that for C-4 plants, this yield is independent of temperature. There is higher photorespiration at higher temperatures decreasing photosynthesis in C-3 plants.

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- [1] J. A. Bassham and B.B. Buchanan (1982) Carbon Dioxide Fixation Pathways in Plants and Bacteria. In: Govindjee (ed.) Photosynthesis, Vol. 2, pp. 141-189. Academic Press, New York.
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- [6] F.R. Tabita (1995) The biochemistry and metabolic regulation of carbon metabolism and CO₂ fixation in purple bacteria. In: R. Blankenship, M.T. Madigan and C. Bauer (eds.) Anoxygenic Photosynthetic Bacteria, pp. 885-914, Kluwer Academic, Dordrecht. [Advances in Photosynthesis, Vol.2, Series editor: Govindjee]

NOTE: If interested in this topic, please watch out for a book on Physiology and Biochemistry of Plants, to be edited by Drs. Leegood (UK), Sharkey (USA) and Von Camerer (Australia), and to be published in 1998 in the "Advances in Photosynthesis" series.

Summary

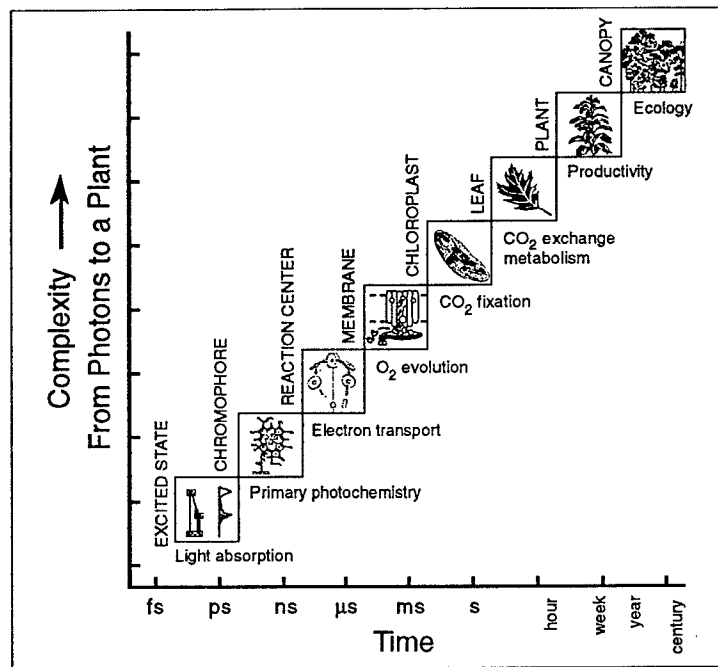


Fig. 12. An arbitrary diagram relating the dimensions of components of a plant with time domains of the associated processes. This diagram incorporates the ideas of Barry Osmond at the higher levels and of the author at the lower levels.

Govindjee

1 1

Efficiency and Global Aspects of Photosynthesis

1. Efficiency aspects

(Whitmarsh and Govindjee, 1996)

The theoretical minimum quantum requirement for photosynthesis is 8 quanta for each molecule of oxygen evolved (four quanta required by photosystem II and four by photosystem I). Measurements in algal cells and leaves under optimal conditions (e.g., low light) give quantum requirements of 8-10 photons per oxygen molecule released (see Emerson, 1958). These quantum yield measurements show that the quantum yields of photosystem II and photosystem I reaction centers under optimal conditions are near 100%. These values can be used to calculate the theoretical energy conversion efficiency of photosynthesis (free energy stored as carbohydrate/light energy absorbed). If 8 red quanta are absorbed (8 mol of red photons are equivalent to about 344 kilocalories/mol, or about 1,400 kJ) for each CO₂ molecule reduced (120 kilocalories/mol or, 480 kJ/mol), the theoretical maximum energy efficiency for carbon reduction is 34%. Under optimal conditions, plants can achieve energy conversion efficiencies within 90% of the theoretical maximum. However, under normal growing conditions the actual performance of the plant is far below these theoretical values. The factors that conspire to lower the quantum yield of photosynthesis include limitations imposed by biochemical reactions in the plant and environmental conditions that limit photosynthetic performance. One of the most efficient crop plants is sugar cane, which has been shown to store up to only 1% of the incident visible radiation over a period

of one year. However, most crops are less productive. The annual conversion efficiency of corn, wheat, rice, potatoes, and soybeans typically ranges from 0.1% to 0.4% (Odum, 1971).

2. Global Aspects

The amount of CO_2 removed from the atmosphere each year by oxygenic photosynthetic organisms is massive. It is estimated that photosynthetic organisms remove 100×10^{15} grams of carbon (C)/year (Houghton and Woodwell, 1990). This is equivalent to 4×10^{18} kJ of free energy stored in reduced carbon, which is roughly 0.1% of the incident visible radiant energy incident on the earth/year. Each year the photosynthetically reduced carbon is oxidized, either by living organisms for their survival, or by combustion. The result is that more CO_2 is released into the atmosphere from the biota than is taken up by photosynthesis. The amount of carbon released by the biota is estimated to be $1\text{-}2 \times 10^{15}$ grams of carbon/year. Added to this is carbon released by the burning of fossil fuels, which amounts to 5×10^{15} grams of carbon/year. The oceans mitigate this increase by acting as a sink for atmospheric CO_2 . It is estimated that the oceans remove about 2×10^{15} grams of carbon/year from the atmosphere. This carbon is eventually stored on the ocean floor. Although these estimates of sources and sinks are uncertain, the net global CO_2 concentration is increasing. Direct measurements show that each year the atmospheric carbon content is currently increasing by about 3×10^{15} grams. Over the past two hundred years, CO_2 in the atmosphere has increased from about 280 parts per million (ppm) to its current level of 360 ppm. Based on predicted fossil fuel use and land management, it is estimated that the amount of CO_2 in the atmosphere will reach 700 ppm within the next century. The consequences of this rapid change in our atmosphere are unknown. A brief discussion of the effect of increased CO_2 on plant life is available in a popular article by Bazzaz and Fajer (1992). Because CO_2 acts as a greenhouse gas, some climate models predict that the temperature of the earth's atmosphere may increase by 2-8°C. Such a large temperature increase would lead to significant changes in rainfall patterns. Little is known about the impact of such drastic atmospheric and climatic changes on plant communities

and crops. Current research is directed at understanding the interaction between global climate change and photosynthetic organisms.

References cited above:

Bazzaz, F.A. and Fajer, E.D. (1992). Plant life in a CO₂-rich world. *Scientific American* 266 (1), 18-24.

Emerson, R. (1958) The quantum yield of photosynthesis. *Annu. Rev. Plant Physiol.* 9: 1-16.

Houghton, R.A. and G.M. Woodwell (1989) Global climatic change. *Scientific American* 260:36-44.

Odum, E.P. (1971) *Fundamentals of Ecology*. W.B. Saunders Co. Philadelphia.

Outline for global aspects:

◆ 1. The Carbon Cycle

- Global Carbon cycle (Fig. 1, these Notes)
- Accounting of C: mostly in Oceans (Fig. 2, these Notes)
- Carbon fluxes; more going to the atmosphere (Fig. 3, these Notes)
- Distribution of carbon (Table1, these Notes)
- The missing carbon (Table1, these Notes)

◆ 2. The Greenhouse Effect

A. Changes in CO₂ on Earth

- Evidence for CO₂ increase: (Fig. 4, these Notes)
- CO₂ increases as a function of latitude and time (Fig. 5, these Notes)
- Past CO₂ increases (Fig. 6, these Notes)

- Is this mainly due to fossil fuel burning and deforestation? (Fig. 7 A, B, these Notes)
 - But don't forget the "Yo-yo" effect (Fig.8, these Notes)
- B. *Changes in temperature on Earth*
- Global temperature variations (Figs.9, Fig. 10, these Notes)
- Predictions of increases in temperature due to increases in CO₂ levels (Fig. 11, these Notes)
 - Predictions in changes in vegetation due to possible increases in temperature (Fig. 12, these Notes)
- C. Possible predicted changes:
- Increased photosynthesis in some plants, and, thus, increased biomass
 - Long-term damage of some plants
 - Floods due to melting of ice

◆ 3. Biomass and Climate

Two major climatic factors (rain and temperature) play important roles in determining which plants grow where. Biomass of a plant, related to primary productivity, can be estimated from the area index (L.A.I, i.e., area of leaves divided by area of the ground), and LAI can be estimated from the ratio of the reflection of near infra red light (not absorbed by leaves) to the reflection of red light (absorbed by leaves).

- Relationship between Net Primary Productivity and leaf biomass (Fig.13 and 14, these Notes)
- Relationship between Net Primary Productivity and Leaf Area Index (Fig. 15, these Notes)
- Relationship between the ratio of near infra red reflectance/red reflectance to leaf area index (Fig. 16, these Notes)

[In the first approximation, then, these reflectance studies can give some estimate of biomass in certain plants.]

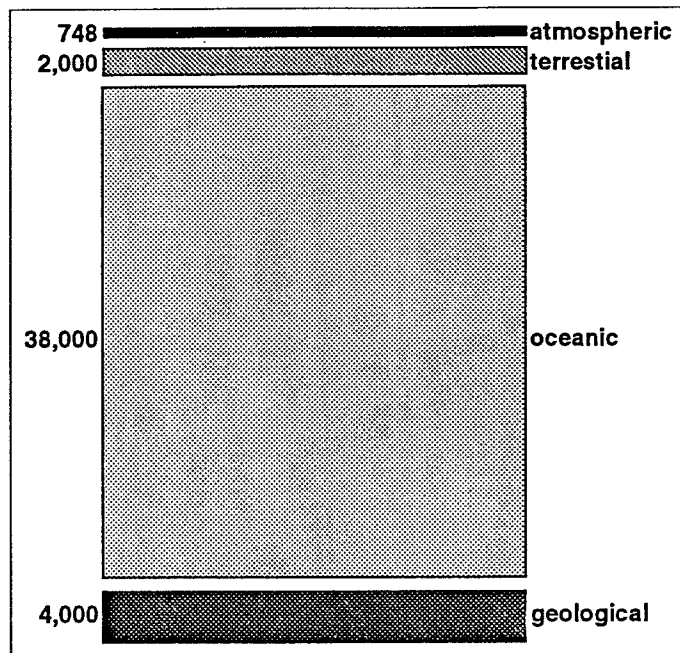


Figure 1 Reservoir sizes in gigatons of carbon in our atmosphere, land (terrestrial), Ocean and buried under Earth (geological). (Data taken from Post et al., American Scientist, 1990, 78, 310-326). Diagram redrawn by SOLS Artist Service for the author.

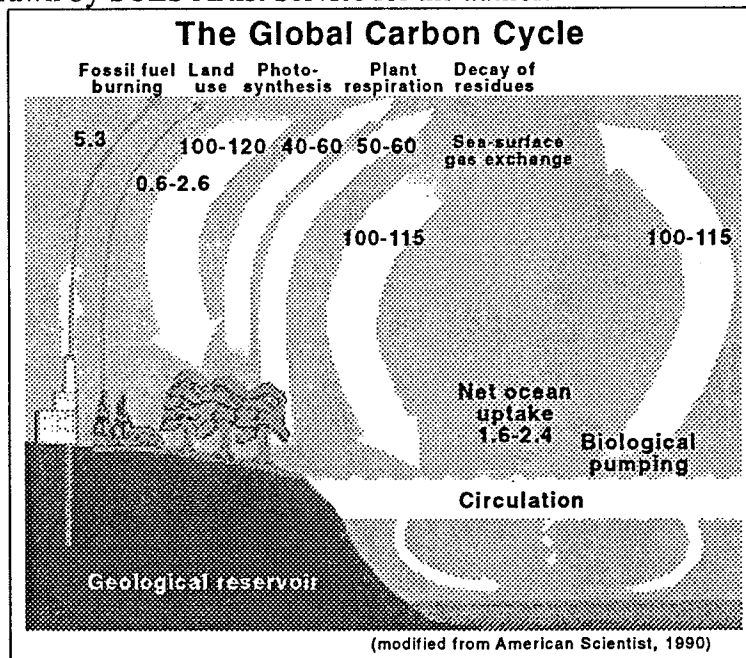


Fig. 2 1980 estimates of carbon fluxes in gigatons. It seems more carbon is estimated to go to the atmosphere than is accounted for by measured increases in carbon there! (Data of Post et al., 1990).

Table 1 The Missing Carbon: Approximately 2×10^{15} grams

<i>Estimates, in grams</i>	
<i>Extra going to atmosphere</i>	<i>Extra found in</i>
Fossil burning: 5.3×10^{15}	Increase in atmosphere: 3×10^{15}
Destruction of land vegetation: $0.6-2-6 \times 10^{15}$ grams	Uptake by Oceans: $1.6-2.4 \times 10^{15}$
Total (A) <u>$5.9-7.9 \times 10^{15}$</u>	Total (B) <u>$4.6-5.4 \times 10^{15}$ grams</u>

A-B= Estimated missing carbon= 2×10^{15} grams; it is suggested to go to some unknown sink? What sink?

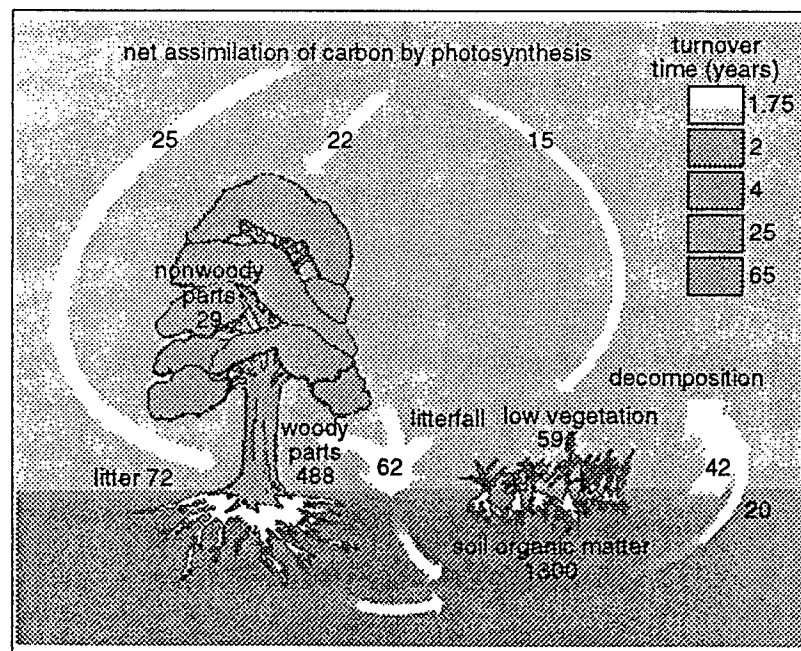


Fig. 3 Accounting of carbon assimilation and turnover times of carbon by terrestrial vegetation. Woody plants of a tree have low turnover but more carbon (Post et al, 1990).

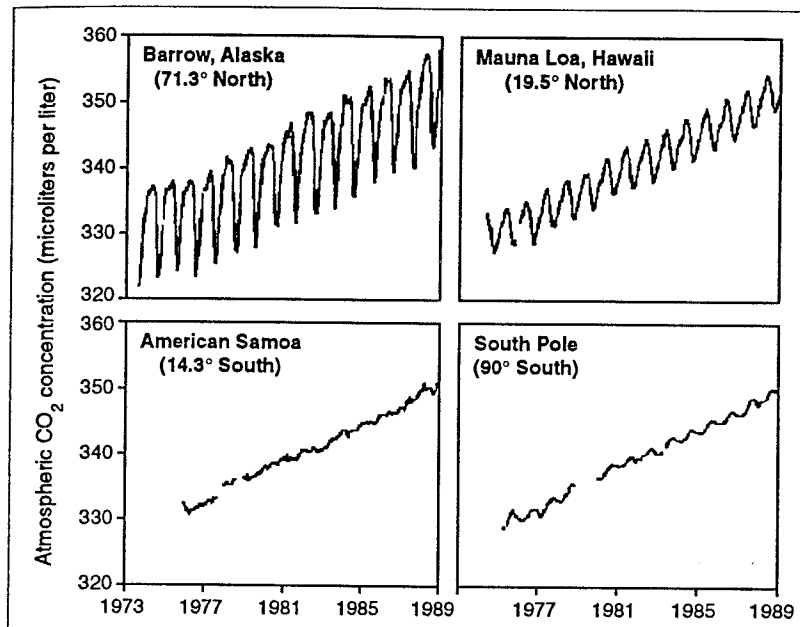


Fig. 4 Data on increases in CO₂ at four sites (Data of National Oceanic and Atmospheric Administration, 1986-1989) (See Post et al., 1990). Note that the depth of seasonal changes (low CO₂ in summer and high in winter) depends upon the site of measurements. **Can you speculate reasons for it?**

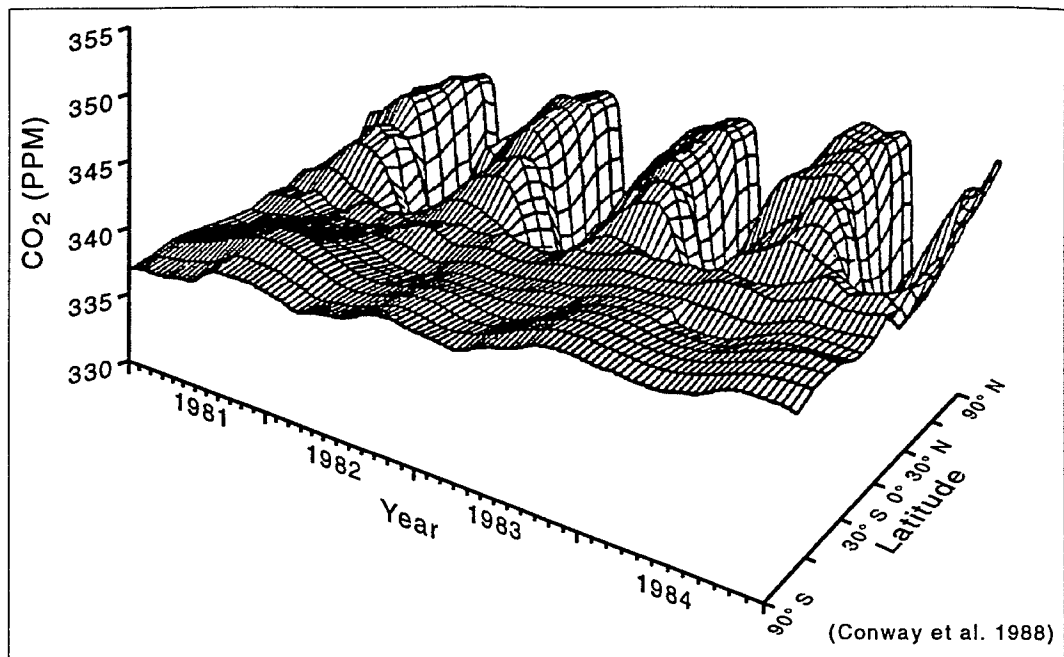


Fig.5 Seasonal fluctuations in atmospheric CO₂ concentrations are largest in the Northern hemisphere (where there is more vegetation) than in the Southern hemisphere (Data of Conway et al., 1988) (See Post et al., 1990). Redrawn by SOLS Artist Service for the author.

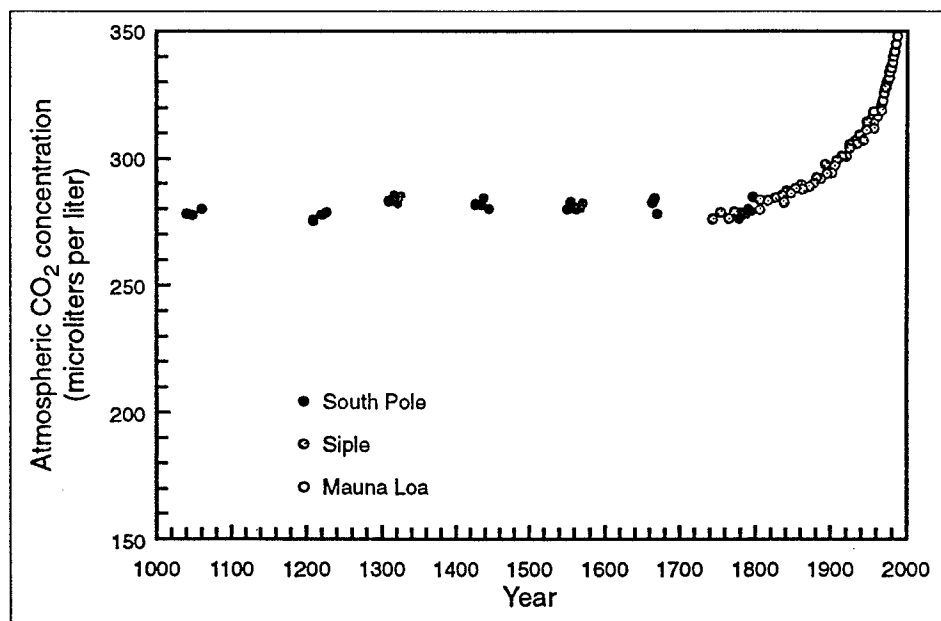


Fig 6 This figure shows that atmospheric CO₂, that remained approximately constant from 1,000 to 1800 A.D., suddenly started increasing and is expected to continue to increase (Post et al., 1990). Redrawn by SOLS Artist Service for the author.

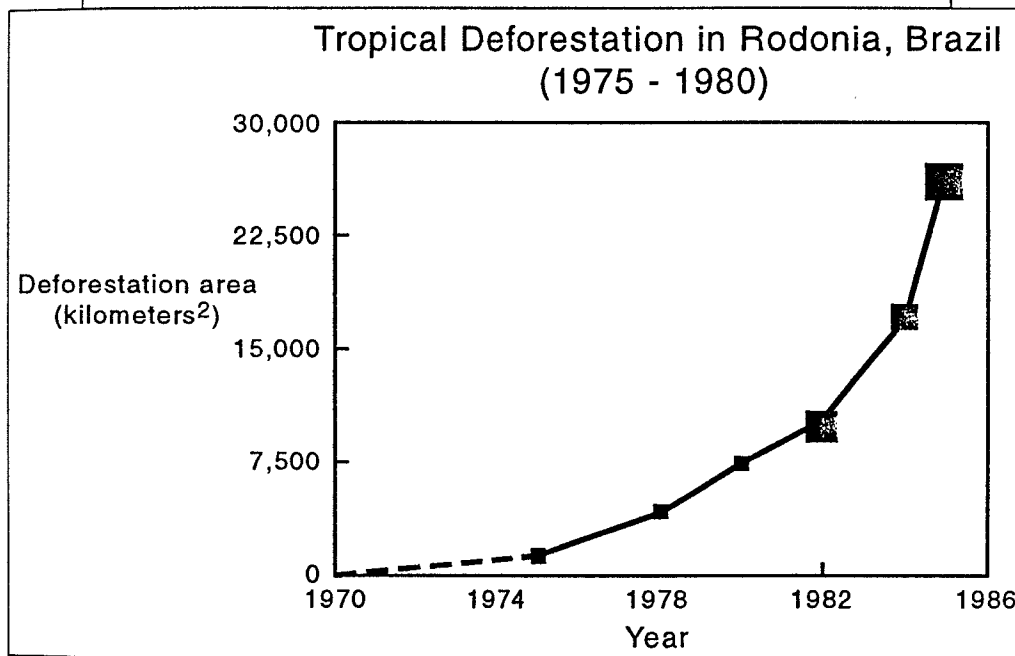
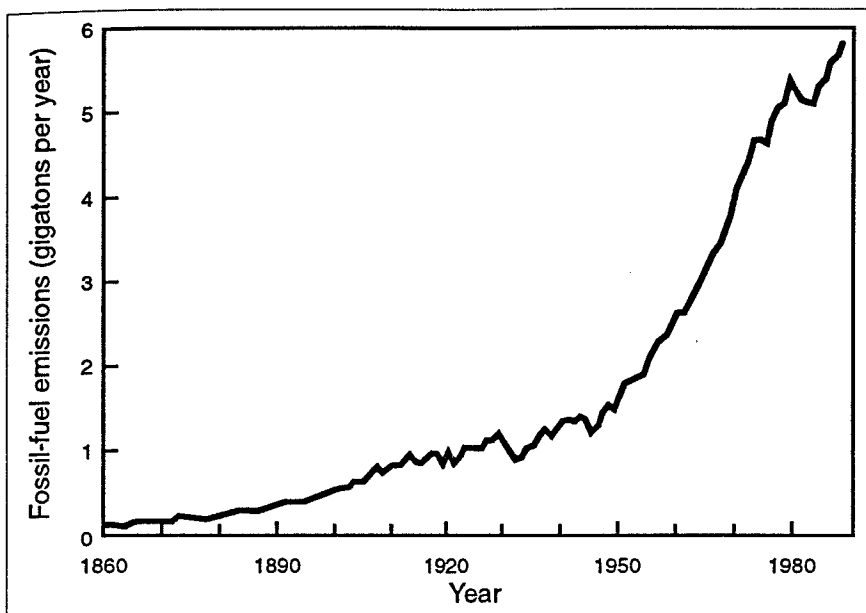


Fig. 7 Increases, during 1860-1980, in atmospheric CO₂ concentrations (also see Fig. 4.6) seem to be correlated with data on fossil fuel burning (top) and deforestation (bottom) (Data of Marland et al., 1988 and Mallingreau and Tucker, 1988). Redrawn by SOLS Artist Service for the author.

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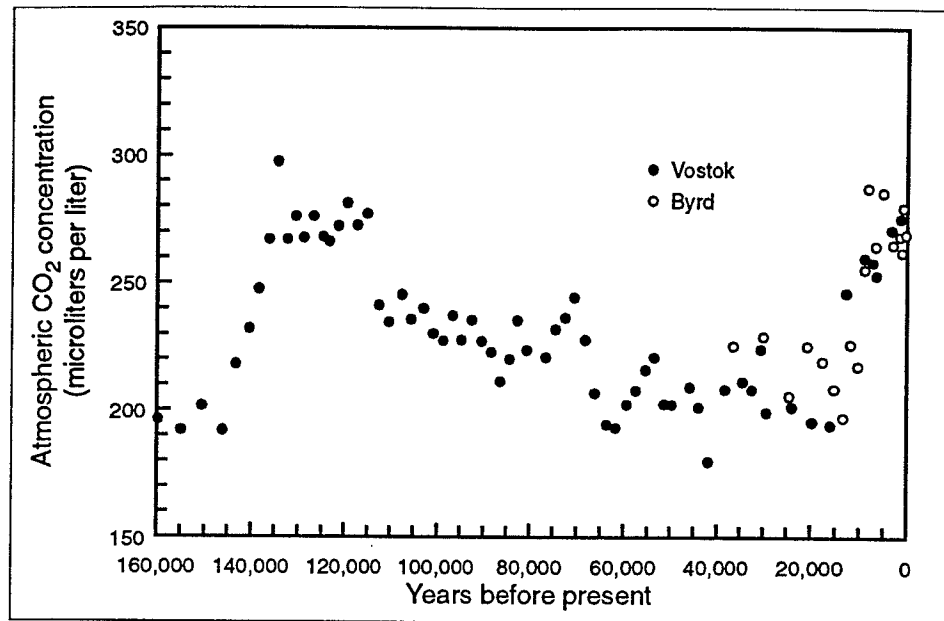


Fig. 8 CO₂ concentrations, measured from "ancient" air bubbles trapped in "ancient" Antarctic ice-cores, peaked 130,000 years ago during interglacial period. These approached the current atmospheric CO₂ levels. Is there a giant "yo-yo" in nature? (Data of Barnola et al., 1987; Neftel et al., 1982).

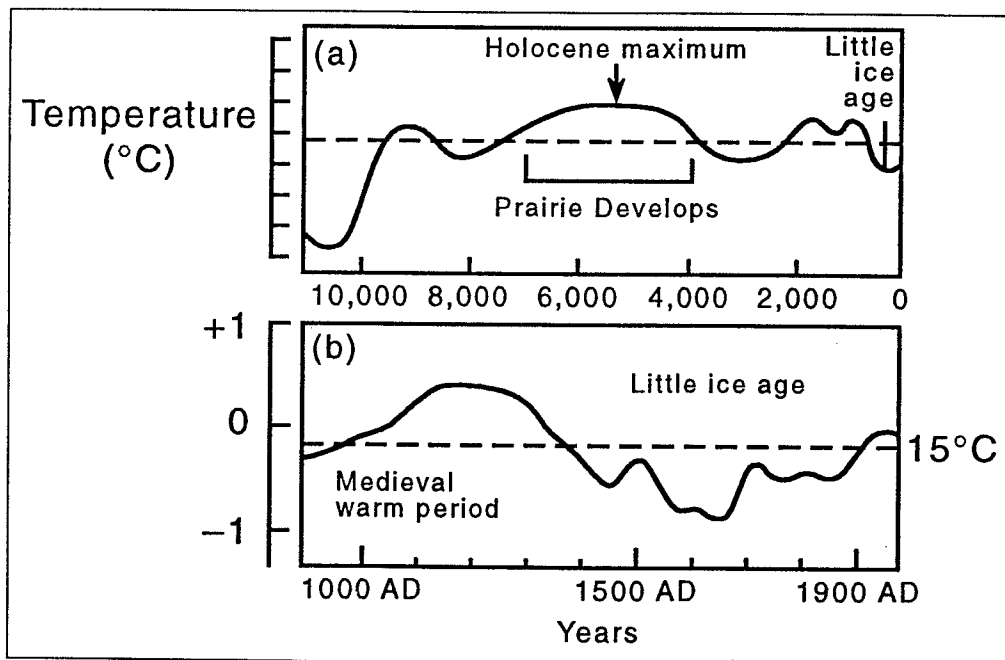


Fig. 9 Temperature data in (a) is during the last 10,000 years; and in (b) is during the last thousand years. Dashed line is for data in 1900. About 5 to 6,000 years ago, the prairie must have developed due to the warming spell.

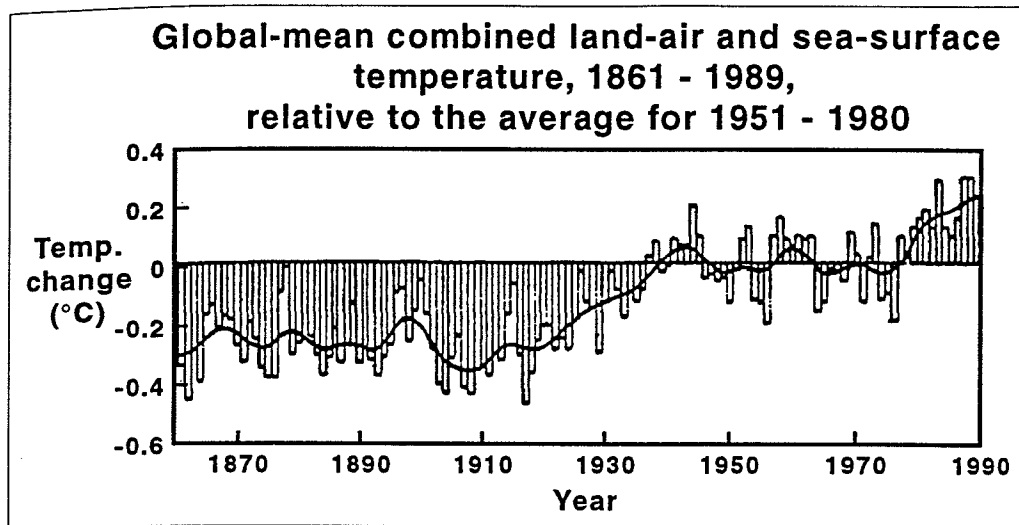


Fig. 10 Has the temperature been increasing on this Earth slightly and steadily since 1900?

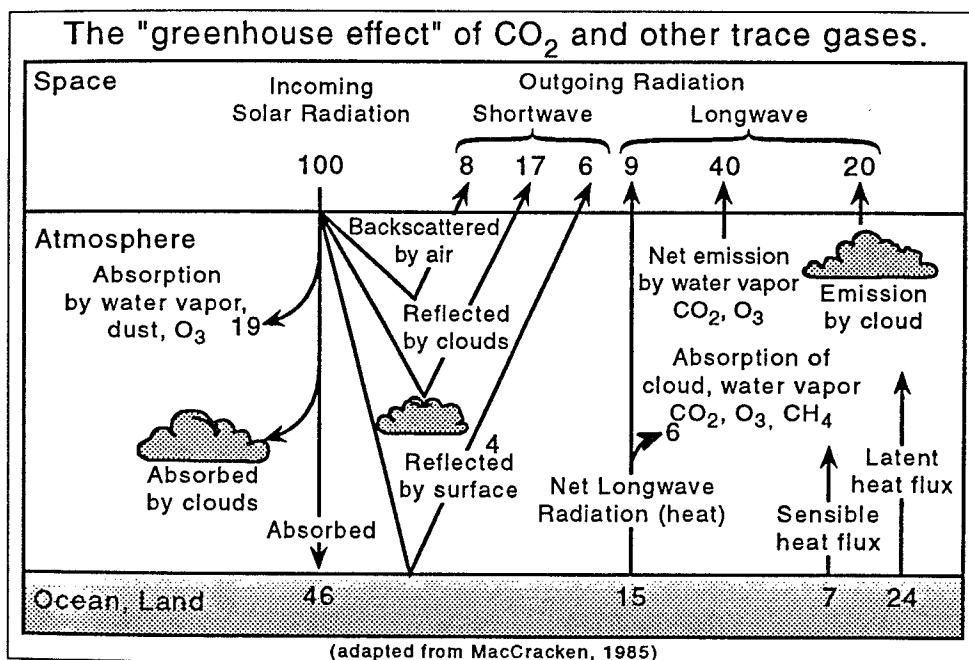


Fig. 11 Data of MacCracken (1985) for the "greenhouse effect" of CO₂ and other gases. Sun's radiation is dominated by shortwavelength light, but the Earth radiates longwavelength light. Since CO₂ absorbs these longwavelength radiations, and, thus, decreases it from going into space, it warms the Earth. Thus, increases in [CO₂] in Earth's atmosphere is expected to increase Earth's temperature. There may be an error in the diagram; an arrow going into the Ocean, Land may be missing (100); and the "15" from the Ocean, Land is "115", and "6" above it is "106"; further, the "4" near "Reflected by surface" belongs to "Absorbed by clouds". Sorry about these errors that I could not correct.

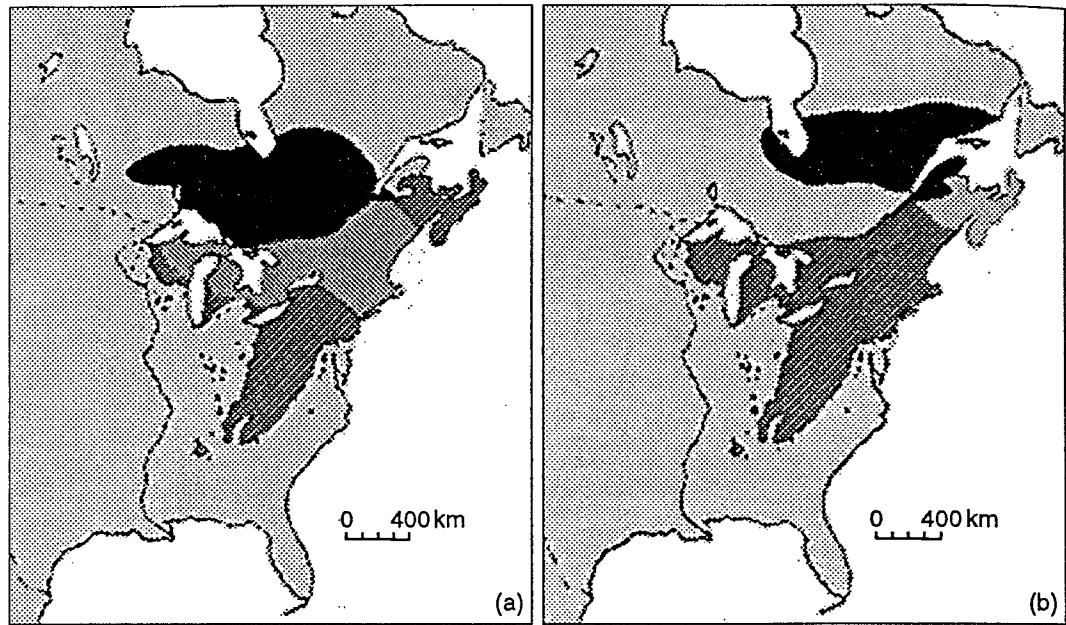


Fig. 12 Predictions of Davis (1988) are shown in cross-hatched area where the Eastern Hemlock trees are expected to be found 100 years from now. Dark diagonals in graphs are based on predictions of range of these trees with CO₂ doubling with climate changes proposed by (a) Hansen et al. (1983) and (b) Manabe and Wetherald (1987), whereas the light diagonal shading is for the current distribution of these trees.

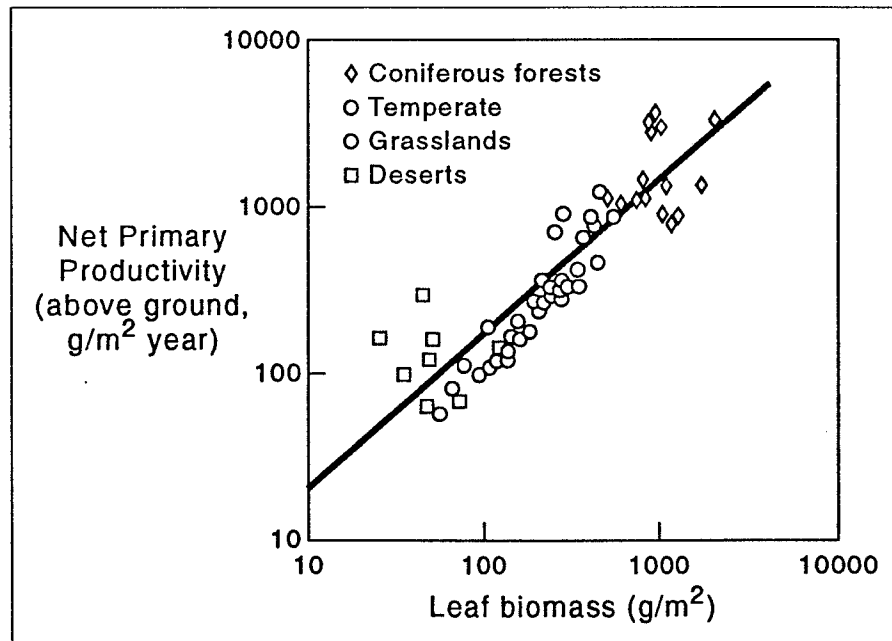


Fig.13 Relationship between leaf biomass and net primary productivity in forests (Data of Webb et al., 1983).

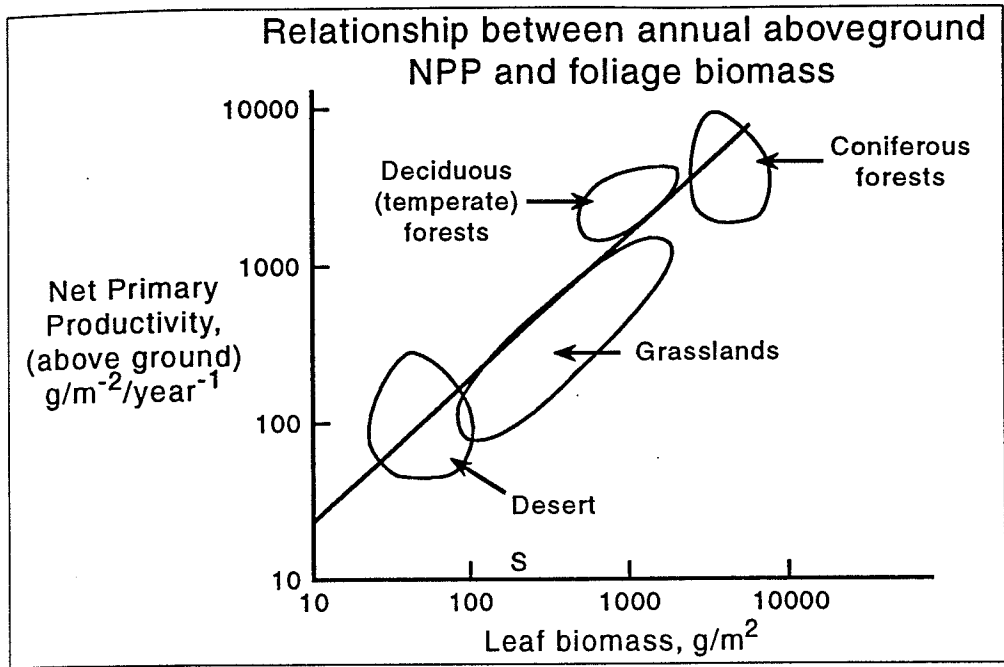


Fig. 14 Relationship between leaf biomass and net primary productivity in various biomes (refer to lecture 1). (Data of Gholz, 1982)

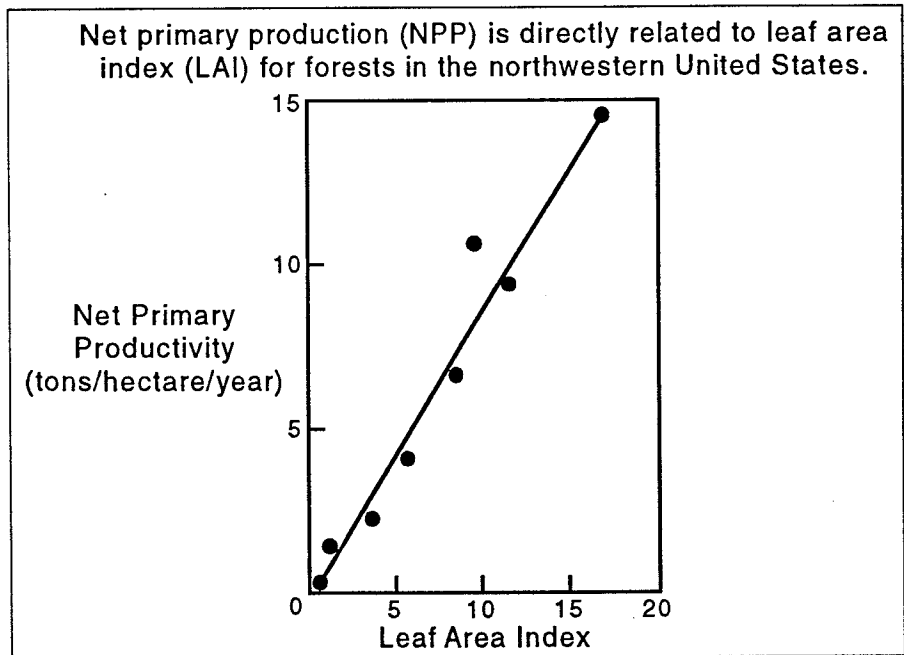


Fig. 15 Relationship between net primary productivity and leaf area index in forests. (Data of Gholz, 1982).

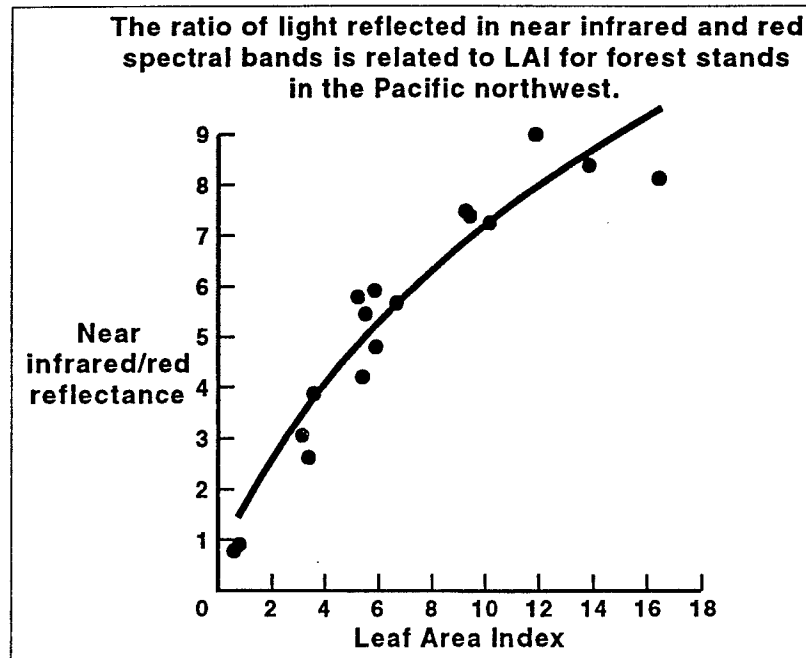


Fig. 16 Satellite data on the ratio of near infrared reflectance to red reflectance plotted against the leaf area index of forest stands. (Data of Peterson et al., 1987)

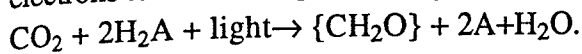
🍏 Dictionary/glossary

1. *Beta factor*: refers to the concept that increased CO₂ in the atmosphere can be used to increase photosynthesis that would increase biomass and would lessen the accumulation of CO₂ in the atmosphere.
2. *Gigaton*: a billion (10⁹) tons.
3. *Greenhouse gases*: are atmospheric gases that are responsible for warming of the lower atmosphere and the Earth: CO₂, methane, nitrous oxide, ozone, water vapor and chlorofluorocarbons.
4. *Leaf area index (LAI)*: area of leaves above a unit of ground surface.
5. *Organic matter*: compounds having a carbon backbone, often with carbon atoms arranged as a chain or ring structure.
6. *Photosynthesis*: the physical-chemical process by which certain chlorophyll (or bacteriochlorophyll) containing organisms use light energy for the biosyntheses of organic molecules.

Oxygenic photosynthesis: Photosynthesis by plants, algae and cyanobacteria in which water is oxidized to molecular O₂ and CO₂ is reduced to carbohydrates.

CO₂+H₂O+light → {CH₂O}+O₂ where {CH₂O} stands for carbohydrate.

Anoxygenic photosynthesis: photosynthesis by certain bacteria in which oxygen evolution does not occur, and the organisms use compounds (such as H₂S or organic compounds, written below as H₂A) other than water to obtain hydrogen atoms or electrons to reduce CO₂ to carbohydrates.



7. *Radiation, long wavelength*: the word radiation refers to the process in which energy is emitted as particles or waves, whereas long wavelength refers to the length of their waves being long. Well, what is long? This is ambiguous. In the context, it was used in these Notes, it refers to infra-red radiation.

8. *Radiation, short wavelength*: see just above; it refers to radiation with short wavelengths. Here again, this is ambiguous. In the context used in lecture 4, it refers to visible (including red) radiation.

9. *Remote sensing*: is the term often used when measurements on biomass, chlorophyll concentration, or leaf area index, etc. of plants are made from a remote site by using optical instruments that measure either reflectance or fluorescence.

10. *Respiration*: is the process by which living organisms oxidize glucose with molecular oxygen releasing CO₂; in an overall which sense, it is the reverse of oxygenic photosynthesis. $\{\text{CH}_2\text{O}\} + \text{O}_2 \rightarrow \text{CO}_2 + \text{H}_2\text{O} + \text{energy}$.

11. *Soil*: the portion of the earth's surface consisting of disintegrated rock and humus. (Humus=dark organic material in soils, produced by the decomposition of vegetable or animal matter, and essential to the fertility of the earth.)

12. *Source/Sink*: A source is where something originates or starts from, and a sink is where something goes to.

Questions:

- ◆ 1. Identify the major pools (reservoirs) and fluxes of the global carbon cycle.
- ◆ 2. Discuss the uncertainties in the balance of the global carbon cycle.
- ◆ 3. Describe the *greenhouse* effect and provide a physical explanation for the same.
- ◆ 4. Relate leaf biomass to net primary productivity (NPP), NPP to leaf area index (LAI), as measured by remote sensing of chlorophyll.
- ◆ 5. Discuss the potential effects of climate change (for example, increased CO₂ concentration, increased temperature) on vegetation.
- ◆ 6. Define and, or describe the following **concepts and terms**:

beta factor, gigaton, greenhouse gases, leaf area index, organic matter, photosynthesis, radiation, respiration, remote sensing, short and longwavelengths, soil, source/sink

Life on Earth requires energy for survival; it is the solar energy, the energy from what some call "Surya Bhagwan", that runs it. But, this is made possible by photosynthesis. Some of the main points to remember are: (1) Photosynthesis is a series of energy transforming reactions that convert light energy into chemical energy in the form of carbohydrates, proteins, etc. (2) Photosynthetic organisms live autotrophically..they are the primary producers. (3) Photosynthesis provides the energy and reduced carbon for almost all life on Earth. (4) It is certain that atmospheric carbon di-oxide will double during the next century. (5) Photosynthetic organisms are expected to change the global atmosphere, whereas the global atmosphere will affect, in turn, photosynthesis.

beta factor, gigaton, greenhouse gases, leaf area index, organic matter, photosynthesis, radiation, respiration, remote sensing, short and longwavelengths, soil, source/sink



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#12

Sunlight, Summary and Comparative Aspects

- 1. About the Sun
- 2. Summary of Photosynthesis
- 3. Comparative Aspects of Photosynthesis

Table 1. Data on Sun, Sunlight and Earth

Our "Suraj"

- **Sun:** It is a typical star in terms of brightness. It is the energy source that drives the evolution and survival of life on Earth. Its output has remained virtually unchanged for the last 4.5 billion years. No one expects any significant change in it in the next 2 billion years. Its current age is about 50% of its age at its death that is expected in about 4 billion years.
- **Diameter:** 1,400,000 km
- **Mass:** 2×10^{33} g (about 60% hydrogen)

- **Energy output** : 4×10^{33} ergs/s (or 4×10^{26} Watts)
- **Mass to energy**: The Sun converts 10^{-11} of its mass into energy by the fusion of hydrogens into helium. [$E = mc^2$]
- **Temperature**: Center: 10^7 K; surface: 5,780 K; blackbody radiation
- **Density**: Center: 10^5 g/cm³; average 1.4 g/cm³!

Our "Prithvi"

- **Distance to Sun**: 150,000,000 km
- **Interception of light**: it takes 8 minutes to receive light here after it is created on the Sun; one part in 2.2×10^9 radiation emitted by the Sun is intercepted by the Earth.

♦ **Sunlight on Earth**:

1.37 kilowatts (kW) meter⁻²
 or 1.37 kJ (kilojoules) m⁻² s⁻¹ (second⁻¹)

On a clear day at noon, this is about 0.85 kW per square meter..

This is equivalent to: 2,000 μmol of photons (also called μEinsteins) m⁻²s⁻¹

- About 45% of this light is the so-called PAR (Photosynthetic Active Radiation, 400- 700 nm range)
- ♦ Out of 100% Sunlight on Earth, only 4% is converted into carbohydrate because the rest is lost as:

(1) not absorbed as it is at wavelengths longer than 700 nm, etc: 60%

(2) energy in other metabolic paths: 20%

(3) reflected or transmitted: 8%

(4) heat : 8%

• **Temperature of the Earth:**

The average is 18 C; this temperature depends on many factors including radiation energy absorbed by the Earth; warming of the Earth's core by nuclear reactions there; and the radiation emitted by the earth to the environment.

Table 2. Summary of Photosynthesis

- ◆ Photosynthesis can be broadly separated into *dark* (or light independent) and *light* (light-dependent) processes. *Traditionally*, *light* reactions are those involved in the oxidation of water to oxygen and reduction of NADP^+ to NADPH with the accompanying formation of ATP; and dark reactions are those involved in the use of these (NADPH and ATP) to fix CO_2 into carbohydrate.
- The light reactions occur on *thylakoid membranes* and the dark reactions in *stroma matrix* of chloroplasts. In **reality**, the only true light reactions are those that occur at the reaction center chlorophylls P680 and P700, all others being "dark" reactions.
- ◆ If we could start photosynthesis with brief flashes of light it will follow the sequence given on the next page in the box

ON THYLAKOID MEMBRANES

- Light Absorption
- Excitation Energy Transfer
- Primary Photochemistry
- Electron Transport leading to
 - oxygen evolution
 - production of reducing power NADPH
 - proton translocation
- Formation of ATP

IN STROMA MATRIX

- CO₂ fixation by RUBISCO and PEPC^{'ase}_(all) _(C-4, CAM)
- Reduction and Phosphorylation of phosphoglyceric acid to triose phosphate using NADPH and ATP
- Regeneration of receptors of CO₂
- Formation of 6-C Sugars and Starch

(sugars are formed outside chloroplasts, but starch is formed inside chloroplasts)

Comparative Aspects of Photosynthesis

Photosynthetic systems; Electron transport pathways; c
Comparative aspects of energy converting processes in various organisms

(1) Photosynthetic Systems

According to Carl Woese and co-workers, all life on Earth can be divided into three **domains: archea; bacteria; and eucarya**. Examples of archea are methanogens and the so-called halobacteria (e.g., *Halobacterium halobium*). Anoxygenic photosynthetic bacteria and oxygenic cyanobacteria and prochlorophytes belong to bacteria, whereas green and other multicolored algae, bryophytes, pteridophytes, gymnosperms and angiosperms belong to eucarya.

A. Anoxygenic Photosynthetic Bacteria

Anoxygenic photosynthetic bacteria are of several types; examples are: (i) purple and (ii) green. Purple bacteria are subdivided into Rhodospirillaceae (examples are: *Rhodospirillum rubrum* and *Rhodospirillum rubrum*) and Chromatiaceae (e.g., *Chromatium vinosum*). Green bacteria are subdivided into Chlorobiaceae (e.g., *Chlorobium limicola*) and Chloroflexaceae (e.g., *Chloroflexus aurantiacus*). Some comments on these organisms and their systems follow.

(1) *Rhodospirillum rubrum*. Reaction center: P960; has bacteriochlorophyll *b* instead of bacteriochlorophyll *a*; there is uphill energy transfer here since "antenna" (light harvesting) pigment molecules absorb with a peak at 1,065 nm. Its reaction center was crystallized by H. Michel; and its structure solved by Nobel laureates Michel, J. Deisenhofer and R. Huber and their co-workers. They contain three proteins: L, M and H. [Bacteriochlorophylls are different from chlorophylls since their ring II is hydrogenated (see an earlier handout); the conjugated (single-double bond) system of bacteriochlorophylls is more symmetrical than that of chlorophylls, the latter have their first excited bands at lower wavelengths than those of bacteriochlorophylls. Thus, they absorb far red and infra-red light.]

(2) *Rhodobacter sphaeroides*. Reaction center: P870 --reaction center has been crystallized and x-ray diffraction patterns and structure are also available. They contain bacteriochlorophyll *a*.

(3) *Chloroflexus aurantiacus*. It has electron transport pathway like that of *R. sphaeroides*. In contrast, however, it lacks the "H" subunit and one of its bacteriochlorophyll monomer is changed into a bacteriopheophtin (without Mg) molecule.

(4) *Chlorobium limicola*. Reaction center: P840; has similarity to that of Photosystem I of plants, algae and cyanobacteria.

NOTE: The reaction centers of all the purple(red) bacteria have similarities to that of Photosystem II of plants, algae and cyanobacteria, but there are also remarkable differences. Photosystem II can oxidize water to oxygen, but the purple and green bacteria do not; they are anoxygenic.

B. Oxygenic cyanobacteria

Oxygenic cyanobacteria and prochlorophytes are prokaryotes just as anoxygenic photosynthetic bacteria are. Cyanobacteria have been called "Blue bacteria" (George Papageorgiou). Several species are being used today for studies on molecular biology of photosynthesis. A particular species *Synechocystis* sp. PCC (*Pasteur Culture Collection*) 6803 has been extensively used as it is a highly transformable strain. It was used to conclude that "tyrosine-161" on the D1 protein IS the redox component "Z" of Photosystem II. L subunit of anoxygenic bacteria is equivalent to the D1 of oxygenic organisms; and, M subunit is equivalent to D2 protein; there is no equivalent protein for the H subunit.

C. Oxygenic Eukaryotes

All other chlorophyll a - containing organisms (red algae, green algae, cryptomonads, diatoms, brown algae, C-3 plants, C-4 plants, CAM plants, etcetra) perform oxygenic photosynthesis. Examples are:

Spinacea oleracea (spinach: a C-3 plant)

Zea mays (maize: a C-4 plant)

Porphyridium cruentum (a red alga)

Navicula minima (a diatom)

(2) Types of Electron Flow

(A.) "Non cyclic" (rather a poor word; it should be simply "linear"): All plants, algae and oxygenic bacteria: there are two photosystems and two light reactions; they are oxygenic; molecular oxygen is produced when water is oxidized. Both iron-sulphur centers and quinone complexes serve as electron acceptors. [Consult an earlier presentation for an exceptional mutant of *Chlamydomonas reinhardtii*]

(B) "Cyclic" , accompanied by reverse electron flow: Most purple and Chloroflexus type (green gliding) bacteria; these are anoxygenic.; oxygen is not produced; they have one light reaction; quinone complexes are used as electron acceptors. A cyclic electron flow produces a proton gradient ("high energy" intermediates) that are then used to "reverse" electron flow from an external hydrogen donor (e.g., succinate) to NAD. This would not normally occur because the free energy change would be positive. ATP synthesis is also via proton gradient, ΔpH and membrane potential, mostly latter in these organisms.

(C) "Non cyclic" of Chlorobium type: This involves a photosystem I type reaction-- however, details are not identical. Here, iron-sulphur centers are used.

(3) Types of Antenna Systems

(A) In Anoxygenic photosynthetic bacteria: Pigment protein complexes B800, B850, B890, etc. in purple bacteria. They contain bacteriochlorophyll *a* (800, 850, 890 stand for wavelength peaks, in nm) or bacteriochlorophyll *b* (B-1065) as in *Rhodospirillum rubrum*. [H. Zuber and R.J. Cogdell (1995) Structure and Organization of Purple Bacterial Antenna Complexes. In: Anoxygenic Photosynthetic Bacteria, edited by R. Blankenship, M.T. Madigan and C. Bauer, pp. 315-348. Kluwer Academic, Dordrecht.] There are alpha and beta polypeptides that have transmembrane orientation.

Green bacteria contain chlorosomes (these are external to the membrane) having bacteriochlorophyll *c*, *d*, and *e* (700- 760 nm absorption); and a base plate in the membrane containing bacteriochlorophyll *a* (800-870 nm). [R. Blankenship, J.M. Olson and M. Miller (1995) Antenna complexes from green photosynthetic bacteria. In: Anoxygenic Photosynthetic Bacteria, edited by R. Blankenship, M.T. Madigan and C. Bauer, pp. 399-435. Kluwer Academic, Dordrecht.]

(B) Cyanobacteria and red algae: They contain phycobilisomes having phycobiliproteins (phycoerythrin, absorbing green light, peak at 500-545 nm region, is red; it transfers energy to blue phycocyanin that absorbs orange light, 600-220 nm; and to allophycocyanin that absorbs 650-670 nm light); phycobilins are open chain tetrapyrroles. Phycobilisomes are external to the membrane, whereas chlorophyll *a* containing protein complexes are in the membrane. [W.A. Sidler (1994) Phycobilisome and phycobiliprotein structures. In: The Molecular Biology of Cyanobacteria, edited by D. Bryant, pp. 139-216. Kluwer Academic, Dordrecht.]

Cryptomonads also contain phycobilins but these are not in phycobilisomes, but are arranged in the membrane itself unlike those in cyanobacteria and red algae.

(C) Other Algae: Green algae and prokaryotic *Prochloron* contain light-harvesting complexes called LHCI and LHCII; both have Chlorophyll *a* and *b*, but LHC I has much less chlorophyll *b*. Chlorophyll protein complexes (CP-43 and CP-47, etc) contain chlorophyll *a* and are present not only in this group, but also those in section (B) above. On the other hand, chlorophyll *c*-containing complexes are present in diatoms and brown algae. Chlorophyll *a* in vivo has its red absorption band in the 670-690 nm range; and chlorophyll *b*'s red band is at 650 nm in vivo.

(D) Higher Plants: These contain complexes similar to that of green algae although there are differences. Kuehlbrandt et al. (Nature **367**: 614-621, 1994) have solved the structure of LHCIIb; there are three membrane-spanning polypeptides, and, the distances and the arrangements of various chlorophylls (*a* and *b*) are now known. LHC-II is of several varieties, some of which are mobile, whereas others are not. The mobile ones can move between the two photosystems. In one theory: Excess light in photosystem II causes preponderance of reduced plastoquinone; they activate a kinase and the mobile LHC become phosphorylated. Due to repulsion of negative charges on the phosphate group, mobile LHCII move out from the grana to the stroma region where Photosystem I is located. On the other hand, excess light in Photosystem I oxidizes reduced plastoquinone and thus the kinase activity decreases. When this happens, phosphatase takes over and mobile LHC is dephosphorylated and it moves back to grana again.

(4) Notes on carotenoids

Carotenoids are present in all photosynthetic organisms; these carotenoids are of varied kind; their functions are two fold: (1) to act as light harvesters; and (2) to protect photosynthesis against excess light. Carotenoids absorb in the blue

to yellow region. Fucoxanthol is certainly a carotenoid that transfers energy very efficiently to chlorophyll *a*. It is present in diatoms and brown algae. It has recently been suggested that some of them may have "virtual" energy bands in the red below the chlorophyll bands allowing energy transfer from chlorophyll *a* to them! A particular role of xanthophylls (zeaxanthin and violaxanthin) in this protection mechanism has been discussed (see e.g., A. Gilmore, T.L. Hazlett and Govindjee (1995) Xanthophyll-cycle dependent quenching of photosystem II chlorophyll *a* fluorescence: formation of a quenching complex with a short fluorescence lifetime. Proc. Natl. Acad. Sci. USA 92: 2273-2277).

(5) A reminder on the arrangement of protein complexes

In Rhodospirallaceae, B-800-850 and B-890 pigment protein complexes are arranged in the membranes to serve as antenna to the reaction center complex. Currently, highly sophisticated detailed molecular structural models are available. A minireview by R.Cogdell and co-workers will contain a picture on one of the bacteria (Photosynthesis Research, available in September, 1996); in addition, Klaus Schulten and co-workers also are going to be presenting another beautiful model soon.

In chlorobiaceae, bacteriochlorophyll *c*, *d* and *e* are in chlorosomes, the latter sticking to the membrane as a knob on the base plate containing bacteriochlorophyll *a*; the reaction center and a bacteriochlorophyll *a* containing complex is in the membrane. The structure of Fenna-Matthews Olson protein is available (see Tronrud, DE, Schmidt, MF and Matthews, BW (1986) X-ray Structure and amino acid sequence of a bacteriochlorophyll *a* protein from *Prosthecochloris aestuarii* refined at 1.9 Angstrom resolution. J. Mol. Biol. 188: 443-454.]

In cyanobacteria and Rhodophyta (red algae), phycobilins are in phycobilisomes sticking out of the membrane. The reaction centers I and II are surrounded by Chlorophyll *a* containing antenna complexes; phycobilisomes are attached mostly near photosystem II reaction centers.

Higher plants and green algae (chlorophyceae) have two reaction centers (I and II), core antenna complexes containing chlorophyll *a* and light harvesting complexes with both chlorophyll *b* and *a*. Although PSI reaction center has been crystallized and X-ray structure available, PSII reaction center needs to be solved. Our current model for PSII reaction center is in press in "Protein Science" (1996).

(6) Redox potentials, E_m , of Reaction Center chlorophylls, the primary donors

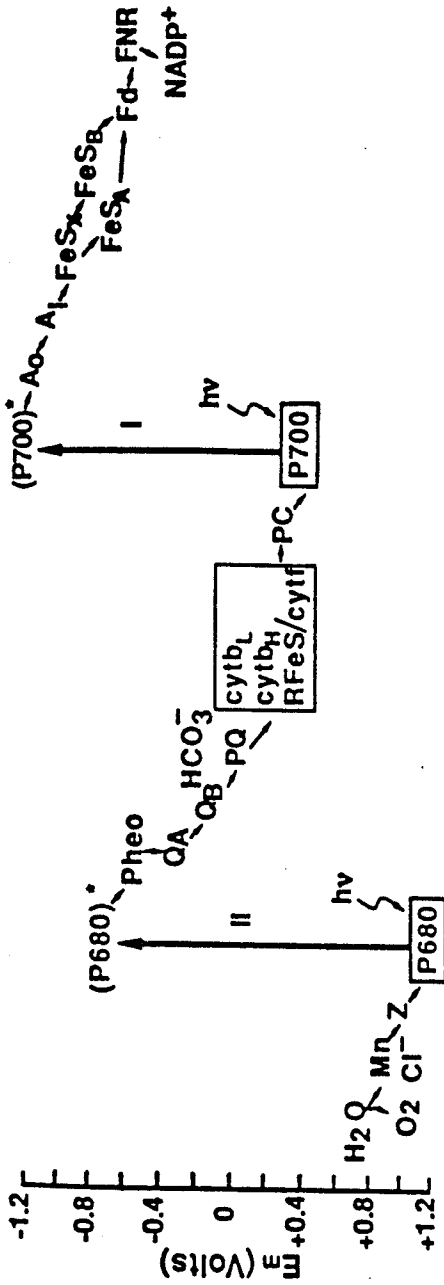
P680/ P680 ⁺ (Photosystem II):	+ 1.2 eV
P700/P700 ⁺ (Photosystem I):	+0.4 eV
P840/P840 ⁺ (Chlorobium reaction center):	+0.2-0.3 eV
P870/P870 ⁺ (R. sphaeroides reaction center):	+0.4 eV
P960/P960 ⁺ (R. viridis reaction center :	+0.4 eV

Questions

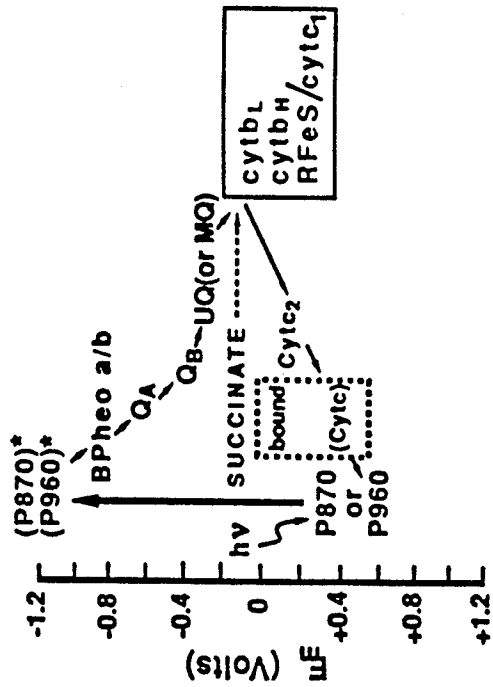
- [1] Why only Photosystem II can oxidize water to oxygen?
- [2] Why does photosynthesis need light?
- [3] Why is a plant leaf green?
- [4] Why is fluorescence spectrum shifted to longer wavelengths than the absorption spectrum?
- [5] What factors determine exciton transfer from one pigment to another?
- [6] Why is there an asymmetry in electron flow in bacterial reaction center when the reaction center itself seems very symmetrical?
- [7] What is the key evidence for the series scheme of two light reactions in photosynthesis?
- [8] How do you distinguish between the "Q-cycle" and the "two-electron gate" of photosynthesis?
- [9] At which step is energy needed during ATP synthesis?
- [10] What is unknown about the mechanism of water oxidation?

[11] Write an essay on "The Photosynthetic Process" emphasizing the energy transduction pathway from Photons to NADPH and ATP.

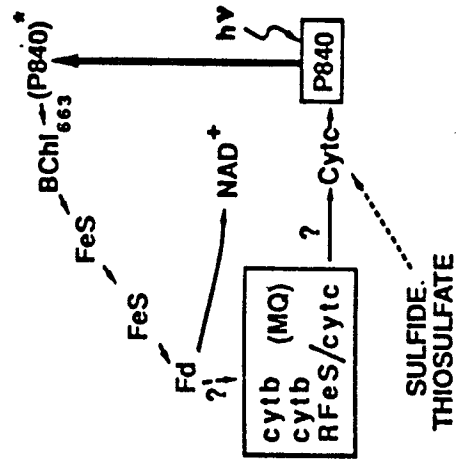
PLANTS AND CYANOBACTERIA



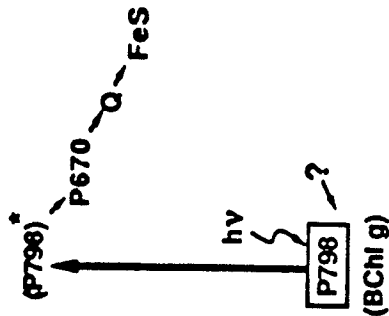
PURPLE (NON-SULFUR)
& FILAMENTOUS GREEN BACTERIA



GREEN SULFUR
(Chlorobium type)



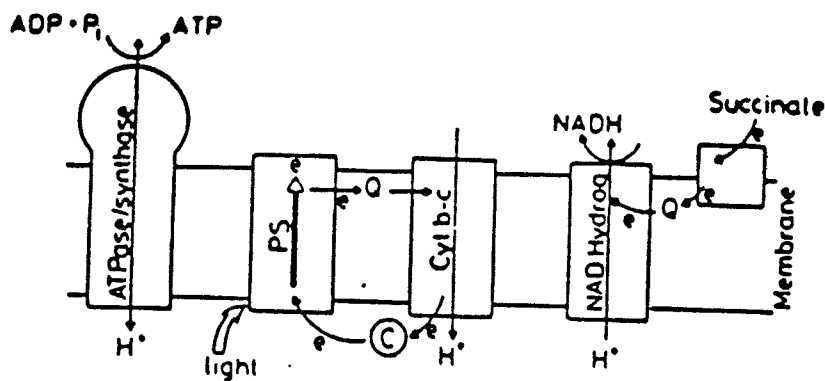
GRAM POSITIVE LINE
(Heliobacterium chlorum)



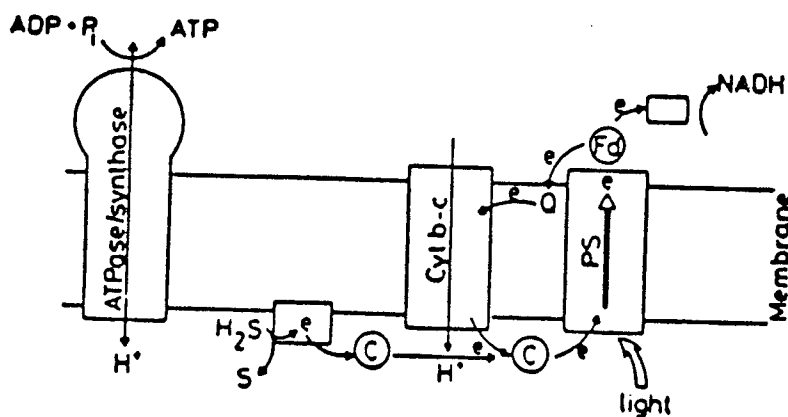
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Figure 1A

A RHODOSPIRILLACEAE



B. CHLOROBIAEAE



C. HIGHER PLANTS

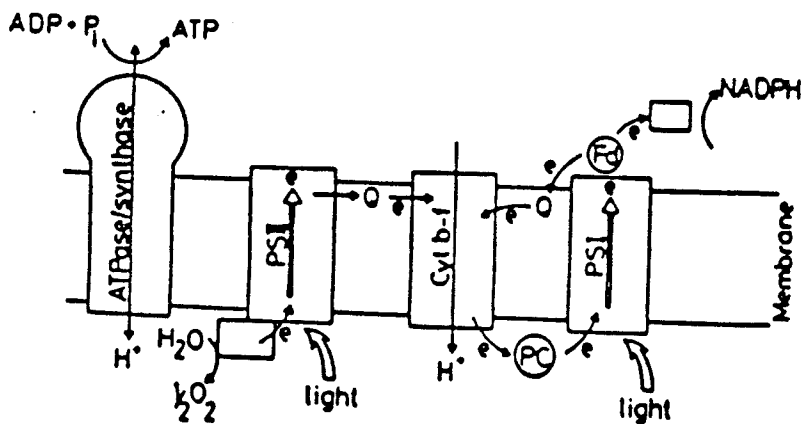
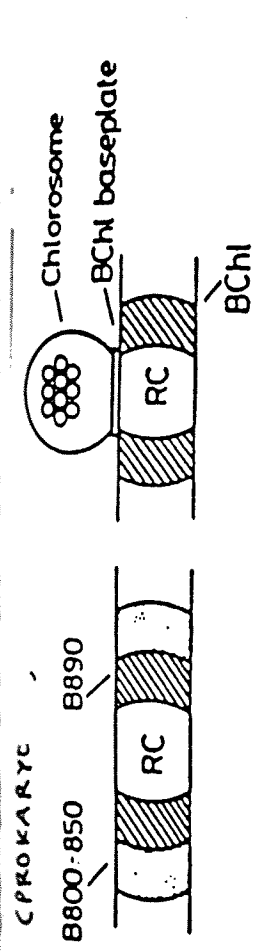


Figure 1B

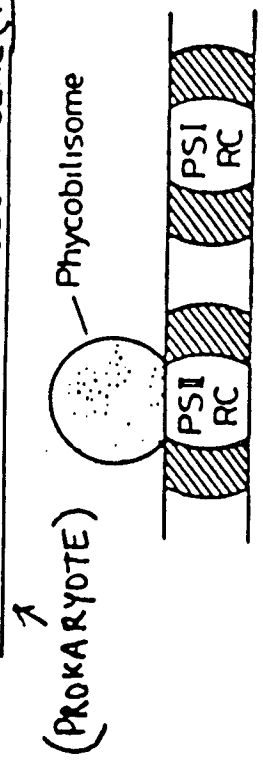
A comparison of various types of electron transport schemes found in photosynthetic membranes emphasising that anoxygenic photosynthetic bacteria possess either PSII- or PSI-type reaction centres and that all contain cytochrome *b/c* and ATPase/synthase complexes. The scheme shown for higher plants is also applicable for all oxygenic organisms including Cyanophyceae and eukaryotic algae. Q, quinone; PS, photosystem; e^- , electron; C, soluble cytochrome *c*; PC, plastocyanin; Fd, ferredoxin.

From Jim Barber's book

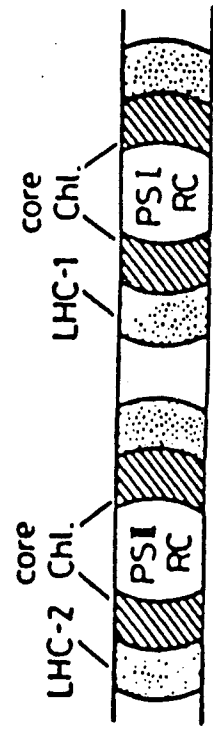
synthetic
PSI-type
scheme
ceae and
, plasto-



C. CYANOPHYCEAE AND RHODOPHYCEAE (RED ALGAE)
(EUKARYOTE)



D. HIGHER PLANTS AND ALGAE (EUKARYOTES)
GREEN



From Jim
Barber's book

Figure 2

A diagrammatic representation showing the relationship between various light-harvesting systems of oxygenic and anoxygenic photosynthetic organisms. In all cases there seems to be an inner light-harvesting 'core antenna' system tightly associated with the reaction centre (hatched). This core antenna system is serviced by a secondary light-harvesting system (dotted) which is more variable in amount, organisation and pigment type, being characterised by a particular class of photosynthetic organism and its growth conditions

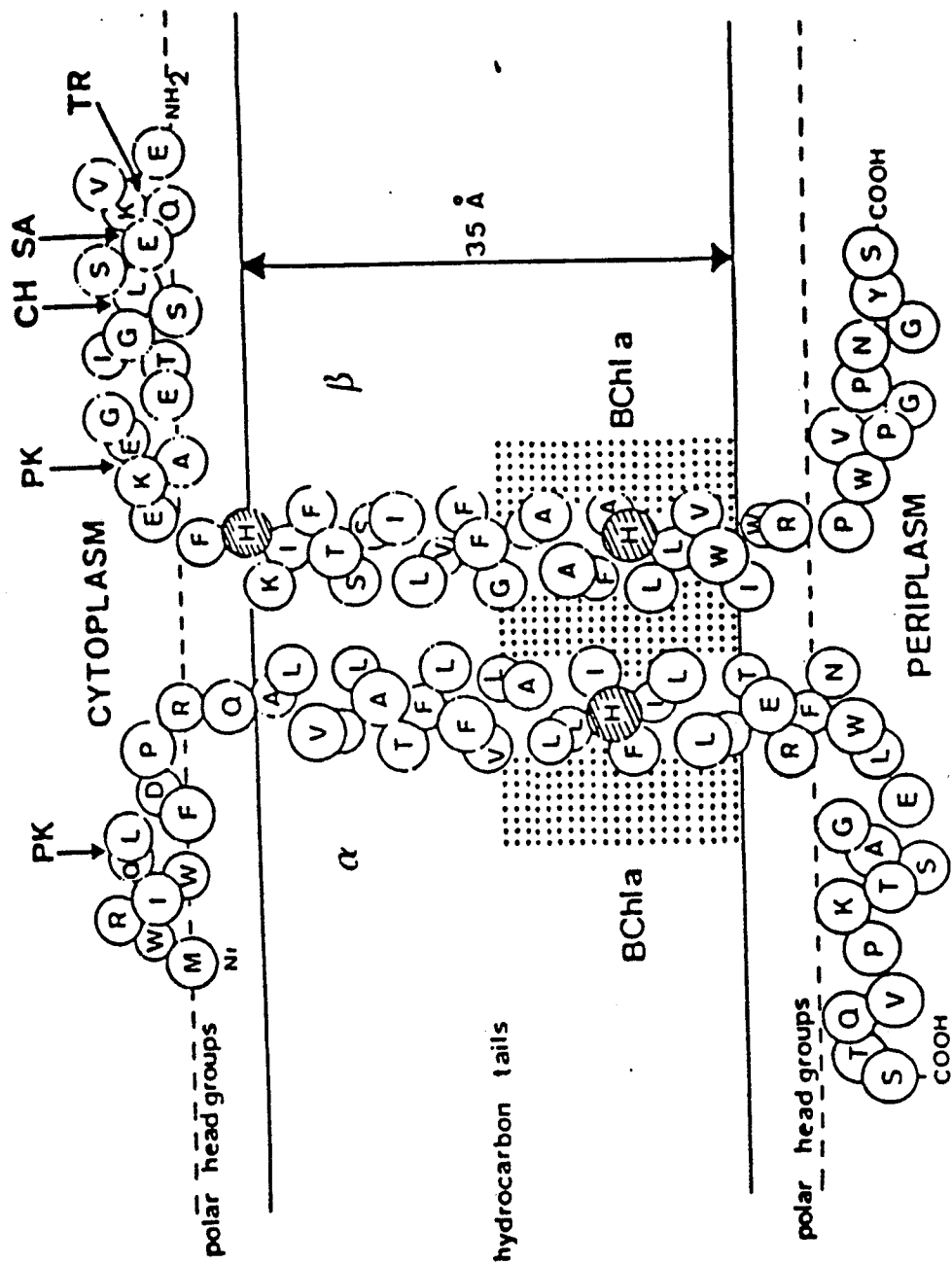


Figure 3

Transmembrane orientation of the α - and β -antenna polypeptides (R_s). Length of the α -helix in the hydrocarbon tail region is 35 Å. BChl a: binding sites of BChl a at the central His residues. The N- and C-terminal domains of the α - and β -polypeptides are located in the polar head region or at the membrane surface (cytoplasm, periplasm). In vesicles with the cytoplasmic site outside, the N-terminal domains of the α - and β -polypeptides are split off. PK, TR, CH and SA: splitting points with proteinase K, Trypsin, Chymotrypsin, Staph. proteinase.

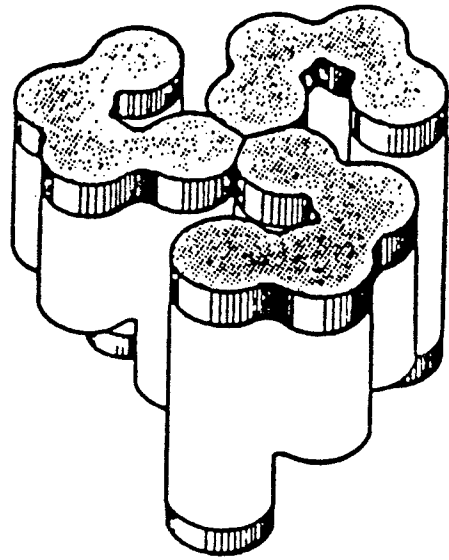
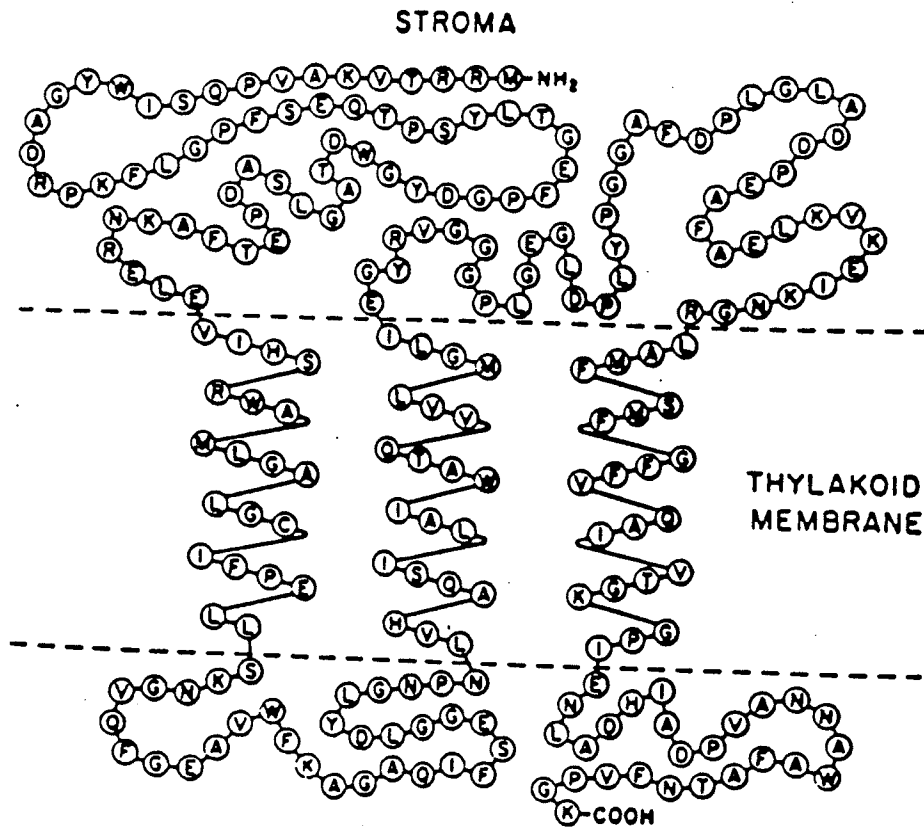


Figure 4

Schematic drawing of the three-dimensional structure of the LHCII complex as proposed by Kühlbrandt (1984). The 3-fold symmetry of the LHCII indicates that it is composed of three structurally equivalent subunits (trimer). The transverse diameter of the complex (thickness of the crystalline sheet, which has been investigated by electron microscopy and image analysis) is 59 Å. The shaded regions of the asymmetrical complex would presumably protrude by 10–15 Å into the stroma side of the 45 Å thylakoid membrane in vivo (functional role in grana stacking).



Model of the three-dimensional structure of the Chl *a/b* binding protein (or the LHCII complex) of *L. gibba* as proposed by Karlin-Neumann et al. (1985). Three segments (α -helices) span the thylakoid membrane. 48% of the polypeptide chain (including the N-terminal region) are located on the membrane surface at the stroma side, a smaller part of the polypeptide (including the C-terminal region) is located in the luminal membrane surface region.

split off. PK, TR, CH and SA: splitting points with proteinase K, Trypsin, Chymotrypsin, Staph. proteinase.

APPENDIX

Research of Robert Emerson and coworkers

1. *Robert Emerson* (Nov. 4, 1903 - Feb. 4, 1959)

E.I. Rabinowitch (1959). Robert Emerson 1903-1959. *Plant Physiol.* **34**:179-184.

E. Rabinowitch (1961). Robert Emerson 1903-1959. *Biographical Memoirs, National Acad Sci. USA.* **35**:112-131.

2. *Ph.D. Thesis*: R. Emerson (1927). Über die Wirkung von Blausäure, Schwefelwasserstoff und Kohlenoxyd auf die Atmung verschiedener Algen. Inaugural-Dissertation zur Erlangung Doktorwürde, Philosophischen Fakultät, Friedrich-Wilhelms-Universität, Berlin, 32 pages. (Sept. 25, 1925 - Oct 11, 1927) (under Otto Warburg). (25 references, out of which 10 were to Warburg and 3 to Dr. E. Negelein), (Referees: Prof. Dr. Kniep and Prof. Dr. Diels).

3. *Discovery of Photosynthetic Unit*: 102 Kirchhoff Lab. Cal. Tech: [10₂/2400 Chl; first use of repetitive flash method; limiting dark reaction, 10ms.]

R. Emerson and W. Arnold (1932a). A separation of the reactions in photosynthesis by means of intermittent light. *J. Gen. Physiol.* **15**:391-420.

R. Emerson and W. Arnold (1932b). The photochemical reaction in photosynthesis. *J. Gen. Physiol.* **16**:191-205.

[H. Gaffron and K. Wohl (1936) Zur Theorie der Assimilation. *Naturwissenschaften.* **24**:81-90, 873-890.]

[K. Wohl (1937). Zur Theorie der Assimilation. *Z Phys. Chem.B.* **37**:169-185.]

[J. Myers 1994). The 1932 experiments. *Photosynth. Res.* **40**:303-310.]

4. *The Minimum Quantum Requirement is 8 per O₂*: Carnegie Inst. Wash; Stanford, CA. [Discovery of CO₂ "gush": first explanation of Warburg's low quantum requirement data.]

R. Emerson and C.M. Lewis (1941). Carbondioxide exchange and the measurement of the quantum yield of photosynthesis. *Amer. J. Bot.* **28**:789-804.

W. Arnold (1949; experiments were done in 1936-1937). A Calorimetric determination of the quantum yield in photosynthesis. In: Franck, J. and Loomis W.E. (eds) *Photosynthesis in Plants*, pp 273-276. The Iowa State Press, Ames, Iowa.

R. Emerson (1958). The quantum yield of photosynthesis. *Annu. Rev. Plant Physiol.* **9**:1-24 (Emerson's latest views).

R. Govindjee, E. Rabinowitch and Govindjee (1968). Maximum quantum yield and action spectra of photosynthesis and fluorescence in *Chlorella*. *Biochim. Biophys. Acta* **162**:530-544.

5. *Discovery of the "Red Drop" (1943) and the Enhancement Effect (1956-1959): the concept of the two light reactions and two pigment systems: 155-157 Natural History Building, UIUC, Urbana, IL.*

R. Emerson and C.M. Lewis (1943). The dependence of the quantum yield of *Chlorella* photosynthesis on wavelength of light. *Am. J. Bot.* **30**:165-178.

R. Emerson, R. Chalmers, C. Cederstrand and M. Brody (1956). Effect of temperature on the longwave limit of photosynthesis. *Science* **123**:673. [A brief mention of results with red alga *Porphyridium* appears in this abstract.]

R. Emerson, R. Chalmers and Cederstrand (1957). Some factors influencing the long-wave limit of photosynthesis. *Proc. Natl. Acad. Sci. USA* **43**:133-143.

R. Emerson (1957). Dependence of yield of photosynthesis in long-wave red on wavelength and intensity of supplementary light. *Science* **125**:746. [A brief mention of results on *Chlorella* appears in this abstract.]

R. Emerson (1958) Yield of photosynthesis from simultaneous illumination with pairs of wavelengths. *Science* **127**:1059-1060. [This abstract clearly shows enhancement effect.]

R. Emerson and R.V. Chalmers (1958). Speculations concerning the function and phylogenetic significance of the accessory pigments of algae. *Phycol. Soc. Amer. News Bull.* **11**:51-56.

- * R. Emerson and E. Rabinowitch (1960). Red drop and role of auxilliary pigments in photosynthesis. *Plant Physiol.* **35**:477-485. [*:This contains action spectra of Enhancement effect obtained by Emerson, and shown by Emerson & Chalmers (1958-see above) at the meeting, but, not published there; Emerson was scheduled to present this work at the International Botanical Congress in Montreal in August, 1959; the paper was written by E. Rabinowitch after Emerson's death in February, 1959.

[B. Kok (1959) Light-induced absorption changes in photosynthetic organisms II. A split beam difference spectrophotometer. *Plant Physiol.* **34**:184-192 (see pp. 190-192, where the first antagonistic effect of red and far-red light on P700 was discovered and related to Emerson's discovery).]

Govindjee and E. Rabinowitch (1960). Two forms of Chlorophyll *a* *in vivo* with distinct photochemical function. *Science* **132**:355-356. [Discovery that Chl *a* is in both photosystems.]

Govindjee, S. Ichimura, C. Cederstrand and E. Rabinowitch (1960). Effect of combining far-red light with shorter wavelength on the excitation of fluorescence in *Chlorella*. *Arch. Biochem. Biophys.* **89**:322-323. [Discovery of two-light effect by fluorescence].

R. Govindjee, Govindjee and G. Hoch (1962). The Emerson enhancement effect in TPN-photoreduction by spinach chloroplasts. *Biochem. Biophys. Res. Commn.* **9**:222-225. [Discovery of Emerson enhancement effect in NADP reduction.]

Govindjee, O.H. Owens and G. Hoch (1963). A mass spectroscopic study of the Emerson enhancement effect. *Biochim. Biophys. Acta* **75**:281-284. [The effect is on photosynthesis not respiration in green alga *Chlorella*.]

[Duysens, L.N.M. (1989). The discovery of the two photosynthetic systems: a personal account. *Photosynth Res.* 21:61-79.]

Govindjee (1996). The role of chlorophyll *a* in oxygenic photosynthesis: two photosystems and two light reactions. Revision of *Scientific American* article by Rabinowitch and Govindjee, vol. 213, pp 74-83, 1965. (available from the author)

Discoveries of Eugene Rabinowitch and his collaborators: A Partial list

1. *E. Rabinowitch (April 27, 1901- May 15, 1973)*

Govindjee (updated) Eugene Rabinowitch: his life-primarily based on an interview with him on Jan. 5, 1964 at 1021 West Church, Champaign (mostly in his words). For circulation only as it is an unedited copy. 44 manuscript pages, with errors.

T.T. Bannister (1972). The careers and contributions of Eugene Rabinowitch. *Biophysic J.* 12:707-718. Govindjee (1972) Publications of Eugene Rabinowitch. *Biophysic. J.* 12:719-~~722~~

722. [This is an unusually short and rather abnormal list; complete list is available from the author.]

S.S. Brody (1995). We remember Eugene. *Photosynth. Res.* 43:67-74.

2. *Franck-Rabinowitch Effect, the Cage Effect:*

J. Franck and E. Rabinowitch (1930). Kinetics of photochemical reactions. "Chlorknallgas" reaction. *Z. Elektrochem* 36:794-

3. *First difference absorption spectroscopy measurement:*

E. Rabinowitch and H.L. Lehman (1935). Kinetics of recombination of bromine atoms. *Trans. Faraday Soc.* 32:547- . See Fig. 1 in E. Rabinowitch and W.C. Wood (1936) *J. Chem. Phys* 4:358-362.

4. *Discovery of Photogalvanic effect in thionine and methylene blue (storage of light energy by dyes):*

E. Rabinowitch (1940). The Photogalvanic effect I and II. The photochemical properties of the thionine-iron system. *J. Chem. Phys.* 8:551- , 8: 550- .
[This area was followed, markedly improved; and published extensively till 1970 with his many postdocs: S. Ichimura; S. Ainsworth; K.G. Mathai; D. Franckowiak, G. Singhal and V. Srinivasan]

*5. *First description of a series scheme (the "Z" scheme):*

E. Rabinowitch (1945). *Photosynthesis*, vol. I, see p. 162 (scheme 7.V)

E. Rabinowitch (1956). *Photosynthesis*, vol. II (Part II), see p. 1862 (discussion of role of cytochrome f).

6. *Description of the "Sieve effect" (the flattening effect in absorption spectroscopy of pigments in vivo) (also LNM Duysens):*

E. Rabinowitch (1951, 1956) see : *Photosynthesis*, vol. 2 (part 1), p. 714 and vol. 2 (part 2), p. 1865.

M. Das, E. Rabinowitch, L.Szalay and G. Papageorgiou (1967). The "Sieve" effect in *Chlorella* suspensions. *J. Phys. Chem.* 71:3543-3549.

7. *First study of crystals of chlorophylls:*

E.E. Jacobs, A. E. Vatter and A.S. Holt (1953). Crystalline chlorophyll and bacteriochlorophyll. *J. Chem Phys.* 21:2246-2247.

E.E. Jacobs, A.S. Holt, R. Kromhout and E. Rabinowitch (1957). Spectroscopic properties of crystals and monolayers of chlorophyll and related compounds. *Arch. Biochem. Biophys.* 72:495-511 (see also *J. Chem. Phys.* 20:1326 (1953); *Z. Phys.* 33:261 (1953); *J. Chem Phys.* 22:142 (1957).)

8. *First accurate measurement of quantum yield of fluorescence of pigments in vivo and in vitro.:*

P. Latimer, T.T. Bannister and E. Rabinowitch (1956). Quantum yields of fluorescence of plant pigments. *Science* 124:585-586.

9. *Discovery of selective scattering in algal cells:*

P. Latimer and E. Rabinowitch (1956). Selective scattering of light by pigment containing plant cells. *J. Chem. Phys.* 24:480.

10. *Discovery of absorbance changes at 705nm, and at 680nm--they missed the discovery of P700 and P680 although this was the intent of this work.*

J.W. Coleman, A.S. Holt and E.I. Rabinowitch (1955 but published in 1957) Reversible bleaching of chlorophyll *in vivo* paper presented at Gatlinburg conference, October 25-29, 1955; published in 1957 in *Research in Photosynthesis*, edited by H. Gaffron et al., Interscience, pp. 68-74. (also see *Science* 123:795, 1956.) At this conference, the future discoverer of P700 talked about transitory rates, kinetics of photosynthesis, and *photoinhibition*. Koř published his discovery of P700 in *Biochim. Biophys. Acta* 22:399-401 (1956); *Nature* 179:583-584 (1957); and *Acta Botan Neer.* 6:316-336 (1957). L. e. K

[Changes at 680nm turned out to be mainly due to fluorescence changes; and the smaller change at 705nm was not given much attention. J.W. Coleman did not succeed in finishing his Ph.D.]

11. *First measurements of lifetime of fluorescence of photosynthetic pigments (independent of the Russian work):*

S.S. Brody and E. Rabinowitch (1957). Excitation lifetime of photosynthetic pigments *in vitro* and *in vivo*. *Science* 125:555. (also see G. Tomita and E. Rabinowitch (1962) *Biophysic. J.* 2:483.) m /

12. *Discovery of a new emission band at 77K: F720*

S.S. Brody (1957). New Excited state of chlorophyll. *Science* 128:838-839.

13. *Two-light Reaction - Two Pigment System Work: 1960*

See *Discoveries of Emerson* for a 1960 paper with Emerson; and papers with Govindjee (Govindjee and R. Govindjee, students of Emerson, finished their Ph.D.s under Rabinowitch in 1960 and 1961, respectively)

Biology 251
Govindjee
1996 Version

The Role of Chlorophyll *a* in Oxygenic Photosynthesis: Two Photosystems and Two Light Reactions*

Original by Eugene I. Rabinowitch and Govindjee

Based on an article first published in:
Scientific American

July 1965

Vol. 213, No. 1 pp. 74-83

Revised by Govindjee (1996)

*This is an extensively revised version of the original article with a changed title; it is for personal use and cannot be reproduced without permission; not for sale.

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THE ROLE OF CHLOROPHYLL A IN PLANT AND CYANOBACTERIAL PHOTOSYNTHESIS

Any effort to understand the basis of life on this planet must always come back to oxygenic photosynthesis: the process that enables plants to grow by utilizing light, carbon dioxide (CO_2), water (H_2O) and a tiny amount of minerals. Oxygenic photosynthesis is the one large-scale process that converts simple, stable, inorganic compounds into the energy-rich combination of organic matter and oxygen and thereby makes abundant life on earth possible. Photosynthesis (both oxygenic and anoxygenic) is the source of almost all living matter on earth, and of almost all biological energy.

The overall reaction of oxygenic photosynthesis can be summarized in the following equation: $\text{CO}_2 + \text{H}_2\text{O} + \text{light} \rightarrow (\text{CH}_2\text{O}) + \text{O}_2 + 112,000 \text{ calories of energy per mole}$. (CH_2O) stands for a carbohydrate; for example, glucose: $(\text{CH}_2\text{O})_6$. "Mole" is short for "gram molecule": one gram multiplied by the molecular weight of the substances in question—in this case carbohydrate and oxygen.

When one of us first summarized the state of knowledge of photosynthesis in 1948, the whole process was still heavily shrouded in fog [see "Photosynthesis," by Eugene I. Rabinowitch; *Scientific American*, August, 1948]. Five years later investigation had penetrated the mists sufficiently to disclose some of the main features of the process [see "Progress in Photosynthesis," by Eugene I. Rabinowitch; *Scientific American*, November, 1953]. Since then much new knowledge has been accumulated; in particular the sequence of chemical steps that convert carbon dioxide into carbohydrate is now understood in considerable detail [see "The Path of Carbon in Photosynthesis," by J.A. Bassham; *Scientific American*, June, 1962]. The fog has also thinned out in other areas, and the day when the entire sequence of physical and chemical events in photosynthesis will be well understood seems much closer.

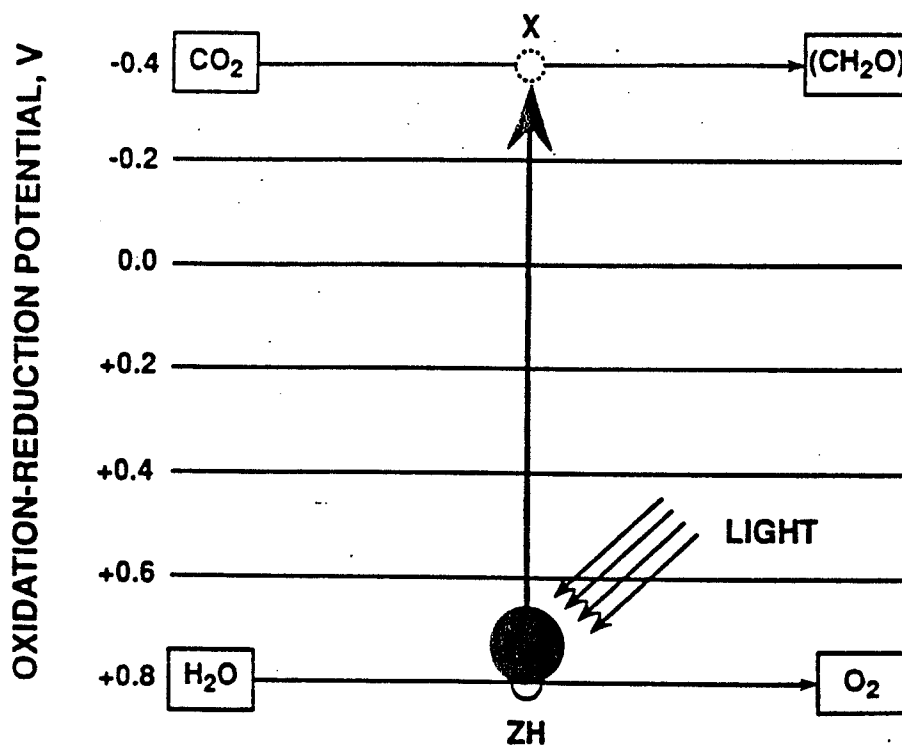
The oxygenic photosynthetic process apparently consists of three main stages: (1) the removal of electrons and protons from water and the production of oxygen molecules; (2) the transfer of electrons from an intermediate compound in the first stage to one in the third stage, and (3) the use of electrons (and protons) to convert carbon dioxide into a carbohydrate [see Figure 1]. Note that an electron plus a proton is a hydrogen atom.

The least understood of these three stages is the first: the removal of protons and electrons from water with the release of oxygen. It is known that it entails a series of steps requiring several polypeptides, chloride and calcium ions and manganese. (It has been summarized by Govindjee and W. Coleman in February 1990 issue of *Scientific American*.) The third stage—the production of carbohydrates from carbon dioxide—is the best understood, thanks largely to the work of Melvin Calvin and his co-workers at the University of California at Berkeley. (See his personal perspective: M. Calvin: Forty years of Photosynthesis and related activities, *Photosynthesis Research* 21 (1):3-16, 1989.) The subject of our article is the second stage: the transfer of

electrons from the first stage to the third. This is the energy-storing part of photosynthesis; in it, to use the words of Robert Mayer, a discoverer of the law of the conservation of energy, "the fleeting sun rays are fixed and skillfully stored for future use."

The light energy to be converted into chemical energy by plant and cyanobacterial photosynthesis is first taken up by plant pigments, including the green pigment chlorophyll *a*. Certain special chlorophyll *a* molecules (called reaction center chlorophyll *a*) function as photocatalysts: when they are in their energized state, which results from either the transfer of excitation energy to them or from the direct absorption of light, they catalyze an energy-storing chemical reaction. This reaction is the primary photochemical process; it is followed by a sequence of secondary "dark"—that is, nonphotochemical—reactions in which no further energy is stored. The photochemical events have been summarized by Govindjee and Rajni Govindjee ("The Primary Events of Photosynthesis", *Scientific American*, Dec., 1974; an updated and corrected version).

Figure 1. The tripartite scheme of plant and cyanobacterial photosynthesis. Three stages are (1) the removal of electrons and protons from water with the release of oxygen (bottom arrow); (2) the transfer (vertical arrow) of the electrons by energy from light trapped by the reaction center chlorophyll *a* (Chl *a*); and (3) the use of the hydrogen atoms (electrons and protons) to reduce carbon dioxide to carbohydrate (top arrow). In this scheme the oxidation reduction potentials involved (at pH 7) are indicated by the scale at the left, and the "primary reductant" and "primary oxidant" intermediates are designated as ZH and X, respectively. Their identity and nature has been changing since the original article in 1965 (see text here and the revised version of Govindjee and Govindjee, *Scientific American*, December, 1974).



Once it was thought that in plant and cyanobacterial photosynthesis the primary photochemical process is the decomposition of carbon dioxide into carbon and oxygen, followed by the combination of carbon and water. For quite some time it had been suggested that the energy of light serves primarily, although indirectly, to dissociate water, presumably into entities equivalent to hydroxyl radicals (OH) and hydrogen atoms; the hydroxyl radicals would then react to form oxygen molecules. It is better than either of these two formulations to say that the primary photochemical process in oxygenic photosynthesis is the boosting of electrons from a stable association with oxygen in water molecules to a much less stable one with carbon in organic matter. Although it does not happen in reality, the "oxygen atoms" "left behind" can be imagined to combine into oxygen molecules, an association also much less stable than the one between oxygen and hydrogen in water. *The replacement of stable bonds (between oxygen and hydrogen) by looser bonds (between oxygen and oxygen and between hydrogen and carbon) obviously requires a supply of energy, and it explains why energy is stored in oxygenic photosynthesis.*

The transfer of electrons or hydrogen atoms (equivalent to electrons and protons) from one molecule to another is called oxidation-reduction. The electron is transferred from a donor molecule (a "reductant") to an acceptor molecules (an "oxidant"); after the reaction the donor is said to be oxidized and the acceptor to be reduced. Usually, after the transfer of an electron occurs, it is followed by protonation: in an aqueous system (such as the interior of the living cell) there are always hydrogen ions (H^+), and if such an ion combines with the electron acceptor, the acquisition of an electron becomes equivalent to the acquisition of a hydrogen atom (electron + H^+ ion \rightarrow H atom).

The chain of oxidation-reduction reactions in photosynthesis has some links that involve electron transfers and others that involve hydrogen-atom transfers. For the sake of simplicity we shall speak of electron transfers, with the understanding that in some cases what may be transferred is a hydrogen atom. Indeed, the end result of the reactions undoubtedly is the transfer of hydrogen atoms.

In the oxidation-reduction reactions of oxygenic photosynthesis the electrons must be pumped "uphill"; that is why energy must be supplied to make the reaction go. The tiny chlorophyll *a*-containing chloroplasts of the photosynthesizing plant cell act as chemical pumps; they obtain the necessary power from the absorption of light by chlorophyll *a* (and to some extent from absorption by other pigments in the chloroplast *e.g.*, carotenoids and chlorophyll *b*). Cyanobacterial cells are prokaryotic and do not have organelle like chloroplast, but chlorophyll *a* is in the thylakoid membranes just as in plant cells. Other pigments, such as phycobilins, are in phycobilisomes attached to the thylakoid membranes. (See Figure 3 in the revised version of 1974 Scientific American article.) It is important to realize that the energy is stored in the two products organic matter and free oxygen and not in either of them separately. To release the energy by the combustion of the organic matter (or by respiration, which is slow, enzyme-catalyzed combustion) the two products must be brought together again.

How much energy is stored in the transfer of electrons from water to carbon dioxide, converting the carbon dioxide to carbohydrate and forming a proportionate amount of oxygen?

Oxidation-reduction energy can conveniently be measured in terms of electrochemical potential. Between a given donor of electrons and a given acceptor there is a certain difference of oxidation-reduction potentials. This difference depends not only on the nature of the two reacting substances but also on the nature of the products of the reaction; it is characteristic of the two oxidation-reduction "couples." For example, when oxygen is reduced to water (H_2O) its potential is about +0.8 volt at pH 7, but when it is reduced to hydrogen peroxide (H_2O_2) the potential is +0.27 volt. **The more positive the potential, the stronger is the oxidative power of the couple; the more negative the potential, the stronger is its reducing power.**

When two oxidation-reduction couples are brought together, the one containing the stronger oxidant tends to oxidize the one containing the stronger reductant. In oxygenic photosynthesis, however, a weak oxidant (CO_2) must oxidize a weak reductant (H_2O), producing a strong oxidant (O_2) and a strong reductant (a carbohydrate). *This calls for a massive investment of energy.* The specific amount needed is given by the difference between the oxidation-reduction potentials of the two couples involved in the reaction: oxygen-water and carbon-dioxide-carbohydrate. The oxygen-water potential is about +0.8 volt; the carbon-dioxide-carbohydrate potential, about -0.4 volt. The transfer of a single electron from water to carbon dioxide thus requires +0.8 minus -0.4 or 1.2 electron volts of energy. For a molecule of carbon dioxide to be reduced to (CH_2O)—the elementary molecular group of a carbohydrate — four electrons (or hydrogen atoms) must be transferred; hence the minimum total energy needed is 4.8 electron volts. This works out to 112,000 calories of energy per mole for each set of four electrons transferred.

We know the identity of the ultimate electron donor in plant and cyanobacterial photosynthesis (water) and of the ultimate electron acceptor (carbon dioxide), but what are the intermediates involved in the transfer of electrons from the first stage to the third? This has become the focal problem in recent studies of this photosynthetic process. It appears quite certain that a special chlorophyll a molecule donates electrons in the first stage and a pyridine nucleotide nicotinamide adenine dinucleotide phosphate, $NADP^+$, receives them in the third stage. The ZH, shown in figure 1, is currently labeled simply as Z; it is a tyrosine residue (#161) within a protein (D1) and is suggested to donate electrons to the oxidized chlorophyll a ($P680^+$). However, X, shown in figure 1, may represent an electron carrier (e.g. an iron-sulphur center F_x or FeS_x) that precedes $NADP^+$.

From the study of the mechanism of respiration we have been familiar with the important oxidation-reduction catalyst $NADP^+$ (formerly known as triphosphopyridine nucleotide, or TPN). $NADP^+/NADPH$ has an oxidation-reduction potential of about -0.32 volt at pH 7; thus, in itself it is not a strong enough reductant to provide the -0.4-electron-volt potential needed to reduce carbon dioxide to carbohydrate. $NADPH$, the reduced form of $NADP^+$, can achieve this feat, however, if it is supplied with additional energy in the form of the "high-energy" compound

adenosine triphosphate, or ATP. A molecule of ATP supplies about 10,000 calories per mole when it is hydrolyzed and its terminal phosphate group is split off, and this is enough to provide the needed boost to the reducing power of NADP⁺/NADPH system. Furthermore, we know that NADP⁺ is reduced when cell-free preparations of chloroplasts are illuminated. Put together, these two facts led to the now widely accepted result that the second stage of oxygenic photosynthesis manufactures both ATP and reduced NADP (NADPH) and feeds them into the third stage.

At first it was assumed that NADP⁺ is identical with X, the primary acceptor in our scheme. Subsequent experiments by various workers—notably Anthony San Pietro, then at Johns Hopkins University, and Daniel I. Arnon and his colleagues at the University of California at Berkeley—suggested, however, that NADP⁺ is preceded in the “bucket” “brigade” of electron transfer by ferredoxin, a protein that contains iron. This compound has an oxidation-reduction potential of about -0.42 volt; therefore if it is reduced in light it can bring about the reduction of NADP⁺ by a “dark” reaction requiring no additional energy supply.

Bessel Kok of the Research Institute for Advanced Studies in Baltimore had later found evidence suggesting that compound X may be a still stronger reductant, with a potential of about -0.6 volt. If this is so, plants may have in principle, the alternative of either applying this stronger reductant directly to the reduction of carbon dioxide or letting it reduce first ferredoxin and then NADP⁺ and using reduced NADP to reduce carbon dioxide. It seems a roundabout procedure to create a reductant sufficiently strong for the task at hand, then to sacrifice a part of its reducing power and finally to use ATP to compensate for the loss. It is not unknown, however, for nature to resort to devious ways in order to achieve its aims. It is now known (see later discussion) that there are two light reactions between Z and X: light reaction II takes electrons from Z to plastoquinone and light reaction I takes electrons from a copper protein plastocyanin to a special chlorophyll a molecule A₀. Between A₀ and ferredoxin are: a phylloquinone (A₁) and three iron sulfur centers F_X (FeS_X), F_A (FeS_A) and F_B (FeS_B). The reaction is completed by electron flow from plastoquinol to plastocyanin via a Rieske iron-sulphur protein and a cytochrome f (see later discussion).

For photosynthesis to be a self-contained process the required high-energy phosphate ATP must be itself manufactured by photosynthesis. The formation of ATP has in fact been detected in illuminated fragments of anoxygenic bacteria by Albert W. Frenkel of the University of Minnesota and in chloroplast fragments by Arnon and his co-workers [see *“The Role of Light in Photosynthesis,”* by Daniel I. Arnon: *Scientific American*, November, 1960]. As a matter of fact, ATP is needed not only to act as a booster in the reduction of an intermediate in the carbon cycle by reduced NADP but also for another step in the third stage of photosynthesis. According to a sequence of reactions worked out in 1951 by Andrew A. Benson, James Bassham and Melvin Calvin and their colleagues at the University of California at Berkeley, carbon dioxide enters photosynthesis by first reacting with a “carbon dioxide acceptor,” a special sugar phosphate called ribulose bisphosphate. It turns out that the production of this compound from its precursor—ribulose monophosphate—calls for a molecule of ATP.

ATP is produced in chloroplasts, cyanobacterial thylakoids and in mitochondria, the tiny intracellular bodies that are the site of the energy-liberating stage of respiration in animals as well as plants. The mitochondria produce ATP as their main function, exporting it as packaged energy for many life processes. The chloroplasts (see an electron micrograph of a chloroplast in the original Scientific American article of 1965 on p. 74), on the other hand, make ATP only as an auxiliary source of energy for certain internal purposes. The energy of the light falling on the chloroplasts is stored mostly as oxidation-reduction energy by the uphill transfer of electrons. Only a relatively small fraction is diverted to the formation of ATP, and this fraction too ultimately becomes part of the oxidation-reduction energy of the final products of photosynthesis: oxygen and carbohydrate.

Let us now consider the uphill transport of electrons in greater detail. Recent investigations have yielded considerable information about this stage. Apparently the pumping of the electrons, as briefly mentioned above, is a two-step affair, and among the most important intermediates in it, not yet mentioned, are the catalysts called cytochromes.

The idea of a two-step electron-transfer process grew from a consideration of the energy economy of oxygenic photosynthesis. Precise measurements, particularly those made by the late Robert Emerson and his co-workers at the University of Illinois, showed that the reduction of one molecule of carbon dioxide to carbohydrate, and the liberation of one molecule of oxygen, requires a minimum of eight quanta of light energy. The maximum quantum yield of plant and cyanobacterial photosynthesis, defined as the maximum number of oxygen molecules that can be released for each quantum of light absorbed by the plant cell, is thus 1/8, or 12 percent. Since the transfer of four electrons is involved in the reduction of one carbon dioxide molecule, it was suggested by one of us (E.R.) in 1945 that it takes two light quanta to move each electron. This scheme suggested that one light reaction takes electrons from ZH to an intermediate Y, and the oxidized Z reacts with water to evolve O₂. Another light reaction takes electrons from reduced Y to X, and the reduced X reduces CO₂ to (CH₂O) [see figure 2].

Emerson and his colleagues determined the maximum quantum yield of oxygenic photosynthesis (number of O₂ molecules evolved per quanta absorbed) in low intensity monochromatic light of different wavelengths throughout the visible spectrum. They found that the quantum yield, although it remained constant at about 12 percent in most of the spectrum, dropped sharply near the spectrum's far-red end [see Figure 3]. This decline in the quantum yield, called the "red drop," begins at a wavelength of 680 nanometers in green plants and at 650 nanometers in red algae.

There are two chlorophylls present in green plants: chlorophyll *a* and chlorophyll *b*. Only chlorophyll *a* absorbs light at wavelengths longer than 680 nanometers; the absorption of chlorophyll *b* rises to a peak at 650 nanometers and becomes negligible at about 680 nanometers. Emerson found that the quantum yield of oxygen evolution at the far-red end of the spectrum beyond 680 nanometers can be brought to the full efficiency of 12 percent by simultaneously

exposing the plant to a second beam of light with a wavelength of 650 nanometers. In other words, when light primarily absorbed by chlorophyll a was supplemented by light primarily absorbed by chlorophyll b, both beams gave rise to oxygen at the full rate. This relative excess in photosynthesis when a plant is exposed to two beams of light simultaneously, as compared with the yield produced by the same two beams separately, is known as the *Emerson effect*, or the *enhancement effect*.

The "red drop" can be more easily "grasped" by noticing the difference between the action spectrum of photosynthesis (rate of O₂ evolution per incident quanta plotted as a function of wavelength of light) and the absorption spectrum of the sample: the action spectrum falls at shorter wavelengths than the absorption spectrum. Similarly, the "Emerson effect" can be easily observed by measuring the rates of O₂ evolution in the far-red and shorter wavelength light beams given separately and together: the sum of the rates of O₂ evolution obtained with the two beams given separately is smaller than when the two beams are given together.

On the basis of his discovery Emerson concluded that photosynthesis involves two photochemical processes: one using energy supplied by chlorophyll a, the other using energy supplied by chlorophyll b or some other "accessory" pigment. Experimenting with various combinations of a constant far-red beam with beams of shorter wavelength, and using four different types of algae (green, red, blue-green* and brown), Emerson and coworkers found that the strongest enhancement always occurred when the second beam was absorbed mainly by the most important accessory pigment (the green pigment chlorophyll b in green cells, the red pigment phycoerythrin in red algae, the blue pigment phycocyanin in blue-green algae or the brownish pigment fucoxanthol in brown algae). Such results suggested to Emerson that these other pigments are not mere accessories of chlorophyll a but have an important function of their own in photosynthesis [see *Figure 4*].

Certain findings concerning the behavior of pigments in living plant cells, however, seemed to make this conclusion untenable. Illuminated plant cells fluoresce; that is, pigment molecules energized by the absorption of light quanta reemit some of the absorbed energy as fluorescent light. [For other details, see *Govindjee and R. Govindjee, Scientific American, Dec. 1974, and its updated version.*] The source of fluorescence can be identified, because each substance has its own characteristic fluorescence spectrum. The main fluorescing pigment in plants always proves to be chlorophyll a, even when the light is absorbed by another pigment. This has first been indicated for brown algae in a study conducted in 1943 by H.J. Dutton, W.H. Manning and B.B. Duggar at the University of Wisconsin; later the finding was placed on a firm basis and was extended to many other organisms, in 1952, by L.N.M. Duysens then of the University of Utrecht. Known as sensitized fluorescence, the phenomenon indicates that the initial absorber has transferred its energy of excitation to chlorophyll a; the transfer is effected by a kind of resonance process. Careful measurements have shown that certain accessory pigments—

*blue-green algae are actually cyanobacteria

chlorophyll *b*, phycoerythrin, phycocyanin and fucoxanthol—pass onto chlorophyll *a* between 80 and 100 percent of the light quanta they absorb. For some other accessory pigments—for example carotene—the transfer may be less efficient.

Figure 2. Plant and cyanobacterial photosynthesis with oxidation-reduction reactions between three intermediary catalysts (X, Y and Z) utilizing two primary photochemical reactions. One light reaction (bold lines) functions to take an electron (or hydrogen atom) from HZ and place it on Y; the oxidized product Z reacts with H₂O to evolve O₂ (right side). Another light reaction (bold lines) functions to take an electron (or hydrogen atom) from HY and place it on X; the reduced product HX reduces CO₂ to carbohydrate (CH₂O) (left side). The E's with subscript indicate that they are enzymatic reactions catalyzed by different enzymes (after E. Rabinowitch, 1945). In current language, electron transfer from HZ to Y involves photosystem II, and that from HY to X involves photosystem I. Now, Z could be equated with a specific tyrosine residue in photosystem II, Y with a cytochrome, and X with NADP⁺ or an equivalent intermediate (see text).

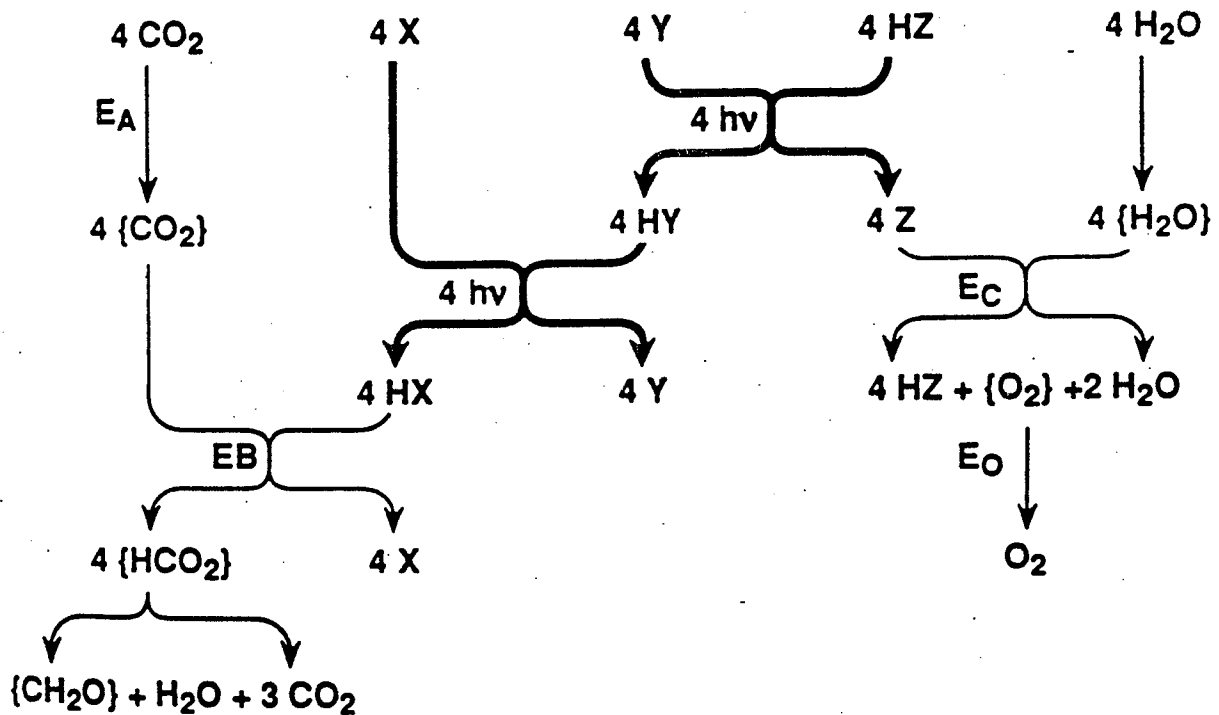
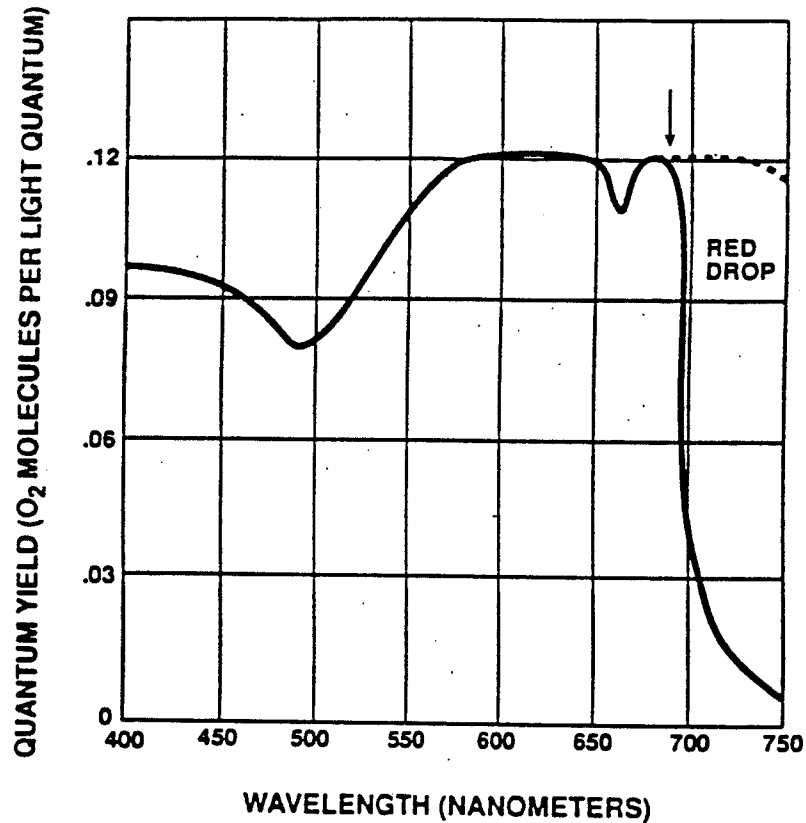


Figure 3. The quantum yield of oxygen evolution (O_2 molecules per absorbed quantum) as a function of wavelength of light showing the "Red Drop" beginning in the long wave region (at approximately 680 nanometers) in the green alga *Chlorella pyrenoidosa*. If plant photosynthesis was run by one pigment system and one light reaction and if absorption at all wavelengths was equally effective, the fractional absorption (0 to 1) spectrum would parallel the action spectrum (rate of O_2 evolution per incident quantum) and the quantum yield spectrum (rate of O_2 evolution per absorbed quantum = action spectrum divided by fractional absorption spectrum) would be a straight line without any dip or drop. The drop at wavelengths greater than 680 nm is now explained by the absorption of light preferentially in the so-called photosystem I, the long-wavelength system. Since plant photosynthesis is now shown to require two photosystems, absorption in only one system is not sufficient for efficient photosynthesis. However, the system can be restored to full efficiency in the "red drop" region if supplementary shorter wavelength light, absorbed in the so-called photosystem II, is added simultaneously (see dashed curve); this enhancement in the quantum yield is known as the *Emerson enhancement* effect after its discoverer Robert Emerson of the University of Illinois at Urbana. The "dip" in the quantum yield curve in the green region (500-550 nm) of the spectrum is due to the absorption by carotenoids that are either not in the chloroplast (but in the cell wall) or because of their relative inefficiency in transferring energy to chlorophyll; a small "dip" at 660 nm is due to imbalance in absorption by photosystems I and II. This illustration and the next one is based on the data of the late Robert Emerson and coworkers; the quantum yield of oxygen evolution data was published in 1943, and the enhancement effect data in 1957.



This puts accessory pigments back in the role of being mere adjuncts to chlorophyll *a*. True, they can contribute, by means of resonance transfer, excitation energy to photosynthesis, thereby improving the supply of energy in regions of the spectrum where chlorophyll *a* is a poor absorber. Chlorophyll *a*, however, collects all this energy before it is used in the primary photochemical process. Why, then, the enhancement effect? Why should chlorophyll *a* need, in order to give rise to full-rate photosynthesis, one "secondhand" quantum obtained by resonance transfer from another absorber in addition to the one quantum it had absorbed itself?

A better understanding of this paradox resulted from the discovery that there apparently exist in the cell not only chlorophyll *a* and chlorophyll *b* but also, at least, two forms of chlorophyll *a*. These two forms have different light-absorption and fluorescence characteristics, and they probably also lead to different photochemical functions. Duysens had suggested that two types of chlorophyll *a* exist: fluorescent and non-fluorescent, but, in the original work, the latter was considered inactive in photosynthesis.

In the living plant cell chlorophyll *a* absorbs light most strongly in a broad band with its peak between 670 and 680 nanometers. In our laboratory at the University of Illinois we (Govindjee and Rabinowitch) undertook, in 1959, to plot the Emerson enhancement effect more carefully than before as a function of the wavelength of the enhancing light. We found that for green algae and diatoms the resulting curve showed, in addition to peaks corresponding to strong absorption by the accessory pigments, a peak at 670 nanometers that must be due to chlorophyll *a* itself. This was particularly clear for the diatom *Navicula* [see Figure 5]. It was this finding that suggested that different forms of chlorophyll *a* may sensitize different light reactions. Although the Emerson effect spectrum measures a sort of difference between the action spectra of the two light reactions, we suggested that the form that absorbs light at the longer wavelengths—mainly above 680 nanometers—belonged mostly to one pigment system, called System I by Duysens *et al.* in 1961. The form that absorbs at 670 nanometers seemed to belong mostly to another pigment system: System II. In the second system the form of chlorophyll *a* that absorbs at 670 nanometers is strongly assisted by accessory pigments, probably by resonance transfer of their excitation energy. Careful analysis of the absorption band of chlorophyll *a* by C. Stacy French of the Carnegie Institution of Washington's Department of Plant Biology, and also in our laboratory, confirmed that the band is double, with one peak near 670 nanometers and another at 683 nanometers [see Figure 5].

If chlorophyll *a* is extracted from living plants, there is only one product¹; we must therefore assume that in the living cell the two forms differ in the way molecules of chlorophyll *a* are clumped together, or in the way they are associated with different chemical partners (*e.g.*, proteins). Be this as it may, the important implication of our finding, published in 1960, was that

¹There is, however, evidence in the literature that plants contain more than one chemical species of chlorophyll *a*; there are reports for the existence of a divinyl chlorophyll *a*; a chlorinated chlorophyll *a*; and an epimer called chlorophyll *a*'. A positive identification of any of these chemical species with any of the chlorophyll intermediates (P680, P700, and A₀) is not available.

photosynthesizing cells possess two light-absorbing systems, one containing a form of chlorophyll *a* absorbing at longer wavelengths and the other a form absorbing at shorter wavelengths. The latter system includes most of chlorophyll *b* (in green-plant cells) or other accessory pigments (in brown, red and blue-green algae). Further investigation—particularly of red algae—suggested, however, that the distribution of these two components in the two systems may be less clear-cut. In red algae a larger fraction of all the chlorophyll *a* seems to belong to System I rather than System II.

Figure 4. Emerson enhancement effect as a function of the wavelength of the supplementary light for the green alga *Chlorella pyrenoidosa* (top left), the cyanobacterium (or blue-green alga) *Anacystis nidulans* (top right), the red alga *Porphyridium cruentum* (bottom left) and the diatom *Navicula minima*. In each case the solid curve shows the action spectrum of the Emerson enhancement effect, plotted as a function of supplementary wavelength, calculated as a ratio:

$$\frac{RO_2(\lambda_1 + \lambda_2) - RO_2(\lambda_2)}{RO_2(\lambda_1)}$$

where $RO_2(\lambda_1 + \lambda_2)$ is the rate of O_2 evolution in both the far red (λ_1) and the supplementary (λ_2) beams given together, $RO_2(\lambda_2)$ the same in the supplementary beam alone and $RO_2(\lambda_1)$ in the far red beam alone. These curves were observed to be almost parallel to the fractional absorption (see dashed curves) by the various accessory pigments: chlorophyll *b* in *Chlorella*, phycocyanin in *Anacystis*, phycoerythrin in *Porphyridium* and fucoxanthol (peaking at ~ 525 nm) and chlorophyll *c* (about 635 nm) in *Navicula*. Data obtained by Robert Emerson and coworkers during 1957-1958 at the University of Illinois at Urbana.

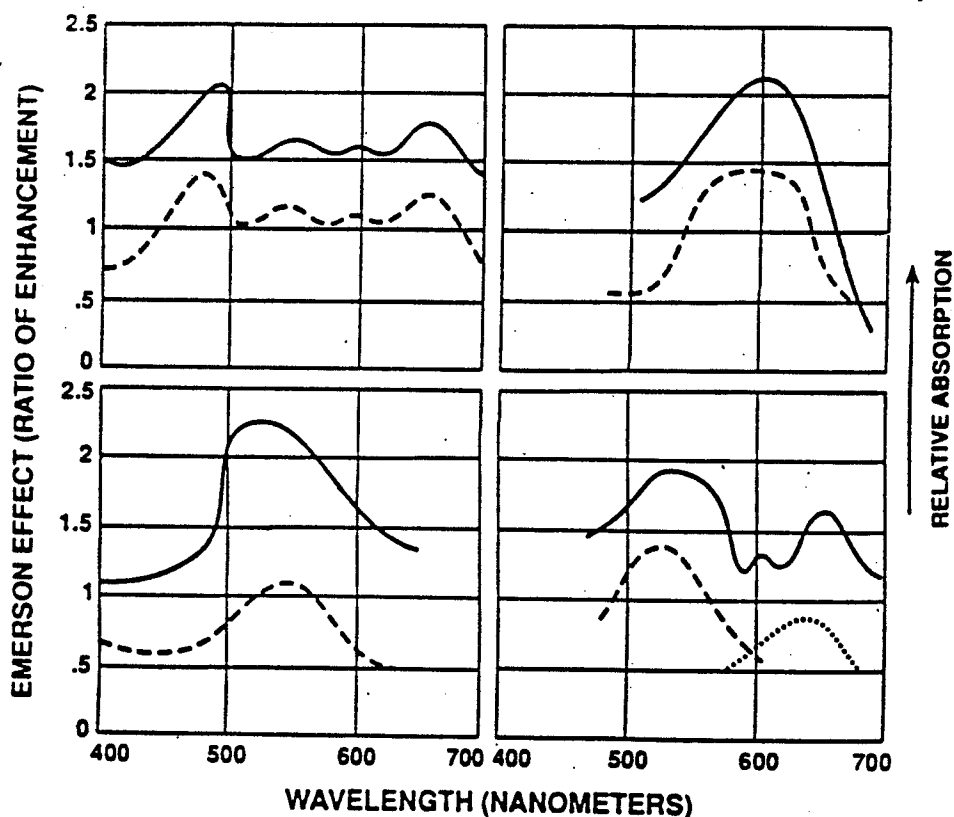
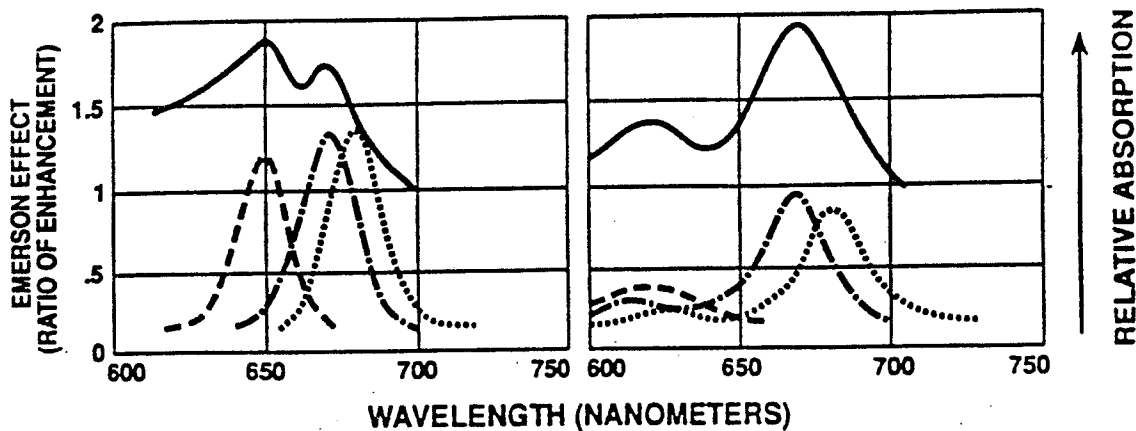


Figure 5. Detailed action spectra of the Emerson enhancement effect in the green alga *Chlorella* (left) and the diatom *Navicula* (solid curves). Also shown are fractional absorption spectra for chlorophyll *b* (dashed, left), chlorophyll *c* (dashed, right), a spectral form of chlorophyll *a*-*Chla* 670 (dots and dashes) and *Chla* 680 (dots). The Emerson effect peaks coincide not only with the absorption peaks of *Chlb* and *Chlc*, but also with *Chla* 670. These experiments by Govindjee and E. Rabinowitch (1960), particularly in *Navicula*, were the first ones to show that chlorophyll *a* belongs to both pigment systems of photosynthesis. This solved the dilemma of efficient energy transfer from the accessory pigments to chlorophyll *a* (L.N.M. Duysens, 1952) and of a proposed separate reaction by accessory pigments (Emerson and coworkers, 1957): one light reaction was preferentially sensitized by long wavelength spectral forms of chlorophyll *a* and the other was preferentially sensitized by accessory pigments and a shorter wavelength spectral form of chlorophyll *a*.



The two systems may provide energy for the two different photochemical reactions suggested earlier by E. Rabinowitch in 1945, and the high efficiency of photosynthesis requires that the rates of the two reactions be equal. What are these reactions? This question brings us to another significant finding, which suggested the participation of cytochromes in photosynthesis.

Cytochromes are proteins that carry an iron atom in an attached chemical group. They are found in all mitochondria, where they serve to catalyze the reactions of respiration. Robert Hill and his co-workers at the University of Cambridge first found that chloroplasts also contain cytochromes—two kinds of them. One, which they named cytochrome *f*, has a positive oxidation-reduction potential of about 0.4 volt. The other, which they named cytochrome *b₆*, has a potential of about 0 volt. On the basis of an experiment of Duysens in which light-induced oxidation of a cytochrome was studied, Rabinowitch, in 1956 (see p. 1862, Vol. 2.2), had sug-

gested that one light reaction may oxidize cytochrome and another light reaction may reduce it; this scheme was tantamount to the suggestion that "Y" in the 1945 scheme may be the cytochrome. In 1960 Hill, together with Fay Bendall, proposed an ingenious hypothesis as to how the two cytochromes might act as intermediate carriers of electrons and connect the two photochemical systems [see Figure 6]. They suggested that cytochrome b_6 receives an electron by a photochemical reaction from the electron donor ZH; the electron is then passed on to cytochrome f by a "downhill" reaction requiring no light energy. (The oxidation-reduction potential of cytochrome f is much more positive than that of cytochrome b_6 .) A second photochemical reaction moves the electron uphill again, from cytochrome f to the electron-acceptor X in the third stage of photosynthesis. In this sequence the photochemical reactions store energy and the reaction between the two cytochromes releases energy. Some of the released energy, however, can be salvaged by the formation of an ATP molecule; this type of reaction was known to occur in the transfer of electrons among cytochromes in respiration. In this way ATP is obtained without spending extra light quanta on its formation. Although the concept of the energy drop has been confirmed, the role of cytochrome b_6 as proposed by Hill has been proven to be *wrong*. Furthermore, our understanding of the mechanism by which ATP is synthesized has undergone tremendous changes.

Independent experiments by Duysens and his associates confirmed part of this hypothesis, by showing that the absorption of light by System I causes the oxidation of a cytochrome f , whereas the absorption of light by System II causes its reduction. This is exactly what we would expect and what was implied in 1956 by Rabinowitch. Figure 6 shows that the light reaction of System II should flood the intermediates between the two photochemical reactions with electrons taken from ZH; the light reaction of System I should drain these electrons away, sending them up to the acceptor X and into the third stage of photosynthesis. This is then the push-pull, or the antagonistic, effect of light I and II on the intersystem chain of electron carriers.

This, then, describes in a general way the oxidation-reduction process by which the chloroplasts store the energy of light in plant photosynthesis. Several other investigators have contributed evidence for the two-step mechanism; notable among them are French, Kok, Horst Witt of the Max-Volmer Institute in Berlin and their colleagues. Experiments of Kok, performed before the publication of the Hill and Bendall paper, deserve special mention. In cyanobacteria, far-red light oxidizes a special chlorophyll a molecule (P700, the reaction center of system I), whereas orange light, absorbed by phycocyanin reduces the oxidized P700. This antagonistic effect of two wavelengths of light is totally consistent with the series scheme, discussed above. In detail the process is much more complex than the scheme of Figure 6 suggests. Its "downhill" central part includes, as mentioned earlier, certain compounds of the group known as quinones, Rieske Fe-S center and also plastocyanin, a protein that contains copper, in addition to the cytochrome f [see figure 7]. This scheme is commonly known as the Z-scheme. For a description of the development of the concept of two light reactions and two photosystems, see the Personal Perspective of L.N.M. Duysens. (The discovery of the two photosynthetic systems: a personal account, *Photosynthesis Research*, 21 (2) 61-79, 1989.)

Figure 6. Electron (or hydrogen atom) transfer in photosynthesis as conceived in 1965 (see original Scientific American article, p. 80) as a two light reaction process involving two pigment systems. According to Robin Hill and Fay Bendall (1960) electrons from the donor ZH were boosted to cytochrome b_6 ; this reaction (light reaction II of L.N.M. Duysens et al., 1961) was sensitized by energy collected in pigment system II and trapped by, then, a hypothetical pigment Rabinowitch and Govindjee (1965) had named P680; the pigments of system II included accessory pigments and a short-wavelength form of chlorophyll a —Chla 670—as suggested by the data of Govindjee and Rabinowitch (1960). According to Hill and Bendall, electrons were passed downhill to cytochrome f , synthesizing adenosine triphosphate (ATP) in the process; this was the main contribution of Hill and Bendall. Rabinowitch had already stated in 1956, while explaining the earlier results of Duysens on cytochrome f oxidation, that one light reaction oxidized cytochrome f and another could reduce it bringing electrons from H_2O . Duysens and coworkers (1961), independently of Hill and Bendall scheme, had provided evidence for the antagonistic effect of light absorbed in System I and II on the redox state of cytochrome f in the red alga *Porphyridium*; this remains the key evidence for the series scheme shown here. An earlier experiment by Bessel Kok (1959) had shown an antagonistic effect of light I and II on the redox state of the reaction center pigment P700 of system I although the results were, then, not clearly interpreted in the scheme shown here. Energy from system I (primarily long wavelength form of chlorophyll a with some accessory pigments), trapped by P700, boosts electrons to an acceptor that was labeled X, whence they move via many intermediates (not shown) to ferredoxin (Fd) to nicotinamide adenine dinucleotide phosphate (NADP). Energy from NADPH and ATP is used to reduce phosphoglyceric acid (PGA) into sugars. The above scheme of 1965 has now been replaced by a new detailed scheme [see figure 7]. Three main points to note are:

- (1) Cytochrome b_6 *does not* occupy the position suggested by Hill and Bendall; it is used elsewhere in a sort of “side” pathway now called the “Q” cycle.
- (2) The redox potential of P680 is suggested to be close to +1.2 volts (at pH 7) and, thus, there is another energy drop between H_2O and P680 that could provide energy for another ATP formation. (As shown later in figure 8, ATP is not *directly* made during the two drops between H_2O and P680 and between cytochrome b_6 and cytochrome f , but, instead, by the proton gradients made available during water oxidation ($2H_2O = O_2 + 4H^+ + 4$ electrons) and during plastoquinol oxidation by the cytochrome b_6/f complex: $PQH_2 \rightarrow PQ + 2H^+ + 2$ electrons.)
- (3) The light reactions are excitation of P680 to P680* and of P700 to P700*; thus, the beginning and the end of the two vertical arrows in the scheme should be placed at the redox potentials of P680 (+1.2 V) and P700 (+0.4 V) and P680* (-0.6 V) and P700* (-1.2 V), respectively.

Figure 6.

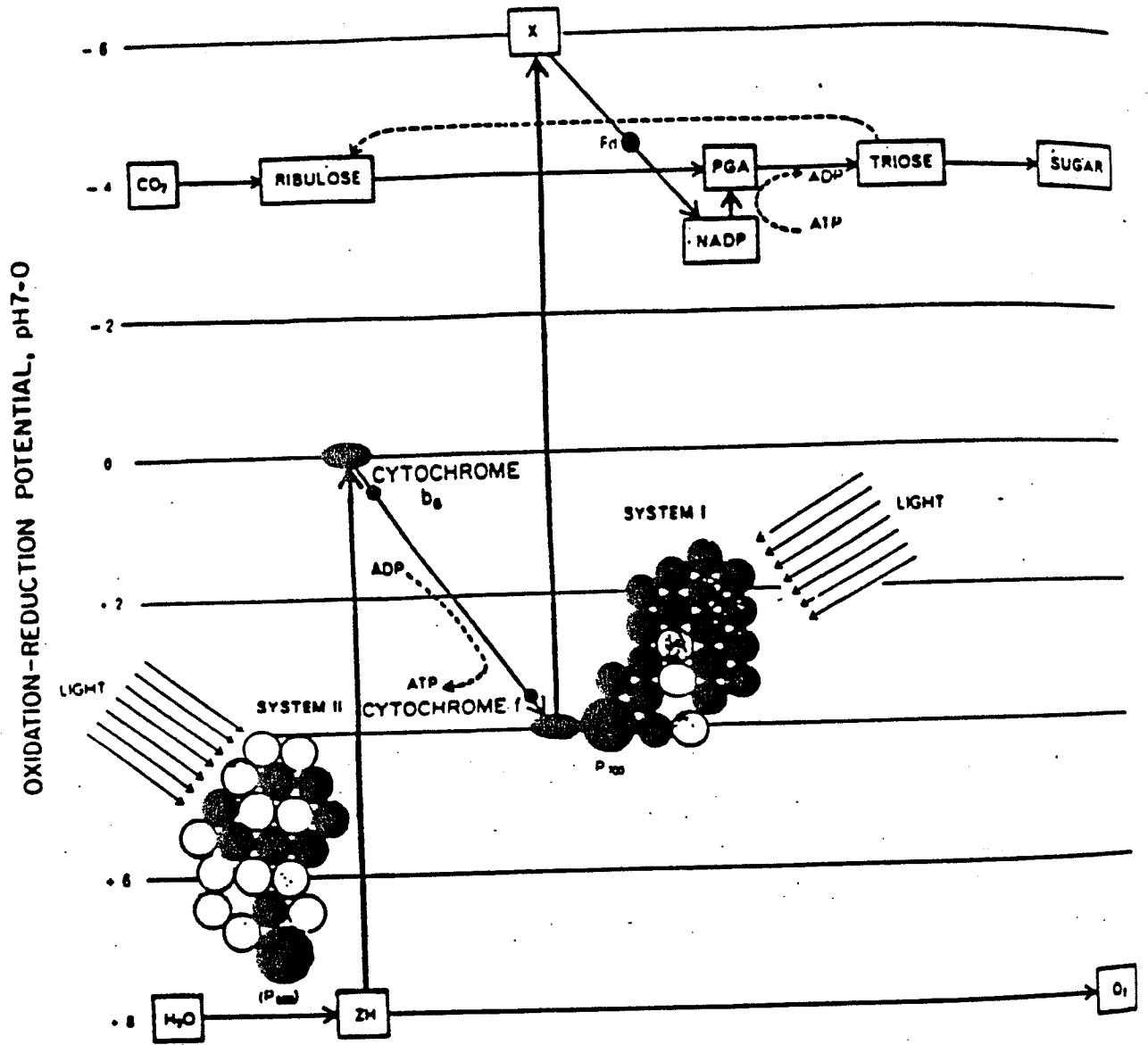


Figure 7. The Z scheme of electron transport in plant and cyanobacterial photosynthesis. Dashed rectangles: Three major multiprotein complexes (PSII, photosystem II; *cyt b/f*, cytochrome *b*₆/*f* complex; and PS I photosystem I), located in the thylakoid membrane, and containing the photosynthetic components required for electron flow from H₂O to NADP⁺.

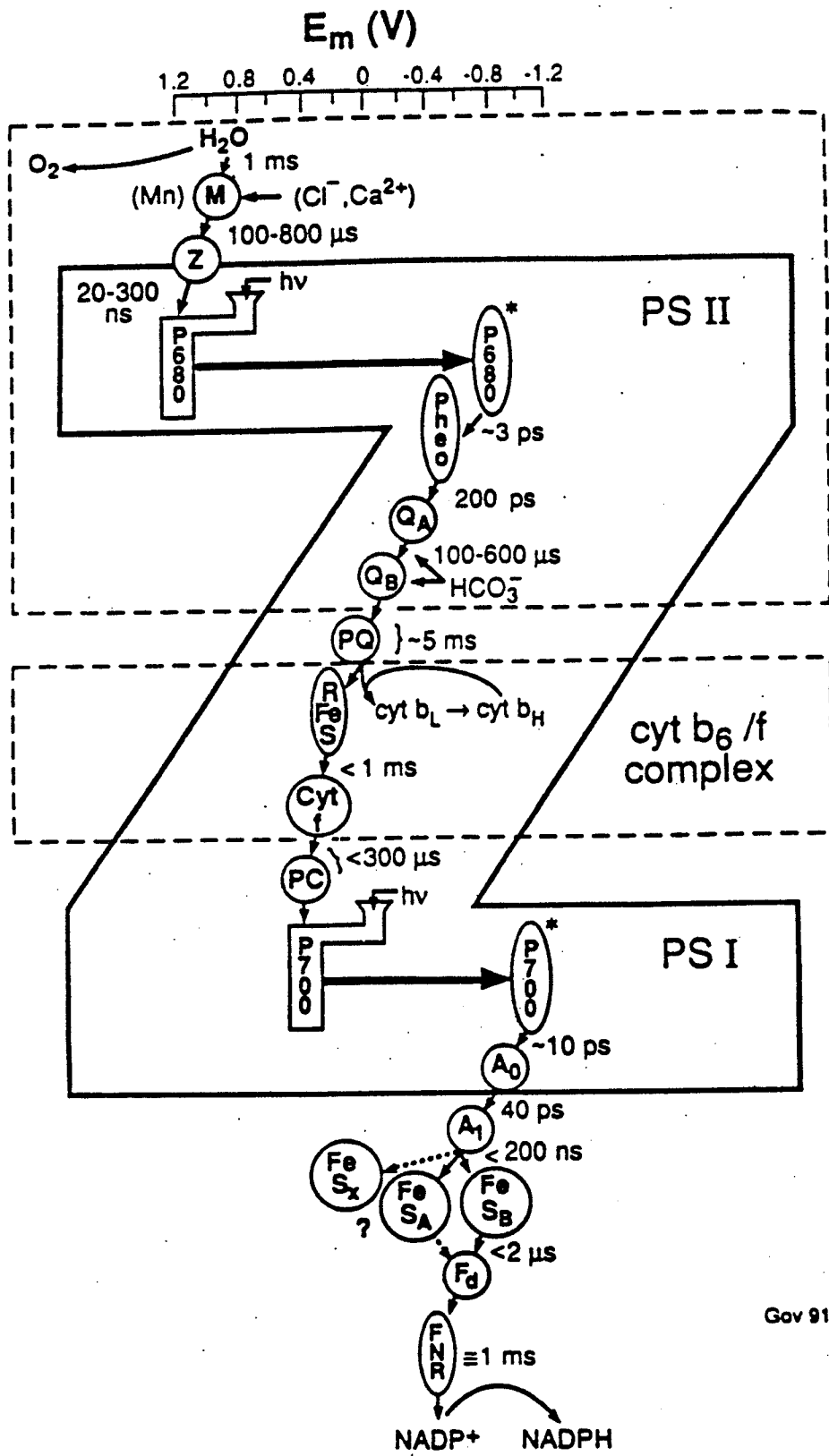
Primary reactions: The electron carriers are placed horizontally according to their midpoint redox potentials at pH 7.0 (Em.7). A 90° turn would clearly bring this scheme in the proper direction of energy (see Figure 1). Electron flow is initiated when a photon or exciton reaches the reaction center chlorophyll a P680 (in PSII) and P700 (in PS I) (see *hν* going into the funnel). P680* and P700* (see ovals) indicate the first singlet excited states of P680 and P700. The *first* reaction of P680* is the conversion of excitonic energy into chemical energy: charge separation, *i.e.*, the formation of the cation P680⁺ and anion pheophytin⁻ (Pheo⁻) within a few ps. The first reaction of P700*, the charge separation into P700⁺ A₀⁻, may also occur in a few ps. Here, A₀ is a special chlorophyll a molecule. The P680⁺ recovers its lost electron from Z, now thought to be tyrosine-161 of the D1 polypeptide of PS II.

Secondary reactions of PSII. The positive charge on Z is then transferred to the charge accumulator M, or the water oxidizing complex (WOC). It is suggested that M is nothing other than a Mn-cluster located on the luminal portions of the D1 and D2 polypeptides of PS II. The intrinsic binding site of Cl⁻ lies somewhere in this region. Water oxidation seems to require, for full efficiency, another polypeptide, an extrinsic 33 kDa polypeptide. Four positive charges must accumulate before an O₂ molecule is evolved. The Pheo⁻ delivers the extra electron to a bound plastoquinone electron acceptor, Q_A, located on the D2 polypeptide of PS II; Q_A delivers its electron to a second plastoquinone electron acceptor Q_B, located on the D1 polypeptide of PS II. Bicarbonate ions seem to be involved in the Q_A-Fe-Q_B region, where Fe is an iron atom (Fe²⁺) between Q_A and Q_B. After reduction to plastoquinol, *i.e.* after two turnovers of the reaction center P680, Q_B (H₂) exchanges with a mobile plastoquinone (PQ) molecule.

Reactions of the *cyt b/f* complex. Plastoquinol (PQH₂) delivers one electron to the Rieske Fe-S center (R-Fe-S), and the other to a cytochrome *b* (*b*_L). The electron on R-Fe-S reduces cytochrome *f* (*cyt. f*) and the one on *cyt. b*_L is transferred to cytochrome *b*_H (*cyt. b*_H), returning back in a cyclic process (called the Q-cycle).

Secondary reactions of PS I. Reduced *cyt. f* delivers its extra electron to a copper protein, plastocyanin (PC), which delivers the electron to P700⁺ (produced in the primary PS I reaction). On the other hand, A₀ passes its electron to A₁ (perhaps a phylloquinone). The rest of the electron carriers are: FeS_X (F_X) (an iron sulfur center X), FeS_B (F_B) (an iron sulfur center B), FeS_A (F_A) (an iron sulfur center A), Fd (ferredoxin) and FNR (ferredoxin-NADP⁺ reductase). The pathway may simply be: A₀ → A₁ → F_X → F_B, F_A → F_d → NADP⁺.

Reaction times. The diagram shows either measured or estimated times of the various reactions in the Z-scheme, except for the production of P680* and P700* that occur in femtosecond time scale. The bottleneck reaction is of the order of 5 ms and it involves the total time involved in the exchange of Q_B(H₂) with PQ, diffusion of PQH₂ to the *cyt. b/f* complex, and the reoxidation time of PQH₂. [The diagram does not show the steps involved in H⁺ uptake and release.] (After Govindjee and Coleman, 1993.)



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What is known of the submicroscopic structure in which the reactions of the second stage of photosynthesis take place? There is much evidence that the photosynthetic apparatus can be described as statistical "units" within the thylakoid membranes, each unit containing about 300 chlorophyll molecules. This picture first emerged from experiments conducted in 1932 by Emerson and William Arnold on photosynthesis of green algae during flashes of light; it was later supported by various other observations. The pigment molecules are close enough in the statistical unit that when one of them is excited by light it readily transfers its excitation to a neighbor by resonance. The energy goes on traveling through the unit, rather as the steel ball in a pinball machine bounces around among the pins and turns on one light after another. Eventually the migrating energy quantum arrives at the entrance to an enzymatic "conveyor belt," where it is trapped and utilized usually to load an electron on to the belt. (The steel-ball analogy should not be taken literally; the migration of energy is a quantum-mechanical phenomenon, and the quantum's location can only be defined in terms of probability; its entrapment depends on the probability of finding it at the entrance to the conveyor belt.) How is this statistical unit related to the physical components in the membrane is not quite understood. Four protein complexes (photosystem II; cytochrome *b/f* complex; photosystem I; and ATP synthase, also called CF_1 - CF_0 complex, where CF stands for coupling factor), located on the thylakoid membrane, are involved in the electron transport from H_2O to $NADP^+$ and for ATP synthesis [see figure 8]. In plants, both photosystems II and photosystem I are served by light-harvesting pigment-carrying complexes called light-harvesting complexes (LHC) II and I, respectively; the latter are composed of several polypeptides. LHC-II contains most of chlorophyll *b*, whereas chlorophyll *a* is present in both LHC I and LHC II. In addition to LHCs plants contain other chlorophyll *a* containing antenna labeled as CP43, CP47, etc.. The reaction center chlorophyll P680 is located in the photosystem II complex and P700 in the photosystem I complex. The light harvesting complexes (LHCs) of plants are replaced by phycobilisomes, containing phycobilins in cyanobacteria. These are attached to the thylakoid membranes and serve mostly, but not exclusively, photosystem II. It is these pigments—protein complexes—that must constitute the statistical units.

How is the quantum trapped? The trap may be a pigment molecule with what is called a lower excited state; the migrating quantum can stumble into such a molecule but cannot come out of it easily. In the current picture, this process may be reversible. The high efficiency is due to the tremendous numbers of visits to the trap and to the high efficiency of the photochemistry at the trap. Kok has found evidence that System I contains a small amount of a special form of chlorophyll *a* called pigment 700 because its red-most absorption has a maximum at a wavelength of 700 nanometers; this pigment could serve as a trap for the quantum bouncing around in System I. There seems to be a proper amount of pigment 700: about one molecule per unit. Furthermore, Kok's experiments showed, as already noted earlier, that pigment 700 is oxidized by light absorbed in System I and reduced by light absorbed in System II. It has an oxidation-reduction potential of about +0.4 volt. All these properties fit the role we have assigned pigment 700 in our scheme: collecting energy from a 300-molecule statistical unit in System I, using it to transfer an electron to the acceptor X and recovering the electron from cytochrome *f* via plastocyanin [see Figure 7].

We suspected in our original 1965 version of this article (*Scientific American* 213, 74-83, 1965) that there should be a counterpart of pigment 700 in System II, but then none had been convincingly demonstrated. We believed, however, that a pigment we had tentatively named pigment 680—from the anticipated position of its absorption band—did serve as an energy trap in System II. P680 was discovered in 1969 in the laboratory of H.T. Witt in West Berlin. In 1989, Mike Wasielewski, Mike Seibert and Govindjee showed that in stabilized isolated reaction center preparations of photosystem II, P680 is oxidized within a few picoseconds at 4° Celsius.

Figure 8. A schematic diagram of the four major protein complexes. From left to right: Photosystem II [OEC = oxygen evolving complex, P680 = reaction center chlorophyll a, Ph = functional pheophytin, Q_A = first plastoquinone electron acceptor (on D-2), Q_B = second plastoquinone electron acceptor, PQ (PQH₂) = mobile plastoquinone (plastoquinol)]; cytochrome b₆/f complex (cyt b₆ = cytochrome b₆, Cyt_f = cytochrome f, FeS = Rieske Fe-S center, PC = plastocyanin); Photosystem I (P700 = reaction center chlorophyll a, A₀ = chlorophyll a, first electron acceptor, FeS = iron sulfur centers, Fd = ferredoxin); and the ATP synthase (the membrane portion is called CF₀; and the hydrophilic portion is called CF₁). A diagram by Donald R. Ort (1988, personal communication) (see also Govindjee and Eaton-Rye, 1986).

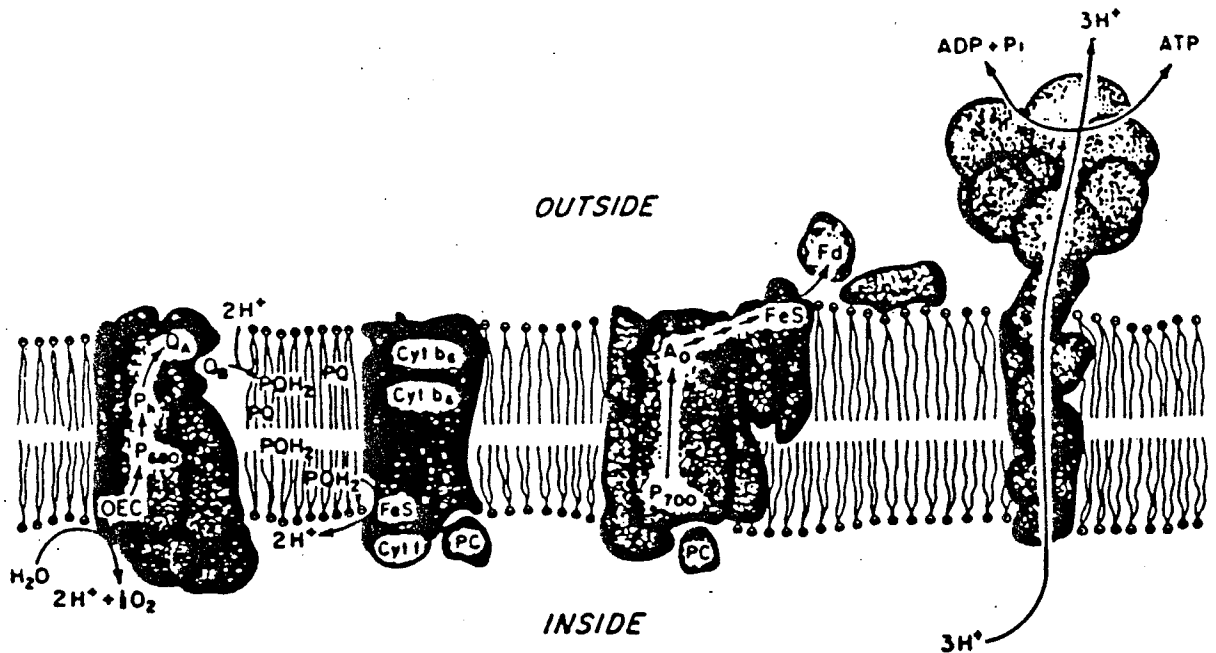


Figure 8.

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No doubt these pictures will change as more information emerges. It is merely an effort to penetrate the inner sanctum of plant and cyanobacterial photosynthesis, the photocatalytic laboratory in which the energy of sunlight is converted into the chemical energy of life ($\{CH_2O\}$ and O_2 from CO_2 and H_2O).

What is the spatial organization of the pigment systems in the electron-boosting mechanism of the second stage of photosynthesis? The light harvesting complexes of both photosystem I and photosystem II span the thylakoid membranes just as the P680 and P700-containing photosystem complexes do. The chloroplasts are known from electron microscope studies to consist of thin layers of membranes containing two layers of lipid molecules studded with proteins piled one atop the other. In higher plants, Photosystem II is located in regions where these membranes are appressed, *i.e.*, they form what is called grana (like stacks of coins), whereas photosystem I is located in regions where these membranes are unappressed, *i.e.*, they are located in what is called stroma lamellae. In many algae, there is no such distinction between grana and stroma lamellae. The units comprising Systems I and II may operate independently or they may be sufficiently close together to exchange energy by resonance, when such exchange is needed to maintain a balanced rate of operation by the two systems.

The picture of the energy-storing second stage of photosynthesis presented in this article is, of course, still only a working hypothesis. Alternative hypotheses are possible. Moreover, it is possible that under certain artificially created circumstances, one light reaction may be enough to reduce $NADP^+$ since photosystem II alone is capable of producing intermediates that have enough reducing power to reduce $NADP^+$. If that were the case and if photosystem I were used to undergo cyclic reactions only producing ATP, the minimum quantum requirement for O_2 evolution in photosynthesis maybe 6, not 8: 4 photons to evolve 1 O_2 molecule and reduce 2 $NADP^+$ to 2 NADPH, and 2 photons to produce enough ATP—needed to fix CO_2 to (CH_2O) . Such results have been claimed in the past, but they need to be rigorously established by fool-proof independent methods. A recent work by E. Greenbaum and coworkers has shown this possibility in a mutant of *chlamydomonas reinhardtii*.

No doubt these pictures will change as more information emerges. It is merely an effort to penetrate the inner sanctum of plant and cyanobacterial photosynthesis, the photocatalytic laboratory in which the energy of sunlight is converted into the chemical energy of life ($\{CH_2O\}$ and O_2 from CO_2 and H_2O).

Govindjee

June 26, 1996

The Primary Events of Plant (and Cyanobacterial) Photosynthesis*

by Govindjee and Rajni Govindjee

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THE PRIMARY EVENTS OF PLANT (AND CYANOBACTERIAL) PHOTOSYNTHESIS

When the sun shines on the leaves of a green plant, some of the radiant energy is utilized to promote chemical reactions, with the ultimate result that water and carbon dioxide are converted into oxygen and organic compounds. Plant (and cyanobacterial) photosynthesis, the oxygenic photosynthesis, has been summarized in this way since the end of the 18th century. The summary is essentially correct, but it describes the process only in terms of what flows into and out of the plant. Today a more detailed and more precise explanation is sought; we want to know what happens inside the illuminated leaf or a cyanobacterial cell. It is not sufficient to say that light "promotes chemical reactions." Rather, the molecular mechanism by which light is absorbed and by which its energy is utilized must be identified.

In this article we are concerned mainly with the first steps in plant and cyanobacterial photosynthesis; the absorption of light by a specific molecule and the transfer of that energy from one molecule to another, as in a bucket brigade, until it is eventually conveyed to those few molecules that participate in chemical reactions. These initial processes are called the "primary events" of photosynthesis. They are physical in nature, and they must be completed before the chemical activities of photosynthesis can begin. We shall be discussing mainly the oxygenic photosynthetic systems, the plant and cyanobacterial systems, not the systems that are anoxygenic (photosynthetic bacteria).

Electron Flow in Oxygenic Photosynthesis

Investigations of the primary events had been hindered in the past by the speed with which the events take place and by their complexity and inaccessibility; many could be observed only in the living cell. Most of the experiments intended to explore their sequence had by necessity been indirect. Many of them have been quite ingenious, however, and they have revealed several important characteristics of the system by which the energy of sunlight is made available to the photosynthetic machinery of the plant. Furthermore, recent achievements in the isolation of pigment-protein complexes and reaction centers and of the availability of femtosecond to picosecond laser flashes have allowed refined and detailed studies on the primary events of both oxygenic and anoxygenic photosynthesis.

The major organic products of plant photosynthesis are carbohydrates: substances, such as sugars and starches, whose composition is some multiple of the empirical formula (CH_2O). Because carbohydrates appear superficially to be compounds of carbon and water, it was thought for many years that oxygenic photosynthesis consisted in splitting carbon dioxide (CO_2), which would allow the oxygen to escape as a diatomic gas (O_2) and free the carbon to combine with water.

It is now known that this scheme is wrong. Neither carbon dioxide *nor* water can properly be said to be split or decomposed in photosynthesis. The net effect of the process is instead to transfer hydrogen atoms from water to carbon dioxide; the oxygen evolved comes from the H_2O , not the CO_2 . We note that hydrogen atom transfer may very well be (and is often) an electron transfer followed by uptake of protons (H^+). There are three major reasons to believe that O_2 comes from H_2O , not CO_2 : (1) When the released O_2 is measured, it is $^{18}\text{O}_2$ with H_2^{18}O , but not with C^{18}O_2 in the system; (2) although bicarbonate ions may affect the chloroplast or the Hill reaction ($\text{H}_2\text{O} + \text{ferricyanide} + \text{light} \rightarrow \text{O}_2 + \text{ferrocyanide}$), they have not been shown to affect the O_2 evolution process; and (3) photosynthetic bacteria, that do not evolve O_2 , have an equivalent process, e.g., $\text{H}_2\text{S} + \text{CO}_2 + \text{light} \rightarrow \text{S} + \{\text{CH}_2\text{O}\}$; thus, by analogy $2\text{H}_2\text{O}$ goes to O_2 .

Since the process takes place in water solution it is not necessary, as mentioned above, to actually move a complete hydrogen atom; if an electron is transferred, a hydrogen nucleus, or proton, can be drawn later from the aqueous medium to complete the atom. Chemical processes of this kind, in which electrons are transferred from one molecule to another, are called oxidation-reduction reactions. The molecule that has lost electrons is said to have been oxidized; the one that has received them is said to have been reduced. Thus in photosynthesis water is oxidized and carbon dioxide is reduced.

Ordinarily, of course, water does not (and cannot) reduce carbon dioxide, and it is not oxidized by it. For the reaction to proceed inside the plant cell, energy must be supplied. The energy requirement of an oxidation-reduction reaction is commonly measured in volts, and the electron transport involved in photosynthesis proceeds against an energy potential of about 1.2 volts.

An electrochemical gradient of 1.2 volts represents a rather large barrier, and there is reason to believe that in oxygenic photosynthetic organisms it is not overcome by a single quantum of light; it appears instead that two quanta are required for the transport of each electron. This hypothesis is supported by numerous recent experiments; moreover, it agrees well with an important observation made 50 years ago by the late Robert Emerson of the University of Illinois at Urbana-Champaign (Emerson was our first Ph.D. advisor): photosynthesis becomes inefficient at far-red light (the so-called "red drop" in photosynthesis). Emerson later (in 1956) found evidence that there are two pigment systems in plants (as well as in cyanobacteria) that preferentially absorb light of slightly different wavelength (or color), implying that electron transport takes place in two main stages and involves two photochemical events [see "The Role of Chlorophyll in Photosynthesis," by Eugene I. Rabinowitch and Govindjee; *Scientific American*, July, 1965].

Although most of the molecules associated with the two pigment systems (more generally, two photosystems) have been identified, all of their relations are yet to be unraveled. It is possible to draw a detailed map of the electron-transport pathway [see figure 1]. The scheme, known as the "Z" scheme, is based on the early (1945, 1956) ideas of the late Eugene Rabinowitch, also of the University of Illinois at Urbana-Champaign, a model proposed by the late Robert Hill and Fay Bendall (in 1960) of the University of Cambridge and experiments by dozens of international scientists [also see "The Mechanism of Photosynthesis," by R.P. Levine; *Scientific American*, December, 1969]. A better way to represent the components involved in the electron transport pathway is to show a model of the thylakoid membrane studded with the four major protein complexes: photosystem II (that transfers electrons from water to plastoquinone); cytochrome b/f complex (that transfers electrons from plastoquinol to a copper protein plastocyanin); photosystem I (that transfers electrons from plastocyanin to the pyridine nucleotide NADP⁺, nicotinamide adenine dinucleotide phosphate; and the ATP synthase (also known as the coupling factor "1", CF₁, and the coupling factor "O", CF₀, complex, both of which cooperate to produce ATP from ADP and inorganic phosphate, see figure 2).

In the current "Z" scheme, light striking the aggregation of molecules designated pigment system II results ultimately in the transfer of an electron from a donor called Z (now known to be a tyrosine residue) to an acceptor called Q_A, a bound plastoquinone molecule. (Note Q_A, not Q⁻_A, is a quencher of photosystem II chlorophyll *a* fluorescence.) The photosystem II complex, where this reaction occurs, is suggested to contain several polypeptides including a 32-kilodalton (kDa) D-1 protein; a 34 kDa D-2 protein; cytochrome b-559; and three extrinsic polypeptides having molecular masses of 17, 23 and 33 kDa, to be labeled here as Y-17, Y-23 and Y-33 in higher plants. In cyanobacteria, Y-17 and Y-23 are replaced by some other proteins. Z is suggested to be located on D-1, and Q_A on D-2. The electron transport from Z to Q_A involves several steps; (1) light energy produces the excited reaction center chlorophyll *a* molecule (P680*) from its unexcited state (P680), located between D-1 and D-2; (2) within a few picoseconds (1 picosecond (ps) = 10⁻¹² second), an electron from P680* is transferred to a pheophytin molecule producing an oxidized P680 (P680⁺) and a reduced pheophytin (Pheo⁻) as first shown in 1989 by Mike Wasielewski at the Argonne National Laboratory in collaboration with Govindjee

and Mike Seibert (*also see section: The Reaction Center*); (3) within 200 picoseconds, the reduced pheophytin transfers the electron to Q_A ; and (4) within 20-300 nanoseconds (1 nanosecond (ns) = 10^{-9} second) Z is oxidized and $P680^+$ is reduced, *i.e.*, electron is transferred from Z to $P680$.

Figure 1. The Z scheme of electron transport in photosynthesis. Dashed rectangles: Three major multiprotein complexes (PSII, photosystem II; cyt b/f, cytochrome b₆/f complex; and PS I photosystem I), located in the thylakoid membrane, and containing the photosynthetic components required for electron flow from H₂O to NADP⁺.

Primary reactions: The electron carriers are placed horizontally according to their midpoint redox potentials at pH 7.0 (Em, 7). (To appreciate, the energetics, turn the diagram 90° so that Em scale shows -1.2V at the top.) Electron flow is initiated when a photon or exciton reaches the reaction center chlorophyll a $P680$ (in PSII) and $P700$ (in PS I) (see hv going into the funnel). $P680^*$ and $P700^*$ (see ovals) indicate the first singlet excited states of $P680$ and $P700$. The first reaction of $P680^*$ is the conversion of excitonic energy into chemical energy: charge separation, *i.e.*, the formation of the cation $P680^+$ and anion pheophytin⁻ ($Pheo^-$) within a few ps. The first reaction of $P700^*$, the charge separation into $P700^+ A_0^-$, also within a few ps. Here, A_0 is a special chlorophyll a molecule. The $P680^+$ recovers its lost electron from Z, now thought to be tyrosine-161 of the D1 polypeptide of PS II.

Secondary reactions of PSII. The positive charge on Z^+ is then transferred to the charge accumulator M, or the water oxidizing complex (WOC). It is suggested that M is nothing other than a tetranuclear Mn-cluster located on the luminal portions of the D1 and D2 polypeptides of PS II. The *intrinsic* binding site of Cl^- lies somewhere in this region. Water oxidation seems to require, for its full efficiency, another polypeptide, an extrinsic 33 KDa polypeptide; this is, however, not an absolute requirement. Four positive charges must accumulate before an O₂ molecule is evolved. The $Pheo^-$ delivers the extra electron to a bound plastoquinone electron acceptor, Q_A , located on the D2 polypeptide of PS II; Q_A^- delivers its electron to a second plastoquinone electron acceptor Q_B , located on the D1 polypeptide of PS II. Bicarbonate ions seem to be involved in the Q_A -Fe- Q_B region, where Fe is an iron atom (Fe^{2+}) between Q_A and Q_B . After reduction to plastoquinol, *i.e.*, after two turnovers of the reaction center $P680$, Q_B (H_2) exchanges with a mobile plastoquinone (PQ) molecule.

Reactions of the cyt b₆/f complex. Plastoquinol (PQH_2) delivers one electron to the Rieske Fe-S center (R-Fe-S), and the other to a cytochrome b (b_L). The electron on R-Fe-S reduces cytochrome f (cyt. f) and the one on cyt. b_L is transferred to cytochrome b_H (cyt. b_H), returning back in a cyclic process (called the Q-cycle).

Secondary reactions of PS I. Reduced cyt. f delivers its extra electron to a copper protein, plastocyanin (PC), which delivers the electron to $P700^+$ (produced in the primary PS I reaction). On the other hand, A_0^- passes its electron to A_1 (perhaps a phylloquinone). The rest of the electron carriers are: FeS_X (F_X) (an iron sulfur center X), FeS_B (F_B) (an iron sulfur center B), FeS_A (F_A) (an iron sulfur center A), Fd (ferredoxin) and FNR (ferredoxin-NADP⁺ reductase). The pathway here may simply be $FeS_X \rightarrow FeS_B, FeS_A \rightarrow Fd, etc.$

Reaction times. The diagram shows either measured or estimated times of the various reactions in the Z-scheme, except for the production of $P680^*$ and $P700^*$ that occur in femtosecond time scale. The bottleneck reaction is of the order of 5 ms and it includes the total time involved in the exchange of $Q_B(H_2)$ with PQ, diffusion of PQH_2 to the cyt. b₆/f complex, and the reoxidation time of PQH_2 . [The diagram does not show the steps involved in H⁺ uptake and release.] (After Govindjee and Coleman, 1993.)

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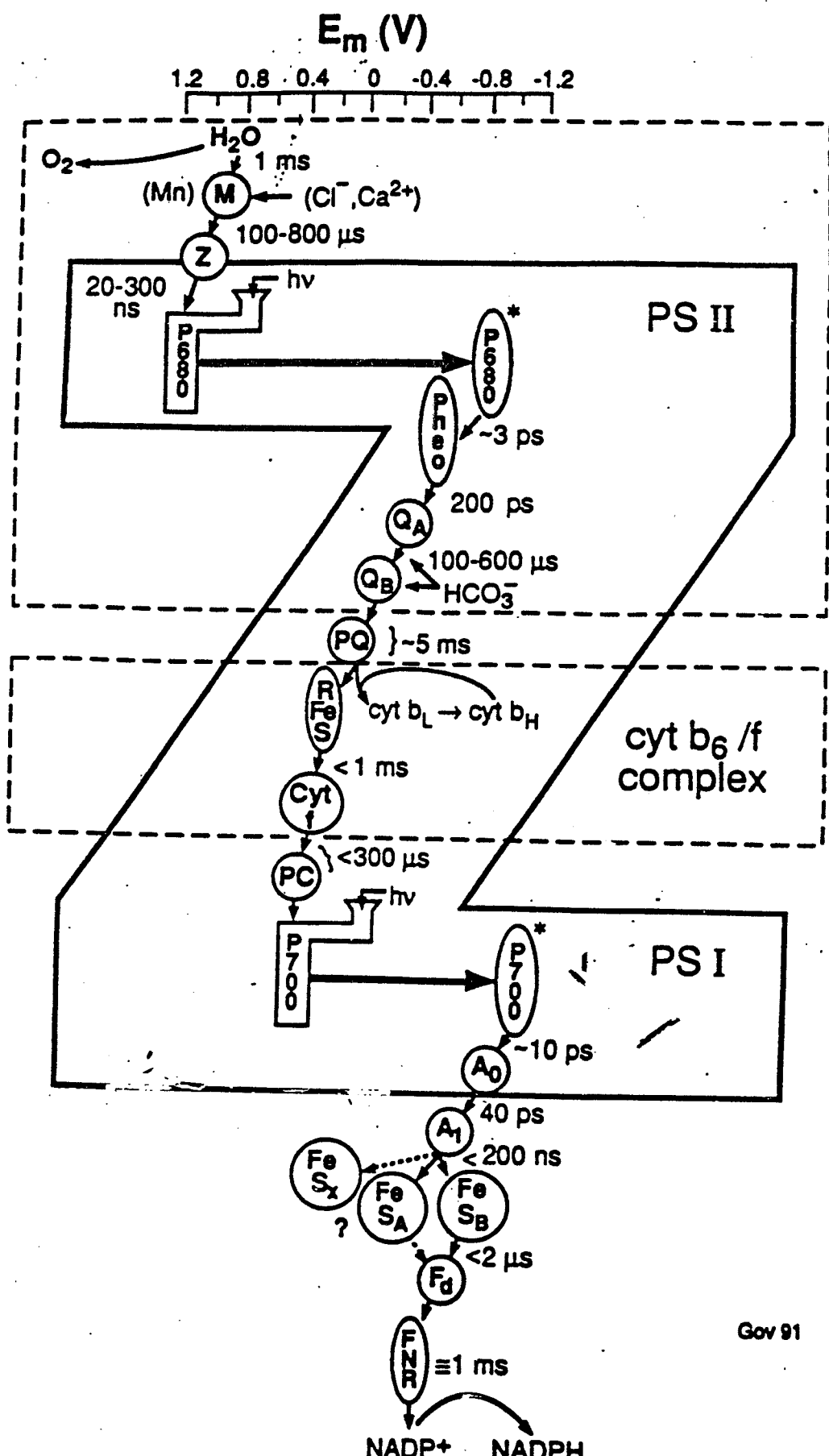
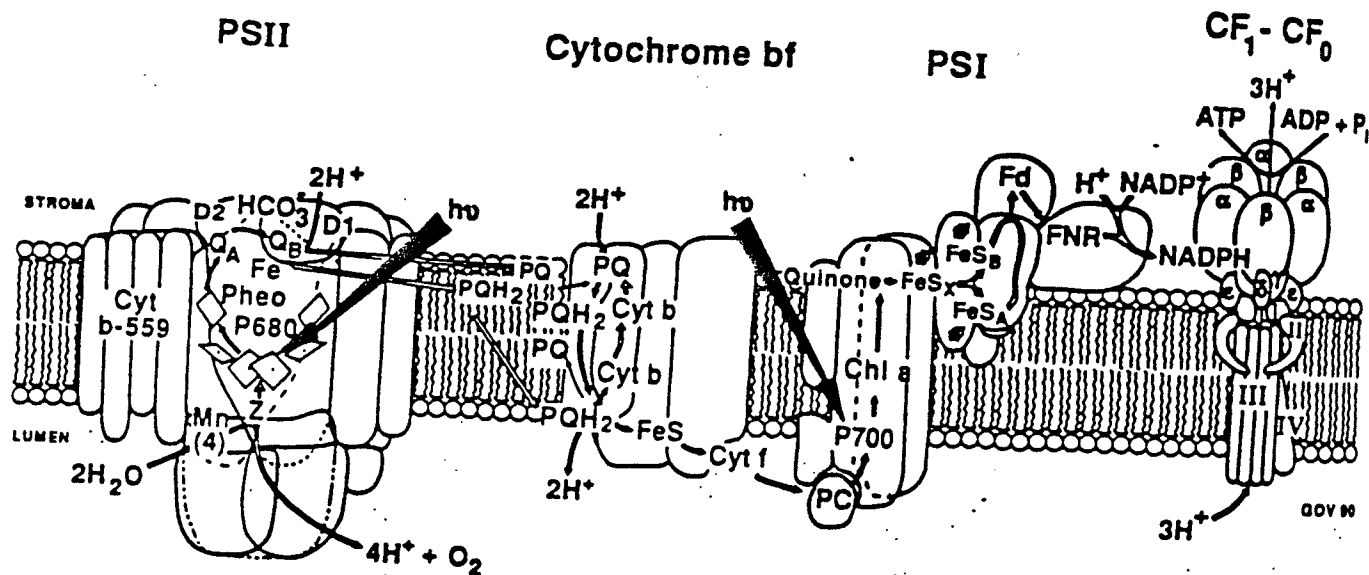


Figure 2. A working model for the arrangement of the 4 major protein complexes involved in electron transfer from H_2O to $NADP^+$ and in phosphorylation of ADP to ATP. Photosystem II (PSII) is in the appressed (grana) membrane; cytochrome b-f are found in both appressed and non-appressed (stroma) membranes; Photosystem I (PSI) and CF_1-CF_0 (coupling factor "1": sticking out as a door-knob and coupling factor "0": in hydrophobic or membrane portion that act as ATP synthase) are in the non-appressed membrane. All symbols have the same meaning as in the legend of Figure 1, α , β , γ , Δ , and ϵ are subunits of CF_1 and I, II, III, and IV are subunits of CF_0 ; D-1 and D-2 are proteins of the reaction center II complex. Protons are transported across the membrane during electron flow -- from stroma (the N-side) to lumen (the P-side) side. This proton gradient is dissipated through CF_1-CF_0 producing ATP on the stroma side. (Diagram by Govindjee and Coleman, based on a 1986 diagram by Govindjee and Eaton-Rye.)



Work on the kinetics of oxygen evolution by Pierre Joliot of the Institut de Biologie Physico-Chimique in Paris (France) and the late Bessel Kok of the Research Institute for Advanced Studies (Martin Marietta Company) in Baltimore has shown that a molecule of oxygen is evolved after the "oxygen evolving complex" M (that donates electrons to Z) has given up four electrons, and thus accumulated four positive charges; M is eventually restored to neutrality by scavenging four electrons from two water molecules. Although the nature of M is not fully clear, it definitely includes a manganese complex. The oxygen atoms from the water form an O₂ molecule and the four protons enter the solution on the lumen side as positive ions. The mechanism of water oxidation is the least understood step in the "Z" scheme. Several polypeptides (D-1, D-2, Y-17, Y-23 and Y-33) and several cofactors (manganese, chloride and calcium) are involved. As noted earlier, in cyanobacteria, Y-17 and Y-23 are, however, absent, and replaced by some other proteins. Several laboratories, including that of Barbara Zilinskas and of L. Sherman have obtained a mutant of a cyanobacterium that lacks Y-33, but it still produces some O₂. Thus, even Y-33 may not be an absolute requirement of O₂ evolution, as also mentioned earlier. Experiments suggest that manganese ions undergo oxidation-reduction reactions. This topic is the subject of another article by Govindjee and W. Coleman (also in Scientific-American, see February, 1990.)

From reduced Q_A the electrons are transferred, down the electrochemical gradient, to pigment system I. Several kinds of molecule serve as intermediaries, including at least another plastoquinone molecule Q_B located on D-1, mobile plastoquinone molecules, a Rieske iron sulphur protein, a cytochrome f (a type of protein that also serves as electron-carriers in cellular respiration), and a copper-containing protein, plastocyanin. The cytochrome b/f complex (figure 2) contains two types of cytochrome b and a 17kDa protein in addition to Rieske iron-sulphur protein and cytochrome f, mentioned here.

On absorbing a quantum of light, pigment system I promotes the transfer of an electron from plastocyanin to another acceptor molecule, an iron-sulphur protein labeled F_X or FeS_X (see figure 2 for the various polypeptides). The electron transport from plastocyanin to F_X (FeS_X) also involves several steps: (1) light energy produces the excited "reaction center" chlorophyll a molecule (P700*) from its unexcited state (P700); (2) within about 10 picoseconds, an electron from P700* is transferred to a chlorophyll a molecule (A₀) producing an oxidized P700 (P700⁺) and a reduced chlorophyll a molecule (A₀⁻), as shown in 1987 by Wasielewski in collaboration with James Fenton and Govindjee; this time may include the time of excitation energy transfer within the auxilliary chlorophyll a molecules that are not part of the "reaction center core" since reaction centers of photosystem I without the auxilliary pigments do not exist; recent estimates suggest that the charge separation time may be 3 ps; (3) within 40 to 200 picoseconds, the reduced A₀, A₀⁻, transfers its electron to A₁ (most likely, a phylloquinone); (4) the reduced A₁, A₁⁻, transfers its electron to the iron sulfur center F_X (FeS_X) within, perhaps, a nanosecond or so; and (5) within few microseconds P700⁺ recovers an electron from plastocyanin. The oxidized plastocyanin is then reduced by an electron that flows from reduced Q_A via the intermediates mentioned above. The reduced F_X (FeS_X) then donates the electron to the iron-sulfur-center F_A (FeS_A) or F_B (FeS_B), which transfers electron to ferredoxin, reducing it. The ferredoxin, with the help of the enzyme ferredoxin-NADP⁺ reductase (FNR), ultimately reduces NADP⁺, a molecule with many roles in metabolism. (In its reduced form it is abbreviated NADPH.) This sequence of events is repeated for each of the four electrons donated ultimately by H₂O, so that eight electron transfers, and eight quanta of light, are required for each molecule of oxygen evolved.

Energetically the transfer of electrons from reduced Q_A to the oxidized reaction-center molecule (P700⁺; figure 1) is a downhill process and is "coupled", through the proton and membrane potential gradient, with the production of adenosine triphosphate (ATP), the ubiquitous energy-carrying molecule, from adenosine diphosphate (ADP) and inorganic phosphate. According to the chemiosmotic hypothesis of the Nobel-laureate Peter Mitchell, ATP is synthesized by the membrane-bound ATP-synthase (figure 2) using the energy of the proton motive force; the



proton motive force includes the proton gradient and the membrane potential created by the electron transport process. The electron transport process occurs in the thylakoid membrane that has an "outside" and an "inside" (the lumen), *i.e.*, there is a vesicle. The charge separations in photosystems I and II lead to vectorial transport of electrons across the membrane from P700 to F_X and from P680 to Q_A , creating a membrane potential. Since reduction of plastoquinone pool by Q_A^- occurs near the "outerside" of the membrane, and since plastoquinol formation requires protons, protons are picked up from the outside (figure 2). And, plastoquinol reduces the electron carrier Rieske iron-sulfur center in the cytochrome b/f complex on the "innerside" releasing protons into the lumen. This, then, creates the proton gradient across the membrane. Furthermore, as mentioned earlier, the oxidation of water by the oxidized "M" also leads to the release of protons into the lumen: $e.g., 2 H_2O + 4 M^{4+} \rightarrow O_2 + 4 H^+ + 4 M^{3+}$.

The existence of a cyclic flow of electrons around photosystem I has been suggested, but not yet proven in normal healthy plants. It is highly likely that it occurs under stress conditions. The electrons from reduced F_X , instead of reducing $NADP^+$, could cycle back to the reaction center P700; when this cyclic flow goes via plastoquinone it could be coupled with the production of ATP. (Another "site" where the downhill process occurs is between water and the oxidized reaction-center molecule P680⁺, see figure 1. This is coupled to ATP synthesis in the same fashion, *i.e.*, by the formation of proton gradient, as that between Q_A and P700.) The importance of cyclic electron flow may vary from plant to plant, but its contribution to photosynthesis in the so-called C-4 plants¹

may be significant. It is ATP and NADPH that are required for the reduction of carbon dioxide and they mediate its introduction into a carbohydrate cycle [see "*The Path of Carbon in Photosynthesis*," by J.A. Bassham; *Scientific American*, June, 1962].

Pigment Molecules

The photosynthetic apparatus of the higher plants is organized inside chloroplasts, the cellular organelles that give plants their characteristic green color. (See p. 71 in the original *Scientific American* article for a photograph of green chloroplasts.) The chloroplasts are complex structures, separated from the cytoplasm of the cell by a membrane and apparently having some autonomy; each has a bit of the genetic material DNA and is able to synthesize some of the important proteins it requires independent of the cell nucleus.

Inside the chloroplast is an elaborately folded network of membranes termed lamellae. In chloroplasts from most higher plants the lamellae form structures called grana (having appressed membranes), which are separated by a material known as stroma matrix, but connected by unappressed membranes, the stroma lamellae or the frets. The grana appear to be dense stacks of membranous sacs. (See p. 71 in the original *Scientific American* article for electron micrographs of chloroplasts and lamellae.) These sacs, called thylakoids, contain lipids, proteins and pigments. It is in the thylakoid membrane that the business of the electron transport in photosynthesis is transacted; carbon fixation occurs in the stroma matrix. Most photosystem II complexes are located in the appressed regions of the grana and most photosystem I and the ATP-synthase complexes are in the unappressed regions (the stroma lamellae; and the "ends" of the grana).

¹There are two types of plants: C-3 (example: spinach) and C-4 (example: maize). In C-3 plants, CO_2 is added to 5-carbon sugar ribulose biphosphate to form two molecules of 3-carbon containing organic acid phosphoglyceric acid. However, in C-4 plants, CO_2 is added to a 3-carbon acid phosphoenolpyruvic acid to form 4-carbon containing acids malic and aspartic via the 4-carbon oxaloacetic acid. C-4 pathways require more ATP molecules than the C-3 pathways and, thus, it is suggested that in these plants extra ATP may come from the operation of "cyclic" electron flow, mentioned in the main text.

A further level of statistical organization can be inferred in the chloroplast, even if it cannot be physically resolved. Experiments performed in 1932 by the late Robert Emerson and William A. Arnold, who now lives in Tennessee, suggested that a minimum of 2,400 chlorophyll molecules are required to evolve one molecule of oxygen. An aggregate of this size could be considered the statistical photosynthetic unit. We now know that eight photochemically driven electron transfers are required to generate one molecule of oxygen, so that a smaller statistical unit is plausible, one containing 300 pigment molecules per photoact. This is the new statistical photosynthetic unit, the smallest unit capable of photochemical action. The relationship of this functional unit to the actual pigment-protein complexes, isolated from thylakoids, remains to be resolved.

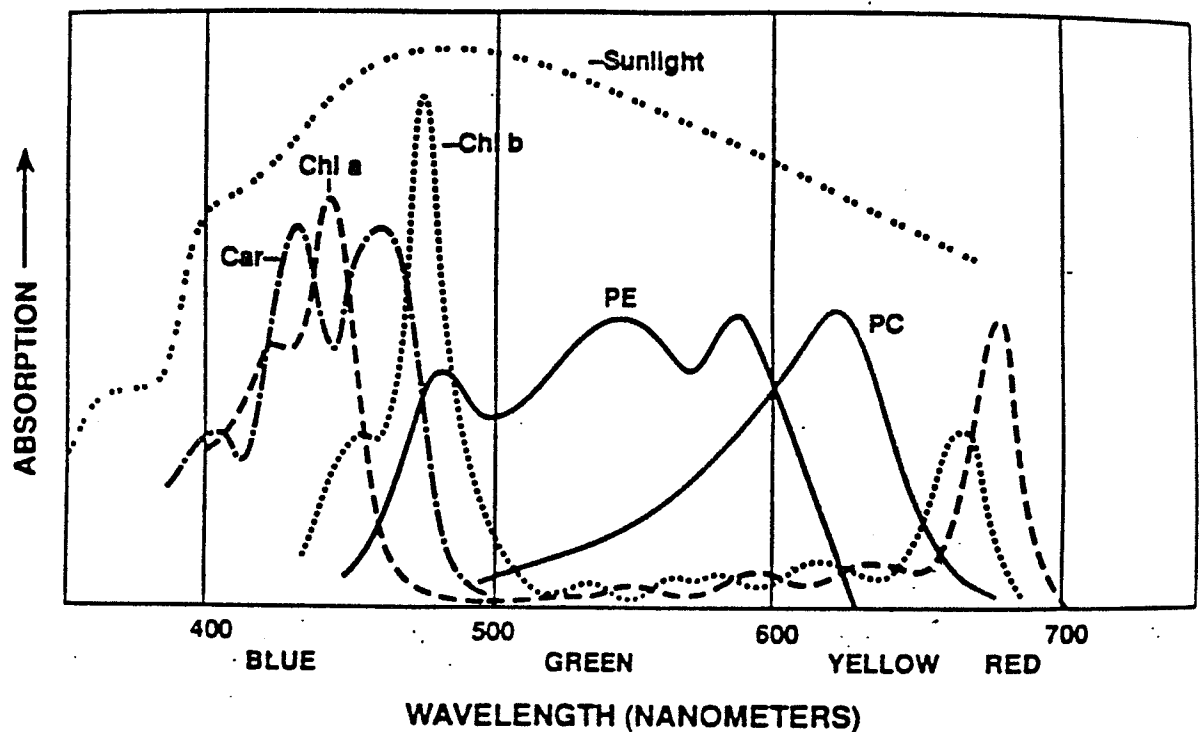
Embedded in the thylakoid membranes are the pigment molecules that initiate the process of photosynthesis. In vivo, they are associated with specific proteins. Pigments are substances that by definition strongly absorb visible light. Most absorb only in certain regions of the spectrum and transmit light of all other wavelengths; as a consequence they appear colored. For example, chlorophyll, the most important plant pigment, absorbs both the longer and the shorter waves in the visible spectrum: red and orange and blue and violet. The transmitted wavelengths, chiefly the yellow and green in the middle of the visible spectrum, combine to yield the green of grass and trees [see figure 3].

The majority of organic molecules absorb most strongly in the ultraviolet; the various pigments of the chloroplast absorb at the longer wavelengths of visible light because they have chains or rings of carbon atoms connected by "conjugated," or alternating, single and double bonds. In chlorophyll the system of conjugated bonds is located in a ring, and it encompasses nitrogen atoms as well as carbon atoms [see figure 4]. The ring structure is one of the class of compounds called porphyrins, which are found widely in both plants and animals. In chlorophyll the central cavity of the ring is occupied by a magnesium atom; the function of the magnesium is not understood. In pheophytins, magnesium is replaced by hydrogen atoms. Porphyrin rings in the blood protein hemoglobin and in the cytochromes contain an atom of iron instead of magnesium.

Attached to the porphyrin ring in chlorophyll (a and b) is a long hydrocarbon "tail," the phytol chain. It consists of carbon atoms linked together, but only one of the bonds is double; for this reason the phytol chain does not appear to play an important part in determining the chlorophyll absorption spectrum. Its function is unknown. The phytol chain is hydrophobic, that is, it repels water but has an affinity for oils and fats; the porphyrin ring, on the other hand, is hydrophilic, being drawn to water.

The two major varieties of chlorophyll are distinguished by small differences in structure in one region of the porphyrin ring. Chlorophyll a, the most abundant form, found in all higher plants and in all algae and in cyanobacteria, has a methyl group ($-\text{CH}_3$) at that position. In chlorophyll b, which is found in higher plants, in green algae and prochloron, the methyl group is replaced by an aldehyde group ($-\text{CHO}$). On the other hand, in bacteriochlorophyll a, the form of the pigment found in most photosynthetic bacteria, a single bond is substituted for one of the double bonds in the conjugated ring, and two hydrogen atoms are added.

Figure 3. Estimated *in vivo* absorption spectra of several photosynthetic pigments (chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), carotenoids (Car), phycoerythrin (PE), and phycocyanin, (PC) by Govindjee (unpublished). An absorption spectrum of a pigment measures the amount of light it absorbs at various wavelengths. The chlorophylls have two major absorption bands, one in the blue (called the Soret band) and one in the red; they are green because they transmit the intermediate wavelengths in the green region of the spectrum. The carotenoids absorb blue to green light and appear yellow or red. Phycoerythrin, which absorbs blue through yellow light, is red and phycocyanin, absorbing yellow to orange light, appears blue. Cyanobacteria and red algae do not contain Chl *b*. Together the pigments absorb most of the light in the solar spectrum. Higher plants and green algae do not contain PE and PC.



In addition to these structurally distinct molecules, various spectral forms of chlorophyll and bacteriochlorophyll have been detected in living cells: they can be distinguished only by differences in their absorption spectra. The late C. Stacy French of the Carnegie Institution of Washington's Department of Plant Biology had detected forms of chlorophyll whose maximum absorption of red light is at wavelengths of 660, 670, 680, 685, 690 and 695 to 720 nanometers. The variations are probably produced by chlorophyll molecules in different environments: aggregated differently with other chlorophyll molecules, or associated differently with different proteins. All chlorophylls are, however, associated with proteins.

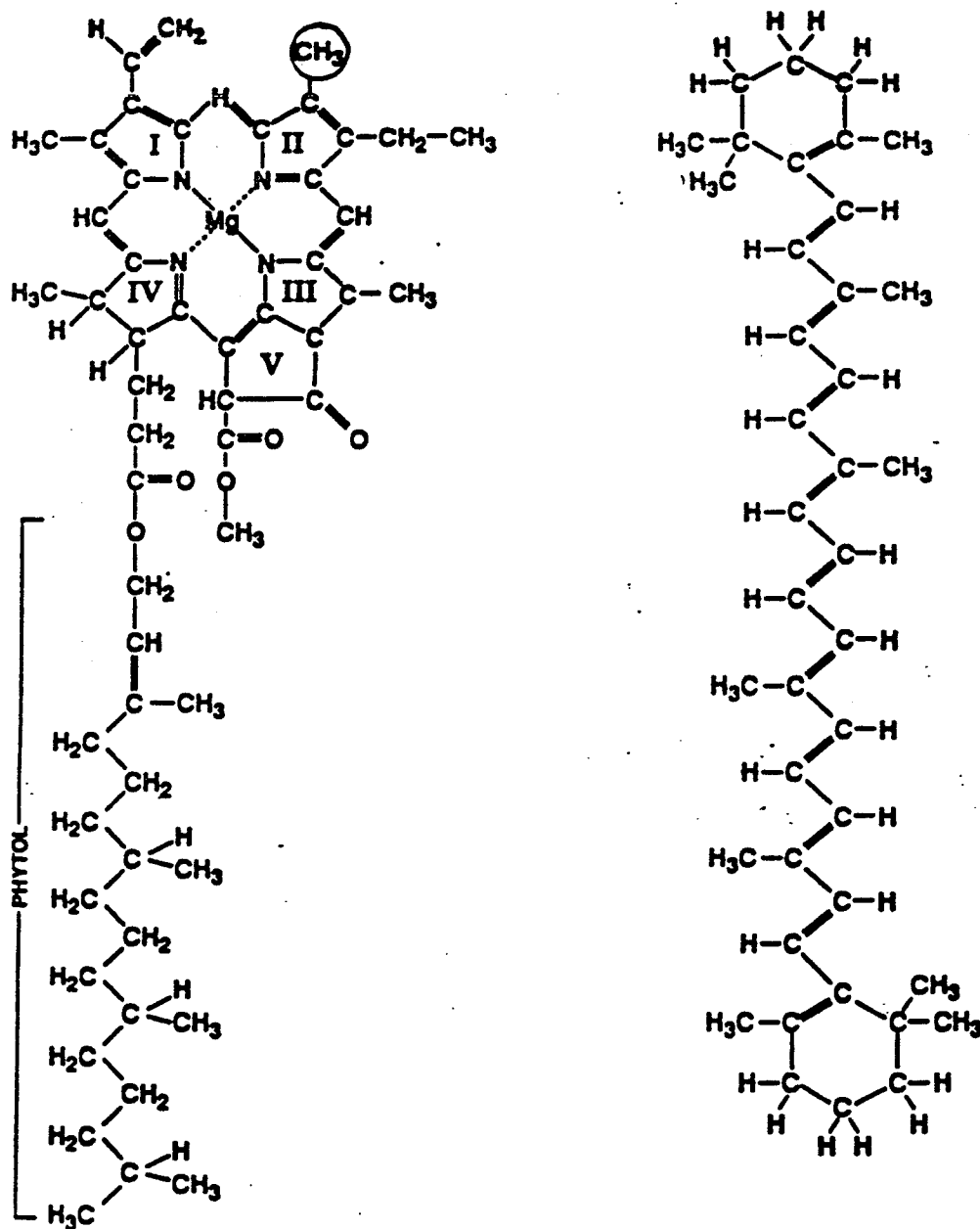
Although the chlorophylls are undoubtedly the most important plant pigments, they are not the only ones present and they are not the only ones that participate in light absorption. The carotenoids, found in bacteria, algae and higher plants, absorb mainly blue wavelengths and are yellow, orange or red in color. (Their name derives from the same root as "carrot," and they give the carrot its orange color.) They consist of long chains of carbon atoms linked by conjugated single and double bonds and bearing a six-carbon ring at each end [see figure 4]. The carotenoids are divided into two classes: the carotenes, which are hydrocarbons and therefore consist of hydrogen and carbon only, and the carotenols (commonly called xanthophylls), which are alcohols and ketones and contain oxygen as well as carbon and hydrogen. Another category of pigments is the phycobilins, named for their resemblance to pigments in the bile of animals. They include the red phycoerythrins, the blue phycocyanins and allophycocyanins and are found mainly in the blue-green algae (cyanobacteria) and red algae (for absorption spectra, see figure 3). The molecules of both types have an open ring related to the porphyrin structure of the chlorophylls, but in the phycobilins the ring is bound to a protein component.

Light Absorption

When a photon is absorbed by an atom or molecule, its effect is to change the configuration of the electronic charge associated with the valence, or outer, electrons surrounding the nucleus (or nuclei). Because the new configuration has more energy than the "ground," or lowest, state, the atom or molecule is said to be in an excited state. The transition from the ground state to an excited state can take place only under certain conditions prescribed by the laws of quantum mechanics. The electrons can occupy only specified, distinct energy states; regions between states are forbidden to them (see figure 5). Moreover, the energy of the absorbed photon must exactly match the energy of the transition. Since the energy (E) of a photon is inversely proportional to its wavelength (λ), only certain wavelengths can be absorbed by a particular atom or molecule [$E = hc/\lambda$, where h = Planck's constant, and c = velocity of light; see figure 6].

In atoms these restrictions are quite confining, since the permitted energy levels are few and transitions between them are large. As a consequence the absorption spectra of atoms usually consist of a relatively few narrow lines. In molecules, however, and particularly in pigment molecules, a number of factors tend to broaden the lines into bands. For one thing, the conjugated bonds in pigments bring about a great proliferation of allowed states because each double bond adds a pair of electrons shared by the conjugated system as a whole. The effect of a large system of conjugated bonds is therefore to decrease the transition gap between the ground state and the first, or lowest, excited state and to create many additional excited states just below the first. It is this effect that is responsible for shifting the absorption spectrum of pigment molecules from the ultraviolet region of the spectrum into the visible region; because of the smaller transition to the first excited state pigments are able to absorb photons of lower energy and hence greater wavelength.

Figure 4. Chemical structures of chlorophyll a (left) and a carotenoid (right). Pigment molecules are distinguished by systems of conjugated, or alternating, single and double bonds. When the pigment absorbs light an electron circulating throughout the system of bonds enters an excited state. In chlorophylls the conjugated bonds are in a complex ring porphyrin. Attached to the ring is a "tail" of phytol, made up of carbon atoms joined mostly by single bonds. Chlorophyll b differs from chlorophyll a in replacing $-CH_3$ in ring II with $-CHO$. In the carotenoid pigments the conjugated bonds are located in a straight chain of carbon atoms that has a cyclic ring at each end.



Two other factors also increase the number of possible quantum states in molecules: the vibrational and the rotational energy of the molecules. These motions are also confined to discrete energy levels, but the levels are much more closely spaced, so that the ground state and each of the excited states broaden into a manifold of substates. In approximate terms the transition between the ground state and the first excited state in chlorophyll a represents an energy difference of between one and two electron volts. (An electron volt is the energy acquired by an electron when it is accelerated through a potential of one volt.) The vibrational substates are separated by about 0.1 electron volt and the rotational substates by about 0.01 electron volt.

When a photon of appropriate energy strikes a molecule of chlorophyll a, the pigment enters an excited state almost instantaneously, within a femtosecond (10^{-15} second). This time is approximated from the interaction of light waves with matter. For example, the frequency ν (i.e., number of cycles per second) of 700 nanometer light is 0.4×10^{15} ; this means that an interaction during one cycle can take 2.5×10^{-15} s. This is an extremely brief interval; in a vacuum light travels only about three ten-thousandths of a millimeter in 10^{-15} second.

There are two major excited states available in chlorophyll a. The absorption of red light (with a wavelength of about 680 nanometers for the pigment in the protein complex) raises the molecule to the lowest and most important of the levels, called the first excited singlet state. Blue light (with a wavelength of about 440 nanometers) promotes the molecule to the third excited singlet state. (Transition to the second excited singlet state, between the first and the third, is weak.) The upward transition may begin from any of the various vibrational and rotational substates of the ground state and end on any of the substates of the excited state.

Having reached an excited state, the chlorophyll molecule cannot immediately and directly utilize its energy to the tasks of biochemistry, nor is useful work the only possible outcome of the absorption of a photon. The excited molecule can give up its energy in any of a number of ways, all of which conform to the same laws of quantum mechanics that govern light absorption.

The Fate of Excitation Energy

For the purposes of photochemistry the most important energy level in chlorophyll a is the first excited singlet state. Molecules elevated to higher excited states return to the first excited singlet state rapidly (in from 10^{-14} to 10^{-13} second), so that they reside in the higher state too briefly to enter into any competing processes. The return to the first excited state is achieved through the small transitions separating the vibrational and rotational sublevels. Each of these transitions is so small that the wavelengths associated with them are not perceived as radiation, only as heat. By the same mechanism a molecule that has been raised to one of the higher sublevels of the first excited state will quickly decay to one of the lower substates. This process is called internal conversion; since it results in the degradation of the received energy to heat, it contributes only to the kinetic motion of the molecules, not to photochemistry.

Because of internal conversion a high-energy photon of blue light is of no more use to a plant than a relatively low-energy red one. The squandering of the energy of short-wavelength light, however, is less extravagant than it may at first seem. Although individual photons of short wavelength are the most energetic, most of the energy of the solar flux is distributed in the yellow and orange and beyond. There are simply greater numbers of photons at longer wave-lengths, and their aggregate energy is much greater.

Figure 5. Energy level (or a Jablonski) diagram of a molecule. Excitation of an electron in a pigment molecule promotes the electron to one of a few discrete energy states; intermediate levels are forbidden. Superimposed on the permitted electronic energy states are substates representing the vibrational and rotational energy of the molecule; the substates are discrete, but they are closely spaced. When an electron absorbs a photon, it is first elevated to an energy level called an excited singlet state. Depending on the energy of the photon, the electron may reach the first or some higher excited singlet state; it quickly subsides to the first, however, by dissipating part of its energy as heat in a process called internal conversion. In the first excited singlet state the molecule can utilize its energy in a chemical reaction, can transfer the energy to another molecule, lose energy by internal conversion, reradiate it as fluorescence or enter another excited state, the triplet state. Because the triplet state is longer lived than the excited singlet state, light is emitted by molecules in the triplet state only after a delay and is called phosphorescence instead of fluorescence. In the reaction center molecules of photosynthesis, most of the first singlet states undergo photochemistry, *i.e.*, charge separation, mentioned in the text.

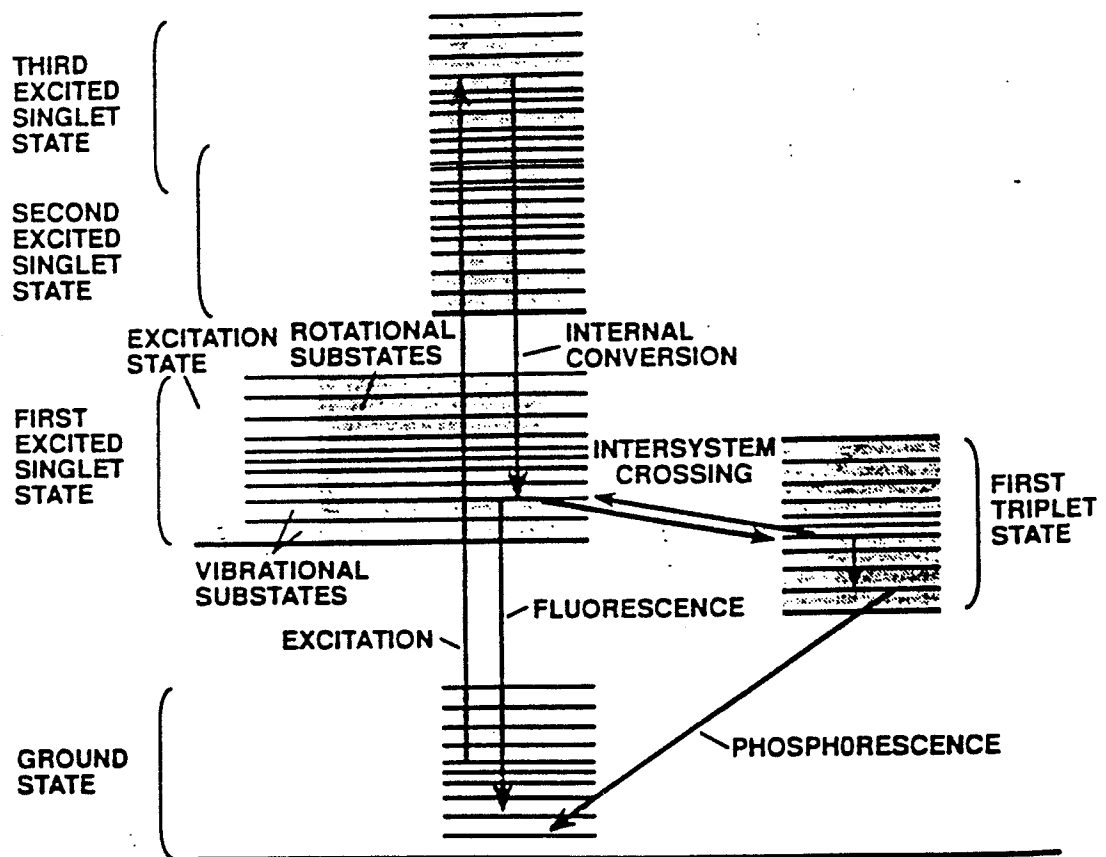
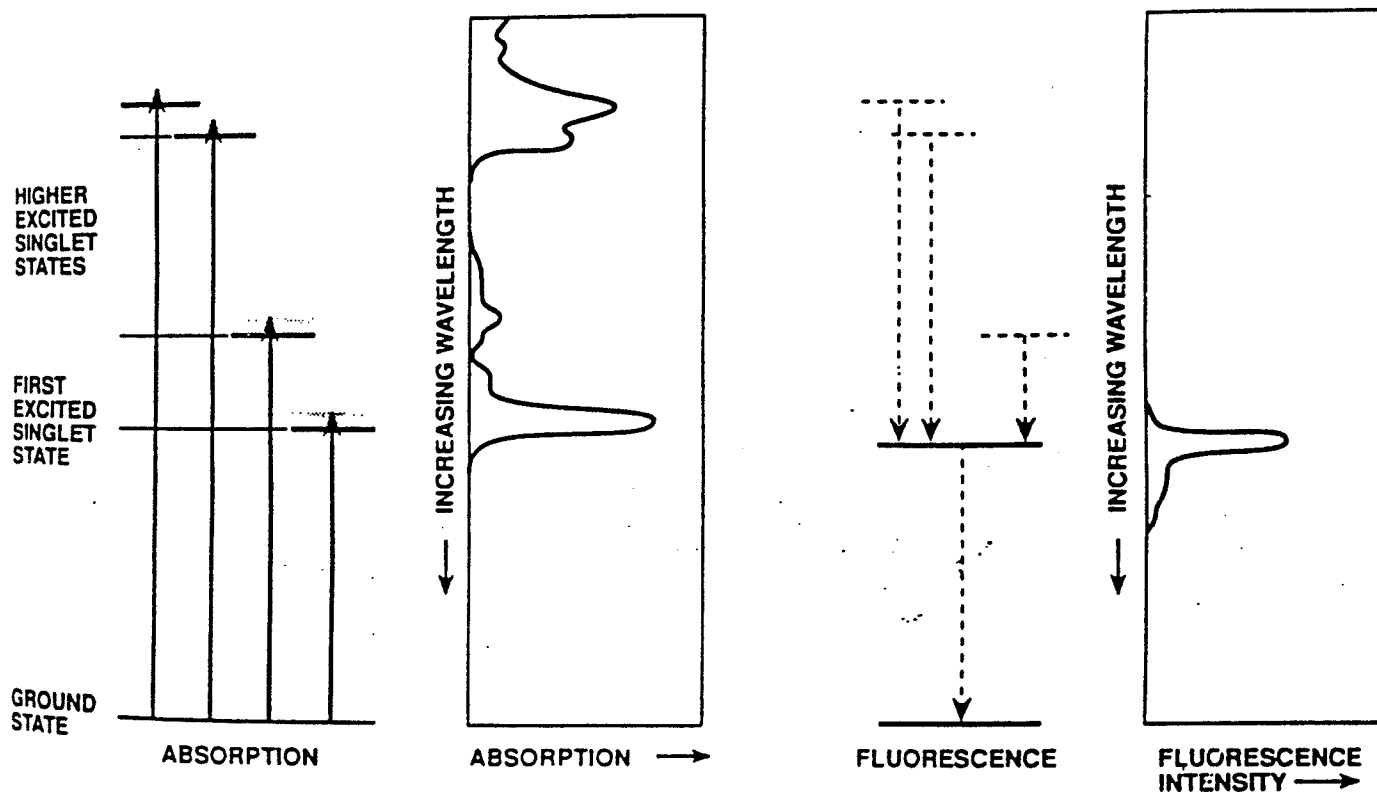


Figure 6. Relationship of energy levels to absorption and fluorescence spectra. Electronic (energy) transitions associated with absorption and fluorescence by chlorophyll are constrained by the requirement that the molecule occupy only discrete energy states. Only photons whose energy corresponds to the energy difference between the ground state and an excited state can be absorbed; since the energy of a photon is inversely related to its wavelength, the selective absorption of a photon is reflected in the absorption spectrum of the pigment (diagram and graph at the left). Even though chlorophyll absorbs light in several parts of the spectrum, it fluoresces only in the red (diagram and graph at the right). The fluorescence is from the first excited singlet state; molecules in higher states decay to this one by internal conversion (energy is lost as heat to the surrounding or the environment). Fluorescence is at longer wavelengths than the lowest-energy absorption band because molecules relax to a low substate of the first-singlet state before fluorescing; this shift is known as the **Stokes' shift**. (Original diagram was based on a diagram by K. Sauer in Govindjee, ed., *Bioenergetic of Photosynthesis*, 1975.)



Once an excited chlorophyll molecule has subsided to one of the lower sublevels of the first excited singlet state, several pathways are open to it (see figure 7). Through further internal conversion it can dissipate its remaining energy of excitation as heat; it can enter another excited state, of a somewhat different character, called the triplet state; it can return to the ground state by emitting a quantum of light as fluorescence; it can transfer its energy to an appropriate neighboring molecule, or it can enter into a chemical reaction, such as those characteristic of photosystems I and II. The last process is obviously the most important, but it is by no means the only one that takes place.

Singlet and triplet states are distinguished by a quantum number called the total spin number, which has to do with the directions in which the electrons in a molecule "spin." In singlet states the spins of a pair of electrons are antiparallel, or opposite; in triplet states the spins are parallel. Doublet states, which are also found in some photosynthetic pigments, occur when there are unpaired electrons. The triplet state is so named because electron pairs spinning in parallel can align themselves in three ways with an external magnetic field; moreover, they superimpose three additional energy sublevels on the quantum state of the molecule. In the singlet state all orientations with respect to an external field are equivalent, and no additional sublevels are introduced [see figure 8]

The triplet state is metastable, that is, it is stable for a comparatively long time. Events that can reverse the spin of an electron are relatively uncommon, and transitions between the ground state and the triplet state are therefore rare. The triplet state can be readily entered, however, from an excited singlet state. There is considerable evidence that molecules in the triplet state are present in the chloroplast, especially at very high light intensities. An analysis made in our laboratory at the University of Illinois had suggested that they are present, but in low concentration; our data suggested that at any given moment less than one chlorophyll molecule in 10 million is in the triplet state. Evidence exists for the existence of triplet states of chlorophyll a or bacteriochlorophyll in photosynthetic systems, but they occur in high concentrations only when the photochemical oxidation-reduction processes driven by the pigments are blocked, as at very high light intensities.

Even if the triplet state is present in illuminated plants, there is no evidence that it forms a part of the main photosynthetic pathway; there are many other ways in which its energy could be dissipated, such as internal conversion and a return directly to the ground state with the emission of light. The latter is termed phosphorescence (see figure 5): it is characterized by light emitted after a delay and at a considerably longer wavelength than the light absorbed. A molecule in the triplet state can also return to the first excited singlet state if it absorbs a small quantum of energy; it then has before it all the possible fates of other molecules at that energy level.

Light emitted during a transition from a singlet state directly to the ground state is termed fluorescence instead of phosphorescence. Ordinarily there is only a brief (nanosecond or subnanosecond) delay between absorption and reemission, and the wavelength of the fluorescent light is only slightly longer than that of the light absorbed. The small increase is caused by the loss of energy through internal conversion (see figure 6).

Fluorescence

Fluorescence has provided some of the most valuable techniques available for the investigation of photosynthesis, but for the plant it is an entirely wasteful phenomenon. (For a photograph of fluorescence, see the original article in *Scientific American*, Dec, 1974.) Ordinarily, however, the magnitude of fluorescence is rather small. For every 100 photons absorbed, only from three to six are reemitted. For this reason fluorescence in living plants is faint and can rarely be detected by the unaided eye. The fluorescence of chlorophyll in solution, on the other hand, can be quite bright, since the process that would normally drain away most of the energy of excitation—photosynthesis—is absent.

Figure 7. Fates of excitation energy are of varying probability and of varying importance to the living plant. The pathway indicated in heavy boxes and with heavy arrows is the only one known to contribute to photosynthesis. Other transitions take place in the plant but do not result in useful chemical reactions. Transitions indicated by broken lines are of the lowest probability.

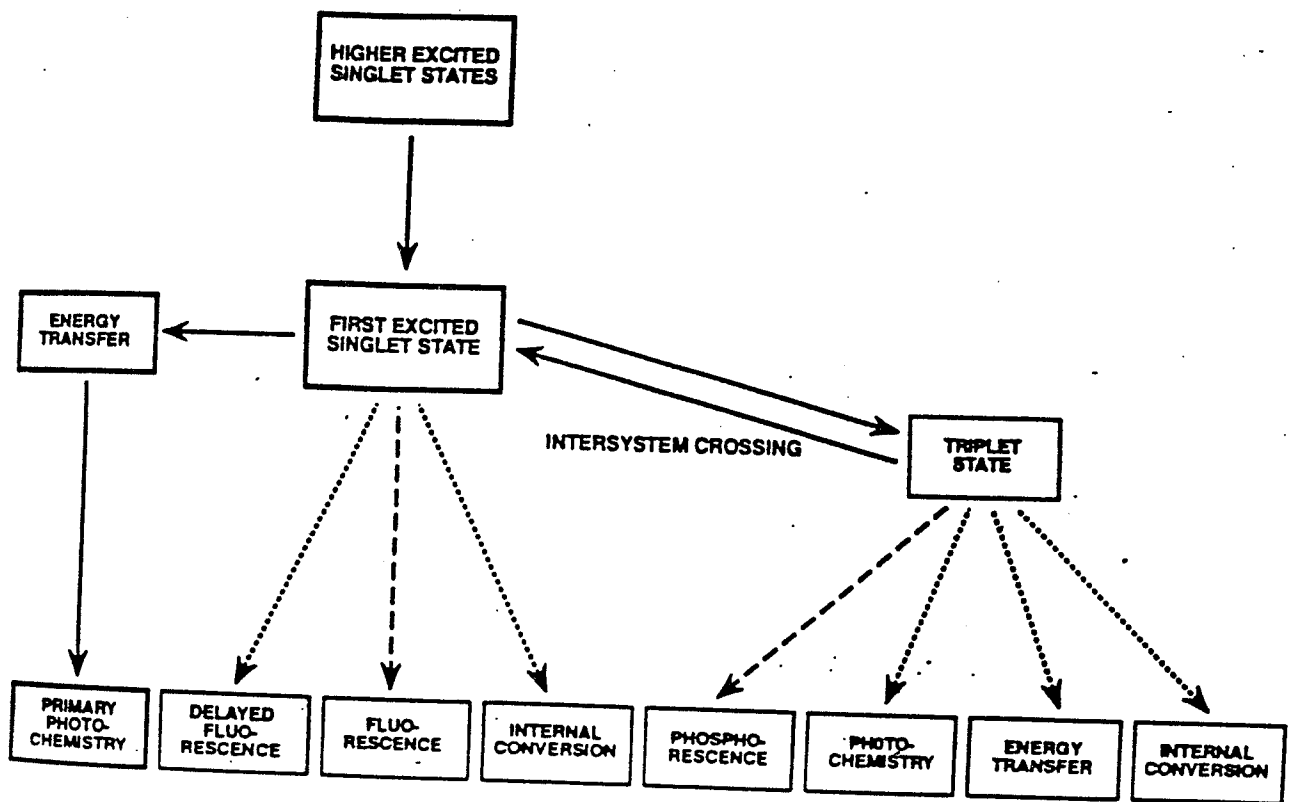
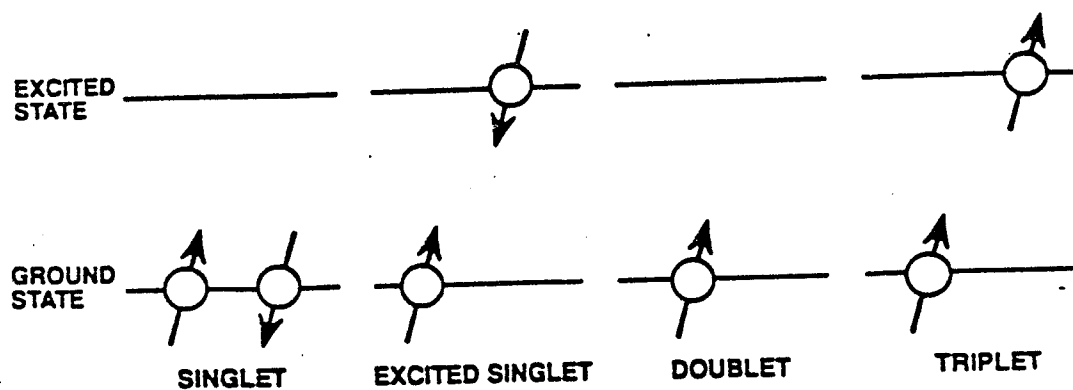


Figure 8. Definitions of singlet, doublet and triplet states. These states are defined by the "spin" of the outer electrons in an atom or a molecule. In this diagram the arrows represent the spin axis. If the spins are antiparallel the molecule is in a singlet state; if they are parallel it is in a triplet state. The doublet state requires an unpaired electron. "Singlet", "doublet" and "triplet" refer to the number of ways the electrons can orient themselves with respect to a magnetic field. (Numerically they are defined by what is called multiplicity that equals 2 times the total spins plus one, where each electron has a spin of $+1/2$ or $-1/2$, depending on the direction of the spin.)



Chlorophyll fluorescence is invariably red, even if the exciting light is blue or green or yellow. The fluorescent light is emitted in a transition from the first excited singlet state, and molecules promoted to higher states by more energetic photons merely dissipate part of their energy as heat before fluorescing [see figure 6]. At room temperature the fluorescence spectrum consists of a major electronic band at 685 nanometers and a minor vibrational band at about 740 nanometers. When the spectrum is measured at low temperature (e.g., liquid nitrogen temperature, 77 degrees Kelvin), bands appear at 685, 695, 720 and 735 nanometers. Most of the fluorescence (about 80%) at room temperature is from pigment system II. However at 77 degrees Kelvin, pigment system II is primarily responsible for the bands at 685 and 695 nanometers, and pigment system I for the 720-735-nanometer bands [see figure 9].

In living cells the intensity of chlorophyll a fluorescence changes as a function of time. This effect, discovered by the late Hans Kautsky of the University of Heidelberg, consists of a fast change, completed in about two seconds after the plant is illuminated, and a slower fluctuation that achieves a steady state only after several minutes. Both temporal patterns could reflect, among many other things, the shifting relation between pigment systems I and II. The fast change is sensitive to the rate of electron flow through the photosystem and might be explained as indicating that pigment system II briefly gets ahead of system I, and wastes the surplus energy in fluorescence.

The slow change is thought to be related to temporary alterations in the thylakoid membrane as well as to the changes in CO₂ fixation. One hypothesis suggests that fluorescence changes also monitor short-term regulation processes. If the two pigment systems are spatially close together, energy from pigment system II, which is more fluorescent, might "spill over" into the weakly fluorescent system I, thereby diminishing the overall light emission. If the two systems were then separated, the spillover would be eliminated and the fluorescence would increase. The conformational change in the thylakoid membrane required by this theory might be caused by the movement of ions. An alternative explanation postulates changes in the density or spacing of chlorophyll molecules in pigment system II. Separating the molecules from one another could be expected to decrease internal conversion and thus to increase fluorescence. A specific theory for a short-term regulatory process is as follows [see figure 10]. It is suggested that excess light into photosystem II (called state II) leads to overreduction of the plastoquinone (PQ) pool between the two systems (figure 1). This leads to the activation of a membrane-bound enzyme kinase, which very rapidly phosphorylates light-harvesting chlorophyll a/b protein complex. This phosphorylation results in a net increase of negative charges that force some of the light-harvesting complexes to dissociate from photosystem II and to move to the photosystem I area in the non-appressed region of the thylakoid membrane. Such a process would decrease fluorescence from photosystem II and increase fluorescence from photosystem I. When excess light goes into system I (state I), the PQ pool is oxidized, kinase is no more activated, and another enzyme phosphatase can dephosphorylate the above-mentioned light-harvesting complexes, which then migrate back to the appressed thylakoid regions and rejoin photosystem II. Thus, fluorescence from photosystem II increases accompanied by a decrease in photosystem I fluorescence. It remains to be seen if these processes really occur *in vivo* or not.

The lifetime of an excited state in a fluorescent molecule is an important parameter in photosynthesis, since it gives an indication, albeit an indirect one, of how much time is available for the energy of excitation to reach a chemically active molecule. The lifetime is defined as the time required for fluorescence to decay to 1/e of its maximum intensity (e is approximately 2.7). As we have mentioned, the lifetime of the higher singlet states is extremely brief, from 10⁻¹⁴ to 10⁻¹³ second; these states, however, do not enter into photochemistry. The first excited singlet state is much longer lived. Its lifetime was first measured by Seymour S. Brody at the University of Illinois and by Aleksander N. Terenin and his colleagues at Leningrad State University.

Figure 9. Fluorescence emission spectra of photosynthetic systems. These spectra can discriminate between different pigment molecules as they are "fingerprints" of various molecules. Chloroplasts at room temperature (solid line) have a strong fluorescence peak at 685 nanometers and a much smaller peak at about 735 nanometers; most of this fluorescence originates in the Photosystem II of plants -- the 735 nm band being due to one of the major vibrational bands of the main electronic band. At low temperature (e.g. liquid nitrogen temperature, 77 degrees Kelvin), isolated pigment system I complex (also solid line) fluoresces most strongly in the 720-735 nanometer region, with minor bands at 685 and 695 nanometers. On the other hand, isolated pigment system II complex (dashed line) peaks at 685 and 695 nanometers.

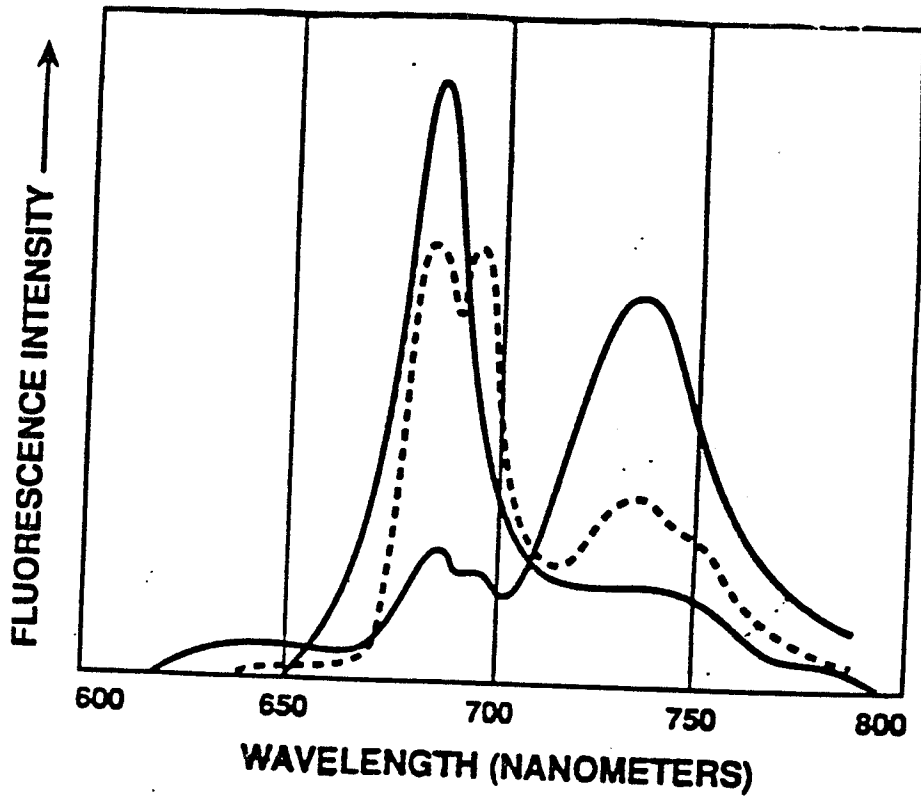
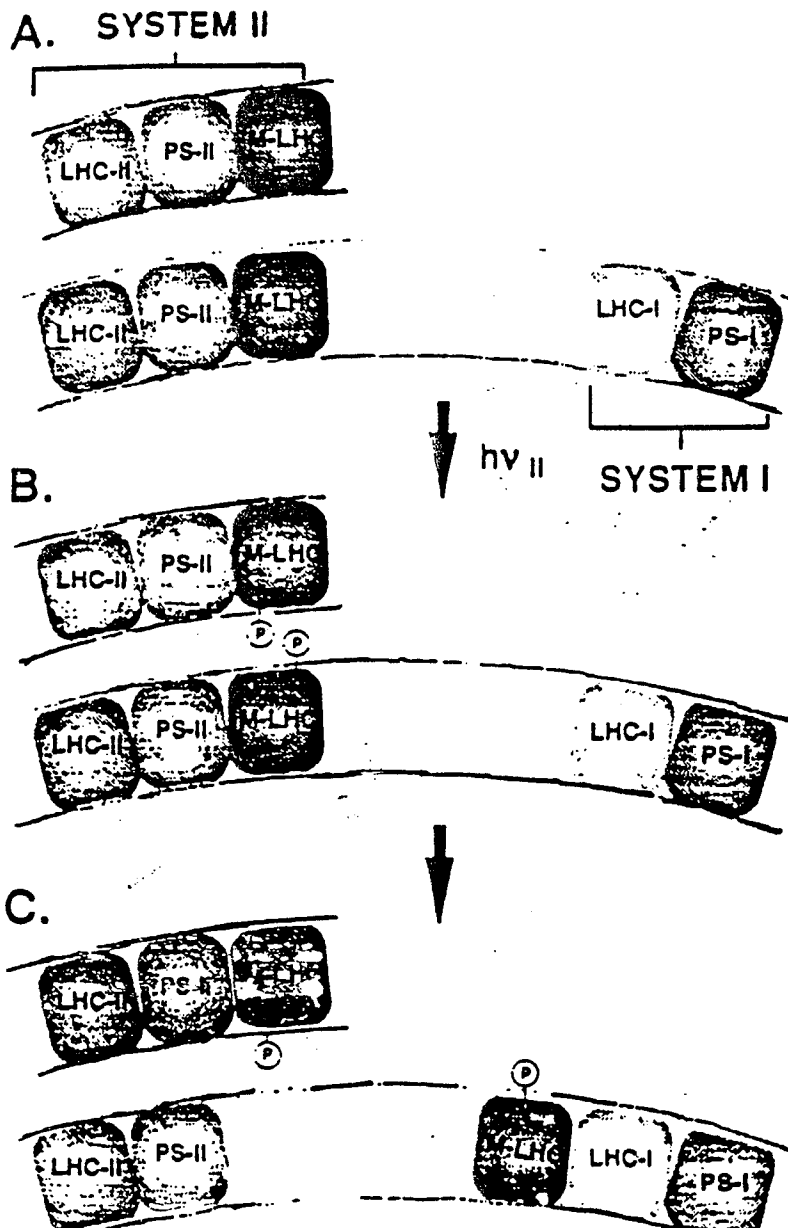


Figure 10. A model for the control of excitation energy redistribution in photosynthesis. A. The pigment-protein complexes in the appressed regions of thylakoid membranes are: LHC-II (chlorophyll a/b containing light-harvesting complexes of pigment system II); PS-II (pigment system II containing the various chlorophyll a-protein complexes—29, 43 and 47 kilodalton masses and the reaction center complex); and M-LHC (mobile light harvesting complex). In the unappressed regions we have LHC-I (chlorophyll a/b containing complexes of pigment system I although there is very little chlorophyll b here) and PSI (various pigment system I complexes and the reaction center complex). Exposure with photosystem II light II ($h\nu_{II}$) leads to the accumulation of plastoquinol (PQH_2) between the two systems (see figure 1); this, in turn, is suggested to activate a kinase that leads to phosphorylation of the mobile light-harvesting complex (see B). These phosphorylated complexes physically move to the unappressed regions in the LHC-I/PSI area (see C). Thus, the antenna size of the system I region increases at the expense of the decrease of the system II region. It is said that $h\nu_{II}$ produces state II (C) from state I (A). At low light intensity, and in comparison to state I, state II would have a lowered activity of system II and an elevated activity of system I. State II would have a decreased chlorophyll a fluorescence intensity at room temperature as most fluorescence originates in system II. Conversely, oxidation of PQH_2 by exposure to light absorbed in system I ($h\nu_I$) would inactivate the kinase; the phosphatase would then dephosphorylate the mobile light harvesting complex and it will move back to the system II region. This will convert state II to state I leading to an increased system II activity and a decreased system I activity; this will be accompanied by an increased room temperature chlorophyll a fluorescence. It is suggested that such a regulation can cause 15-20% changes in energy redistribution that could play significant part in the energetics of plants or plant parts under shaded environment.



Measurements of the lifetime of chlorophyll a in living cells, made with photosynthesis stopped by inhibitors or by low temperature, or made in light so bright it saturates the photochemical system, yield a lifetime of fluorescence of about 2×10^{-9} second (2 nanoseconds:2ns). Recent picosecond fluorescence spectroscopic and kinetic measurements in several laboratories around the world suggest the existence of, at least, three lifetime components: an 80 picosecond component from photosystem I, and two (ns) components (approximately 1.3 ns and 2.3 ns) from photosystem II. If the photosynthesis is not stopped, that is measurements are made when photochemistry is highly efficient, the photosystem II components become faster (approximately 0.5 ns and 0.25 ns, respectively).

The Transfer of Energy

Most of the pigment molecules in the chloroplast do not take part directly in the chemical processes of photosynthesis. Of the 300 molecules in the basic photosynthetic unit, almost all are thought to serve merely as "antenna" molecules [see figure 11]. (The metaphor is not far-fetched; after all, the molecules are tuned to receive signals of a particular wavelength.) The antenna pigments transfer the energy they absorb to a reaction center, which might consist of a single specially deployed molecule or a dimer of chlorophyll a. This molecule then introduces the energy into the oxidation-reduction cycle.

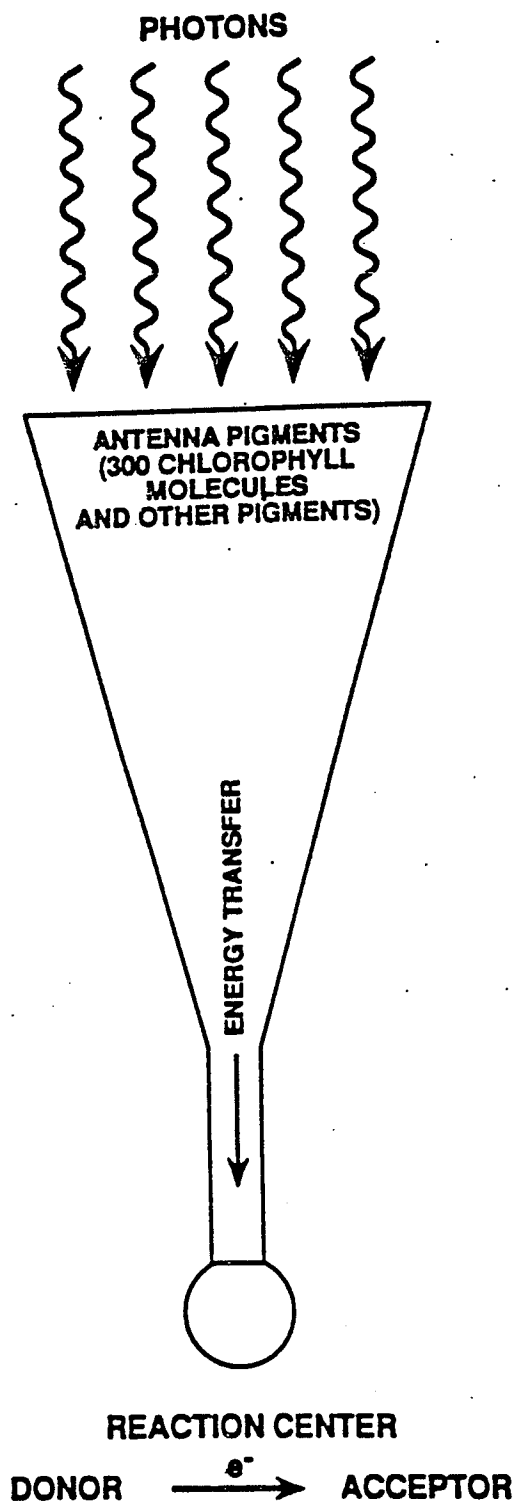
If such a system is to operate efficiently, some method of rapidly transferring the energy of excitation from one molecule to another must be provided. That energy is indeed transferred is suggested by a simple observation: green leaves contain chlorophyll b, which is fluorescent in isolation, yet in living cells only the fluorescence of chlorophyll a can be detected. Evidently this accessory pigment transfers its energy to chlorophyll a so efficiently that its own fluorescence is quenched. The effect can be demonstrated directly in monomolecular layers of mixed chlorophyll a and b; even when the mixture is illuminated at a wavelength that will stimulate only chlorophyll b, only chlorophyll a fluorescence is observed.

Among the accessory pigments in red algae or in cyanobacteria a sequence of energy-carriers has been worked out. It begins with the carotenoids, which pass their energy to Chl a. Energy absorbed by phycoerythrin, passes through phycocyanin and allophycocyanin (another phycobilin) to chlorophyll a. Each transfer from one kind of pigment to another involves a modest loss of energy as fluorescence or heat [see figure 12].

Once the excitation energy has been passed to a chlorophyll a molecule it can migrate through the entire population of chlorophyll a molecules without loss. That the migration does take place was suggested by Arnold and E.S. Meek at Oak Ridge in 1956. Arnold and Meek illuminated chloroplasts with plane-polarized light and observed the resulting fluorescence. If the exciting energy had been retained by each molecule during its fluorescence lifetime, the polarization would have been maintained in the fluorescent light; it was in fact destroyed entirely, indicating energy migration.

One possible explanation of the mechanism underlying energy transfer is called the Förster resonance theory, formulated in 1948 by the late Theodor Förster of the Technical University in Stuttgart. The theory holds that a molecule must first subside to the lowest vibrational sublevel of the first excited singlet state before its energy can be transferred; once this requirement is met the molecule is coupled to its neighbors by a resonance fundamentally dependent on their distance ($1/R^6$, where R is the distance) and orientation and on the overlap between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor.

Figure 11. A statistical photosynthetic unit. It is thought to consist of about 300 chlorophyll molecules; in most higher plants and algae that seems to be the minimum number. The unit includes the bulk pigments, which serve as an energy gathering "antenna" and a reaction center chlorophyll, where energy is trapped and utilized to promote chemical reactions. The antenna and reaction center molecules are physically located in different polypeptides; the antenna molecules are distributed in several polypeptides.



Faster mechanisms, in which the molecule need not first relax to its lowest vibrational state, have been proposed. In that case, the energy belongs to all the molecules and one need not speak about transfer. They cannot yet be excluded, but calculations based on measurements of fluorescence lifetime support Förster's theory. An upper limit to the time required for energy transfer had been provided by an experiment devised by Peter M. Rentzepis and coworkers of Bell Laboratories. Working with bacterial photosynthetic systems that had been stripped of all antenna pigments, so that only the reaction centers remained, they observed an energy exchange within 6 ± 2 picoseconds. Many new results are now available.

The path taken by the migrating excitation energy in the antenna pigments is thought to resemble a "random walk" that continues until the energy quantum happens to encounter an available reaction center, or until it is reradiated as fluorescence. The reaction centers themselves might be considered to be dispersed randomly in a monolithic bed of bulk pigment, so that any quantum of energy could wander throughout the mass and in principle could eventually reach any of the reaction centers. Alternatively, each reaction center might be surrounded by, and served exclusively by, a fixed aggregate of pigment molecules. These two hypotheses are called the "lake model" and the "isolated puddle model." The actual situation appears to be a hybrid of the two, calling for a "connected puddle model" [see figure 13].

The Reaction Center

The elaborate system of pigmentation in plant cells is intended solely to convey the energy of light quanta to reaction centers where the central events of photosynthesis take place. In the past, learning the nature and the exact business of the reaction center had been an intractable problem in the study of plant photosynthesis; now, some of the molecules active there have not only been labeled spectrally, but a protein complex has now been isolated by O. Nanba and Kimiuki Satoh in Japan in 1987 that contains the reaction center function of photosystem II. This complex contains 4 polypeptides (two 32-34 kilodalton polypeptides called D-1 and D-2, a cytochrome b-559, and a 4.5 kilodalton polypeptide), 6 chlorophyll a and 2 pheophytin molecules.

Louis N.M. Duysens of the University of Leiden had discovered the first of the reaction center molecules when he observed a light-induced change in the absorption spectrum of photosynthetic bacteria at 890 nanometers; the change was later shown to be produced by bacteriochlorophyll in the bacterial reaction center. Kok identified a similar change in the absorption of green plants at 700 nanometers; this light-induced absorption change is now known to be caused by the reaction-center molecule of pigment system I, designated P700. Exposure to light has been demonstrated to oxidize P700. The analogous molecule for pigment system II was discovered by G. Döring and Horst Witt of the Technical University in West Berlin and is labeled P680. At the Argonne National Laboratory, Mike Wasielewski, Mike Seibert and Govindjee have measured, in 1989, the time for the primary charge separation in a stabilized preparation of the reaction center of photosystem II: the reduction of pheophytin and the oxidation of P680 was found to occur in a few picoseconds at 4 degrees Celsius.

A major breakthrough in the field of anoxygenic bacterial (not cyanobacterial) photosynthesis has been the crystallization and the x-ray analysis of the reaction center from *Rhodospseudomonas viridis*, a photosynthetic bacterium. This pioneering work by H. Mitchell, J. Deisenhofer and R. Huber, for which they received the Nobel Prize in Chemistry in 1988, has led to the first 3-dimensional picture of a membrane protein. We now know the structural details, at the atomic level, of this efficient energy-storing device (see D. Youvan and B. Marrs, *Scientific American*, June, 1987). Such a detailed model has now permitted investigations on the mechanism of electron transport in the reaction center. An important question, that is being tackled in many laboratories, is why the electrons flow on one side of the reaction center when there is an almost perfect symmetry in the arrangement of the chromatophores: two bacteriochlorophylls form the special pair; then there are two monomer bacteriorchlorophylls on two sides followed by two bacteriopheophytins one on each side. (See Figure 14.) This is followed by two quinones Q_A and Q_B, also one on each side; in the center of the symmetrical axis lies an iron (see Figure 14). The reaction center contains three polypeptides (labeled as L, M and H) in addition to the cytochrome

Figure 12. Energy transfer sequence in plants and cyanobacteria. Transfer of energy from one kind of pigment to another follows an established sequence. The carotenoids, which absorb blue light, are suggested to pass their energy of excitation to Chl *a*. The energy of green light, absorbed by phycoerythrin, is transferred to phycocyanin in these systems. Phycocyanin absorbs orange light, and passes the accumulated energy to chlorophyll *a* in these systems. In each of these transitions some energy is lost by internal conversion, and in transfers from phycoerythrin to chlorophyll *a* via phycocyanin and allophycocyanin some energy is also lost as fluorescence at each step. In diatoms, carotenoid fucoxanthol transfers energy to chlorophyll *a* via another chlorophyll, chlorophyll *c*. In higher plants and green algae, chlorophyll *b* contributes its energy directly to chlorophyll *a* without loss. Chl *b* is not present in cyanobacteria, red algae, and diatoms, but Chl *a* is common to all oxygenic photosynthesizers. Higher plants do not contain phycoerythrin or phycocyanin.

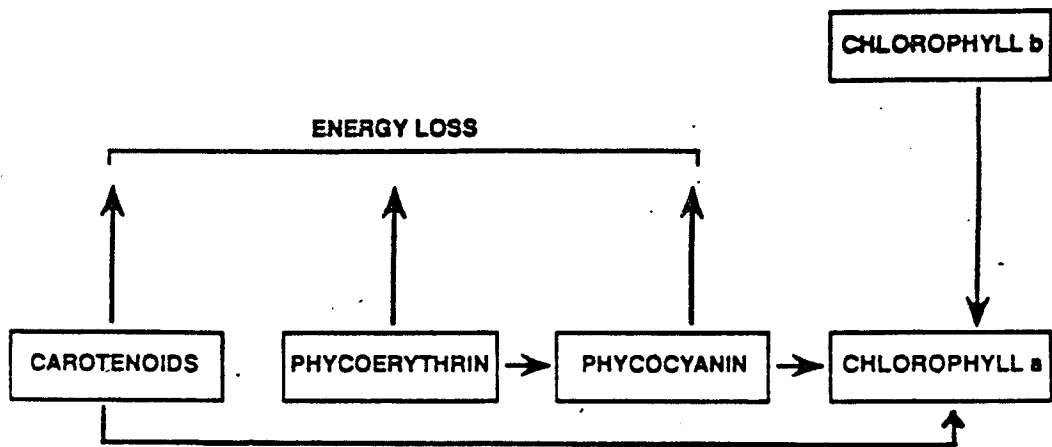
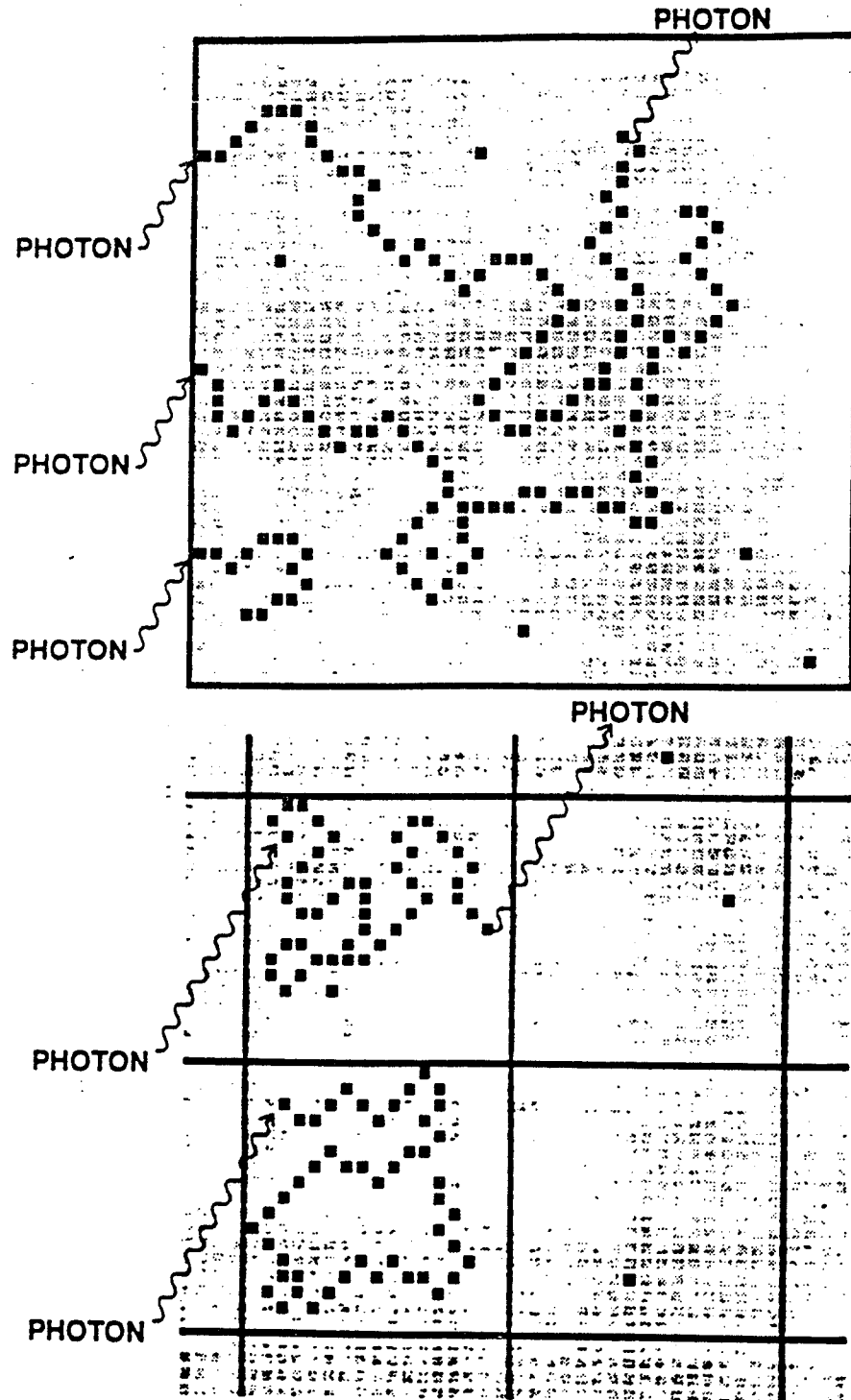


Figure 13. Flow of energy through chlorophyll a molecules is rapid and efficient. Although directionality may exist when energy is being transferred from chlorophyll b to chlorophyll a, or from a short-wave absorbing form of chlorophyll a to a long-wave absorbing form of chlorophyll a, energy transfer among similar chlorophyll a molecule must be undirected and random. In the diagram at the top each square represents a pigment molecule; there is one reaction center (black square) for hundreds of antenna molecules. In the "lake model" (top), energy absorbed by any molecule can wander through the entire mass of pigments until it is trapped in a reaction center or until it is reemitted. In the "isolated puddle model" (bottom), a single reaction reaction center is committed to each aggregate of several hundred chlorophyll molecules. The energy again meanders randomly, but only within the confines of the individual units. The mechanism of energy transfer, which probably involves resonance between nearby molecules, operates with almost perfect efficiency.



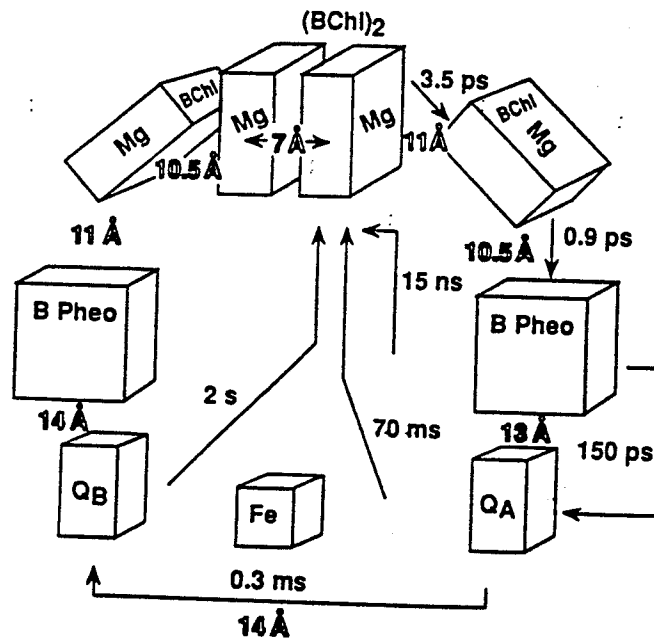
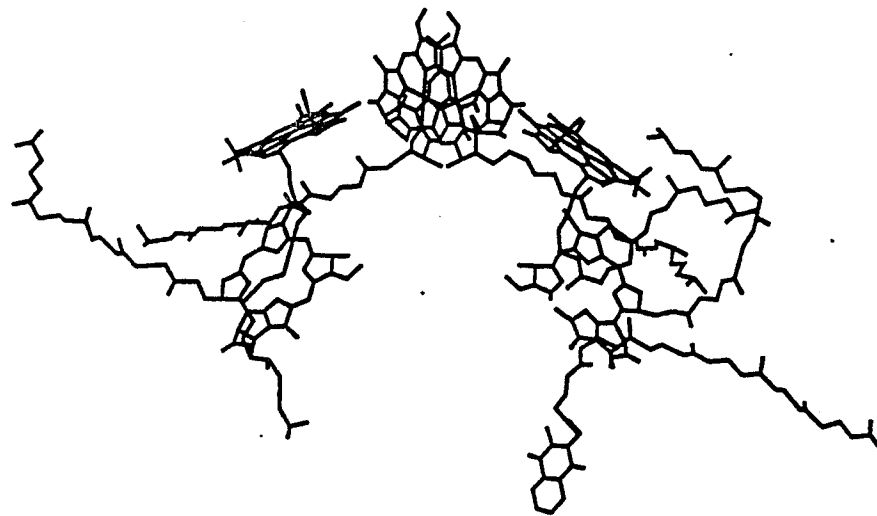
that is on the special pair side. The L and M polypeptides traverse the membrane 5 times, but most of the H polypeptide is on the opposite side of the cytochrome. Sequence similarities have been discovered between the L and M and the D-1 and D-2 polypeptides. However, differences must exist since plants evolve O_2 and bacteria do not.

These specialized reaction center molecules appear to stand at the culmination of the physical, energy-gathering stage of photosynthesis, and at the beginning of the chemical, energy-storing phase. There are a number of ways of determining whether or not they do mark that intersection. The chemical processes they participate in should be the first to take place after a brief flash of light, and they should be in the main pathway of photosynthesis. Primary photochemical reactions should also continue even at very low temperatures (at liquid nitrogen temperature, 77 degrees Kelvin, or at liquid helium temperature, 4 degrees Kelvin). These tests have been made and the evidence that has accumulated is clear. P700 is in fact oxidized by light at 77 degrees Kelvin (although the pigment cannot be fully reduced at that temperature). The absorption change associated with P680 has been observed at low temperatures, as mentioned above. Similar results in reaction centers from photosynthetic bacteria have been published: J. Breton and coworkers in France showed, in 1988, that the reactions get much faster when the samples are cooled to liquid helium temperatures ($4^\circ K$). A series of experiments and calculations, beginning with early measurements made by Kok and others, show that the oxidation of P700, of P680 and of all bacterial reaction centers, are a highly efficient reaction, and therefore they must be all part of the main pathway.

Finally, if P680 and P700 are the active molecules of the reaction center, they must be capable of initiating the sequence of events that leads to the transfer of electrons. They could do so, when they are in the photoexcited state, by donating one of their own electrons to the acceptor molecule, then recovering it when in the ground state from the donor [see figure 15]. In the case of pigment system II, for example, P680, after absorbing a photon, transfers one of its valence electrons to the molecule labeled pheophytin and in the process would return to the ground state. It could then accept an electron from the donor molecule Z, which, in turn, accepts electrons from the intermediate M. This hypothesis implies that the reaction-center pigments bear a positive charge during the electron transfer, and the presence of chlorophyll ions in the chloroplast has indeed been demonstrated by electron-spin-resonance techniques that monitor unpaired electrons. Reduced pheophytin transfers electrons to Q_A .

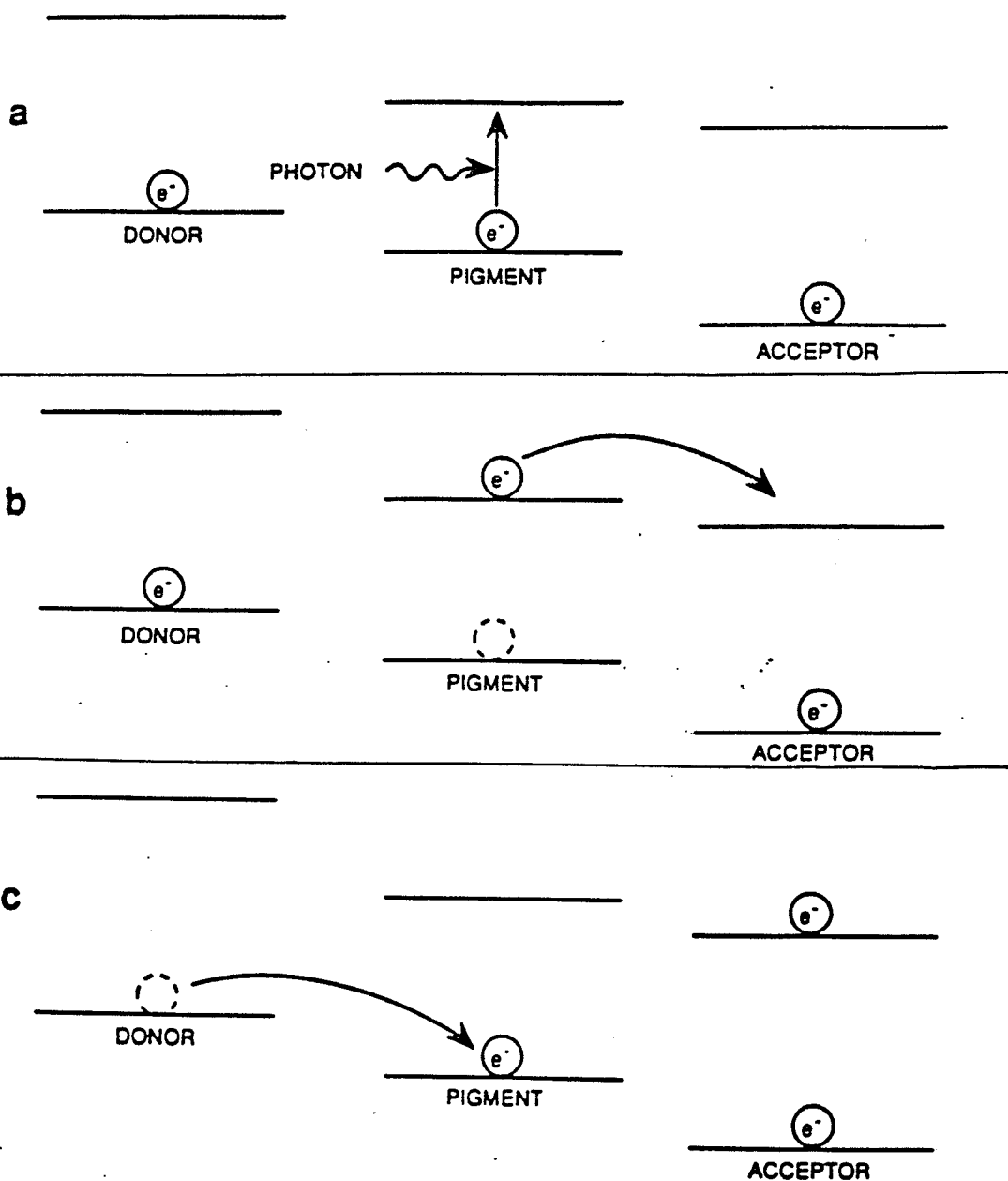
If this sequence of events is correct, some means must be provided to prevent oxidized M and reduced Q_A from reacting with each other to annul the charges developed on them. A direct reaction between them is thermodynamically the most favorable of all reactions, and it is known to take place, although only to a minor extent. (It is responsible for the delayed light emission discovered in 1951 by Arnold and Bernard L. Strehler at Oak Ridge.) The back-reaction between oxidized M and reduced Q_A must not be allowed to dissipate a significant amount of the absorbed energy, however, if photosynthesis is to achieve useful results. What mechanism intervenes to separate them is for now unknown. That is only one of several mysteries, however, that remain to be solved by further study of photosynthesis.

Figure 14. (Top) Structure of the chromophore portion of the reaction center of the purple nonsulfur photosynthetic bacterium *Rhodospseudomonas viridis* as determined by X-ray analysis of its crystals (based on the work of H. Michel, J. Deisenhofer, R. Huber and coworkers). (Bottom) A simplified representation of the reaction center chromophores based on data of several investigations. Only the right side is active. $(Bchl)_2$ is the special pair, the reaction center bacteriochlorophyll; Bchl is the monomer bacteriochlorophyll; Bpheo is bacteriopheophytin; Q_A is a bound ubiquinone; Fe is the non-heme iron; and Q_B is a bound ubiquinone or menaquinone. The cytochrome c lies on the special pair side; the H-polypeptide on the Q_A -Fe- Q_B side—one helix crosses the membrane where the shown structure sits; the L and M polypeptides cross the membrane 5 times. It is suggested that photosystem II reaction center has quite a bit of similarity to the bacterial reaction center; they are, however, different in several respects. For example, they must have binding sites for manganese and chloride needed for water oxidation. Also, a bicarbonate-reversible formate effect exists on the Q_A -Fe- Q_B side of plants and cyanoobacteria, but, not on photosynthetic bacteria (Govindjee and coworkers). Diagram is based on a diagram by J. Norris in Govindjee (ed.) *Light Emission by Plants and Bacteria* (1986).



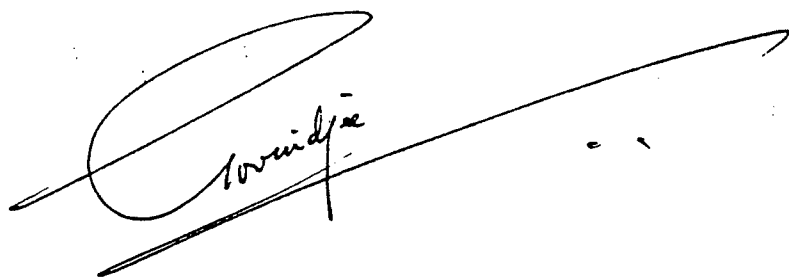
Gov35b Bacterial Reaction Ctr.

Figure 15. The primary photochemistry involves the conversion of light (or excitonic) energy into chemical energy: An electron transfer from a "reluctant" donor to a "reluctant" acceptor, that is "uphill" transfer. This transfer, against an electrochemical gradient, occurs by the use of light energy in the reaction center in three stages. On absorbing a photon or upon receiving an exciton from the antenna molecules (a) the reaction center chlorophyll molecule is promoted to an excited state. The electron is then transferred to an acceptor molecule, e.g., pheophytin in photosystem II (b); because the reaction center chlorophyll is in an excited state this process is "downhill", that is, it is favored by the electrochemical gradient. In the final step (c), the pigment regains an electron from a donor molecule (e.g., Z, a tyrosine residue in photosystem II), another downhill process.



Efforts are now underway to probe further the structural and functional similarities and the differences between the plant and cyanobacterial (oxygenic) and bacterial photosynthetic (anoxygenic) reaction centers. A major similarity lies in the charge separation reactions and in the operation of electron flow on the electron acceptor side, *e.g.*, in the existence of a "two" electron gate. After one light reaction, Q_A^- (bound on the D-2 protein of plants, or the "M" subunit of photosynthetic bacterial reaction center) transfers electrons to Q_B (bound on the D-1 protein of plants, or the "L" subunit of photosynthetic bacterial reaction center) reducing it, but Q_B^- remains tightly bound to the reaction center in both oxygenic and anoxygenic systems. It takes another light reaction before Q_B is doubly reduced to Q_B^{2-} and only then it is released from the site as plastoquinol Q_BH_2 , after acquiring 2 protons; this is, thus, the two electron gate. Several herbicides (*e.g.*, atrazine and diuron) inhibit photosynthesis by displacing Q_B from its binding site. A subtle difference lies in the ability of formate ions to inhibit the Q_A^- to Q_B^- electron flow in plants and cyanobacteria, but not in photosynthetic bacteria, as shown recently in our laboratory; bicarbonate ions fully reverse the formate effect in both plants and cyanobacteria. A major difference, however, lies on the electron donor side. In photosynthetic bacteria, a cytochrome *c* delivers an electron to the oxidized reaction center, $P890^+$, *etc.* However, in oxygenic photosystem II, electrons ultimately come from H_2O via a Mn-complex (M) and a tyrosine residue (Z). Site-directed mutagenesis of individual amino acids is being actively pursued in many laboratories, including our own, to find the binding sites and to understand the molecular function of the various cofactors, polypeptides and the chromophores. The function of the "H" subunit of the bacterial reaction center is unknown; it is not clear if any polypeptide in plants serves an equivalent function, whatever that maybe, to that of the "H" subunit.

An entire field is to open up before our eyes and we are beginning to learn about the roles of individual and groups of amino acids in the binding of various chromophores and other chemical components, and in their involvement in various electron and proton transport pathways. A discussion on this topic, however, calls for another article: this article has already been too long. Good hunting!

A handwritten signature in black ink, appearing to read "David", is written over a large, sweeping horizontal line that extends across the width of the page.

How Plants Make Oxygen¹

A biochemical mechanism called the water-oxidizing clock enables plants and some bacteria, the cyanobacteria, to exploit solar energy to split water molecules into oxygen gas, protons and electrons

by Govindjee and William J. Coleman²

Because molecular oxygen is a necessity of life for human beings, it is easy to forget that simple organisms lived without it for hundreds of millions of years. For those early anaerobic organisms, oxygen was a toxic substance that could steal essential electrons from the molecules in their cells. It may seem surprising, then, that many of these anaerobic cells engaged in a form of photosynthesis (the anoxygenic photosynthesis), because plant and cyanobacterial photosynthesis produces all the oxygen in the atmosphere. The exact process by which oxygen is generated in these organisms has been something of a mystery, but now the mechanism can be described in some detail. It is a "water-oxidizing clock" that generates a molecule of oxygen with every four ticks.

One of the fundamental tasks of photosynthesis is to make it possible for cells to convert carbon dioxide into carbohydrates with energy absorbed from the sun. The production of oxygen is not crucial, which is why anaerobic cells could photosynthesize without making molecular oxygen long ago and why they have continued to do so to this day.

If oxygen is toxic, why and how did green plants and their ancestors ever begin producing it through photosynthesis? The answer to the first question involves energy metabolism. Sunlight provides the energy that drives life on the earth, but cells cannot store or employ that light energy directly; it must be converted into a more usable, chemical form. Electrons are part of the common "currency" of biological energy conversion: many energetic reactions in cells can be generally understood as the transfer of electrons between molecules.

¹ Govindjee and W.J. Coleman. 1990. How plants make oxygen. *Scientific American* 262(2):50-58. **Revised**, 1994.

²GOVINDJEE and WILLIAM J. COLEMAN have collaborated on studies of the mechanism of oxygen evolution in green plants. Govindjee is professor of biophysics and plant biology at the University of Illinois at Urbana-Champaign since 1969. He was born in Allahabad, India, and received his Ph.D. in biophysics from Urbana in 1960. He has written many books, papers and reviews on the subject of photosynthesis, including two previous articles for *Scientific American*. Coleman is a postdoctoral research fellow studying the photosynthesis, with the aid of magnetic resonance, at DuPont (Wilmington, Delaware). He received his bachelor's degree in biology and literature from the University of Pennsylvania in 1979 and his Ph.D. from Urbana in 1987 for his work with Govindjee.

To live, therefore, cells need a source of electrons. Anoxygenic photosynthetic bacteria typically oxidize, or draw electrons from, organic acids and simple inorganic compounds (*e.g.*, H_2S). These substances are relatively rare, however; consequently, anoxygenic bacteria survive today mostly in sulfur springs, lake bottoms and similar environments where such molecules are sufficiently plentiful.

Approximately three billion years ago, however, some photosynthetic cells learned how to spread into virtually any environment by tapping the electrons in a nearly ubiquitous substance: water. They evolved the ability to split pairs of water (H_2O) molecules into electrons, protons (H^+ 's, or hydrogen nuclei) and molecular oxygen (O_2). The electrons and protons were energetically useful, the O_2 was simply a by-product. In short, the evolution of O_2 was a breakthrough for photosynthetic organisms not because O_2 was important in itself but because it meant that photosynthetic cells could exploit water and invade new, more diverse environments.

How cells produce oxygen is a far more complicated question. Developing the ability to draw on water as an electron source was no simple feat, and it demanded several modifications to the established photosynthetic mechanism. Because water molecules give up their electrons only grudgingly, the relatively weak oxidant (electron-accepting molecule) that anoxygenic photosynthetic bacteria were able to generate with sunlight had to be replaced with a much stronger one. (One of the most important events must have been the evolution of a highly oxidizing reaction center chlorophyll *a* capable of oxidizing water to O_2 , *i.e.*, having a midpoint redox potential at pH 7 ($E_{m,7}$) more positive than +0.8 eV. In 1977, Paul Jursinic and one of us (G) calculated its $E_{m,7}$ as $\sim 1.2\text{V}$.) Even so, the energy available from a single photon, or a quantum, of visible light is not sufficient to split a water molecule. This problem appears to have been solved by drawing the energy from four photons to split two water molecules, thereby releasing four electrons and four protons. Such a mechanism creates yet another difficulty, however, because the photochemical reaction center can handle only one electron at a time.

To solve that problem, photosynthetic cells developed the special water-splitting catalyst that we call the water-oxidizing clock: a unique biochemical ratcheting mechanism for stabilizing intermediates of the water-splitting reaction so that the electrons could be transferred one by one. In recent years much has been learned about the workings of the water-oxidizing clock and its place in the overall process of oxygenic photosynthesis.

In higher plants, the primary reactions of photosynthesis take place within specialized thylakoid membranes inside the cell structures called chloroplasts. Embedded in the thylakoid membranes are various protein complexes, each of which contributes to the total photosynthetic reaction. (See figure 2 in the revised version of "The Primary Events of Photosynthesis" in *Scientific American*.) The generation of O_2 takes place entirely within the complex of proteins and pigments known as photosystem II, which

is found in the cells of all oxygenic photosynthesizers: cyanobacteria, algae and other plants containing chlorophyll *a*.

The essential task of photosystem II is to act as a tiny capacitor, storing energy by separating and stabilizing positive and negative charges on either side of the thylakoid membrane. To do this, an array of specialized pigments in photosystem II absorbs a photon and efficiently converts this light energy into a widening separation of charge.

Orchestrating the movements in the complex process of converting light energy into a separation of charge requires the collaboration of many specialized polypeptides and proteins in the photosystem. Polypeptides are linear polymers of amino acids arranged in a defined sequence; they are often many hundreds of amino acids in length. Proteins consist of one or more polypeptides folded into intricate, orderly structures.

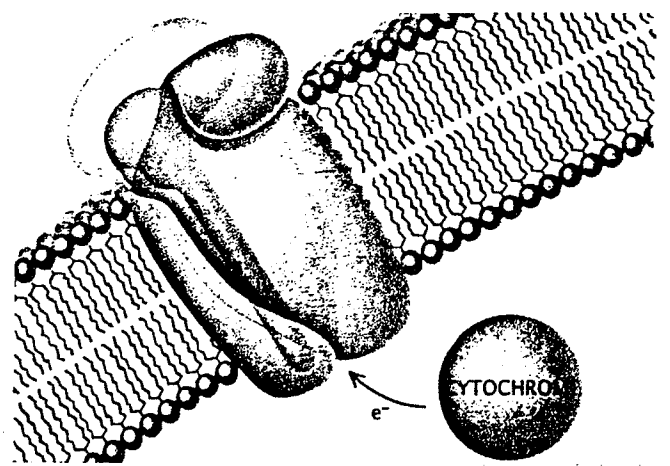
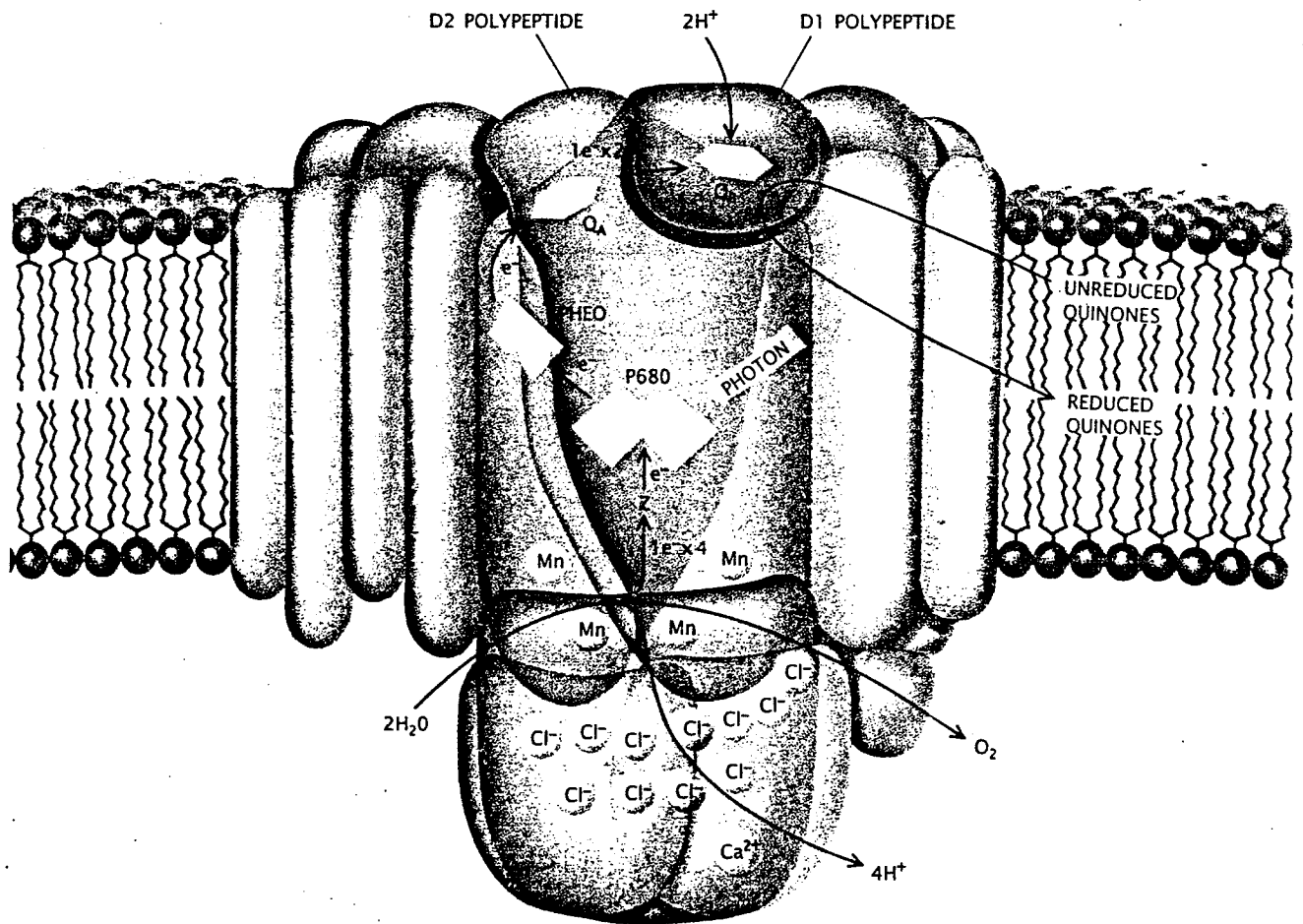
The electron-transfer reactions in photosystem II take place within the so-called reaction center containing about 6 chlorophyll *a* molecules. The major structural components of the reaction center are the two larger polypeptides named D1 and D2 and a smaller flanking protein named cytochrome *b*₅₅₉. A polypeptide with a molecular weight of 33 kilodaltons and at least two others of different weights are bound to the inner surface of higher plant thylakoid membranes. (See the "blob" sticking into the lumen, Figure 1; see p. 52 of the original article for a good colored picture.) These polypeptides serve as a stabilizing matrix for the pigments and other molecules in photosystem II that perform the electron-transfer and oxygen-producing reactions. Other small polypeptides are associated with photosystem II, but their functions are still unknown. Several organic ions and charged atoms—manganese, chloride, calcium, iron and bicarbonate—are involved in catalyzing electron transfer, maintaining the protein structure or regulating the photosystem's activity.

In addition, large numbers of "antenna" chlorophyll molecules collect light energy and funnel it efficiently to the reaction center. Several hundred antenna pigment molecules contained in antenna complexes, labeled LHC-II (light-harvesting chlorophyll *a*/ chlorophyll *b* complex of photosystem II), CP-43 (chlorophyll *a*-protein complex of 43 k Da molecular mass), CP-47 (chlorophyll *a* protein complex of 47 k Da molecular mass), *etc.*, transfer energy to chlorophyll *a* in reaction center complex II.

The structure of photosystem II is very complicated. Many advances in understanding it, however, have come from studies of equivalent complexes in anoxygenic photosynthetic bacteria. The work of Johann Deisenhofer, Robert Huber and Hartmut Michel, who determined the structure of the reaction center in the anoxygenic photosynthetic bacterium *Rhodospseudomonas viridis*, earned them the Nobel prize for chemistry in 1988.

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separate
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Figure 1: Photosystem II is the protein-pigment complex, in the thylakoid membrane, that traps light energy and produces oxygen . The flow of electrons through the photosystem is driven by the absorption of a photon by a special pair of chlorophylls (P680). The process involves many other molecules, including pheophytin (Pheo) and two plastoquinone (Q_A , Q_B) molecules, and such metal atoms and ions as manganese (Mn), chloride (Cl) and calcium (Ca^{2+}). In this simplified model, several entities (2 Chl molecules, 1 Pheo molecule and 1 non-heme iron atom) have been omitted for clarity. Photosystem II reactions near the inner side of the membrane take four electrons from two water molecules and make molecular oxygen; these reactions constitute the water-oxidizing clock. Protons released from these reactions contribute ultimately to the synthesis of adenosine triphosphate. A simpler photosystem from an anoxygenic bacterium (below) lacks a water-oxidizing clock. Electrons from compounds other than water are passed to this photosystem by a cytochrome protein.



There are many differences between anoxygenic bacterial and oxygenic cyanobacterial and green-plant photosynthetic complexes. As previously noted, (anoxygenic) bacteria do not produce molecular oxygen; moreover, they depend not on chlorophyll but on the pigment bacteriochlorophyll, which absorbs light maximally at a much longer wavelength and is a much weaker oxidant. It just cannot oxidize water. Yet bacterial photosynthetic complexes do catalyze the essential reaction that converts light into an electrochemical potential gradient across a biological membrane [see "Molecular Mechanisms of Photosynthesis," by Douglas C. Youvan and Barry L. Marrs; *SCIENTIFIC AMERICAN*, June, 1987].

From these bacterial studies, it appears that the electron-transporting mechanism in the reaction center of photosystem II has, at least, five components: a "special pair" of chlorophyll *a* molecules that act as the primary donor of an electron; a secondary electron donor named Z that reduces the oxidized chlorophyll (that is, it replaces the electron that the chlorophyll has lost); pheophytin *a*, a pigment that accepts an electron from the chlorophyll; a primary plastoquinone electron acceptor, Q_A ; and a secondary plastoquinone electron acceptor, Q_B . The function of four other chlorophyll *a* molecules and another pheophytin *a* molecule is not known.

As just noted, the reaction center chlorophyll is believed to consist of a special pair of chlorophyll *a* molecules that seem to be chemically identical to many of the antenna pigments but are functionally different. This pigment is named P680 because it most strongly absorbs light at a wavelength of 680 nanometers, its longest wavelength peak. It does, of course, have its so-called Soret peak in the blue region of the spectrum.

Beginning in 1988 the site-directed mutagenesis of a cyanobacterium *Synechocystis* sp. PCC 6803 by Richard J. Debus, Bridgette A. Barry, Gerald T. Babcock and Lee McIntosh at Michigan State University, and Willem F. Vermaas of Arizona State University and his colleagues in France (A.W. Rutherford and O. Hansson) helped to identify D (a slow donor to P680⁺) as one of the amino acids (known as a tyrosine) within the polypeptide D2. More recently, Debus and co-workers and Bruce Diner and his research group, at DuPont Company, have proved, also by site-directed mutagenesis of *Synechocystis* sp. PCC 6803, that "Z" (the fast and natural donor to P680⁺) is a tyrosine residue (Tyrosine 161) within the D1 polypeptide. The quinone Q_A (on D2) is tightly bound to the photosystem II complex, but Q_B (on D1) can diffuse between the protein complex D1 and the lipid in the membrane when it is Q_B^- or when it has accepted two electrons. However, it is tightly bound when it is in the Q_B state.

During photosynthesis the antenna pigments (located in several pigment-protein complexes) absorb a photon and funnel this energy to the P680 in the reaction center. There, the excitation energy is converted into a charge separation when the P680 enters an excited state and quickly passes one electron to a nearby pheophytin molecule [see figure 2]. The pheophytin now carries an excess negative charge, whereas there is a positively charged "hole" on the P680 because it has lost an electron; the

P680 has become a P680*. The separation of the charges gets wider when the pheophytin passes its extra electron to Q_A . The "electrical distance" increases still more when Z donates an electron to the P680* and picks up the positive charge and the Q_A donates its extra electron to Q_B .

The transfers of charge take place rapidly, especially the initial transfer of an electron from the excited P680 to pheophytin, which occurs within a few trillionths of a second (3ps at 4 C). This was shown by one of us (Govindjee) in collaboration with Michael R. Wasielewski and Douglas G. Johnson of the Argonne National Laboratory and Michael Seibert of the Solar Energy Research Institute in Golden, Colorado.

The stepwise transfer of electrons succeeds in pulling far apart the mutually attractive positive and negative charges. Yet the photosynthetic cycle in photosystem II is not complete until all the components of the reaction mechanism are electrically neutral again and ready to begin the charge-separation process anew. How does the Q_B eliminate its negative charge, and how does the Z regain the electron it has lost?

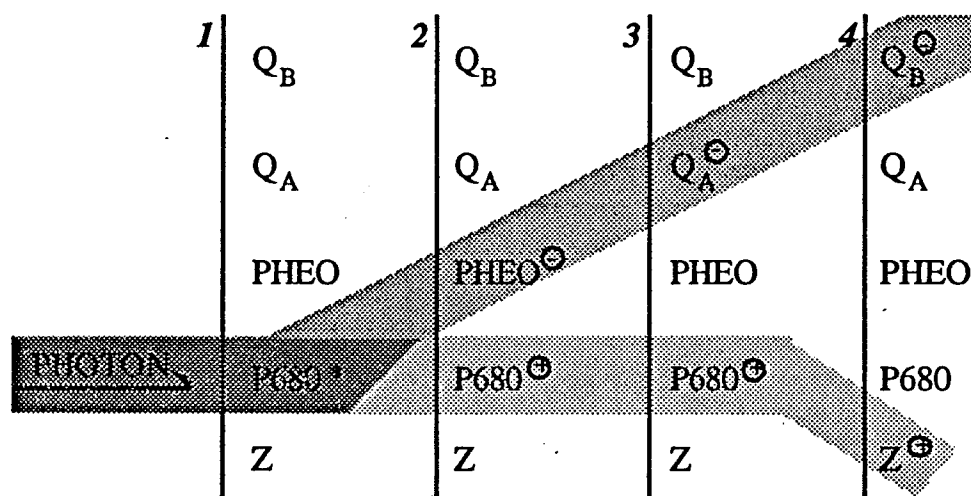


Figure 2

Figure 2: Stepwise electron transfer in the photosystem II reaction center stores light energy in the form of separated positive and negative charges. When the P680 absorbs a photon or an exciton, it enters an excited state and becomes P680* (1). The P680* donates an electron to a pheophytin molecule and is left with an electron deficit, or positive charge (2). The pheophytin's negative charge is passed to a primary plastoquinone molecule, Q_A (3). Finally, the P680* takes an electron from Z, an amino acid tryosine, in the D1 protein and Q_A passes its extra electron to Q_B , another plastoquinone molecule (4). The electron-transfer chain returns to its original state when Z accepts an electron from the water-oxidizing clock and the doubly reduced Q_B (formed after two light reactions) is replaced by an unreduced plastoquinone Q_B .

At the Q_B end of the system, the answer is relatively simple. After the Q_B has acquired two electrons and two protons through two photon-absorption cycles, the doubly reduced Q_B diffuses out of the photosystem II complex and is replaced by an unreduced Q_B . The Q_B is, thus, known as the "two electron gate". The electrons and protons on the freely moving Q_B are carried to still another complex (the cytochrome b_6/f complex) in the photosynthetic pathway. The protons released on the inner side of the thylakoid membrane are eventually exploited to make adenosine triphosphate, an energy-storing compound essential for cellular metabolism.

At the opposite end of photosystem II, it is much harder for the Z^+ to obtain the electron it needs to return to its original state. The electron must come from some oxidizable substance that is available in the cell's environment. Organic acids (such as acetate, malate and succinate) and simple inorganic compounds (such as sulfide and thiosulfate) can be good electron sources, and they are the ones that anoxygenic photosynthetic bacteria exploit; in these bacteria, which lack Z , a cytochrome shuttles an electron to the oxidized special pair of bacteriochlorophylls in their reaction centers. A molecule far more abundant than organic acids—and therefore a potentially richer source of electrons—is water. Yet, although the oxidizing strength of $P680^+$ is great, it is not sufficient by itself to strip water molecules of their electrons.

The problem is that the water-oxidizing reaction releases four electrons simultaneously, whereas $P680^+$ can accept only one electron at a time. It therefore became clear to investigators a few decades ago that there must be a catalytic site near Z and the $P680$ that can, in effect, prolong the oxidation reaction. This water-splitting catalyst must associate with pairs of water molecules and stabilize them during a gradual oxidation process in which electrons are taken away one at a time. The search for this mechanism eventually led to the discovery of the water-oxidizing clock.

An important clue to how this mechanism works was derived from the observation in several laboratories that not all the electrons reach the $P680^+$ chlorophyll molecule at the same rate. Instead the observed transfer time for the electrons varies periodically. This has been demonstrated by experiments in which membranes containing photosystem II reaction centers are placed in darkness and then exposed to brief flashes of light. Each flash is not only very intense but also as brief as possible, so that it sends (on average) only one photon into the photosystem. (Such a light flash is called a "single turn-over" flash.) The observed result is that $P680^+$ recovers an electron in darkness at different rates, depending on the number of light flashes. For example, the time for half of the $P680^+$ s to convert back to $P680$'s is approximately 20 billionths of a second (20 ns) after the first and fifth flashes but is much longer after the second, third and fourth flashes. The change in recovery time varies cyclically every four flashes. The four-point periodicity suggests that a cyclic reaction with four steps donates electrons to the reaction center.

These studies of the behavior of P680 were especially important. Pierre Joliot (grandson of Marie and Pierre Curie) of the Institute of Physicochemical Biology in Paris had previously demonstrated in 1969 that there was a four-point periodicity in the photosynthetic production of oxygen. With a highly sensitive platinum-electrode measuring device that responded to traces of oxygen, Joliot measured the amount of gas that evolved after a series of flashes. There was no O_2 evolution after the first flash and none (or very little) after the second, but there was a maximum release after the third. Thereafter, the amplitude of the O_2 yield oscillated with a period of four until the differences gradually were damped out, or diminished. (See Figure 3)

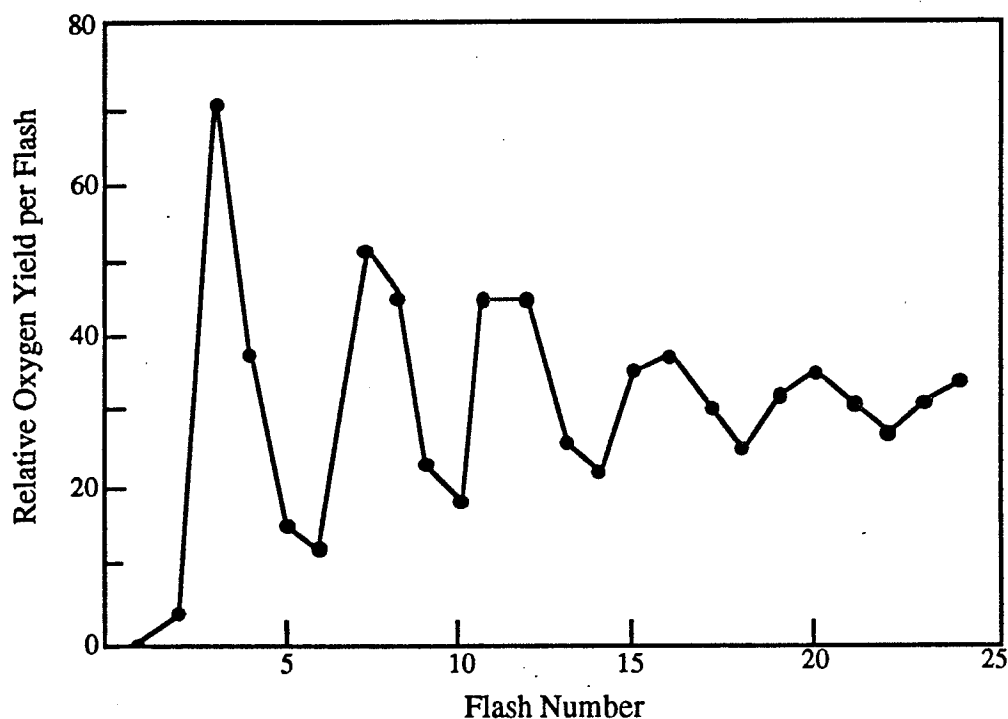


Figure 3

Figure 3: Yield of oxygen from photosynthetic membranes exposed to a series of brief flashes oscillates with a four-point periodicity. It is highest after the third flash and peaks again four flashes later, but the variation in the amplitude gradually decreases as the number of flashes increases. The occurrence of the peaks and the damping of the oscillation are explained by the four-step cycle of the water-oxidizing clock.

Bessel Kok (who had earlier discovered P700, the reaction center chlorophyll *a* of photosystem I) of the Martin Marietta Laboratories in Baltimore proposed a simple hypothesis in 1970 to explain Joliot's results, an idea that came to be known as the water-oxidizing clock or cycle. Kok suggested that the oxygen-producing complex in

photosystem II could exist in several different, transient states of oxidation that he called S states. He could not define the chemical nature of the S states precisely, but he hypothesized that each S state made a unique contribution to a four-stage cyclic mechanism.

Kok suggested that, in darkness, the clock settles into one of two S states: S_0 or S_1 . The predominant (and more stable) state is S_1 , which has one more oxidizing equivalent than S_0 ; in other words, the complex of molecules corresponding to S_1 has one fewer electron than does the S_0 complex. The chemical basis for the predominance of S_1 is not known.

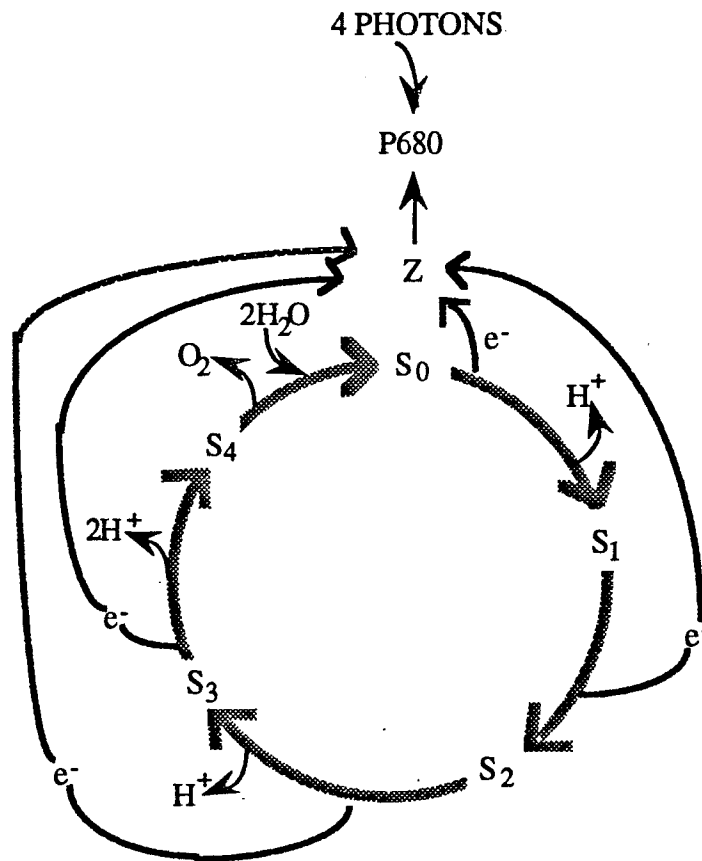


Figure 4

Figure 4: Water-oxidizing clock is a cyclic mechanism that supplies electrons to the P680 chlorophylls in the photosystem II reaction center. As each photon is absorbed by the P680, the clock advances by one S state, or oxidation state, and thereby releases one electron (e^-). When the clock reaches S_4 , it spontaneously releases an oxygen (O_2) molecule and reverts to the S_0 state to close the cycle. The proton (H^+) release pattern shown as 1, 0, 1, 2, starting with S_0 , is observed in thylakoids at a particular pH. It is now known that this is not directly related to the intrinsic release of H^+ 's during the O_2 clock. Under many experimental conditions, the H^+ release pattern is 1, 1, 1, 1.

After one flash of light, P680 becomes P680⁺ and must eventually be reduced by an electron. Kok hypothesized that the clock must undergo a change that boosts it into the next highest oxidation state: a clock that starts at S₁ goes to S₂, and one that starts at S₀ goes to S₁. The transition occurs because one electron is released from the clock to convert P680⁺ back to P680. A second flash creates another P680⁺ and boosts the S₂s to S₃s, and a third flash converts the S₃s to S₄s. When the clock reaches the S₄ state, it has released four electrons and is ready to complete the water-splitting reaction. The clock then removes four electrons from the two bound water molecules, releases O₂ and drops from S₄ back to S₀, making it possible for the cycle to begin again.

This situation is not unlike that of a base runner in baseball: the player must tag all four bases in sequence to end up where he started. If the player misses a tag, he may be forced to retreat; similarly, there is a possibility that the clocks will not progress smoothly from one state to the next. A small probability exists, for example, that S₁ will not change to S₂ after a flash because the photosystem did not utilize the photon efficiently either because it was not freed of P680⁺ or Q_A⁻ or because it back reacted right away. We call these "misses". There is also a low probability that a photosystem will absorb two photons during one flash (if the flashes are not extremely short) and that the water-oxidizing clock may advance in one step from S₁ to S₃ via S₂. We call these "double hits".

Kok's mechanism explained Joliot's observations of the clock's oxygen-producing behavior. Because most of the clocks in a dark-adapted sample are in the S₁ state, the maximum release of oxygen takes place after the third flash, when the clocks change from S₃ to S₄ to S₀ and spontaneously release oxygen. The clocks that began in the S₀ state release O₂ after the fourth flash, which is why there is a small oxygen release then.

Those random "errors" (called misses and double hits) that occur when a few clocks fail to advance during a flash or when they advance by two S states can account for the gradual damping-out in the O₂-release oscillations. These processes slowly desynchronize the turnover of the clocks in the sample. After many flashes, there is an equilibrium such that the numbers of S₀, S₁, S₂ and S₃ clocks are roughly equal, and the yield of oxygen after each flash remains steady. The situation is analogous to that of a room filled with grandfather clocks: initially they may all chime loudly and synchronously on the hour, but as the clocks variously gain or lose time, the room begins to reverberate with a continuous soft chime.

Joliot and Kok's discovery of the water-oxidizing clock replaced the black box of oxygen production with a new theoretical mechanism. The theory did not explain, however, the physical makeup of the clock or the interactions of the clock with water molecules. A long search soon began for the chemical nature of the charge accumulator in the clock—the material or materials whose variable oxidation states constitute each of the S states.

From the beginning it was assumed that this elusive chemical entity was a metal atom. Protein-bound atoms of transition metals, such as manganese, iron and copper, are good candidates for catalyzing oxidation-reduction reactions because of their ability to donate and accept electrons alternately. Manganese (Mn) is believed to make up at least part of the charge accumulator because, as has been long known, O₂ production does not take place unless there are four atoms of manganese in photosystem II for every P680 molecule. Manganese is known to catalyze electron-transfer reactions in other enzymes. It can also assume several relatively stable oxidation states, from +2 to +7; that is, manganese ions can variously share between two and seven electrons with other atoms. When the metal is bound to a large molecule such as a protein, these oxidation states are usually abbreviated as Mn(II), Mn(III) and so on. In photosystem II, only Mn (II), Mn (III) and (IV) have been suggested to be involved.

Metal-containing proteins have been analyzed extensively by the general technique called spectroscopy because some metal complexes absorb particular forms of electromagnetic radiation. If this absorption is measured carefully, it can serve as a spectroscopic "fingerprint" of the protein-bound metal and provide clues to its nuclear or electronic structure. Spectroscopy is especially well suited to the study of manganese compounds. Many of the biologically relevant manganese complexes are "paramagnetic": the manganese atom contains electrons with unpaired spins, and these electrons, like tiny bar magnets, interact strongly with applied external magnetic fields.

Several highly sensitive measuring techniques have exploited the paramagnetic properties of manganese, most notably electron paramagnetic resonance (EPR) spectroscopy. With EPR, changes in the electronic structure of the manganese complex that follow the absorption of light by photosystem II have been studied. Another informative approach has been nuclear magnetic resonance (NMR) spectroscopy, which can measure the properties of the manganese atoms indirectly by monitoring protons in the water molecules that are in contact with the manganese. While working at the University of Illinois at Urbana-Champaign in the mid-1970's, Thomas J. Wydrzynski (then a graduate student of one of us, Govindjee) pioneered, in 1975, the use of NMR to study dynamic changes in the oxidation state of manganese.

X-ray spectroscopy techniques have made valuable contributions to the study of oxidation states and of the physical environment of the manganese atoms in photosystem II. Other studies of the chemical composition of S states have used optical spectroscopy, because manganese complexes have unique absorption bands in the ultraviolet region of the electromagnetic spectrum.

It is worth pointing out, however, that despite the wide range of applicable spectroscopic techniques, two major difficulties have humbled scientists studying the photosynthetic membrane. First, the membrane is complex, and many of its components have overlapping (congested) absorption spectra. Second, because neither the structure nor the chemical nature of the photosystem II complex is known precisely, the

data from spectroscopic analyses of the water-oxidizing clock cannot be interpreted definitively. As a result, we do not yet know conclusively what chemically constitutes the various S states. It has been possible, nonetheless, to develop a tentative, although a quite reasonable, picture.

Pioneering studies on low-temperature EPR by G. Charles Dismukes and Yona Siderer of Princeton University showed, in 1981, that the S_2 state indeed was from a Mn complex, as it oscillated with maxima after flash 1 and 5. Those authors suggested that the S_2 and S_3 states involve multinuclear complexes with as many as four manganese atoms. For example, the S_2 state may be a mixed-valence group consisting of three Mn(III) atoms and one Mn(IV) atom.

It is clear that the manganese atoms undergo dynamic changes, including changes in their oxidation states, during the S-state transitions. A four-point periodicity has been observed in the manganese oxidation state changes, as Kok's model had suggested. In spite of this observation, particularly that of T. Ono and coworkers in Japan (1992), a possibility remains that the manganese atoms do not become consistently more oxidized throughout the cycle. S_2 is more oxidized than S_1 , and S_1 is more oxidized than S_0 , but there is still a question whether manganese oxidation states change from S_2 to S_3 . It seems, then, that the positive charge the clock acquires during its transition from S_2 to S_3 may be carried on some feature of the clock other than the manganese atoms. One of us (Govindjee), together with Subhash Padhye, Takeshi Kambara and David N. Hendrickson of the University of Illinois at Urbana-Champaign, proposed in 1986 that the amino acid histidine in one of the proteins in the clock could possibly store a positive charge. This concept has received experimental support from recent observations in the laboratories of A.W. Rutherford and Jerome Lavergne in France, at least in Ca-depleted Photosystem II membranes.

Equally pioneering work by Melvin P. Klein, Kenneth Sauer and their co-workers at the University of California at Berkeley and by Robert R. Sharp and his colleagues at the University of Michigan at Ann Arbor has helped define the oxidation states of some of the manganese atoms more precisely. Tentatively, S_0 has been identified with the presence of Mn(II), S_1 with Mn(III) and S_2 with Mn(IV). Both Mn(II) and Mn(III) appear to be stable and long-lived in photosystem II; these observations corroborate Kok's prediction of stable S_0 and S_1 states. In contrast, the Mn(IV) associated with S_2 is a relatively transient intermediate. Recent evidence collected in the laboratory of Horst T. Witt of the Technical University in Berlin indicates that during the S_0 -to- S_1 transition, an Mn(II) ion converts to an Mn(III) ion. The only conversions observed during subsequent transitions are from Mn(III) to Mn(IV).

In summary, dynamic changes in the oxidation states of the manganese atoms bound within photosystem II unquestionably correspond to changes in the S states of Kok's clock. The precise chemical and electronic configurations of these states are still uncertain and are under study.

Various experiments have indicated that manganese is probably not bound directly to

any of the small extrinsic polypeptides in the photosystem II complex. This leaves the large D1 and D2 polypeptides as the most likely sites for manganese binding. The two of us had proposed in 1987 that manganese-binding sites may exist on the D1 and D2 polypeptides on the inner side of the thylakoid membrane, but some others had suggested that manganese is bound across the interface between the D1, D2 and 33-kilodalton polypeptides. Recent results suggest that among other amino acids, Aspartic-171 (on D1) and Glutamic-69 (on D2) may be involved in Mn-binding.

X-ray spectroscopy by Klein, Sauer and their collaborators at Berkeley has revealed some details of the arrangement of the manganese atoms. In the S_1 state, two of the atoms appear to be part of a binuclear complex and are separated by only 2.7 angstroms. (One angstrom (\AA) is one tenmillionth of a millimeter.) The other pair of manganese atoms is separated by a larger (3.3\AA) distance. The atoms can be imagined as being at the four corners of a trapezoid. Because of all these studies, much more is now known about how manganese atoms might catalyze the removal of electrons from water to reduce $P680^+$.

Electrons, however, are not the whole story: the water-splitting reaction also produces four protons. Are all four protons released at once, simultaneously with the release of O_2 , or are they liberated sequentially along with the electrons? This question could, in principle, be answered by careful measurements of proton release in response to a series of flashes. Because the release of protons increases the acidity of the surrounding fluid, the timing of proton release can be studied with electrodes and dyes that are highly sensitive to acidity. C. Frederick Fowler of Martin Marietta and, soon thereafter, Satham Saphon and Anthony R. Crofts, then at the University of Bristol in England, discovered that the four protons are released sequentially. One is released during the S_0 -to- S_1 transition, none during S_1 -to- S_2 , one during S_2 -to- S_3 and two during the S_3 -to- S_4 -to- S_0 transition. Recent results in several laboratories, particularly W. Junge in Germany suggest that, depending upon experimental conditions, this release pattern could be 1, 0.5, 1, 1.5 or 1, 1, 1, 1 without much change in the efficiency of O_2 evolution.

These findings could have important implications for the mechanism of the water-oxidizing clock, although their interpretation depends on whether the released protons come from the water molecules directly or from some other source, such as the polypeptides that bind the manganese atoms. If the protons come from the water, then the water molecules must be undergoing some chemical changes prior to the S_4 state. Conversely, if the sequentially released protons come directly from the polypeptides (and are later replaced by protons from the water molecules), then no water oxidation may occur until the final S_4 -to- S_0 transition. In view of the non-constancy of the proton release, mentioned above, it is now considered likely that these proton releases are from the proteins involved, not directly from water. Of course, it must be water that restores these lost protons. What it means is that these proton releases may not provide any clue as to water chemistry.

Regardless of the source of the protons, it now seems likely that the higher S states (particularly S_2) accumulate some net positive charge. It is possible that a negatively charged ion may be needed to stabilize this positive charge, which could explain the observation that ions such as chloride are essential for keeping the water-oxidizing clock running. Seikichi Izawa of Wayne State University in Detroit was one of the first to demonstrate that chloride ions can turn on the water-oxidizing clock.

In collaboration with Herbert S. Gutowsky and his colleagues at the University of Illinois at Urbana-Champaign in 1982, we introduced NMR techniques to monitor the binding of chloride ions to photosynthetic membranes. In early studies, Ion C. Baianu, Christa Critchley and one of us (Govindjee) showed that chloride ions associate with and dissociate from isolated thylakoid membranes freely and rapidly. These findings led us to speculate in 1983 that the binding of a negatively charged chloride ion might be linked with the arrival of a positive charge on the water-oxidizing clock from $P680^+$ and that the release of the chloride ion might coincide with the release of protons. NMR experiments by Christopher Preston and R. J. Pace of the Australian National University in Canberra suggested that chloride ions bind more tightly in the S_2 and S_3 states than in the S_0 and S_1 states. This finding is consistent with the more positively charged character of the higher S states. Unfortunately, no firm conclusions are yet possible.

Peter H. Homann of Florida State University and his associates and the present authors have suggested that chloride probably binds to positively charged amino acids on the proteins of the clock. Working with Gutowsky, we have made observations of chloride binding in photosystem II complexes from spinach. Our measurements indicate that several chloride ions bind to the clock. It had been suggested that the function of chloride ions in the water-oxidizing clock may be to expedite the release of protons from water. In doing this, the chloride ions may increase the efficiency of the water-oxidation reactions, or they may stabilize the charged manganese ions in the higher S states, or they may do both. The role of chloride is, however, still controversial; it may turn out that chloride only functions to organize the photosystem II proteins into a stable structure.

Another ion, calcium (Ca^{2+}), has been suggested to be essential for both the oxidation of water and the operation of the photosystem II reaction center, and it also appears to be intimately involved with the function of chloride. The present authors pointed out in 1987 that there was a regional homology between the 33 kDa extrinsic polypeptide and the various intestinal calcium binding proteins. Experiments in several laboratories suggest that calcium and chloride ions can functionally replace two of the polypeptides at the bottom of photosystem II that are involved in the production of molecular oxygen. It has also been observed that the removal of calcium ions seems to block both the turnover of the water-oxidizing clock (by interrupting the S_3 -to- S_4 -to- S_0 transition) and the fast reduction of $P680^+$ to P680. The discussed role of Ca^{2+} has included it acting as a "gate" to bring H_2O molecules to the Mn complex of the oxygen involving complex. It seems likely, therefore, that calcium has a structural or

regulatory role in photosystem II. Calcium has been shown to play an important part in controlling a wide variety of proteins in other biological systems: it switches the activity of the proteins on and off and maintains their three-dimensional structure. The calcium ions in photosystem II may put the polypeptides of the water-oxidizing clock into the correct functional conformation.

The elaborate mechanism that makes oxygen during photosynthesis is only one small part of the full photosynthetic pathway in oxygen-producing organisms. Although the general details are similar among all photosynthetic species, significant differences have arisen in the course of evolution. Most analyses indicate that the differences between the photosystem II of prokaryotic cyanobacteria and that of eukaryotic plants are relatively minor, which suggests that cyanobacteria are ancestors of, or otherwise closely related to, plants. An interesting difference lies in the fact that cyanobacteria have replaced the 17 and 23 k Da extrinsic polypeptides with a 9-12 kDa polypeptide and a cytochrome *c* and that a cyanobacterial mutant can even function without the extrinsic 33 k Da polypeptide. The differences between the reaction centers of oxygenic cyanobacteria and those of many other anoxygenic photosynthetic bacteria are much more pronounced, revealing a clear division in the evolutionary pathway. More detailed studies of the photosystems by molecular genetics, X-ray crystallography and spectroscopy will undoubtedly refine understanding of the evolution of life.

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The structural basis of photosynthetic light reactions in bacteria

Johann Deisenhofer, Hartmut Michel and Robert Huber

The primary events of photosynthetic light reactions excel in efficiency and speed. They occur in highly organized protein-pigment aggregates that have been well characterized, both chemically and functionally. The recent structural characterization at the atomic level of some important components of the primary photosynthetic processes in bacteria helps our understanding of the underlying physical principles.

Simple biochemical reactions are catalysed by small proteins with simple polypeptide chain folds. More complicated reactions require the cooperative action of distinct domains into which a protein molecule is folded. These domains may move or change conformation during a catalytic cycle to provide the proper environment for a particular catalytic step or to bring reacting components into proximity (for review see Ref. 1). In oligomeric proteins like hemoglobin² cooperation of the closely associated subunits influences their functional properties profoundly; in particular, it allows homotropic or heterotropic regulation. Generally, complex biochemical processes seem to require the interaction and cooperation of protein domains and protein subunits.

Photosynthetic light reactions are complex processes brought about by the cooperation of proteins and pigments organized in a highly ordered manner. Photosynthesis occurs in bacteria (photosynthetic green and purple bacteria

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and cyanobacteria) and in chloroplasts in specialized photosynthetic membranes, which are either infoldings of the cell membrane (in prokaryotes) or of the inner chloroplast membrane (in eukaryotes). Plants, algae and cyanobacteria (blue-green algae) carry out oxygenic photosynthesis with water as the electron donor. Other bacteria, including purple bacteria, utilize an alternative to water as the primary electron donor for photosynthesis. There are two photosystems in chloroplasts and cyanobacteria. Green and purple bacteria use only one photosystem. In photosynthesis, light energy is absorbed and leads to charge separation across the photosynthetic membrane. In secondary reactions the absorbed energy is further converted into chemical energy and stored. The primary events of photosynthesis are the focus of interest for biologists, chemists and physicists who seek to explain the speed and high yield of these reactions. Recent progress in the structure analyses of protein complexes involved in the primary photosynthetic reactions has extended our understanding of these processes and is discussed here^{3,4}.

Several *TIBS* articles deal with other aspects of photosynthesis⁵⁻⁸. Series of reviews referred to in this article are collected in Refs 9 and 10.

Light-harvesting

Only a small portion of incident light would be absorbed solely by the chlorophyll pigments associated with reaction centers, simply because of their limited numbers. To enable them to absorb more light, the reaction centers are associated with light-harvesting complexes (LHC), which may be located within the photosynthetic membrane, or form layers or antenna-like organelles in association with the photosynthetic membrane. Rhodophytes and cyanobacteria have particularly intricate light-harvesting systems, the phycobilisome organelles peripheral to the thylakoid membrane. These phycobilisomes have been characterized functionally and structurally in considerable detail. Because they show maximal absorption of light at a shorter wavelength than photosystems I and II, a large part of the spectrum of sunlight is used. The phycobilisomes are mostly hemi-discoidal organelles, with a central core of two to three cylindrical units from which six rods radiate. They are large aggregates of protein subunits containing open-chain tetrapyrrole pigments. Within the antenna-like rods there is a polar arrangement of three compounds - phycocerythrin (PE), phycocyanin (PC) and allophycocyanin (APC). PE is located at the tips, PC in the center and APC at the bottom of the rods (for a review see Ref. 11). The light energy flows along an energy gradient in the antenna to the photosynthetic membrane, as the wavelength of the absorption maxima of the antenna components increases from PE to APC (Fig. 1). From the antenna, the

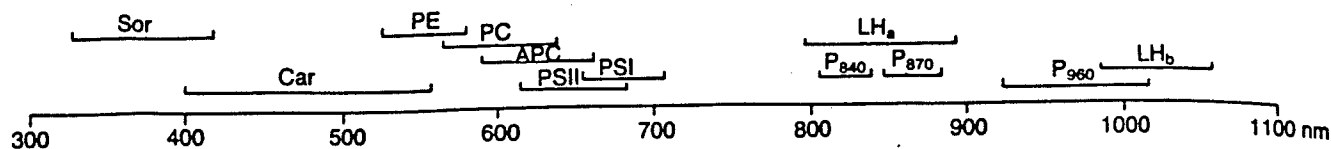


Fig. 1. The main absorption bands of light-harvesting protein complexes and photosystems in bacteria and plants. PE, phycoerythrin in cyanobacteria; PC, phycocyanin in cyanobacteria; APC, allophycocyanin in cyanobacteria; LH_a, light-harvesting pigment-protein complexes in bacteriochlorophyll a-containing purple bacteria; LH_b, light-harvesting pigment-protein complexes in bacteriochlorophyll b-containing bacteria; P₈₇₀, photosynthetic reaction center in bacteriochlorophyll a-containing purple bacteria (absorption at primary donor); P₈₄₀, photosynthetic reaction center in green bacteria (absorption at primary donor); P₉₆₀, photosynthetic reaction center in bacteriochlorophyll b-containing purple bacteria; PS I,II, photosynthetic reaction center in chloroplasts and cyanobacteria; Car, carotenoids of various bacterial light-harvesting pigment-protein complexes; and Sor, sorbent bands of chlorophylls and bacteriochlorophylls in various photosynthetic systems.

light energy flows into the photosynthetic membrane to membranous light-harvesting protein-pigment complexes, which distribute the light energy in the plane of the photosynthetic membrane until it is trapped primarily by photosystem II.

From crystallographic analyses, a very detailed picture of the tetrapyrrole chromophores and the protein components in the phycobilisome antenna has emerged³. The building blocks of the rods are trimeric aggregates of (αβ) protein units that form hollow discs. The protein-linked chromophores are in extended conformation and absorb light of long wavelengths. Isolated tetrapyrroles prefer a cyclic conformation and absorb light of short wavelengths. The

arrangement of the chromophores seems to be optimally adapted to their two functions: light absorption and conduction of light energy along the antenna rods. The three different sets of chromophores, two in the β- and one in the α-subunit, have their transition dipole moments approximately aligned along, and perpendicular to, the antenna axis, respectively. This arrangement is repeated in 3-fold symmetry and ensures absorption of light from all directions. Efficient energy transfer along the antenna axis by inductive resonance (Foerster mechanism) requires parallel alignment of the dipole moments of the chromophores along the stacked discs. This is indeed found (Fig. 2). The triad covers a wider range of

directions than a diad or a tetrad, and is thus clearly the preferred symmetry element in protein aggregates involved in light reactions. It occurs in aggregates of a light-harvesting protein from a green photosynthetic bacterium¹², in crystalline layers of the membranous light-harvesting chlorophyll *ab* protein complex from chloroplasts¹³ and in layers of bacteriorhodopsin¹⁴.

In purple bacteria, small bacteriochlorophyll (BChl) binding peptides, and carotenoids within the photosynthetic membrane, harvest light and transmit its energy to the reaction center. The protein-pigment complexes provide additional absorbing chromophores which, if appropriately oriented, are able to absorb light from all directions. They extend the range of absorbed light to wavelengths of 800–900 nm (in bacteria containing BChl *a*). In bacteria containing BChl *b*, like *Rhodospseudomonas viridis*, the reaction center has its longest wavelength absorption band at 960 nm – a slightly shorter wavelength than the associated light-harvesting complexes which absorb maximally at 1020 nm (Fig. 1).

The reaction center

In the photosynthetic reaction center charge separation occurs upon absorption of light energy. An electron is transferred from the primary donor to the primary acceptor, a quinone (Qa). Qa reduces a secondary quinone (Qb), and Qb equilibrates with the quinone pool of the membrane. The reduced quinones interact with other electron carriers which, after a complex sequence of reactions, transfer an electron back to the oxidized primary donor of the reaction centers. The cyclic electron flow is associated with vectorial H⁺ transport across the photosynthetic membrane and eventually coupled to ATP synthesis. The primary electron donor was assumed (correctly) to be a bacteriochlorophyll dimer on the basis of its spectral properties, but other arrangements were discussed. The excited primary donor

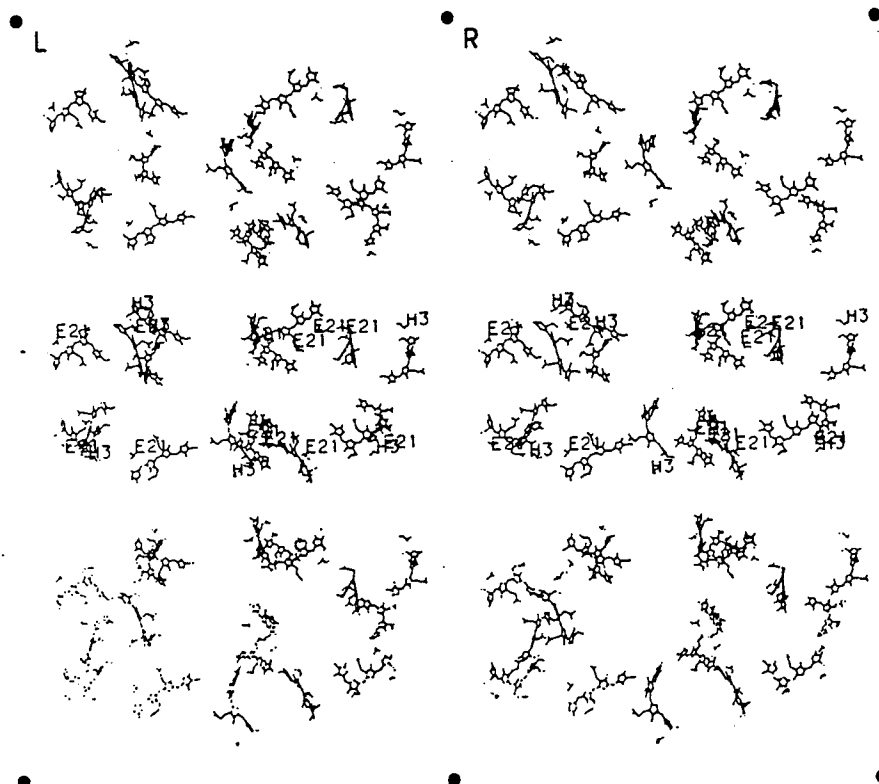


Fig. 2. The arrangement of the tetrapyrrole pigments in stacked disks in crystals of C-phycocyanin from the cyanobacterium *Agmenellum quadruplicatum* derived from the structure of C-phycocyanin from the cyanobacterium *Mastigocladus laminosus*^{3,20}. E21 indicates the pigment attachment sites to helical residues E21 of the α and β subunits. H3 is the attachment at helical residue H3 of the β subunit. (R = right, L = left.) Three hexamer disks are shown corresponding to an antenna rod in the phycobilisome.

releases an electron which is transferred to an intermediate acceptor, a bacteriopheophytin. This reduces Qa which is in close proximity to an inorganic iron. From Qa the electron is transferred to Qb. The role of the inorganic iron in the process is unclear. This picture was derived from biochemical investigations and spectroscopic and kinetic analyses of the photosynthetic reactions of bacteria, which have provided a wealth of functional and structural information (for reviews see Refs 9 and 10).

The reaction center crystals

Crystal structure analysis of the reaction center complex of the purple bacteria *R. viridis*⁴ provides the means to evaluate the information on reaction centre reactions in terms of the three-dimensional atomic structure of the components. The crystals of the reaction centers of *R. viridis* contain protein, pigments, detergent, water and inorganic salt¹⁵. They are surprisingly well ordered for a protein complex of this size and complexity. Although the crystals grow at room temperature they are kept slightly below 0°C to preserve the crystalline order during X-ray diffraction experiments. It is interesting to note that crystals of porin, an integral membrane protein of bacterial cells, also require sub-zero temperatures for optimal ordering¹⁶. This may be a requirement of the detergent phase within the crystals. Heavy-metal derivatives of reaction centers were prepared by soaking with water-soluble heavy-metal compounds. These experiments followed the same course as those with crystals of water-soluble proteins, indicating that the reaction center crystals contain water-filled channels through which the compounds can reach parts of the reaction center complex. A preliminary analysis of the crystal packing showed that the crystal contacts are mainly between parts of the *H*-subunits and the cytochromes of symmetrically related molecules (see Fig. 5). The contacting segments are probably exposed to water when the protein is in solution. The contacts in the crystals of the reaction center of *R. viridis* are probably similar to those of water-soluble proteins, explaining in part the 'normal behaviour' in the preparation of heavy-metal derivatives. This finding supports the strategy used to crystallize this protein complex¹⁷.

The structure of the reaction center

The reaction center *R. viridis* consists of four different protein components. It has a molecular mass of about 150 kDa

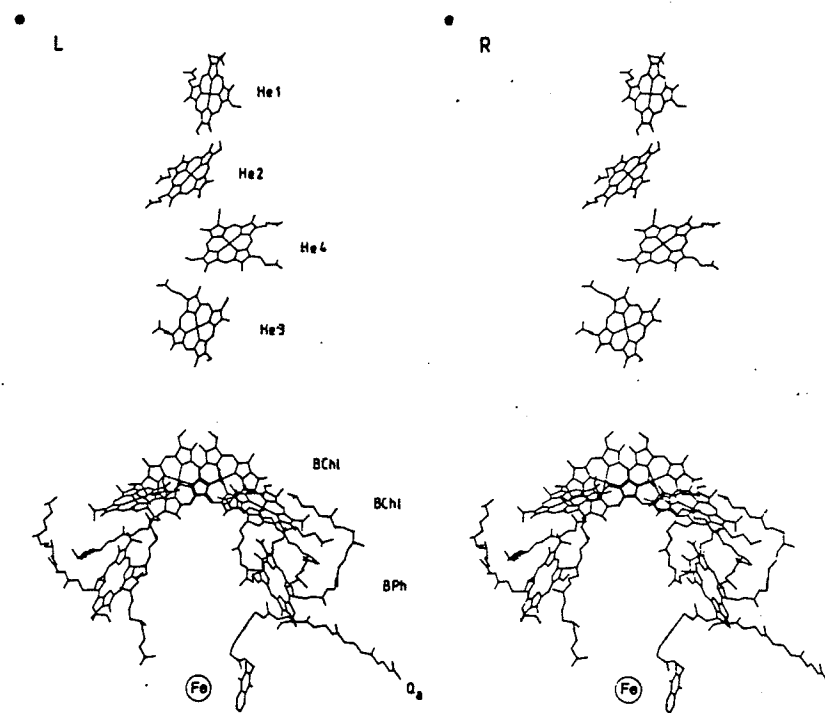


Fig. 3. Stereo-view of the reaction center chromophores. The local diad is vertical. The four heme groups (He1-He4), the bacteriochlorophylls (BChl), the bacteriopheophytin (BPh), the quinone (Qa) and the iron (Fe) are shown.

and a volume of approximately $30 \times 70 \times 130 \text{ \AA}^3$. The four components are: a *c*-type cytochrome, the L- and M-subunits associated with the bacteriochlorophyll chromophores, and the H-subunit. The chemical characterization of the protein components of *R. viridis* and their genes by sequence analysis is still incomplete, but the fragmentary analysis was important in identifying the polypeptide chains in the electron density map. The amino acid sequences of reaction center proteins of *R. capsulata* and *R. spheroides* have been determined and it is clear that there is considerable conservation between sequences of these species¹⁸⁻²⁰. Comparison with partial sequences of *R. viridis* reaction center proteins also shows homology²¹. This similarity extends to proteins of green plants, as shown by the homology between the L-subunit sequences of *R. capsulata* and the photosystem II thylakoid membrane protein from chloroplasts involved in quinone- and herbicide-binding²².

The arrangement of the chromophoric groups of the reaction center *R. viridis* in stereo is shown in Fig. 3. The complex contains four heme groups, four bacteriochlorophylls (BChl), two bacteriopheophytins (BPh), an inorganic iron and a quinone with well defined electron density (see Fig. 2 in Ref. 4). It is obvious that the por-

phyrin systems of the BChls and BPhs are related by an approximate diad which passes through the iron; the symmetry does not hold for the phytol side chains. The branch on the right-hand side is coordinated to the L-subunit, the left-hand side branch to the M-subunit. Two symmetry-related BChls form a closely associated dimer, in which pyrrole rings I of the porphyrins are in contact. The acetyl groups of the BChl rings are coordinated to the central magnesium ions and strengthen the association. The sixth coordination is to amino acid side chains of α -helices of the L- and M-subunits respectively. The dimer is the postulated special BChl pair, the primary electron donor. The planes of the aromatic rings are only slightly inclined to each other by about 15°. The close association and the parallel orientation results in resonance between the electronic transitions of the two BChls to produce large exciton effects. The ring planes of the monomeric BChl and the special pair are inclined by about 70°. The BPh is inclined to the BChl by 64°. A recent theoretical analysis of the absorption spectra of the *R. viridis* reaction center on the basis of the three-dimensional structure shows satisfactory correspondence with the experiment²³. This analysis indicates that the low frequency absorption at 960 nm (P_{960}) is solely due to the lower exciton state of

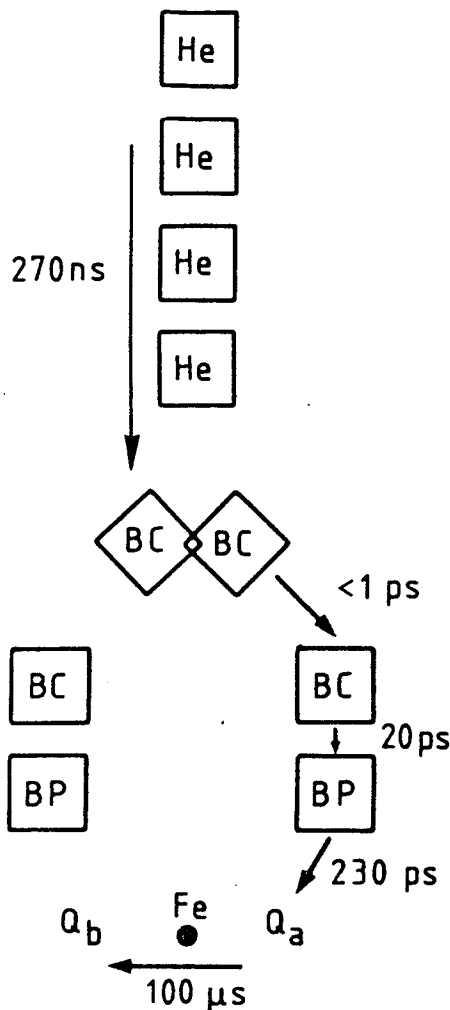


Fig. 4. Electron transfer times ($t_{1/2}$) between the components of the reaction center from *R. viridis* overlaid on a scheme of the structure²⁷. The $Q_a \rightarrow Q_b$ electron transfer time is from data from *R. sphaeroides*²⁸.

the special pair and the higher exciton state of the special pair has no intensity. When P_{960} is oxidized, the spectral properties change considerably. In view of the tightly packed chromophore protein complex, a substantial geometric rearrangement of the BChl components of the special pair upon oxidation seems improbable and is therefore unlikely to explain the spectral alterations.

The symmetrically branched arrangement of the BChl chromophores is intriguing, as it suggests that the electron may use both pathways. However, the chromophore arrangement is not exactly identical, at least with respect to the phytol side groups and the protein environment. Spectroscopic experiments show that only one BPh is bleached²⁴, indicating that only one branch is used. A further essential difference between the two branches is the presence of a quinone only in the right-hand side branch. Functional and spectroscopic studies have differentiated

between strongly- (Q_a) and loosely-bound (Q_b) quinones. The binding site of the well defined quinone has the characteristics expected for strong binding: multiple protein-ligand interactions with residues preferentially from the M-, but also from the L-subunits, and shielding of the ligand from the outside by the protein. The well defined quinone is therefore probably Q_a , which is in contact with the iron by mediation of an amino acid side-chain forming one of the coordinating ligands to the iron. The 'logical' binding site for Q_b is the site related to Q_a by the local diad. This site is without defined electron density in the Fourier map, possibly because part of Q_b is lost during isolation and crystallization. Recent crystallographic studies (present authors in cooperation with O. Epp) of the binding of *o*-phenantroline and terbutryn, competitive inhibitors of Q_b , showed that there is indeed a high degree of occupancy at this binding site. The closest distance between Q_a and the presumed Q_b position is 14 Å. The inorganic iron is located approximately mid-way between Q_a and Q_b and liganded to residues of the L- and M-subunits, which touch the quinones. A plausible route for the electron transfer is therefore via the iron.

This observation provided the final evidence for the spatial location of all the components involved in primary

electron transfer which have been identified by spectroscopic and kinetic measurements. The principal arrangement seems well designed for the dual purpose of the reaction center in absorbing light energy and in transferring electrons across the photosynthetic membrane. The light energy is supplied to P_{960} by the membranous LHC. Efficient energy transfer between LHC and the special pair affords parallel alignment of the transition dipole moments of the LHC chromophores with the special pair of the reaction center. A detailed structural analysis may be possible in the future when well ordered crystals of LHC become available. In contrast, the optical isolation of the special pair from the other chromophores of the reaction center mentioned above serves to trap the light energy at the special pair. On the other hand, efficient electron transfer from the excited special pair requires electrical conductivity by overlap of the electron clouds of the conducting elements, as is indeed the case in the chain from the special pair via BChl to BPh. Electron transfer occurs between components in contact via Van der Waals' forces, except for electron transfer from BPh to Q_a (which have a center-to-center distance of 14 Å). However, the isoprenoid side chain of Q_a touches BPh. The kinetic data for the electron transfer steps identified so far, superimposed on

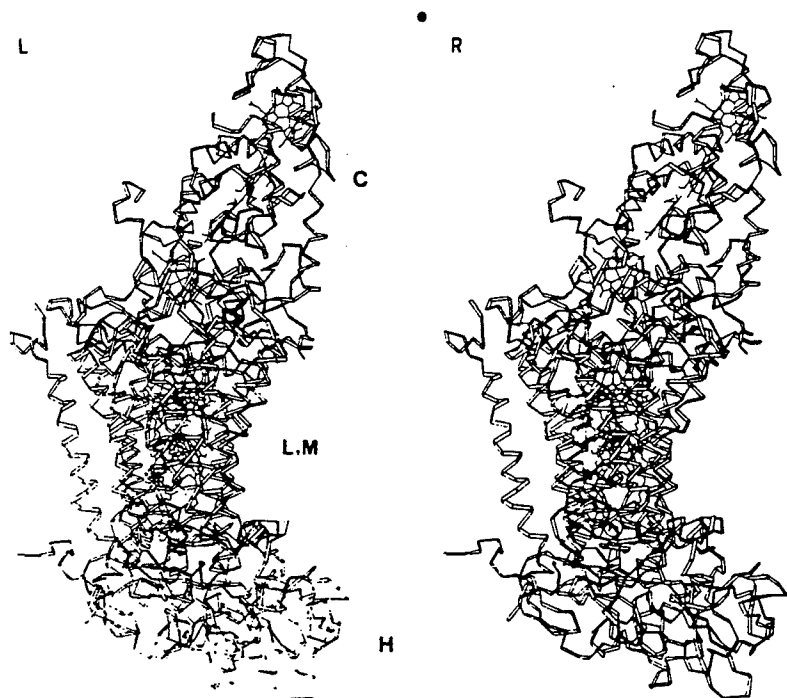


Fig. 5. α band representation of the reaction center components in stereo. The bottom part represents the H-subunit, the middle part the L- and M-subunits. The upper globule is the cytochrome (C). The view is on to the flat side of the L, M module. The L-subunit is toward the observer.

a schematic representation of the spatial arrangement are shown in Fig. 4.

The oxidized special pair is reduced by another chain of conducting elements, the four heme groups of the cytochrome. The separation of the closest heme from the special pair is 21 Å (center-to-center). This relatively large distance may be responsible for the slow transfer rate, about 1000 times less than the electron transfer from the special pair to the primary acceptor.

The forward electron transfer from the special pair to Qa is more than eight orders-of-magnitude faster than the back reaction. This large difference is essential for the high quantum yield of the primary photochemical reaction. An explanation at the atomic level represents a challenge for experimentalists and theoreticians. As already mentioned, the tightly packed three-dimensional structure of the reaction center makes substantial geometric rearrangements between the functional states (P Q) and (P⁺ Q⁻) unlikely. However, even slight changes in the relative arrangements of the conducting elements may destroy the electronic overlap. Analysis of chemically or photochemically oxidized reaction center crystals is possible and may provide a clue; such studies are underway.

The location of the reaction center in the photosynthetic membrane

Crystal structure analysis does not provide direct information on the location of the protein complex in the photosynthetic membrane. The crystals are composed of the well ordered protein phase and a water and detergent phase which is disordered and shows no defined electron density. It may be possible, by further experimentation with neutron scattering, to discern the water and detergent phase in the crystals by contrast variation using deuterated water and detergents.

Some evidence from other sources is available about the location of the reaction center complex in the membrane. Functional properties suggest that the cytochrome is on the periplasmic side and the H-subunit on the cytoplasmic side of the photosynthetic membrane. L- and M-subunits are probably localized in the membrane. Further evidence from amino acid sequencing is discussed below. The approximate diad relating the bacteriochlorophyll chromophores and the L- and M-subunits is most probably normal to the plane of the membrane.

The protein conformation

A complete model has been built of the polypeptide chains of the four subunits of the reaction center using amino acid sequence information. Polypeptide stretches with unknown sequences were built as polyalanine chains. Many amino acid side chains have conspicuous electron density, allowing identification at later stages of the analysis. The polypeptide chain could be traced with confidence. The high content of helical regions, and the similarity of M- and L-subunits were features aiding the interpretation of the map. However, amino acid sequence information is required to exploit in detail the structural features in future studies and also to determine the exact number of residues per subunit. Particularly interesting features of the reaction center structure concern the association with the membrane. A large part of the M- and L-subunits and a short segment of the H-subunit are integrated into the membrane; these segments have a helical conformation. The polypeptide-chain path of the four components of the reaction center is shown in Fig. 5.

The L and M polypeptides have 274 and 320 amino acid residues respectively. Their folding is similar and both have the local diad defined by the BChl, BPh chromophores. Five helical segments are arranged to form a saucer in each subunit. The helical axes are inclined by less than 30° to the local diad. The helices are about 40 Å long, the appropriate width to span the membrane.

The amino acid sequences of the L- and M-subunits of *R. capsulata* and *R. sphaeroides* show five hydrophobic segments¹⁸⁻²⁰ homologous to the five transmembrane helices in *R. viridis*. The sequences of the L- and M-subunits are homologous, indicating similar structures, in accord with structural observations. The role of polar residues in the interaction with the membrane should become clear when the amino acid sequence analysis of the reaction center of *R. viridis* is complete.

The transmembrane helices are connected by segments that are partly helical, form β-ladders or have irregular conformation. These segments presumably lie on the membrane surface, and mediate the contact to the H-subunit and the cytochrome respectively. The M-subunit has a unique C-terminal arm in contact with the cytochrome.

The H (heavy!) subunit is the cytoplasmic cover of the L,M complex. It is a chain of 258 residues, the shortest of

the L,M,H triple. It is folded into a globular C-terminal part, made by two layers of β-sheets and a loosely folded N-terminal part with a prominent transmembrane helix, located in proximity to helical segments of the M-subunit. Much of the H-subunit is in contact with M and L polypeptide segments. The N-terminal transmembrane helix touches, with its tip, a segment of the cytochrome and acts as a transmembrane clamp.

The reaction center of *R. viridis* has a firmly bound cytochrome which does not dissociate under the isolation and crystallization procedures. This contrasts with preparations of reaction centers from most purple bacteria, which contain a complex of L-, M- and H-subunits only¹⁰. The cytochrome forms the periplasmic cover of the L,M complex and is in tight association with it. A C-terminal segment of the M-subunit strengthens the aggregation. The cytochrome is the only component of the complex lacking obvious membrane interaction and is of the c-type as shown by the spectral properties. The polypeptide chain of the cytochrome has been traced in the electron density map and shown to have 332 amino acid residues and is the largest component of the reaction center complex. It binds four heme groups in a way characteristic of c-type cytochromes.

The structural basis of the electron transfer between cytochromes and their macromolecular substrates needs intense study and modelling but so far lacks a solid structural basis (for a review see Ref. 25). The *R. viridis* reaction center has the components in fixed arrangement and the structure of the chromophores clearly indicates that the electron transfer must occur from the I, IV heme edge to the special pair.

Outlook

Reduction of the oxidized special pair by the cytochrome closes the cycle of the electron flow, but we have not yet touched on the events associated with the electron flow from Qb to the cytochrome. These involve a membranous cytochrome *b/c* complex (not yet proven for *R. viridis*) and a soluble periplasmic cytochrome, *c2*. These events are responsible for light-generated proton translocation. Unfortunately the structure of the reaction center can add little to our understanding of these processes, except in generating optimism about the feasibility and productiveness of crystal structure analyses of such complex systems.

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Molecular Mechanisms of Photosynthesis

Spectroscopy, X-ray crystallography and molecular genetics combine to give a detailed picture of events in photosynthesis and show how particular molecules contribute to the process

by Douglas C. Youvan and Barry L. Marrs

Photosynthesis, the process by which light from the sun is converted into the energy necessary for the vital functions of living things, is the keystone of life on the earth. The energy captured by photosynthesis ultimately feeds not only the photosynthesizing organisms themselves but also the animals that feed on photosynthesizing organisms, the creatures that feed on those animals and so on down the food chain. How does photosynthesis work? At the molecular level, what are the interactions that capture the energy of sunlight and turn it into the energy of life? What is the architecture—the organization in space—of the molecules involved? And how does that particular architecture give rise to the speed and efficiency of the photosynthetic process?

These fundamental questions are now yielding answers. Through the separate efforts of workers in such disparate fields as spectroscopy, X-ray crystallography and molecular genetics, the molecular mechanisms of one kind of photosynthesis—the photosynthesis of certain bacteria—have been pictured in great detail.

Each area of research sheds light on a different aspect of the process. Spectroscopists have determined the sequence and timing of molecular events in the so-called light reactions of photosynthesis (the reactions constituting the first stage of the process), as well as the speed with which each interaction proceeds. X-ray crystallographers have visualized the spatial structure of the photosynthetic reaction center (the site where the light reactions take place) and have discovered how the molecules in the reaction center are aligned with respect to one another. Molecular geneticists have located and analyzed—and can now manipulate—the genes that direct the con-

struction of the reaction center's major components.

Knowledge of the molecular interactions, structure and genetic basis of the photosynthetic reaction center makes it possible to ask more detailed questions about its function. How does the presence of each molecule in the reaction center contribute to the function of the whole? Why are certain stages of the light reactions faster or slower than others? How would the process be changed if a particular element had a slightly different shape or composition than it does?

We and others have been trying to answer such questions by applying the powerful tools of molecular genetics. By altering the genetic information that encodes certain elements of the reaction center, one of us (Youvan) has genetically engineered photosynthetic bacteria to produce reaction centers that differ in specified ways from those of unaltered organisms. By testing the photosynthetic function of the altered organisms, it is possible to learn what effect the change has had on the ability of the organism to photosynthesize efficiently.

Such techniques enable researchers to learn about the mechanisms of photosynthesis in minute detail. The results—intriguing in themselves—may also make it possible eventually to design and produce organisms that can photosynthesize in specialized ways or in harsh environments.

The subjects of these studies are bacteria belonging to the genus *Rhodospseudomonas*. The rhodospseudomonads carry out photosynthesis in much the same way as the bacteria that first evolved a method of harvesting the sun's energy more than three billion years ago. The photosynthesis carried out by rhodospseudomonads differs in

certain respects from the photosynthesis of higher plants; for one thing, rhodospseudomonad photosynthesis does not give off oxygen gas. There are nonetheless many similarities between the two processes. For example, both kinds of photosynthesis involve chlorophyll molecules.

A special advantage in studying rhodospseudomonads is that, unlike higher plants, they can obtain the energy required for growth not only by photosynthesis but also by several independent mechanisms, and so they can survive and multiply without photosynthesizing. It is therefore possible to grow colonies of mutants that have defects in their photosynthetic reaction centers and to study, in living organisms, the ways in which specific defects impair photosynthetic function.

The interiors of rhodospseudomonad bacteria are filled with so-called photosynthetic vesicles, which are small, hollow spheres made of lipid bilayers (the material that makes up cell membranes). The photosynthetic reaction centers, composed primarily of proteins, are embedded in the membrane of the photosynthetic vesicles. One end of the reaction center is near the outer surface of the membrane and the other end is near the inner surface.

At the start of the light reactions of photosynthesis, a photon (a packet of light energy) strikes the end of the photosynthetic reaction center nearest the inner surface of the membrane. An electron in that region of the reaction center becomes excited (elevated to a higher energy level) and carries the energy of the photon to the other end of the reaction center (the end near the outer surface of the membrane) by means of a series of chemical interactions. A second photon causes another electron to follow the same route. These interactions produce a separa-

tion of charge: the negatively charged electrons end up near the outer surface of the membrane, and molecules bearing excess positive charge, representing the absence of the electrons, are left near the inner surface of the membrane. The separation of charge represents stored energy, because energy would be released if the electrons and the positively charged molecules were able to come together. Later the charge separation drives chemical reactions that are coupled to, and provide the energy for, certain processes of the bacterial metabolism.

What are the interactions that bring the electrons from one side of the membrane to the other? That question has been answered by spec-

troscopists, who employ electromagnetic radiation to probe the chemical structure of matter.

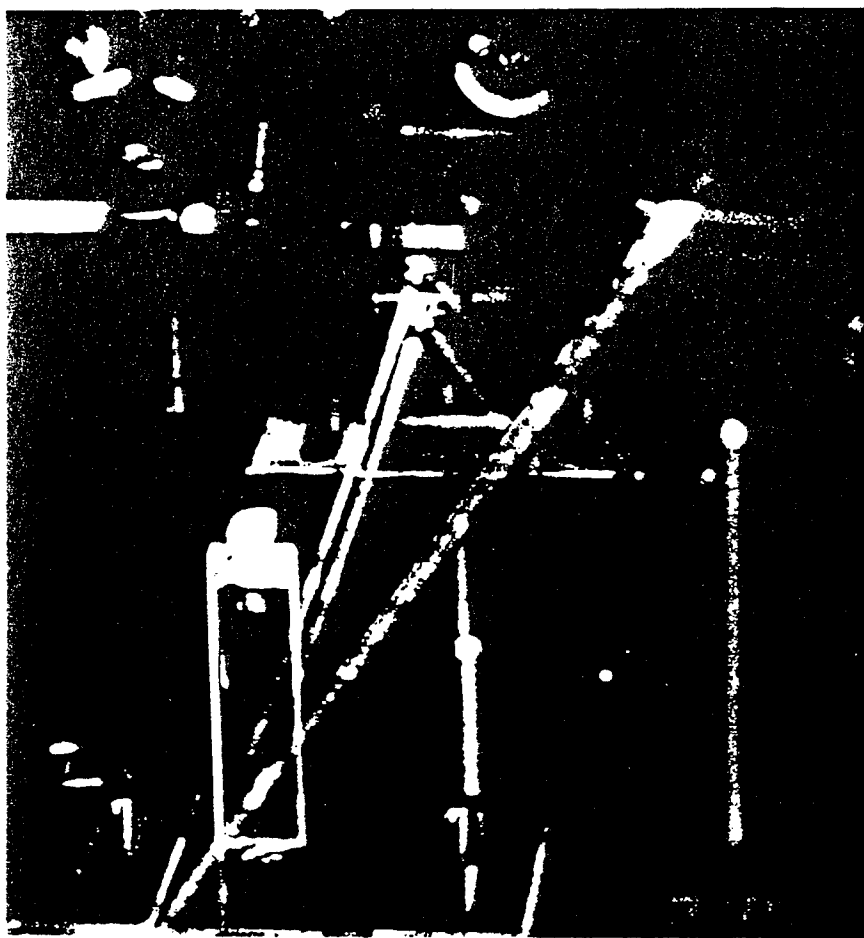
One particularly useful kind of spectroscopy is called optical absorption spectroscopy. The investigator shines a beam of light through a sample and measures how much of the light is absorbed. Each kind of molecule absorbs only certain wavelengths, or colors, of light. The wavelengths absorbed depend on the structure of the molecule and its chemical environment. Every kind of molecule has its own spectroscopic "fingerprint." The spectroscopist can therefore determine the makeup of a sample by finding its absorption spectrum (the degree to which the sample absorbs light of different wavelengths).

When the photosynthetic reaction center absorbs a photon, its chemical structure undergoes a series of changes as the interactions occur that carry the electron through the membrane. Those changes in chemistry can be monitored by noting how the reaction center's absorption spectrum changes after the center has absorbed a photon. In effect, a series of these observations traces the "chemical pathway" the electron follows from one side of the membrane to the other.

Many groups have made such observations. In their experiments the probe beam (the beam that serves to gauge the absorption spectrum of a sample of photosynthetic reaction centers, extracted from a population of bacteria) is usually kept very weak so that it will not cause much photosynthesis. Then the sample is made to photosynthesize by an intense flash of laser light less than one picosecond (a trillionth of a second) long. The laser pulse is so short that each reaction center in the sample absorbs at most a single photon, and the resulting photosynthetic reactions are synchronized: at any given time every reaction center is undergoing the same chemical transformation. Hence the absorption spectrum of the sample as a whole reflects the chemical state of each reaction center, and it is possible with successive flashes to deduce the nature and timing of the reactions in the first stages of photosynthesis.

Another method of spectroscopy is known as electron-spin resonance. Every electron has a certain amount of spin. Like all spinning charged bodies, the electron therefore creates a magnetic field; in some ways the electron can be thought of as a tiny bar magnet. In most stable compounds all the electrons form pairs in which the magnetic fields of the two electrons are aligned in exactly opposite directions. In some molecules, however, there is an unpaired electron. In electron-spin-resonance spectroscopy the investigator gauges how the energy of such unpaired electrons changes in a rapidly varying magnetic field. The results provide clues to the molecular environment of the unpaired electrons.

In the 1970's George Feher and his co-workers at the University of California at San Diego and, independently, James R. Norris and his colleagues at the Argonne National Laboratory traced the initial step of photosynthesis by electron-spin-resonance spectroscopy. They were able to see, for the first time, a signal from an unpaired electron on chlorophyll molecules that had absorbed a photon. The electron was unpaired because the chlorophyll molecules had given up an



PICOSECOND ABSORPTION SPECTROSCOPY can determine, to within a trillionth of a second, the timing and sequence of the reactions that make up the first stages of photosynthesis. Two weak probe beams (*center*) shine through a photosynthetic sample in a cuvette (*foreground*). The beams are so weak that they do not cause much photosynthesis. Photoreceptive instruments (not shown) determine the chemical makeup of the sample by analyzing the light that passes through it. An intense flash of laser light less than a trillionth of a second long (*right*) stimulates photosynthesis in a small region that is also traversed by one of the probe beams. Because the flash is so brief, all the photosynthetic reaction centers (the molecular complexes in which the first reactions of photosynthesis take place) photosynthesize simultaneously. As photosynthesis proceeds, the chemical makeup of the sample changes. The nature of the changes can be determined by analyzing the light from the probe beam. The experimental apparatus in this photograph is from the laboratory of Michael R. Wasielewski at the Argonne National Laboratory.

electron on absorbing the photon. The nature of the electron-spin-resonance signal indicated that the photon-absorbing element in the reaction center consists of two chlorophyll molecules in close association.

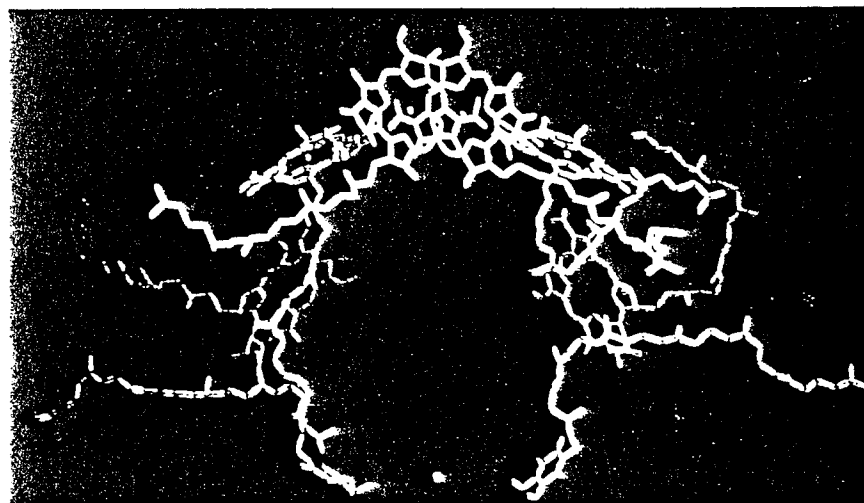
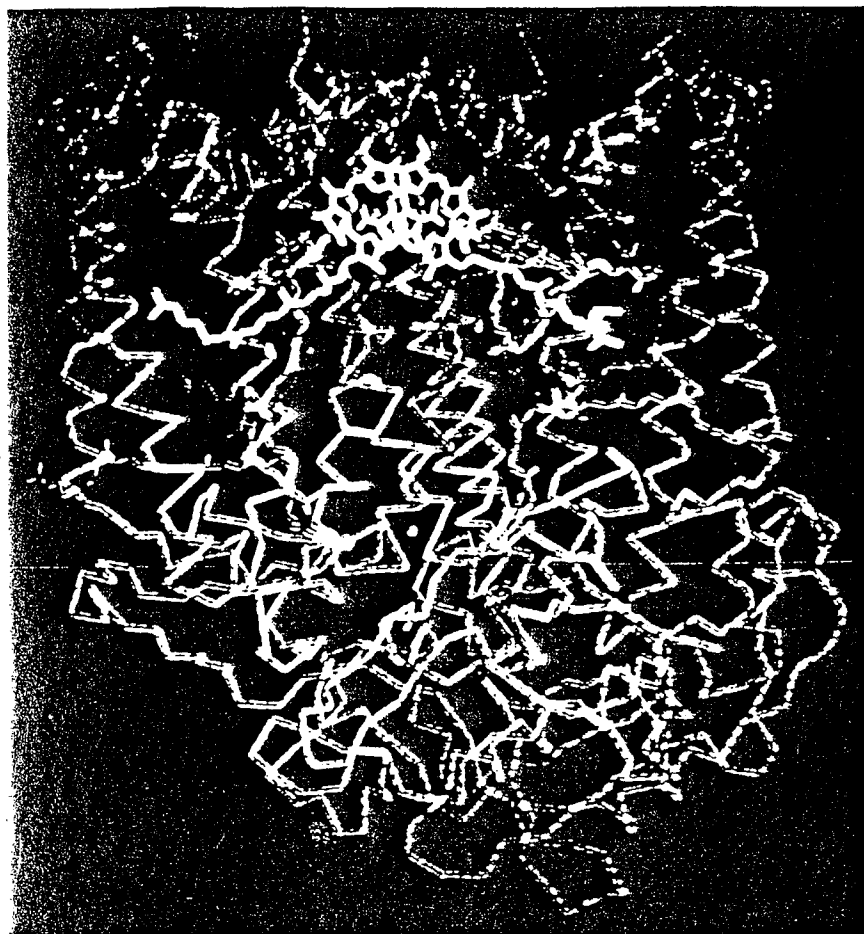
Spectroscopy has revealed the sequence and timing of the electron-transfer reactions that take place in the photosynthetic reaction center after the reaction center absorbs a photon. What about the reaction center's architecture? What physical path does the electron follow from one side of the membrane to the other?

Such questions can be addressed by X-ray crystallography, which is currently the only method capable of revealing the structure of complex biological molecules at the atomic scale. The technique involves aiming a beam of X rays at a crystal and finding the directions in which the crystal diffracts the rays. From the diffraction pattern it is possible to determine the crystal's molecular structure.

The major difficulty in applying X-ray crystallography to the analysis of the photosynthetic reaction center is that the sample to be analyzed must take the form of a well-ordered crystal. Obtaining such a crystal from a protein is a difficult step, and it requires great care and some luck on the part of the crystallographer. Over the past 20 years a few hundred water-soluble proteins have been crystallized and analyzed, but it was only very recently that investigators found methods for crystallizing water-insoluble membrane proteins such as those of the photosynthetic reaction center.

It has become possible to crystallize membrane proteins largely because of technical breakthroughs in the design and use of certain small organic molecules with one end that is hydrophilic (attracted to water) and one end that is hydrophobic (repelled by water). Apparently the hydrophobic ends of these molecules can bind to the hydrophobic parts of membrane proteins, exposing the hydrophilic ends of the small molecules. The resulting conglomeration of small molecules and proteins can then be dissolved in an aqueous (water-based) solution—and so it can be crystallized. The protein molecules and the smaller molecules crystallize together, in a process known as cocrystallization.

In 1983 Hartmut Michel and Johann Deisenhofer of the Max Planck Institute for Biochemistry in Martinsreid applied such techniques to crystallize the proteins of the reaction center of rhodospseudomonads and determine their structure. The work was a tremendous achievement; the reac-



PHYSICAL STRUCTURE of the bacterial photosynthetic reaction center, shown in these computer images, was determined by X-ray crystallography. The top image shows the entire photosynthetic reaction center, the bulk of which is a protein complex (blue). The bottom image shows only the so-called prosthetic, or helper, molecules that are embedded in the protein. The protein itself is embedded in a membrane (not shown) that is part of an internal bacterial structure called a photosynthetic vesicle. The "special pair" of chlorophyll molecules, which absorb the energy of photons (quanta of light energy), is shown in yellow. Other chlorophyll molecules called voyeur molecules (because they are positioned near, but may not take part in, certain reactions of photosynthesis) are shown in green. Molecules of pheophytin are shown in magenta and molecules of quinone are in orange. The small yellow dot near the base of the photosynthetic reaction center represents an ion, or charged atom, of iron. The positions and orientations shown for certain parts of the prosthetic molecules are approximate; the exact coordinates have not yet been determined. The images were produced by Chung-Huan Chang, David M. Tiede, James R. Norris, Jr., and Marianne Schiffer of the Argonne National Laboratory.

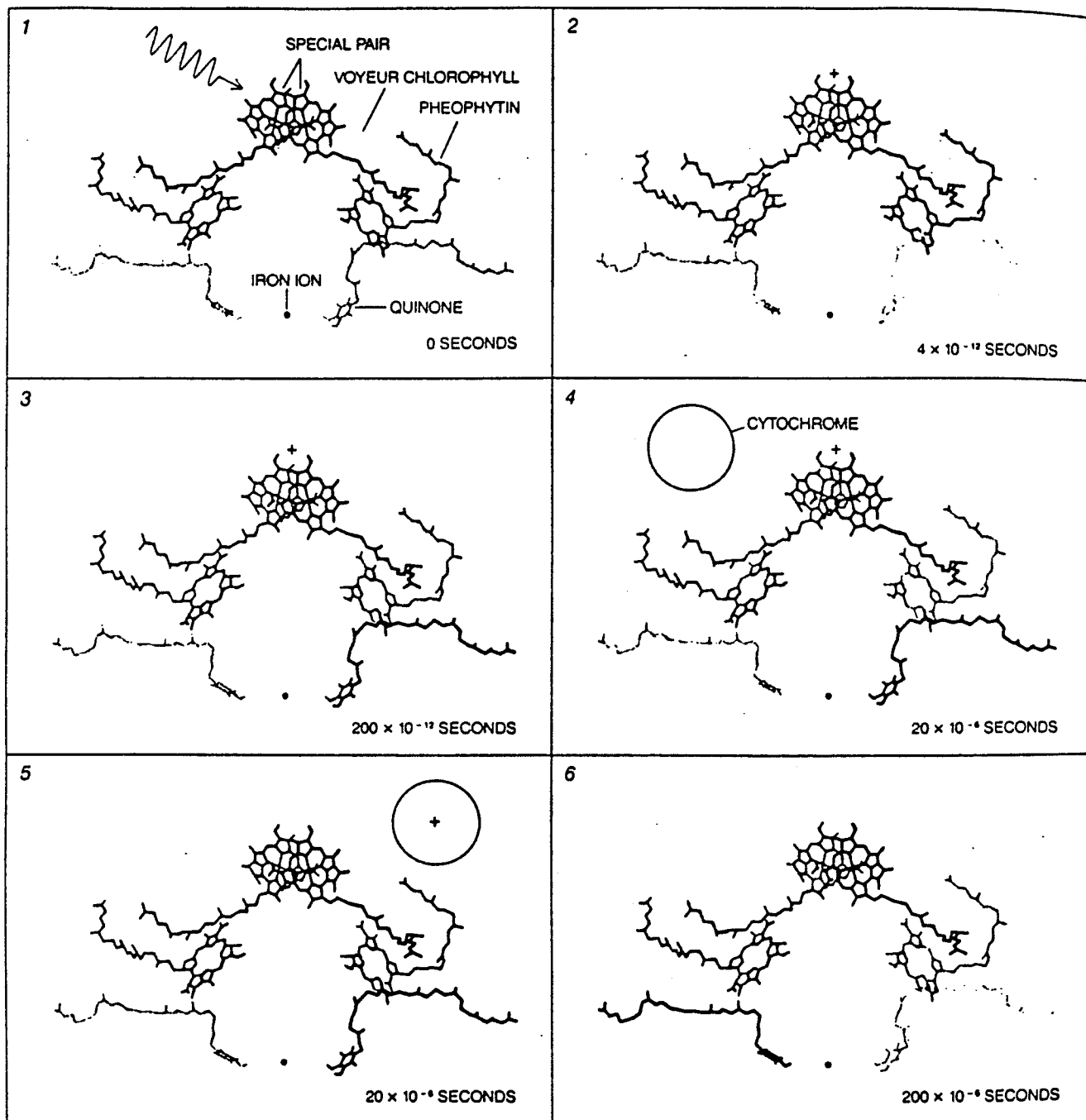
tion center is to date the only integral membrane protein whose structure is known to atomic resolution.

Together the information gleaned from such crystallographic studies and the knowledge gained by spectro-

scopists yield a clear picture, in space and time, of the chemical behavior of the photosynthetic reaction center and the reactions that bring the electron from one surface of the membrane to the other.

The main structural element of the

reaction center is a large protein complex embedded in the membrane. A number of smaller molecules known as prosthetic, or helper, molecules (including molecules of chlorophyll) are in turn partially embedded in the protein complex. The prosthetic mole-



CHEMICAL REACTIONS that make up the early stages of photosynthesis transport an electron to one end of the reaction center while leaving behind a region of positive electric charge at the other end. In the first step of photosynthesis (1) a photon is absorbed by a special pair of chlorophyll molecules and transfers its energy to an electron in the special pair. (In each step the molecules bearing an electron in an excited state are shown in red.) The electron then moves (2) to a pheophytin molecule, passing a

special pair. From there the electron travels (3) to the quinone molecule at the end of one spiraling chain of prosthetic molecules. At this stage a cytochrome molecule (a globular molecule) moving freely in solution approaches the special pair (4) and transfers an electron to it. The cytochrome molecule thereby acquires a positive charge (5) and the special pair is neutralized. Later the excited electron that had traveled to one quinone molecule passes to the second quinone molecule (6). The separation of the electron and the region of positive charge represents stored energy.

cles provide the electrically conductive path that is followed by the photoelectrons during photosynthesis.

One of the most striking features of this structure is that it has nearly perfect twofold rotational symmetry; in other words, a photosynthetic reaction center that had been rotated by 180 degrees, or half of a full circle, about its central axis (the axis running from one surface of the membrane to the other) would look almost identical with an unrotated reaction center. Specifically, the prosthetic molecules are arranged in two spirals situated symmetrically on opposite sides of the central protein. A curious and unexplained experimental observation is that, in spite of the symmetry, the electrons seem to follow only one of these spirals during photosynthesis.

The photosynthetic process begins when a photon strikes a pair of chlorophyll molecules called the special pair, situated at the end of the reaction center that is nearest the inner surface of the membrane. The special pair lies at the junction of the two spirals of prosthetic molecules. An electron within the special pair absorbs the photon's energy and moves to a neighboring prosthetic group, a molecule of pheophytin, which is very similar in composition and structure to chlorophyll. This stage of the process is extremely rapid; it takes place in about four trillionths of a second. During this reaction the electron passes near, but seems not to join, another chlorophyll molecule (known appropriately as a voyeur molecule). The special pair is left with an excess positive charge.

The electron then moves from the pheophytin to a molecule of quinone, which is at the end of the spiraling chain of prosthetic molecules and is near the outer surface of the membrane. From the quinone molecule the electron passes through the central protein to the quinone molecule at the end of the other spiral of prosthetic molecules (the spiral along which electrons are not conducted). This last step of the process is extremely slow: it occurs at a rate approximately 10^8 times slower than the electron-transfer reactions between the special pair and the pheophytin molecule.

In the meantime a globular, water-soluble molecule of cytochrome donates an electron to the special pair, thereby becoming positively charged and neutralizing the special pair. Then the entire process occurs again: another photon strikes the special pair and another electron travels along one spiral arm to a quinone molecule and through the central protein to the quinone molecule at the end of the other spiral arm.

That quinone molecule, which now carries two extra electrons, then pulls away from the protein of the photosynthetic center to participate in later stages of photosynthesis, which take place at the outer surface of the membrane. In addition a second cytochrome molecule donates an electron to the special pair, neutralizing it. The charge separation that stores the energy of the photons is now complete: two cytochrome molecules near the inner surface of the membrane have acquired a positive charge, and two electrons have traveled from the inner to the outer surface of the membrane.

The photosynthetic reaction center is remarkably effective at capturing light energy. It captures the energy of between 98 and 100 percent of the photons it absorbs. As a battery it is about 50 percent efficient: the energy stored in separated charges is about half of the energy inherent in the stimulating photons. The rest of the energy is lost in the reactions that drive the electrons along the chain of prosthetic molecules.

Spectroscopy and crystallography thus provide an intimate view of the process of photosynthesis in rhodospseudomonads. The third vantage from which this process has been examined is that of molecular genetics.

As we have mentioned, it is particularly important, from the geneticist's point of view, that rhodospseudomonads can survive without photosynthesizing. This property makes it possible for mutants that carry genetic defects affecting their photosynthetic centers to survive and multiply. Hence the effect of various mutations can be studied in live organisms.

In the genetic analysis of an organism it is important not only that mutants survive but also that there be some way to transfer DNA, the material of which genes are made, from one organism to another to produce genetic crosses. In that way altered genes can be inserted into individuals. One of us (Marrs) found about 10 years ago that a rhodospseudomonad called *R. capsulata* has just such a mechanism. It occasionally happens that the genetic material of an *R. capsulata* bacterium breaks up into sequences about five genes long. The sites of the breaks seem to be random, and they vary from one individual to another. Each piece of genetic material is packaged into a small, viruslike particle. The cell wall of the bacterium then bursts, releasing these so-called gene-transfer agents. The gene-transfer agents come to rest on other *R. capsulata* bacteria and insert their short strand of genetic material. The trans-

ferred genes are then integrated into the DNA of the recipients.

Gene-transfer agents are useful in making genetic crosses, and they can also help to "map" the genes of the bacteria. By noting how often two different genes are packaged in the same gene-transfer agent, the investigator can estimate how close together they lie on the bacterial chromosome (the strand of DNA that bears most of the bacterial genes). The more often two genes are packaged together, the closer they must be on the chromosome.

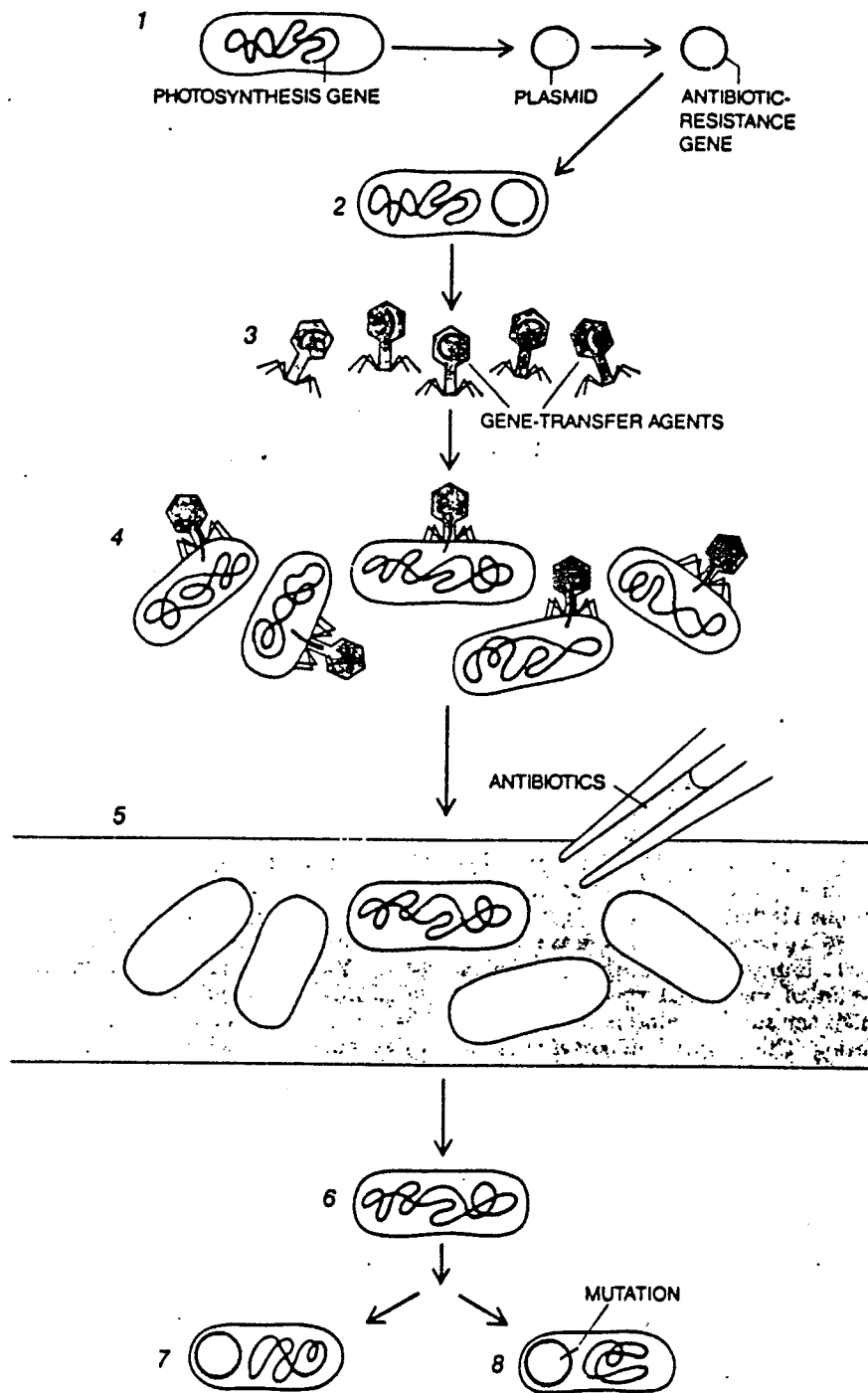
R. capsulata bacteria can also exchange genetic material by "mating." The bacteria couple by means of an appendage called a pilus, and DNA is transferred from "male" to "female" bacteria. The behavior provides another way of creating genetic crosses.

By means of such crosses, one of us (Marrs) was able in 1980 to isolate a fragment of DNA that bears most of the genes necessary for the light reactions of photosynthesis. It included genes for all the proteins within the reaction center and for proteins that make up a separate structure known as the light-harvesting antenna, which helps to capture the energy of photons and funnel it into the reaction center. This was the first time the DNA of a photosynthetic center had been isolated in any photosynthesizing organism.

Building on this work, the other one of us (Youvan) characterized the isolated DNA. He and colleagues from the Lawrence Berkeley Laboratory located each gene within the fragment and determined each gene's sequence of nucleotides (the elements that determine the specific makeup of the protein encoded by the gene). The nucleotide sequence was the basis for a computer analysis of protein structure that predicted aspects of the overall architecture of the reaction center several years before it had been visualized by X-ray crystallography. Knowing the nucleotide sequence for the photosynthetic reaction center in a bacterium also made it possible to compare that sequence with the corresponding sequence in higher plants. The close similarity between the sequences indicates that the makeup of basic photosynthetic structures is nearly universal.

Knowledge of the genetic makeup of the photosynthetic center of *R. capsulata* provides the tools necessary to address a most fundamental question: In what specific ways do the various elements of the reaction center contribute to its function?

One way to answer the question is to create simple mutations in the genes that encode the structures of the photosynthetic center. Organisms carrying



MOLECULAR-GENETIC TECHNIQUES enable an investigator to replace normal genes with mutated ones, producing a bacterium that lacks the gene for the photosynthetic reaction center, and to test the function of altered genes in the bacterium. The photosynthesis gene (purple) from a normal bacterium (1) is inserted into a plasmid (a small ring of DNA). The photosynthesis gene is then deleted, but the sections of DNA that flank it are left in place; a gene for resistance to antibiotics (red) is substituted. The plasmid is inserted into a normal bacterium (2). The bacterium breaks its DNA into small segments and packages them into viruslike entities called gene-transfer agents (3). The gene-transfer agents attach to other bacteria (4), injecting the DNA they carry. Because the antibiotic-resistance gene is sandwiched between DNA sections that usually flank the photosynthesis gene, it replaces the photosynthesis gene in the bacterial DNA. The bacteria are then exposed to antibiotics (5), preventing individuals without the new gene from replicating. The remaining organisms have no gene for photosynthesis (6). When a plasmid containing the normal photosynthesis gene is inserted into these organisms (7), they photosynthesize normally; such bacteria confirm that the genetic manipulations have not irreversibly damaged the organisms' ability to photosynthesize. It is also possible to insert a plasmid containing a photosynthesis gene that has a mutation (8). Testing the photosynthetic function of the resulting bacteria reveals the effect of the altered gene.

the mutations can then be observed spectroscopically to determine how their photosynthetic function differs from that of unmutated bacteria. Such experiments are already providing basic information on certain aspects of bacterial photosynthesis. In some cases the information gained is also applicable to higher plants.

For example, the quinone molecule that ultimately receives the electrons in rhodospseudomonads functions in much the same way, and in a similar environment, in higher plants. The X-ray structure of the protein pocket in the reaction center to which the quinone molecule binds in bacteria has recently been determined by Feher and his colleagues. One of us (Youvan), along with a graduate student (Edward J. Bylina), has introduced mutations in the quinone-binding pocket. Some of the resulting bacteria have no photosynthetic function, others have impaired photosynthetic function and still others have normal photosynthetic function; the degree to which function is affected depends on the particular mutation. In some of the mutated strains the function of the quinone molecule is changed in such a way that these strains are resistant to certain herbicides (such as atrazine) that kill plants by inhibiting the quinone molecule's function.

Such knowledge, gained from the study of bacteria, may soon be applied to higher plants. In the case of herbicide resistance, similar mutations could be made in the photosystems of such higher plants as soybeans, which are not resistant to atrazine. Soybeans made resistant to atrazine by the mutations could be planted in fields formerly planted with such crops as corn, which are naturally resistant to atrazine and are often sprayed heavily with that herbicide to kill weeds.

On a more fundamental level, the work on rhodospseudomonads may lead to discoveries concerning the basic electronic properties of various proteins. By genetic manipulations that would introduce altered reaction centers in the place of natural ones, an investigator might be able to learn how quickly or efficiently the new proteins conduct electrons. Many similar experimental tests can be envisioned.

The profound and detailed knowledge underlying such experiments has been gained by the union of three fields of research often considered to be almost completely unrelated. Each group of investigators, by examining the problem from a distinct angle, has illuminated a different aspect of the process of photosynthesis, so that it is now possible to see the whole.



original

Wavelength and intensity dependent primary photochemistry of isolated Photosystem II reaction centers at 5°C

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Abstract

The long wavelength absorption band of the isolated Photosystem II reaction center was directly excited at five wavelengths between 655 and 689 nm to study the effects of excitation wavelength and intensity on both excitation energy transfer and charge separation processes. Subpicosecond transient absorption measurements were made monitoring principally the bleach of the pheophytin *a* Q_x band at 544 nm. At all pump wavelengths, the kinetics require three exponentials (1–3, 10–25 and 50–100 ps) to be fit properly. The pump energy was varied by a factor of twenty-five (40–1000 nJ), with no apparent effect on either the rates or the amplitude ratios of the three components, although clear evidence of nonlinear behavior was observed at the higher excitation energies. The dependence of both the rates and amplitude ratios of the three components upon pump wavelength will be discussed in terms of excitation energy transfer occurring on a 30 ps timescale. Selective excitation into the short and long-wavelength sides of the composite Q_y band give identical transient spectra at 500 ps, indicating near-unity efficiency of excitation energy transfer. At 1 ps, the spectra are quite different, calling into question the extent of ultrafast (~ 100 fs) excitation energy transfer. The time after the excitation pulse at which the transient crosses ΔA = 0 was found to be a highly sensitive measure of both the excitation energy and the ~~identity~~ identity of the pigment pool that had been excited.

identity

1. Introduction

S

The primary charge separation event in oxygenic photosynthesis occurs in the reaction center (RC) of photosystem II (PSII) [1]. The PSII RC, the D₁-D₂-cytochrome *b*-559 complex, was first isolated by Nanba and Satoh in 1987 [2] (see review, [3]) and understanding its excitation energy transfer and

charge separation processes has been an area of great interest ever since. The original isolation procedure for the PSII RC complex resulted in a preparation containing four-to-five chlorophyll *a* (Chl *a*) per RC. Later, more stable preparations were produced by substituting dodecyl β-maltoside for Triton X-100 [4], resulting in a stoichiometry of six Chl *a*, two pheophytin *a* (Pheo *a*), two β-carotene, and one cytochrome *b*-559 heme per RC [1]. The lowest excited singlet states of the eight chlorophyll-like

One of the two

* Corresponding author.

pigments all absorb within the RC composite Q_y band: the peak wavelength of the combined bands is at about 676 nm at room temperature for a sample immediately after removal from the isolation column. The primary electron donor, P680, is believed to peak at 680–682 nm [5,6] and is considered to be either a Chl a dimer [7,8] (analogous to the bacterial special pair) or a multimer of several Chl a [9,10]. Of the two Pheo a , only one is active (again, analogous to the bacterial system), with the peak of the active one at ~ 682 nm (almost isoenergetic with P680) [11,12] and the peak of the inactive one at ~ 672 nm [12,13]. The accessory Chl a molecules which are not part of P680 (four, if P680 is a dimer) are also believed to peak at ~ 670 nm (see Ref. [1]), although it has been suggested that at least one Chl a not associated with P680 absorbs near 680 nm [14]. Excitation energy transfer links the pigments on the blue side of the composite Q_y band (the “blue” pool) with the active redox pair on the red side of this absorption band (the “red” pool). It has been suggested that the “blue” pool itself is divided into two groups, one of which undergoes rapid (subpicosecond) excitation energy transfer to the “red” pool [15], and the other, weakly coupled to P680, undergoing excitation energy transfer on a tens of picoseconds timescale [16–18]. Only at low temperatures are the two main pools directly observable as separate peaks [9]. This tremendous spectral congestion greatly complicates efforts to understand the PSII RC and is in sharp contrast to the well-resolved absorption bands of the chromophores of the bacterial RC (see Refs. [19,20]).

While the fate of the charge-separated state in the isolated PSII RC has been well established (recombination to yield the excited singlet or the triplet state) [1], the nature of the excitation energy transfer and charge separation processes has been widely debated (see Ref. [21]). Near room temperature, the RC kinetics have been studied by both time resolved Chl a fluorescence [22–24] and transient absorption techniques [15–18,25–33]. While the time-resolved fluorescence measurements are hindered by the presence of processes occurring faster than the time resolution of the instrument, the transient absorption measurements are hampered by the lack of unambiguous indicators differentiating excitation energy transfer from charge separation. Nevertheless, there

are a number of features of transient absorption spectra that have been used to attempt to decipher the kinetics. Much of the work has been done probing the composite Q_y band itself [15–17,25–28,31–34]. Because of the spectral congestion of the six Chl a and two Pheo a pigments in this band, it is difficult to use bleaching as an indicator of ground state depopulation of a specific pigment or group thereof. Furthermore, the small Stokes shift of the fluorescence (3–4 nm) [6] causes the main stimulated emission band to be superimposed upon the bleach, further complicating the interpretation of the kinetics. On the other hand, the $Q_y(1,0)$ stimulated emission sideband at ~ 740 nm has been used as a monitor of the population of the fluorescing state free from the effects of the Q_y bleach [29,32,33]. The loss of Pheo a ground state population due to both excited singlet state formation (i.e., 1 Pheo a) and charge separation can be monitored at the Pheo a Q_x band at ~ 544 nm [18,28,30–33]. While the data from this feature are complicated by the presence of bleaching due to 1 Pheo a (from both direct excitation of and excitation energy transfer to Pheo a), it is still probably the best indicator of charge separation that has been found so far.

Virtually all of the time-resolved experiments on PSII RCs near room temperature have detected both a ~ 3 and a ~ 20 ps component in the kinetics. The controversy as to which component is related to excitation energy transfer and which is related to charge separation is as much about the interpretation of the data as the data itself. Experiments at cryogenic temperatures, however, are all in agreement that 1–2 ps is the measured time for charge separation [6,34,35], and 80–100 ps is the time for excitation energy transfer-limited charge separation (at 77 K) [34]. While several experiments with subpicosecond time resolution have been performed near room temperature that selectively excited each of the two pigment pools [15,16,29,36], the extremely broad bandwidth of the “red”-pool excitation pulse (~ 17 nm) [15] forced an excitation wavelength of 694 nm (i.e., far out into the wing of the band) to achieve any selectivity, thus precluding the use of more than one wavelength for excitation of the “red” pool. We have undertaken a new transient absorption study that utilizes an optical parametric amplifier (OPA) as a pump source. Wavelength tuning with the OPA can

reviews,

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be achieved in a few minutes, allowing the same sample to be utilized for multiple excitation wavelengths. This device provides sub-200 fs time resolution with near transform-limited spectral bandwidths [37]. These narrow bandwidths (~ 6 nm) allow us to investigate the pump-wavelength dependence of the kinetics across the composite Q_y band. Experiments comparing selective excitation of the two pigment pools have the potential to provide insight into the identification of excitation energy transfer and charge separation processes, as selective excitation of the "blue" pool necessitates the occurrence of energy transfer, while selective excitation of the "red" pool minimizes the occurrence of inter-pool energy transfer. Thus, differences in the "blue"- versus "red"-pool kinetics can be ascribed to energy transfer from the "blue" pool to the "red" pool.

Transient absorption kinetics were measured at the peak of the Pheo a bleach, and transient spectra were taken over the 500 to 610 nm wavelength region. At 1 ps, excitation into the "blue" pigment pool of the composite Q_y band results in a significantly different transient spectrum than excitation into the "red" pool. The difference in the two spectra is shown to be very similar to the transient spectrum of $^1\text{Chl } a$ in vitro. At long time (500 ps), the spectra and hence charge separation yields are indistinguishable, indicating near-unity quantum yield for excitation energy transfer from the "blue" pool to the "red" pool. The kinetics of the Pheo a bleach are triple exponential at all excitation wavelengths used. Finally, the time at which the decay crosses $\Delta A = 0$ is shown to be a powerful indicator of pump intensity and the identity of the pigment pool that had been excited.

2. Materials and methods

D_1 - D_2 -cytochrome b -559 PSII RC complex was isolated from PSII-enriched appressed membrane fragments obtained from market spinach [4]. PSII membranes (120 mg Chl a) were ~~pelletized~~ resuspended in 50 mM Tris-HCl (pH 7.2), 30 mM NaCl, and 4% Triton X-100 at 1 mg Chl a /ml, and incubated with stirring for 1 h. All manipulations in this procedure were done at 4°C in the dark. The solubilized material was then centrifuged for 1 h at

32 000 $\times g$, and the supernatant was loaded onto a buffer-equilibrated 1.6×20 cm Fractogel TSK-DEAE 650S (Supelco) column. The column was then washed with 50 mM Tris-HCl (pH 7.2), 30 mM NaCl, and 0.05% Triton buffer using an FPLC system (Pharmacia) until the absorption of the eluent at 670 nm was about 0.03 cm^{-1} (the spectrum of the eluent is like that of an isolated RC except that the red absorption peak was at 670 nm). At that point, the column was washed with 150 ml of 50 mM Tris-HCl (pH 7.2), 30 mM NaCl, and 0.03% n -dodecyl β -D-maltoside (DM) to remove Triton. The RCs were then eluted with a 30–200 mM NaCl linear gradient in the same buffer. Green fractions obtained at around 125 mM NaCl were pooled, desalted and concentrated within 1 h using a Centriprep 50 Concentrator (Amicon), and stored at -80°C until use. These RCs contain about 6 Chl a , 2 β -carotenes, and 1 cytochrome b -559 per 2 Pheo a .

Experiments were performed on Chl a in tetrahydrofuran (THF) solution at room temperature. Chl a was isolated from spinach [38]. The sample was purified on a TLC plate in the dark immediately prior to preparation of the solution. The peak of the Q_y band was at 664 nm, with an absorbance of 0.62. A peak extinction coefficient of 79 000 [39] was used for calculation of the sample concentration (7.8×10^{-6} M). The THF was distilled from lithium aluminum hydride and stored over molecular sieves under N_2 .

A sample temperature of $\sim 5^\circ\text{C}$ for the PSII RCs was maintained by flowing ice-water through the cooling jacket of a 1 cm path length fused silica spectroscopic cell (Starna). The rate of sample degradation is greatly decreased by keeping the sample at this temperature (as compared to room temperature). No significant changes in either the composite Q_y band peak position or the second derivative of the band ~~was~~ discernible after at least an hour of illumination. The sample was continuously stirred; this avoided accumulation of RCs in the triplet state in the sample volume interrogated by the laser beam. Steady-state spectral measurements were done with a Shimadzu UV160 spectrometer.

Transient absorption measurements were performed with an optical parametric amplifier [37] (OPA) pumped by the second harmonic of an ampli-

were

Split

fied Ti:sapphire laser system operating at 1.3 kHz. The pulse length of the pump pulses was ~ 180 fs, and bandwidths (FWHM) of ~ 6 nm (near transform-limited) were typical. A white light continuum was generated from the amplified fundamental in a fused silica block, and was ~~beam split~~ split into a probe beam and a reference beam. The polarization purity of both the pump and probe beams was very high, as both beams pass through calcite polarizers before reaching the sample. Like all of the previous work on PSII RCs done in our laboratory [18,25,31,35], the probe beam was polarized at the magic angle (54.7°) with respect to the pump beam. This polarization configuration, unlike having the two beams with parallel or perpendicular polarizations, allows direct observation of population kinetics without artifacts due to polarization phenomena. The importance of measuring the isotropic kinetics (either by direct measurement with the magic angle configuration or by calculation from both parallel and perpendicular data) should be stressed, as data on PSII RCs using the parallel polarization configuration continue to be presented [16,36].

Pump and probe beams were crossed in the sample with a spot size (diameter) of ~ 250 μm . Pump energies were varied from 40 nJ to 1.0 μJ . At 100 nJ, the energy density of the pump (the parameter of interest when comparing to other work) was ~ 200 $\mu\text{J}/\text{cm}^2$. Both the probe and reference beams were focused into a monochromator with both the entrance and exit slits set at a 4 nm bandwidth. After exiting the monochromator, the intensities of the two beams were measured by separate Si photodiodes. The photodiode output was captured by gated integrators and digitized by a 12-bit A/D board. Every other pump pulse was blocked by a mechanical chopper, and a ΔA value was calculated for each pair of pulses. Kinetic scans were typically the average of 10–20 delayline passes, with each point of each pass being the average of 100 laser shots. The scans were broken down into three temporal regions, with 100 fs per point from -3 to $+17$ ps, 1 ps per point from 17 to 122 ps, and 5 ps per point from 122 to 600 ps. Each data set was individually fit to a triple exponential decay and a nondecaying component using a grid search routine. Transient spectra were measured by setting the time between the pump and the probe pulses at a fixed delay and scanning

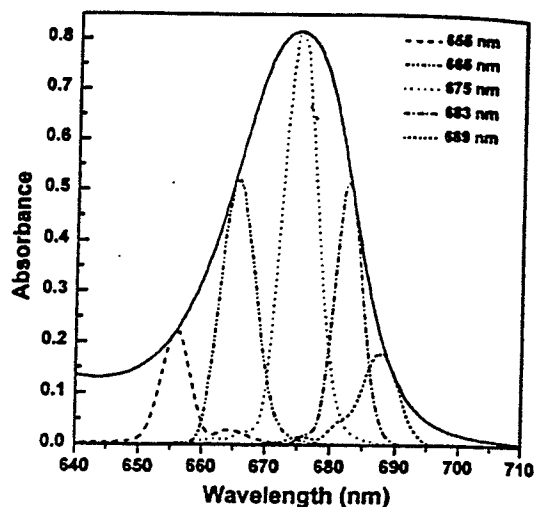


Fig. 1. Steady-state absorption spectrum of the composite Q_y band of isolated PSII RCs in a 1 cm cell. Superimposed are the excitation profiles (product of the pump and RC spectra – see text for details) for the five pump wavelengths. The actual spectral bandwidths for the pump pulses (not shown) are ~ 6 nm (near-transform-limited).

the wavelength of the monochromator (0.75 nm per point). Thus the spectra presented are direct spectra, not ones calculated from the results of global analysis fits of kinetic scans at numerous wavelengths.

Pump wavelengths of 655, 665, 675, 683, and 689 nm were used. The effect of the finite bandwidth (~ 6 nm) of the pump pulses on the absorption of the RCs can be calculated by taking the product of the normalized spectral intensity profile of the pump pulse and the absorption spectrum of the RC. Fig. 1 shows these “excitation profiles” for the five pump ~~pulses~~ wavelengths superimposed upon the spectrum of the composite Q_y band of the RC. Selective excitation of the “blue” side of the Q_y band can be achieved with 655 and 665 nm pump wavelengths, whereas 683 and 689 nm pump wavelengths result in selective excitation of the “red” side of the band. A pump wavelength of 675 nm excites the peak of the band, and no selectivity is achieved. It should be noted that the spectral intensity profile of the pump is similar for all wavelengths, and that the structure of the excitation profiles for 655 and 689 nm in Fig. 1 is due to the much higher sample extinction coefficient at the center of the Q_y band. For all five pump wavelengths, the peak of the excitation profile is within 1 nm of the pump wavelength.

The five pump wavelengths chosen correspond roughly to the peak, half-maxima and quarter maxima wavelengths for the composite Q_y band absorption. The true optical density of the sample for the actual laser pump pulses can be determined by integrating the excitation profile over wavelength and dividing by the integrated pump spectral intensity profile:

$$OD_{\text{effective}} = \frac{\int I_{\text{pump}}(\lambda) OD_{\text{sample}}(\lambda) d\lambda}{\int I_{\text{pump}}(\lambda) d\lambda}, \quad (1)$$

where I_{pump} is the normalized pump spectral intensity profile, $OD_{\text{sample}}(\lambda)$ is the optical density of the sample as a function of wavelength, and $OD_{\text{effective}}$ is the true optical density of the sample for the finite bandwidth laser pulses. Sample concentrations were adjusted to keep the OD near 0.5 at the excitation wavelength, with relative concentrations of 4:2:1:2:4 for 655, 665, 675, 683 and 689 nm, respectively; the calculated optical densities (using Eq. 1) are 0.47, 0.53, 0.38, 0.47, and 0.38. For the 665 and 683 nm pump wavelengths, the OD at the peak of the Q_y band was ~ 0.8 , which corresponds to a RC concentration of $\sim 2 \times 10^{-6}$ M.

Some discussion at this point is warranted about the various ways to display the intensity dependence of observables over the different pump wavelengths. The transient absorption signal is directly proportional to the number of photons absorbed by the sample. For a given pump energy, this quantity changes as a function of wavelength due to two factors: the optical density of the sample (as discussed above), and the change in the number of photons (a small effect – about 5% between 655 and 689 nm). Much of the intensity-dependent data discussed below will compare excitation wavelengths of 665 and 683 nm. As the correction for absorbed photons between these two wavelengths is only 3.5%, the correction will be ignored and the data will be displayed simply as a function of incident pump energy. Because the correction is significantly higher (as much as 20%) between the other pump wavelengths, that data will be presented in terms of absorbed photons per cm². We will also use an alternative way to display the data which clearly show the effects of multiple excitation of RCs at

high pump energies (vide infra), where the data will be displayed in terms of absorbed photons per RC. It should be noted that the experiments on Chl *a* in THF will show that, at high pump energies, the absorption of the sample becomes sublinear. No correction for this phenomena is made in any of the figures: the number of photons absorbed is always calculated assuming linear absorption.

3. Results

3.1. Kinetics at 544 nm with 200 nJ pump pulses

Transient absorption kinetics probing at 544 nm are shown in Fig. 2. The pump energy is 200 nJ and the wavelength is 683 nm. The data are fit to a triple exponential decay (two exponentials did not produce satisfactory residuals) and a component that does not decay on the sub-nanosecond timescale of the experiment. The typical τ of the triple exponential decay with the 683 nm pump are 1–2, 10–15 and 60–70 ps. The nondecaying component (hereafter referred to as the shelf) presumably reflects the fully charge

lifetimes

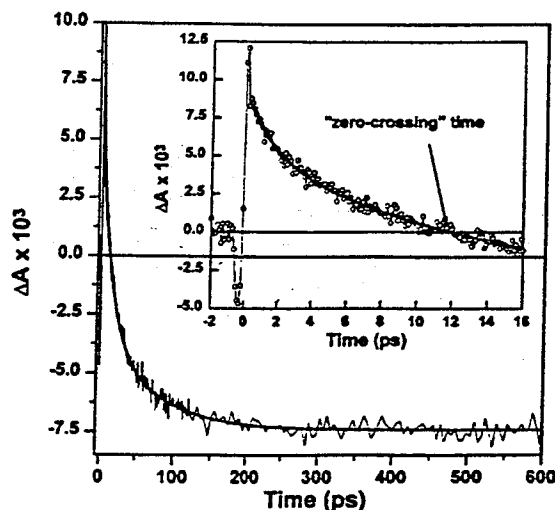


Fig. 2. Transient absorption kinetics probing at 544 nm in isolated PSII RCs. The pump energy and wavelength are 200 nJ and 683 nm, respectively. Inset shows the same data on a shorter timescale. The triple exponential fit to the data is shown in both. The large oscillations near $t = 0$ are due to the buffer/cell system, and are unrelated to the PSII RC dynamics. Note the "zero-crossing" time of ~ 11.5 ps.

Table 1
Averages and standard deviations for the fit parameters to the kinetic data at 544 nm using a pump energy of ~ 200 nJ

λ_{pump} (nm)	τ_{fast} (ps)	A_{fast}	$\%A_{\text{fast}}$	τ_{int} (ps)	A_{int}	$\%A_{\text{int}}$	τ_{slow} (ps)	A_{slow}	$\%A_{\text{slow}}$	Shelf
665	2.7 ± 0.4	0.0044 ± 0.0005	21 ± 2	22 ± 2	0.0125 ± 0.0012	60 ± 3	98 ± 16	0.0039 ± 0.0008	19 ± 4	-0.0069 ± 0.0005
683	1.6 ± 0.4	0.0034 ± 0.0006	20 ± 3	13 ± 2	0.0077 ± 0.0008	46 ± 2	64 ± 5	0.0057 ± 0.0009	34 ± 4	-0.0076 ± 0.0010

The data are fit to a triple exponential decay and a nondecaying component (see text for details).

The amplitudes of the three decay components are given as both absolute amplitudes and as a percentage of the total decay amplitude.

separated state of the RC (P680⁺-Pheo a⁻), which has been shown by nanosecond experiments to have a lifetime of 36 ns at ambient temperature [40]. The difficulties in reliably fitting a triple exponential decay with the modest signal-to-noise (typically about 50 at this pump energy) inherent in transient absorption experiments is well known. It was thus imperative to scan the data until they reached a "flat" (on the timescale in question) baseline, and to record at least several factors of "e" in the decay of the slowest component. This was especially important given that the relative amplitude of the slow component was 20-30% (see below) in our case; i.e., it is weak enough to be difficult to fit, yet strong enough to cause a nontrivial effect on the other components if it is not fit properly. When we fit our data out to only 70 ps, it is well fit to a biexponential with 2 and 21 ps lifetimes. These τ closely match those of Klug et al. [32], whose data extended to only 70 ps in spite of the fact that the transient was still clearly decaying at that time.

The inset to Fig. 2 shows the same data up to 16 ps only. The ultrafast oscillations around $t=0$ are due to an interaction of the pump and probe pulses with the buffer/cell system, and have been observed before by Hong et al. [41]. This signal is roughly linear with pump energy, and as such it cannot be removed by going to lower pump energies. The data are fit only after the oscillations have completely damped out, which is 300-400 fs after the peak of the positive transient. Also shown in the inset is the "zero-crossing" time (see below), which is simply the time after the excitation pulse at which the decay crosses $\Delta A = 0$ while probing at 544 nm.

The result of averages of the fits of multiple scans at pump wavelengths of 665 and 683 nm are given in

Table 1. For all scans used in this table, the pump energy was 200 to 220 nJ, and the probe wavelength was 544 nm. The average at both wavelengths is over six scans, with each scan having a "signal-to-noise" (S/N) ratio ranging from 46:1 to 84:1. The S/N ratio is calculated by dividing the total decay amplitude (i.e., ΔA at ~ 350 fs minus ΔA at long time) by the RMS deviation of the fit. The S/N at 665 nm is slightly better than at 683 nm simply because of the larger amplitude at $t=0$ when pumping into the blue side of the band (vide infra). The amplitudes of the components are given both as absolute amplitudes and as a percentage of the total decay amplitude.

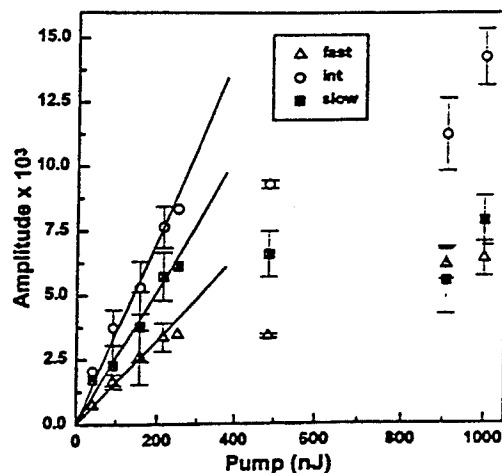


Fig. 3. Absolute amplitudes of the three kinetic components to the triple exponential fit of the transient absorption decay at 544 nm as a function of pump energy with an excitation wavelength of 683 nm in isolated PSII RCs. The lines are linear fits to the data for pump energies up to 200 nJ.

Add new column labels as shown

delete all "%" characters

lifetimes

3.2. Intensity dependence of kinetics

The amplitudes of the three kinetic components as a function of pump energy with an excitation wavelength of 683 nm and a probe wavelength of 544 nm are shown in Fig. 3. Error bars ($\pm 1\sigma$) are given, where applicable. All three components are linear with pump energy up to 200–250 nJ. Above this point, all three amplitudes continue to increase, but at a lesser slope. Similar results were obtained with a 665 nm pump wavelength (data not shown).

In order to determine whether the sublinear behavior at high pump energies as seen in Fig. 3 affects any of the three kinetic components differently, we

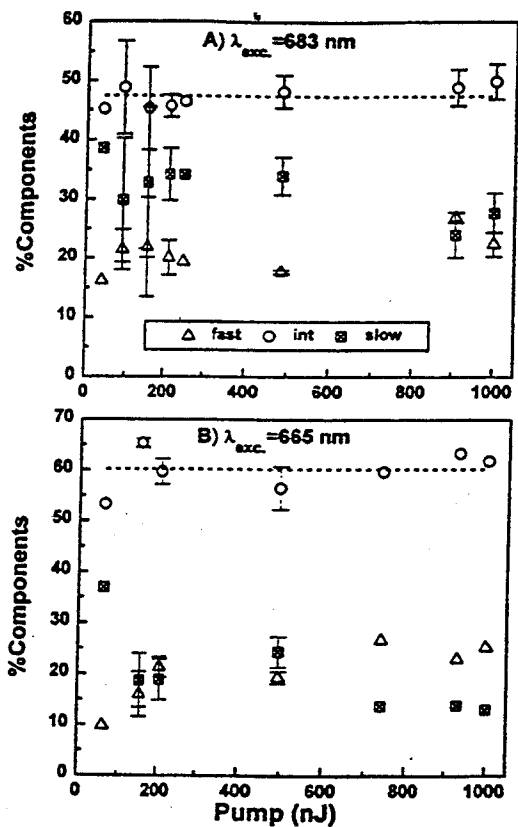


Fig. 4. Relative amplitudes of the three kinetic components to the transient absorption data probing at 544 nm in isolated PSII RCs as a function of pump energy. (A) 683 nm pump. (B) 665 nm pump. The dashed lines are the averages of the values for the intermediate component at each pump wavelength. Note the independence of the data with respect to pump energy, and the larger amplitude of the intermediate component with the 665 nm pump.

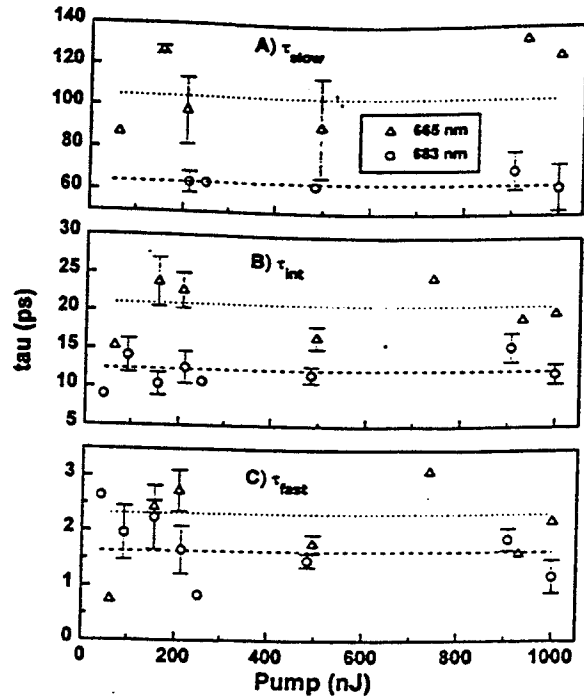


Fig. 5. Time constants for the triple exponential decay of the transient absorption signal at 544 nm in isolated PSII RCs. Data for pump wavelengths of 665 and 683 nm are shown. Dot and dashed lines are the averages over all pump intensities for 665 and 683 nm, respectively. (A) slow component, τ_{slow} ; (B) intermediate component, τ_{int} ; (C) fast component, τ_{fast} . For all three components, the 665 nm time constants are slower than the 683 nm ones. Note the absence of any intensity dependence of the time constants.

have plotted the amplitudes of the components on a *percentage* basis as a function of 683 nm pump energy in Fig. 4A. The largest component is the intermediate component, with $47 \pm 5\%$ of the amplitude. This percentage is independent of the pump energy over the range investigated, and the dashed line is the mean of all of the values. The amplitude of the fast component is $\sim 20\%$, and that of the slow component is $\sim 30\text{--}35\%$. There is no statistically significant trend in the percentages of the fast and slow components up to 500 nJ, and the difference at 0.9–1.0 μJ is fairly small.

Fig. 4B shows similar data, but for a pump wavelength of 665 nm. Once again, the intermediate component is dominant ($60 \pm 4\%$), and its percentage is also independent of excitation energy. The

amplitude percentage of the intermediate component when pumping at 665 nm is significantly higher than when pumping at 683 nm, and will be discussed below in terms of excitation energy transfer. The fast and slow components vary from 15% to 25%, with a change in one at the expense of the other. The trend toward the fast component becoming greater (and the slow component weaker) at pump energies above 700 nJ is probably not statistically significant, as there is only one scan per point in that region.

lifetimes The dependence of the $t = 350$ fs on pump energy for 665 and 683 nm excitation pulses is shown in Fig. 5A–Fig. 5C. With 683 nm excitation, τ_{slow} and τ_{int} are both clearly independent of pump energy, with average time constants of 64 ± 9 and 12 ± 2 ps, respectively. For 665 nm excitation, τ_{slow} and τ_{int} have average time constants of 105 ± 21 and 21 ± 4 ps, respectively, with no apparent dependence upon pump energy. One should note that, for τ_{slow} , all of the 665 nm time constants are slower than the slowest one for 683 nm excitation. This same behavior is also seen for τ_{int} in Fig. 5B. Fig. 5C shows data for τ_{fast} . While the scatter on these data make it difficult to prove the absence of any intensity dependence, none clearly stands out. The time constants for τ_{fast} with 665 nm excitation are generally slower than those for 683 nm excitation, with averages of 2.3 ± 0.7 and 1.6 ± 0.6 ps, respectively.

3.3. Intensity dependence of $t = 350$ fs amplitude

Fig. 6 shows the amplitude of the transient absorption signal at ~ 350 fs after the excitation pulse for pump wavelengths of 665 and 683 nm as a function of pump energy. The amplitude at earlier time cannot be accurately determined, as the large oscillations from the buffer/cell system mask the behavior of the RCs at that time (see Fig. 2 inset). The amplitudes in this figure were derived from the fits to the data. As long as the data are well fit at early time (which it is), this method is preferable to picking the amplitude at a single arbitrary time delay, as the latter is susceptible to the inherently greater noise of a single point. For a given pump wavelength, this amplitude can be used as a measure of the total excited state population (at least in the linear regime). For both pump wavelengths, the amplitude is linear up to 200–250 nJ, and then in-

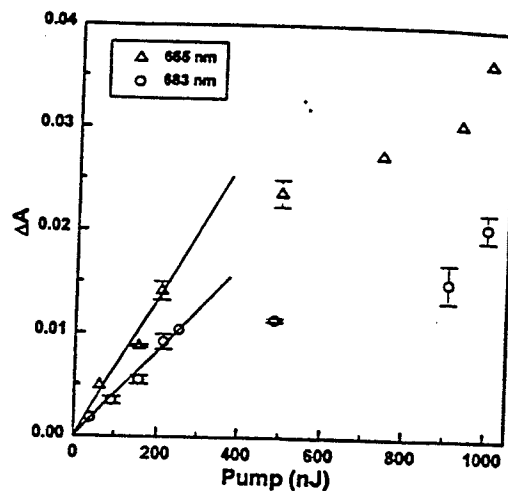


Fig. 6. Amplitude of the transient absorption signal at ~ 350 fs after the laser flash (i.e., the earliest time at which the data can be measured free from artifacts due to the buffer/cell response) as a function of pump energy for pump wavelengths of 665 and 683 nm in isolated PSII RCs. Linear fits to the data for pump energies up to 200 nJ are also shown.

creases at a lesser slope. Note that at all pump energies, the amplitude is greater for “blue”-pool excitation than for “red”-pool excitation.

3.4. Intensity dependence of Chl *a* in THF

In order to gain insight into the cause of the deviation from linearity of the $t = 350$ fs amplitude, we investigated the behavior of Chl *a* in THF upon laser excitation. Although Pheo *a* is also present in the composite Q_y band of the PSII RC, its spectral contribution is relatively minor compared to that of Chl *a*, which dominates because of both the relative pigment oscillator strengths and the stoichiometry. For this reason, only Chl *a* was used for the in vitro experiments, two of which were performed. The first was to make the laser act like simple spectrophotometer: the laser intensity was measured before and after the sample, thus determining the percent transmitted energy, which of course allows a calculation of the absorption of the sample. This was done at many different pump energies from 10 to 850 nJ. At the lowest pump energies, the measured optical density (~ 0.57) matches that measured by a UV-Vis spectrophotometer, taking into account the effect of

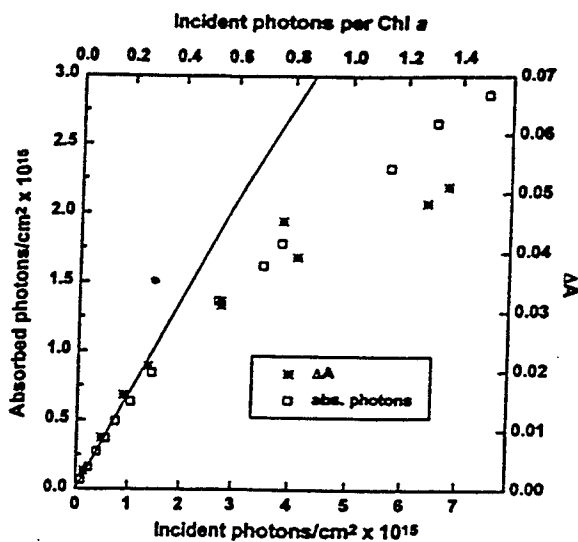


Fig. 7. Chl *a* in THF experiments. Squares show the absorbed photons per cm^2 as a function of incident photons per cm^2 . Deviation from linearity is due to populating a nontrivial fraction of the chromophores in the excited state during the pump pulse. The straight line (73% absorption) is determined from the spectrum of the sample in a UV-Vis spectrometer, adjusting for the effects of the finite bandwidth of the pump pulse. Asterisks show the transient absorption signal at early time probing at 544 nm. Note that the deviation from linearity for the transient absorption data is virtually identical to the absorbance measurement.

the finite bandwidth of the pump pulse as was discussed in ~~the~~ Section 2 for the RCs. As the pump energy was increased, the optical density decreased, getting as low as ~ 0.22 at the highest pump energy used.

The second experiment was to measure the amplitude of the transient absorption signal at early time (the signal decays on a nanosecond timescale) at 544 nm for the Chl *a* in THF solution as a function of pump energy. Fig. 7 shows these data, along with the percent transmission data described above reformulated in terms of absorbed photons/ cm^2 versus incident photons/ cm^2 . As one can see, the two curves have essentially the same form. This should not be surprising, as the transient absorption signal is directly proportional to the number of absorbed photons. The straight line corresponds to 73% absorption (the low-energy limit). Below 1×10^{15} incident photons/ cm^2 , both sets of data are well fit by the line. The sublinear behavior of the sample optical density (and therefore the transient absorption signal) is sim-

ply a result of the pump intensity being high enough to go beyond the linear regime in which Beer's law holds. The deviation occurs because a nontrivial fraction of the sample is in its excited state at high pump intensities [42]. These excited pigments can interact with another photon in the pump pulse and result in stimulated emission, resulting in the "loss" of a previously excited pigment.

Is the sublinear behavior of the $t = 350$ fs amplitude data in the isolated PSII RCs simply a measure of the same phenomena? The highest reasonably linear point for the Chl *a* in THF experiments was at ~ 0.29 incident photons per molecule, which corresponds to 0.21 absorbed photons per molecule (assuming linear, i.e., 73%, absorption). If we multiply this by 3.5 (for simplicity, assuming three Chl *a* per pigment pool, plus one Pheo *a* with half of the oscillator strength), this gives 0.74 absorbed photons per RC for selective excitation of either pigment pool. This is roughly the upper limit of linearity (corresponding to 250 nJ for 665 and 683 nm) for the $t = 350$ fs data in Fig. 6. Since the point of deviation from linearity is quite consistent for the Chl *a* in THF and the isolated PSII RC measurements, and the same sublinear behavior is observed in both samples, it seems quite reasonable to conclude that the behavior is due to the same phenomenon, i.e., a deviation from Beer's law linear absorption. Thus, the nonlinearity of the $t = 350$ fs data for the isolated PSII RCs has nothing to do with the presence of multiple pigments within each RC (e.g., the possibility of annihilation phenomena). The sublinear behavior of the amplitudes of all three of the individual kinetic components (see Fig. 3) is presumably another manifestation of the Beer's law deviation, as the sublinearity begins at the same energy as with the $t = 350$ fs data, and it affects all three components equally (see Fig. 4).

3.5. Intensity dependence of the shelf

With sufficiently high pulse energies, it is possible to excite multiple chromophores in the individual RCs. Of course, each RC is limited to a single charge separated state, as there is only one active redox pair per RC. Therefore, the saturation of charge separation is an excellent indicator of multiphoton

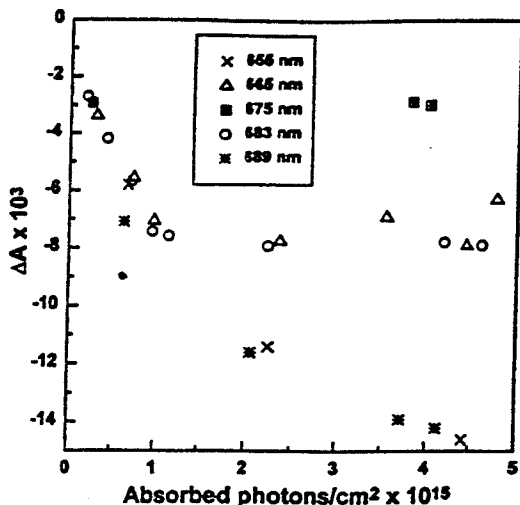


Fig. 8. Amplitude of the nondecaying component (shelf) as a function of absorbed ~~pump~~ photon density at different wavelengths in isolated PSII RCs. For pump wavelengths of 665 and 683 nm, the data are linear up to ~ 250 nJ. Deviations from linearity are due to multiple excitation of individual RCs at high pump energies, and both the point of deviation from linearity and the saturation value of the shelf are dependent primarily on sample concentration, which is higher as the excitation wavelengths gets further from the composite Q_y band peak (see text for details).

excitation of individual RCs, and the amplitude of the Pheo *a* bleach is arguably the best indicator of the extent of charge separation occurring in the sample. The amplitude of the shelf of the transient absorption signal at 544 nm (equivalent to ΔA at ~ 500 ps; i.e., after all excitation energy and charge transfer is complete) is plotted in Fig. 8 as a function of absorbed photons per cm^2 at all five pump wavelengths. The data for 665 and 683 nm are linear up to ~ 250 nJ, and then saturates at a slightly higher value for pump energies between 0.5 and 1.0 μJ . The data for both wavelengths fall on the same curve, with the equivalence in the linear regime indicating that the quantum yield for charge separation is the same for both excitation wavelengths. This implies that the quantum yield for excitation energy transfer (i.e., for excitation at 665 nm) is near unity. At 655 and 689 nm the composite Q_y band oscillator strength is roughly half of that at 665 and 683 nm, and the sample concentration was therefore set to be double. As with the 665/683 nm data, the

655/689 nm data lie on the same curve, thus indicating that the quantum yield for excitation energy transfer and charge separation is unchanged by going out further into the wings of the Q_y band. At the highest pump intensity, the shelf for 655 and 689 nm excitation is twice that of the 665 and 683 nm data, as would be expected since twice as many RCs are excited at saturation at these wavelengths as are excited at 665 and 683 nm. Similarly, the saturation level of the shelf for the 675 nm data is roughly half of that for the 665 and 683 nm data.

3.6. Zero-crossing time

The time at which the transient absorption decay for a probe wavelength of 544 nm crosses $\Delta A = 0$ appears to be a useful monitor of a number of parameters, including the pump energy density, the sample activity, and the identity of the pigment pool that has been excited. Fig. 9A shows the "zero-crossing" time with 655, 665, 683, and 689 nm pump wavelengths for pump energies in the range of 40 to 1000 nJ. The two lines are linear fits to the 665 and 683 nm data. The 655 nm data appear to fit a line of lesser slope but identical intercept as the 665 nm data. Likewise, the 689 nm data appear to have the same intercept as the 683 nm data, but also with a lesser slope. Thus, in the low intensity limit, the zero-crossing time is independent of wavelength *within* each of the pigment pools, but is quite different *between* the two pools. This implies that the zero-crossing time is related to pigment pool-specific phenomena. [18]

The difference in slopes for the two wavelengths within each pigment pool is an indication of what is responsible for this phenomena. Since the slopes are different, it is clear that the zero-crossing time is not simply a function of the pump photon flux. Fig. 9B shows the same data plotted against absorbed photons per RC. Now the 689 nm data actually fall on the same line as the 683 nm data, and the 655 nm data fall on the 665 nm data line. This indicates that the phenomena responsible for the intensity dependence of the zero-crossing time is related to multiple excitation of RCs. It is also unambiguous from Fig. 9B that the zero-crossing time is specific to which pigment pool is excited. Not surprisingly, data at pump wavelengths of 675 nm (data not shown) give

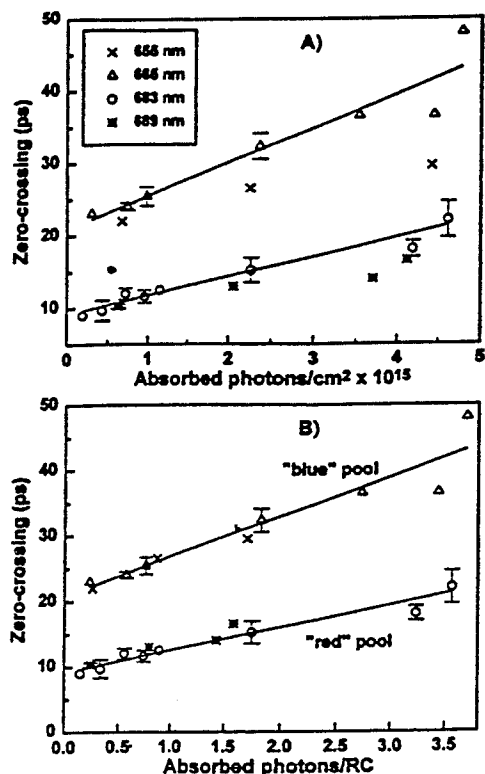


Fig. 9. Zero-crossing time (time after the laser flash at which the transient absorption signal crosses $\Delta A = 0$; see Fig. 2) in isolated PSII RCs for a probe wavelength of 544 nm. Lines in both (A) and (B) are linear fits to the 665 and 683 nm data for all pump energies. (A) As a function of absorbed photons per cm^2 . Note the lesser slope but similar intercept for 655 nm excitation as compared to 665 nm excitation, and similarly with 689 and 683 nm excitation wavelengths. (B) As a function of absorbed photons per RC, which shows effects of multiple excitation of individual RCs. All "blue" pool excitation data now lie on the same line, and all "red" pool data lie on a different line.

zero-crossing times in-between those for selective excitation of the two pools.

The longer zero-crossing time for the "blue" excitation is consistent with the data presented earlier in the paper. Since "blue" excitation results in a higher amplitude at $t = 0$ than "red" excitation, it therefore would take longer to reach $\Delta A = 0$ for "blue" excitation if the decay profiles were identical. As discussed above, all three decay constants are slightly slower for "blue" excitation than for "red", so this too contributes to a longer zero-crossing time for "blue" excitation.

The increase of the zero-crossing time with increasing pump energy is also consistent with the data presented earlier in the paper. In a completely linear regime, doubling the pump energy should be simply equivalent to multiplying the amplitude of the decay curve by a factor of two, which will of course leave the zero-crossing time unchanged. As shown earlier (see Figs. (8) and (6)), increasing the pump energy into the regime where multiple excitation of individual RCs occurs results in complete saturation of the shelf, but the $t = 0$ amplitude continues to increase, albeit at a lesser slope. Since neither the rates nor the relative amplitudes of the kinetic components change to any appreciable extent as a function of pump energy, the decay profile remains fundamentally unchanged. The net result of the same decay profile starting at a higher positive value but ending at the same negative value is that the point at which the decay crosses zero increases in time.

The zero-crossing time is also a measure of sample activity. As the sample degrades and the shelf rises (ΔA more positive, indicating less charge separation), the zero-crossing is pushed out in time. Badly degraded sample do not even cross the baseline (data not shown). The zero-crossing times that we have measured with low pump energies (~ 10 ps with 683 and 689 nm pump wavelengths) appear to be shorter than any previously found in the literature for direct excitation into the "red" pool (18-30 ps) [28,30,32].

3.7. Transient absorption spectra

Transient absorption spectra were recorded at several time delays for both 665 and 683 nm excitation using 250 nJ pump pulses. The same sample was used for all spectra presented here, although other samples were measured and produced identical results. A weak but nonzero transient spectrum before $t = 0$ (due to a small fraction of RCs in the triplet state from the previous pump pulse 1.5 ms earlier) was recorded and subtracted from the transient spectra at positive temporal delays. Fig. 10A shows transient spectra at a delay of 500 ps from 500 to 610 nm. Within the signal to noise of the data, the spectra with 665 and 683 nm excitation wavelengths are identical over the entire region. Thus, after excitation energy transfer and charge separation are com-

transpose

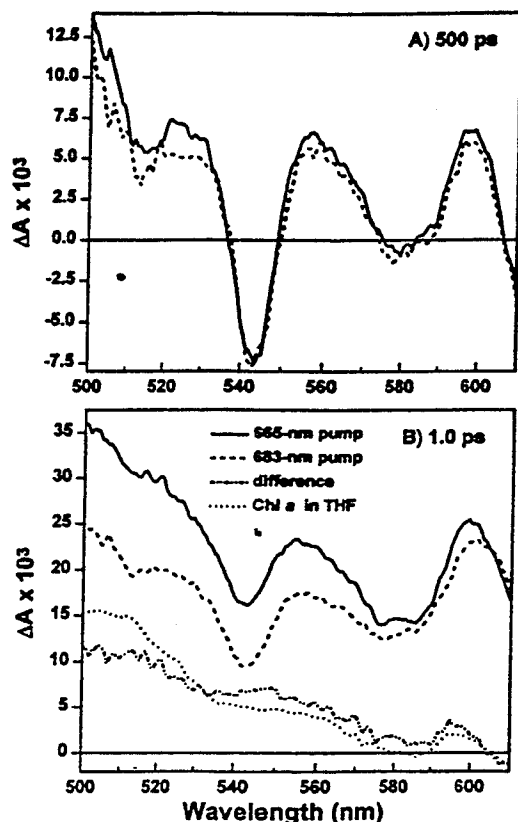


Fig. 10. (A) Transient absorption spectra at 500 ps after the laser flash for pump wavelengths of 665 and 683 nm in isolated PSII RCs. The data, including the Pheo *a* bleach region, are identical to within the S/N, indicating identical degrees of charge separation. (B) Transient absorption spectra at 1.0 ps after the laser flash for pump wavelengths of 665 and 683 nm. Also shown is the 665 minus 683 nm difference spectrum. Note the absence of any feature in the difference spectrum in the region of the Pheo *a* bleach, indicating identical depopulation of the Pheo *a* ground state at this time for both pump wavelengths. The same PSII RC sample was used for all four spectra. Also shown is the transient absorption spectrum of Chl *a* in THF, which is strikingly similar to the 665 minus 683 nm difference spectrum.

plete, the RCs are spectrally indistinguishable. This confirms the conclusion of equal quantum yields for charge separation reached earlier based solely on the data of the shelf value at 544 nm (see Fig. 8). The spectra clearly show that not only is the absolute value of long-time ΔA at 544 nm independent of pump wavelength, but the *depth* of the feature (with respect to the shoulders at either side) is independent as well. This is significant as it is really the depth of the Pheo *a* bleach that is the measure of the popula-

tion of $P680^+ - Pheo a^-$ (and $^1 Pheo a$), not the position of the peak relative to $\Delta A = 0$.

The transient absorption spectra at 1.0 ps for 665 and 683 nm pump wavelengths are shown in Fig. 10B. At this time, the spectra are clearly quite different, and the 665 minus 683 nm difference spectrum is also shown (on the same scale). The general trend is for the difference in the transient absorption spectra to be greater at shorter probe wavelengths. The difference (at 544 nm at 350 fs) was seen earlier in Fig. 6. The absence of any feature in the difference spectrum in the region of the Pheo *a* bleach signifies that the extent of depopulation of the Pheo *a* ground state at 1.0 ps is independent of excitation wavelength. A measurement of the depth of the bleach with respect to the red shoulder at 558 nm confirms that the depth is the same for the two excitation wavelengths. The depth of the bleach at 1.0 ps is already $\sim 50\%$ of the maximum depth (cf. Fig. 10A). The spectrum of the Pheo *a* bleach region at 500 fs (data not shown) is indistinguishable from that at 1.0 ps. Our data are in sharp contrast with that of Rech et al. [16], which show no significant difference in the 520–580 nm region as a function of “red” versus “blue” pigment pool excitation at 3 ps. We have also compared the spectra at both 8 and 50 ps, and the differences between 665 and 683 nm excitation are still present, albeit diminishing (recall at 500 ps, the spectra are indistinguishable). Also shown in Fig. 10B is the transient absorption spectrum of Chl *a* in THF at 14 ps (the *in vitro* Chl *a* spectra do not change on this time scale, so these data can be legitimately compared to the 1.0 ps spectra of the PSII RCs). This spectrum has been rescaled and vertically shifted to match the PSII RC difference spectrum. The features of the PSII RC difference spectrum are very similar to those of *in vitro* Chl *a*, indicating that the state from which excitation energy transfer takes place in the PSII RC is (not surprisingly) an excited state of Chl *a*.

4. Discussion

4.1. Subpicosecond excitation energy transfer

The large differential in amplitude near $t = 0$ (e.g., at ~ 350 fs) that is manifest in Fig. 6 at *all* pump energies for 665 and 683 nm pump wave-

lengths probing at 544 nm is a new observation. The same phenomena exists over virtually the entire 500–610 nm wavelength region at 1.0 ps (see Fig. 10B), and is still present in transient spectra taken at 50 ps. The transient absorption signal at any given wavelength is a combination of stimulated emission, ground state photobleaching, and absorption of the transient species. There is no stimulated emission in this wavelength region. The contribution due to photobleaching of Chl *a* is fairly small because of its weak ground state absorption in this wavelength region. Fig. 10B clearly shows that the depth of the Pheo *a* bleach is identical at 1.0 ps for the two pump wavelengths, so there can be little or no contribution of Pheo *a* bleaching to the 665 minus 683 nm difference spectrum. Assuming that there is no subpicosecond electron transfer occurring (there is no evidence for it), the *differential* in the amplitudes for the two pump wavelengths must be due to differences in the excited state-excited state extinction coefficients of the two pools of excited chromophores. Interestingly, the difference spectrum looks very similar to the transient spectrum of $^1\text{Chl } a$ in vitro, and probably represents the excited singlet state of a “blue”-pool Chl *a* from which the slow energy transfer process occurs (see below). Regardless of the exact nature of the cause of the differences in the transient absorption spectra, the important fact is that there *is* a difference, meaning that excitation energy equilibration at 1 ps is far from complete, in contrast with the 100 fs equilibration proposed in 1992 by Durrant et al. [15]. In 1994, Rech et al. proposed [16] that while the main pool of the “blue” pigments equilibrates on the 100 fs timescale, energy transfer from a secondary pool to the main “blue” pool occurs on a much slower timescale (tens of picoseconds). While our data cannot rule out the existence of subpicosecond energy transfer, the magnitude of the differential between the two spectra at 1.0 ps calls into question the extent of subpicosecond equilibration that could be consistent with our observation. Furthermore, it has been suggested by Schelvis and Aartsma [43] that the observations which Durrant et al. [15] have interpreted as being due to subpicosecond energy transfer between the “blue” and “red” pigment pools may actually be a manifestation of exciton scattering within the P680 dimer itself.

4.2. Slow excitation energy transfer

In order to estimate the time for excitation energy transfer from the “blue” pigment pool to the “red” pool, we have simulated the kinetics based on the average fit values given in Table 1. The 683 nm calculated curve was then subtracted from the 665 nm curve, with the resulting curve shown in Fig. 11. Since the difference between the decays for the two pump wavelengths is due to the absence of the slow energy transfer step for “red”-pool excitation, the decay of the difference curve must be related to energy transfer. The vast majority (89%) of the amplitude of the difference curve decays with a time constant of ~ 29 ps, although a small fraction decays with a > 100 ps time constant (see Fig. 11). While ~~the~~ actual energy transfer rate cannot be determined from this number without knowledge of the kinetic scheme for energy and electron transfer processes in the RC, it does provide an estimate. This energy transfer process also manifests itself as the increase in the amplitude (both absolute and relative) of the intermediate component for 665 nm excitation as compared to 683 nm excitation, as seen in Fig. 4.

the

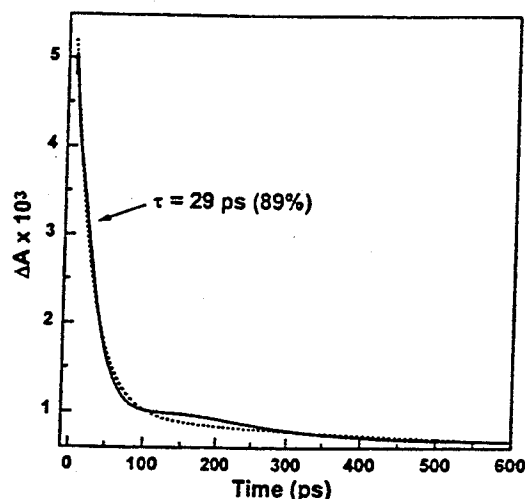


Fig. 11. Simulation of the excitation energy transfer kinetics. The fit parameters in Table 1 were used to calculate “average” decays for 665 and 683 nm pump wavelengths with 200 nJ pulses. The solid curve is the 665 minus 683 nm data. The dashed curve is a biexponential fit to the difference curve, with over 89% of the amplitude of the decay belonging to the 29 ps component.

at
544 nm

4.3. Intensity dependence

Several ways of monitoring the effects of increasing pump intensity on the RC dynamics point to ~ 250 nJ per pulse (for 665 and 683 nm) being the maximum allowable energy for linearity for the spot size used in our experiments. We have both increased (into the nonlinear regime) and decreased (within the linear regime) the intensity by a factor of 4-5 from this point, without any apparent change in either the relative amplitudes or the time constants of the three components of the triple exponential fit to the data (see Figs. 4 and 5). We see no evidence of the appearance of an additional kinetic component that can be ascribed to annihilation phenomena, as has been seen for light harvesting complexes of PSII (LHC II) [44]. Since the isolated PSII RC has significantly fewer pigments than LHC II, it should be less susceptible to annihilation phenomena. Nevertheless, at the highest pump energies used in this study, a significant number of RCs should be multiply excited (see below), and one might expect annihilation to occur in these RCs. A possible explanation for the absence of any observed annihilation at high pump energies may be that it is occurring on a timescale that is difficult to observe given the triple exponential decay of the RCs. For the LHC II complexes, annihilation was easily discernible as a ~ 25 ps component appearing on a "natural" nanosecond long decay. Furthermore, (any) annihilation may not be occurring with a single rate constant, further complicating its detection. There has been recent evidence for the appearance of an additional kinetic component in isolated PSII RCs at higher excitation energies, but this observation is complicated by the use of an excitation wavelength of 400 nm, which excites the high-energy Soret band of the Chl *a* and Pheo *a* molecules without any selectivity [33].

At 200 nJ per pulse with pump wavelengths of 665 and 683 nm, we estimate a sample average of ~ 0.7 absorbed photons per RC, assuming linear absorption (the deviation from Beer's law at this pump level is estimated to be less than 15%). To gain insight into the distribution of unexcited, singly excited, and multiply excited RCs, we have done a simple statistical simulation. We start with a sample of n RCs and $0.7n$ photons, all of which will be absorbed. All RCs start in the ground state (i.e., none

are in the triplet state), which is a reasonable assumption given the 650 Hz repetition rate of our pump pulses and continuous stirring of the sample. Each photon is "absorbed" one-at-a-time, with the probability of absorption by a RC of a given excitation state being proportional to the number of RCs in that state. No attempt to take into account the effects due to sample thickness were made, so the simulation treats the system as an infinitely thin sample. With 0.7 absorbed photons per RC, we estimate 50% of the RCs are unexcited, with almost 70% of the excited RCs being singly excited.

4.4. Charge separation

It is instructive to consider the simplest behavior that could be anticipated from the RCs given a set of experiments that compare direct excitation of P680 at one pump wavelength with excitation of the accessory Chl *a* at a different pump wavelength. Direct excitation of P680 would result in charge separation, and would be observed as a single exponential decay at probe wavelengths appropriate for P680⁺ and Pheo *a*⁻. Excitation of the accessory Chl *a* would result in the serial processes of excitation energy transfer to P680 followed by charge separation, and would be observed as either a single or biexponential decay, depending on the relative rates of energy transfer and charge separation, and whether energy transfer is directly observable at the probe wavelengths. Obviously, neither case resembles the observed data. Even in the relatively straightforward case of (primarily) direct excitation into P680, a triple exponential decay ensues. The simplistic assignment of the relative degree of charge separation to each of the three kinetic components based on their relative amplitudes is probably not warranted, given the large shift to lower ΔA between 1 and 500 ps for the entire transient spectrum (in the 500-600 nm region, see Fig. 10), the exact origin of which is not clear.

Insight into the mechanism of primary charge separation in bacterial reaction centers has been gained from pre-reduction of the bacteriopheophytin primary electron acceptor with chemical reductants, such as sodium dithionite [45]. A similar strategy can be employed to reduce Pheo *a* in the PSII RC prior to flash excitation [2,46]. Pre-reduction of Pheo *a* to Pheo *a*⁻ prevents further photochemical reduction

2, 18, 46

of this species. Under these conditions the transient absorption spectra and kinetics of the PSII RC should reflect only processes involving excited states of the remaining pigments, and possibly, earlier charge separated intermediates (if any exist) such as P680⁺ Chl *a*⁻. Our results indicate that there is a delicate balance between the conditions needed to achieve high yield pre-reduction of Pheo *a* and those necessary to avoid degradation of the protein. We have developed an experimental protocol which allows us to measure the transient absorption spectra and kinetics reproducibly in pre-reduced PSII RCs. Preliminary results using wavelength selective excitation of P680 indicate that both the amplitudes and time constants of each of the three kinetic components change in the "closed" PSII RC relative to those in the "open" PSII RC [18]. It is quite likely that there is a great deal of spectral "heterogeneity" in the ~~sample due to the~~ protein environment of the pigments in the RC. Given this and the close relationship between energy transfer and charge separation within the PSII RC, it is likely that *all three* kinetic components reflect the charge separation process in differing degrees. The data are complex and further experiments are in progress to ascertain whether the pre-reduction strategy will allow us to formulate a model for the charge separation process that accounts for all of the data.

5. Conclusions

Transient absorption kinetic data probing at 544 nm show that for both "red" and "blue" excitation of the composite Q_y band, the ensuing signal has a triple exponential decay. Excitation into the "blue" side of the band results in an increase in all three time constants, and an increase in the relative amplitude of the intermediate component. The difference decay for the two excitation wavelengths decays primarily with a ~29 ps time constant, and is attributed to excitation energy transfer. A very large amplitude difference at early time for the two excitation wavelengths is present, and calls into question the extent of 100 fs energy transfer between the two pigment pools.

The pump energy intensity dependence shows two distinct types of sublinear behavior. The intensity dependence of the amplitude of the transient signal

near $t = 0$ is shown to be similar to that of Chl *a* in vitro, and is ascribed to a simple deviation of Beer's law linear absorption due to a nontrivial population of excited chromophores. The intensity dependence of the shelf amplitude and the zero-crossing time are correlated with the number of absorbed photons per RC, and are related to the limit of one charge separated ion pair per RC. Saturation effects appear at lower energies when pumping into the peak of the composite Q_y band, and at higher intensities when pumping further into the wings. While the highest energies result in the complete saturation of the Pheo *a* bleach, there is no evidence for any kinetic changes that could be attributed to annihilation processes.

The zero-crossing time is shown to be an important indicator of the identity of the pigment pool being excited, the pump energy density, and sample activity, and its value can be determined without fitting the decay. Furthermore, unlike the absolute amplitudes at either $t = 0$ or at long time (i.e., the shelf), which also do not require fitting to be established, the zero-crossing time is relatively independent of the mode matching of the pump and the probe beams, and as such is less susceptible to experimental artifacts.

The results of this work show that a careful examination of the excitation wavelength dependence of the kinetics of the Pheo *a* bleach reveal kinetics sufficiently complex to make assignment of one of the three kinetic components to charge separation very difficult, if such ~~an~~ assignment is even possible. However, these results will serve as a foundation on which to base other, more sophisticated experiments such as liquid He temperature experiments (where excitation energy transfer is unidirectional), studies involving pre-reduction of Pheo *a*, and experiments that measure the actual rate of growth of the Pheo *a* bleach. Hopefully such experiments will enable the development of a model for energy and electron transfer within this protein that is consistent with the data that have already been obtained on this complex nanostructure for photochemical energy conversion.

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Viewpoint

Sixty-three Years Since Kautsky: Chlorophyll *a* Fluorescence

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Abstract. In 1931, using their eyes as instruments, H. Kautsky and A. Hirsch related the time course of chlorophyll *a* fluorescence with photosynthesis in a less-than-one-page article in *Naturwissenschaften* (see Kautsky's photograph). Chlorophyll *a* fluorescence is now being used by hundreds of investigators as a probe for various aspects of photosynthesis—from excitation energy transfer in picosecond time scale to CO₂ fixation in minutes. It is not only a much used, but also a much abused, tool. It is used because of it being a non-invasive, rapid and a highly sensitive probe, and misused because it is sometimes not recognised that it is affected by various photosynthetic and other reactions. I submit that, like any other technique, if it is used with care and with due regard for its time dependence and competing parameters, it will remain as the one-most powerful tool for probing excitation energy transfer, primary photochemistry, electron flow on both the donor and the acceptor side of photosystem



H. Kautsky (ca 60 years old) taken in Marburg, Germany,
(photo courtesy of H. K. Lichtenthaler, Karlsruhe).

II (PSII) of oxygenic PSII. Further, it is very useful in the quick assay of PSII mutations, and down-regulation and other adjustments to stress (excess light, heat, heavy metal, nutrients and certain herbicides). In this paper, I will present my viewpoint, not a review, on the conceptual and experimental developments in this field. Whenever appropriate, and without any shame and humility, I will include some of my involvement in the excitement surrounding this field. I hope that this paper will serve as a starting point for further discussion of not only the history, but the practical use of chlorophyll *a* fluorescence as an intrinsic probe of stresses to plants, as well as individual reactions of oxygenic photosynthesis, when combined with other parallel measurements.

Introduction

Chlorophyll (Chl)* *a* fluorescence is red and beautiful, and is no longer in the purview of specialists alone as it has become a routine probe for information, sometimes misinformation, on the various aspects of photosynthesis. If used properly, it provides information on the identity of the various pigments and pigment complexes, their organisation, excitation energy transfer among them, and on the various electron transfer reactions, specifically of photosystem II (PSII).

It must be stated at the very outset that the quantum yield of Chl *a* fluorescence (Φ_f) of a single species is related to the rate constants (*k*'s) of various pathways of de-excitation (f for fluorescence, h for heat dissipation, t for excitation energy transfer, q for quenching by quenchers (e.g. carotenoids, O₂, triplets, etc.), and p for photochemistry) as follows:

$$\Phi_f = \frac{k_f}{k_f + k_h + k_t + k_q + k_p} \quad (1)$$

The absolute quantum yield of fluorescence (Φ_f) is obtained from the total number of photons emitted (*F*) divided by the total number of photons absorbed (*I_a*), or the lifetime of fluorescence (τ) divided by the theoretical intrinsic lifetime of fluorescence (τ_0) when the only pathway of de-excitation is fluorescence. Measurements of F/I_a will not agree with Φ_f from τ measurements if there is a change in absorption cross-section of the fluorescent pigment bed, such as caused by the formation of non-fluorescent complexes. Further, in view of the homogeneous emission of fluorescence in all directions only a portion of fluorescence is measured with constant intensity of incident light, and thus, only *relative* Φ_f s are reported.

In view of the fact that Chl *a* fluorescence is so rich in information (affected by several de-excitation pathways), it also becomes an ambiguous signal. It is for this very reason, it must be used with caution and in combination with other signals to fully tap its power. In this article, I plan to provide

my viewpoint, not a review, on Chl *a* fluorescence and its use in photosynthesis, particularly PSII since about 90% of Chl *a* fluorescence at room temperature originates in that system. For reviews on Chl *a* fluorescence, readers may consult, among others, Rabinowitch (1951, 1956), Wassink (1951), Butler (1966), Govindjee *et al.* (1967), Govindjee and Papageorgiou (1971), Goedheer (1972), Papageorgiou (1975), Lavorel and Etienne (1977), Govindjee and Jursinic (1979), Govindjee *et al.* (1986) (see various chapters), Lichtenthaler (1988), Krause and Weis (1991), Karukstis (1991) and Holzwarth (1991).

In the Beginning

'It is a noble employment to rescue from oblivion those who deserve to be remembered'—Pliny the Younger, *Letters V*. Althanius Kircher (1646) was the first one to discuss, at length, the bichromatic appearance of an extract of 'lignum nephriticum', that had been recommended for curing kidney ailments. It was yellow in transmitted light and blue in reflected light; the blue light must have been its fluorescence. Sir David Brewster (1834), a Scottish preacher, while discussing his concept of the colour of natural bodies, remarked, almost in passing 'In making a strong beam of the sun's light pass through the green fluid, I was surprised to observe that its colour was a brilliant red, complementary to the green. By making the ray pass through greater thickness in succession, it became first orange and then...'. The green fluid in Brewster's experiment was an alcohol extract of Laurel leaves. It must have contained *chlorophyll*, the pigment of green leaves, as named by Pelletier and Caventou (1818). I consider it likely that this was not only the discovery of (chlorophyll) fluorescence in solution, but also of the reabsorption of this phenomenon in thick samples. However, the clearest discovery of the phenomenon of fluorescence has been that by Sir John Herschel (1845*a*, 1845*b*) in a clear solution of quinine sulfate; it was of 'celestial blue' colour, but was, unfortunately, called *epipolic dispersion*. Brewster (1846) designated it as *internal dispersion*. Professor G. G. Stokes (1852), Professor of Mathematics at Cambridge University,

*Abbreviations used: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; LHCI, light-harvesting complex I; LHCII, light-harvesting complex II; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; NPQ, non-photochemical quenching.

who is known for his discovery that emission bands are shifted to wavelengths longer than the absorption bands (the so-called *Stokes' shift*), used the term *dispersive reflexion*, but quickly added a footnote: 'I confess I do not like this term. I am almost inclined to coin a word, and call the appearance *fluorescence*, from fluor-spar, as the analogous term opalescence is derived from the name of a mineral'. Stokes is the first one to recognise this phenomenon as light emission. Askenasy (1867) has credited Stokes also for the discovery of both phycobilin and Chl *a* fluorescence in fresh red algae. I recommend the book by Harvey (1957) for history of fluorescence prior to 1900.

It was Müller (1874), among others, who noticed that a green living leaf had a much weaker red Chl fluorescence than a dilute Chl solution. Although Müller had predicted an inverse relationship between Chl fluorescence and photosynthesis, his experiments were not done with proper controls. Since both duration of experiment and temperature changed during his measurements, I find it difficult to credit him with the discovery of Chl fluorescence transient (or induction). Further, his concepts on absorption bands cannot be accepted because he used acoustic analogy—vibrations of strings; he expected absorption at all the overtones. Transition dipoles are not strings.

Kautsky's 1931 Paper

'Everything reasonable has been thought of before. We have just to try to think it once anew'—Goethe. On 19 October 1931, Hans Kautsky and A. Hirsch at the Chemisches Institut der Universität in Heidelberg, Germany, submitted a less-than-one page 'Kurze Originalmitteilungen' titled (as translated in English) 'New experiments on carbon dioxide assimilation'. Following illumination of dark-adapted leaves, the time course of Chl fluorescence, observed with the authors' eyes, was correlated, although qualitatively, with the time course of CO₂ assimilation, published earlier by Otto Warburg. The main conclusions illustrated in Fig. 1 were that:

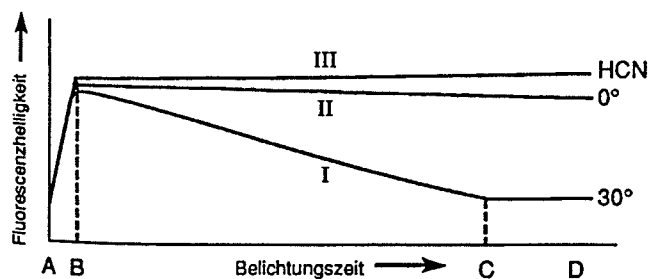


Fig. 1. Schematic representation of Chl *a* fluorescence intensity changes in leaves as observed after turning on the excitation light. I, at 30°C; II, at 0°C; III, poisoned with HCN. Data from Kautsky and Hirsch (1931).

- (a) Chl fluorescence rises rapidly to a maximum, then declines and finally reaches a steady level, all within a matter of minutes.
- (b) The rising portion of the curve was considered to reflect the primary photochemical reaction of photosynthesis, as it was unaffected by temperature (0 and 30°C) and by a poison (HCN). If the light was turned off at the maximum, the fluorescence transient recovered fast.
- (c) The decline in the fluorescence curve was found to be inversely correlated with the increase in the rate of CO₂ assimilation; this suggested to the authors that more chemical energy is produced from photons when less Chl fluorescence is seen.
- (d) The long lag in the carbon assimilation was considered rather strange—it seems that 'light-dependent' processes are required for the full development of the carbon assimilation process; also unexplained was the long time needed for the recovery of fluorescence transient if the light was turned off after the transient was completed.

I consider these observations to be a landmark in the history of photosynthesis that has led to such a 'mess' today that we have to gather 'Downunder' to sort the basics and interpretations of Chl fluorescence at this Robertson Symposium 63 years after the Kautsky and Hirsch paper. Lichtenthaler (1992) has already provided further details about Kautsky and his work on Chl fluorescence induction kinetics.

It is indeed remarkable to note that only one year after this paper, two classical papers by Robert Emerson and William Arnold (1932*a*, 1932*b*) appeared in which the concept of a 'photosynthetic unit' (2500 Chl molecules cooperating to do photosynthesis) was highlighted with highly sophisticated and elegant experiments (see Myers 1994). I shall discuss this concept later in the paper.

The Transient

A dark-adapted leaf or a suspension of higher plant, algal or cyanobacterial cells, shows characteristic changes in Chl *a* fluorescence intensity when illuminated with continuous light. These changes have been called fluorescence induction, fluorescence transient or simply the Kautsky effect. Basically, they can be classified as *fast* (up to 1 s) or *slow* (up to several minutes). These fluorescence transients have been the subject of a vast number of studies and continue to be used as probes for various qualitative effects on photosynthesis. The fast changes have been a bit easier to understand and interpret than the slower changes. However, caution must be exercised even in their interpretations. I remind the readers of a statement by Lavorel and Etienne (1977), 'Any given (fluorescence) response is initially directly related to the closest photochemical step, but subsequently becomes determined by a network of interactions of increasing complexity

ultimately involving the entirety of the apparatus. The analysis of the fluorescence phenomenon is thus made difficult not only because of the number of controlling factors but also because the mode of action of these factors may not simply be reduced to elementary concepts of quenching or non-radiative (heat) dissipation'. Although I fully appreciate this statement, I am not as pessimistic because of the tremendous progress currently being made in integrated approaches in physiology and medicine, in neural networks and in cognitive sciences. These have begun to be applied to Chl *a* fluorescence research.

Nomenclature

In the early literature, the Chl *a* fluorescence transients had been described by inflection points labelled as A, B, C, D, E, or D1, M1, D2, M2, etc. (see e.g. Rabinowitch 1951). However, the currently popular nomenclature of *OIDP* for the fast transient is based on those by Lavorel (1959, 1963; *O*→*P*), and Munday and Govindjee (1969*a*, 1969*b*; *O*→*I*→*D*→*P*) and of *PSMT* for the slow transient by Papageorgiou and Govindjee (1968*a*); a schematic diagram is shown in Fig. 2. The fluorescence increases from *O* (that stands for origin) to *I* (inflection or intermediary peak), decreases to *D* (dip) followed by an increase to *P* (peak) and then a decrease to *S* (quasi-steady state) (Table 1). This is also called the first wave. In the second wave, fluorescence rises from *S* to *M* (a maximum) and declines to *T* (terminal steady state). If there are several additional waves in between, they have been labelled as, e.g. *S*₁ *M*₁ *S*₂ *M*₂ *T* for the slow transient (Yamagishi *et al.* 1978, who partly based this nomenclature on an alternative terminology of Bannister and Rice 1968), or *I*₁ *D*₁ *I*₂ *D*₂ *P* for the fast transient (Neubauer and Schreiber 1987; Schreiber and Neubauer 1987). During weak illumination, the transient rises from *O* to '*PI*', where *PI* stands for plateau (Forbush and Kok 1968) and is considered equivalent to the *O* to *ID* phase. Since during these transient measurements, exciting intensity is kept constant, the fluorescence intensities at

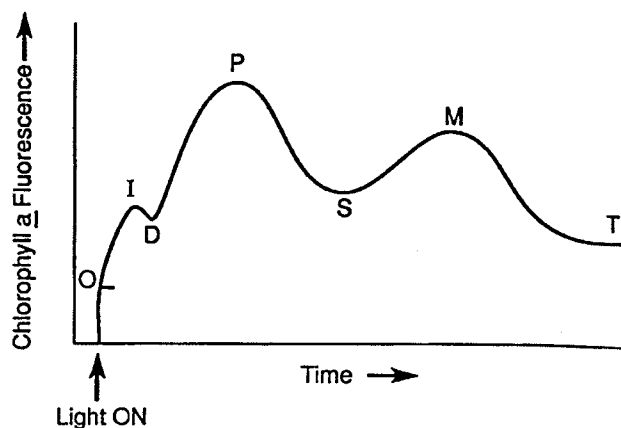


Fig. 2. Nomenclature of the Chl *a* fluorescence transient as used during 1960–1980. Data from Govindjee and Papageorgiou (1971).

these 'inflection' points, F_O , F_I , F_P , etc., may simply be labelled as Φ_{F_O} , Φ_{F_I} , Φ_{F_D} , Φ_{F_P} representing quantum yields of fluorescence. However, such nomenclature should be used with great caution especially during the slow transients (*PSMT*) since changes in absorption cross-section of fluorescent PSII pigments have been suggested to occur in this time scale.

Some Correlations

As a plant physiologist, I had learnt to accept, for pragmatic reasons, correlations that work under defined experimental conditions. Some examples follow.

Complementarity

Kautsky and Hirsch (1931) had already mentioned the antiparallel relationship between Chl *a* fluorescence and photosynthesis. This complementary relationship during the *DPS* transient was quantitatively established by MacAlister and Myers (1940) (Fig. 3). Delosme *et al.* (1959) showed

Table 1. Nomenclature of inflections and in fluorescence transients

<i>The Fast Transient: the first wave</i>		
Origin	'O' (Lavorel)	≡ F_O
Inflection	'I' (Strasser)	≡ I (Delosme) ≡ I_1 (Schreiber)
Intermediary hump	<i>I</i> (Munday)	≡ I_2 (Schreiber)
Plateau	<i>ID</i> (<i>D</i> for dip; Munday)	≡ <i>PI</i> (Kok/Joliot)
Peak	<i>P</i> (Lavorel)	≡ F_m (if maximum)
<i>The Slow Transient: the second wave</i>		
Quasi-steady state	<i>S</i> (Lavorel)	≡ S_1, S_2
A maximum	<i>M</i> (Papageorgiou)	≡ M_1, M_2
Terminal steady state	<i>T</i> (Papageorgiou)	

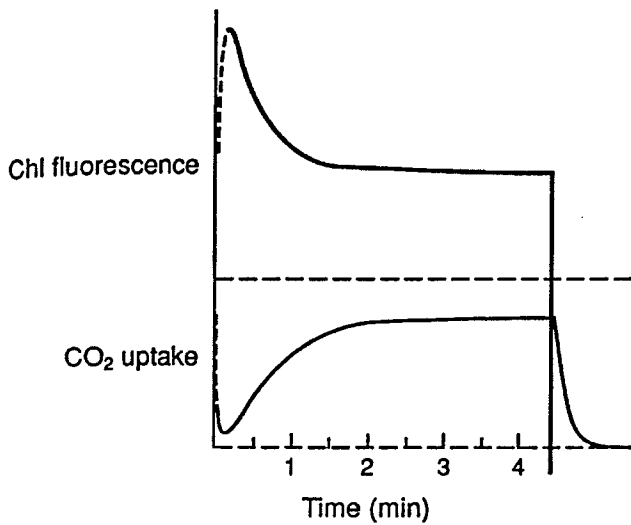


Fig. 3. Simultaneous recording of Chl *a* fluorescence intensity (top curve) and rate of photosynthesis (measured as CO₂ uptake, bottom curve) in a wheat leaf up to 4 min. Note the antiparallel relationship. Atmosphere: 0.03% CO₂ in N₂. Data from McAlister and Myers (1940).

that both O₂ evolution and Chl *a* fluorescence increased during the *OI* phase, and then they were antiparallel during the *DPS* phase; thus, *OID* was an 'activation' phase before O₂ was evolved. Papageorgiou and Govindjee (1968*a*, 1968*b*) and Mohanty *et al.* (1971*a*) showed the parallel increase in fluorescence during *SM* phase, and constancy of O₂ evolution during *MT* decline (Fig. 4). Thus, it is clear that the antiparallel relationship is observed *only* under certain experimental conditions. Looking back at Eqn (1), this happens when *only* two of the parameters change. When other rate constants also change, the antiparallel relationship breaks down. Kautsky and Hirsch (1931) have mentioned that it took a long dark time to restore the transient if the light was turned off at long time after illumination. Duysens and Sweers (1963) showed that the *OPS* transient was not restored if light was turned off at the '*S*' level and turned back on immediately. The hypothesis of *Q* (now called *Q_A*) was that Chl *a* fluorescence increased when *Q_A* was reduced and decreased when *Q_A* was oxidised. If this was the *only* factor controlling *OPS* transient, the transient should have been restored right away. Mohanty and Govindjee (1974) and Briantais *et al.* (1986) discuss the dual nature of this phase extensively: one related to *Q_A*, and the other to some 'high energy state'. Papageorgiou and Govindjee (1971) showed a relationship of the suspension pH, whereas Briantais *et al.* (1979) showed a relationship of proton gradient changes with the *P* to *S* decay. In terms of Eqn (1), this implies that another rate constant (perhaps, *k_h*), besides *k_p[Q_A]*, is affected by pH changes.

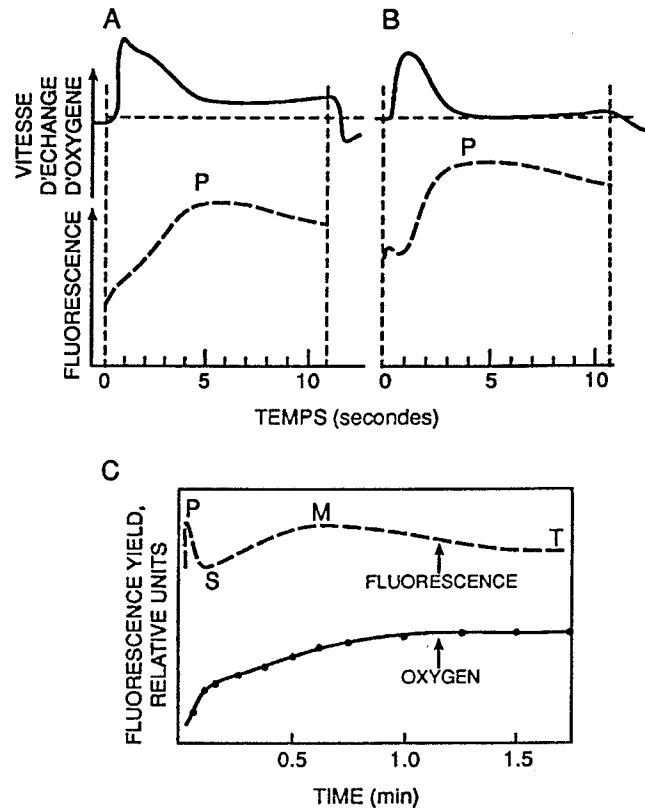


Fig. 4. Simultaneous recording of Chl *a* fluorescence intensity and rate of photosynthesis in algal cells dark-adapted for several minutes. (A) Fast transient, aerobic sample; (B) sample (A), after 3 min of anaerobiosis; (C) slow transient, aerobic sample. Note the initial parallel relationship between O₂ evolution and fluorescence, then antiparallel, followed by parallel (during *S* to *M* rise), and then constancy of O₂ evolution when fluorescence declines. (A) and (B) data from Delosme *et al.* (1959), the original O₂ curves were turned 180° in this figure; (C) data from Mohanty *et al.* (1971*a*); also see Papageorgiou and Govindjee (1968*a*)

PQ pool size

The *OP* rise is mostly due to the net decrease in the concentration of the quencher, *Q_A*, and, thus, the net accumulation of *Q_A*⁻. The area over the Chl *a* fluorescence rise curve of the fast transient (*OIDP*) has been used to measure the size of the electron acceptor pool of PSII, the plastoquinone (*PQ*) pool size, provided, e.g. the same area can be measured, under similar experimental conditions, when the *PQ* pool is 'isolated' from the PSII reaction centre by the addition of inhibitors, such as 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). The earliest papers on the calculations of the acceptor side pool were by Malkin and Kok (1966) and Murata *et al.* (1966*a*). The area over the DCMU curve approximates one electron equivalent on *Q_A*⁻, and the area without DCMU is the total number of electron equivalents on the electron acceptor pool. Such experiments have, in general, provided good estimates

(5–10 PQ molecules) of the PQ pool size. However, see Trissl *et al.* (1993) and Trissl and Lavergne (1995) for a discussion of potential problems.

Sites of inhibition/mutation

A simple-minded, but an effective, use of Chl *a* fluorescence transient has been to use it as a probe for finding out if an inhibitor or a mutation has caused a lesion on the electron donor or the electron acceptor side of PSII. For example, inhibitors or mutations that cause a block in the electron flow beyond PSII, e.g. after the electron acceptor Q_A (Duysens and Sweers 1963), cause a faster fluorescence rise ($O \rightarrow P$) and the fluorescence yield remains high. In course of such experiments, Vernotte *et al.* (1979) discovered that Chl fluorescence was often about 10–20% higher when the PQ pool was all reduced (saturating light, no DCMU). This suggests that oxidised PQ pool can also directly quench antenna Chl *a* fluorescence, an alternative mechanism that does not involve Q_A —a concept that often comes as a surprise to many students. A block in the electron flow on the donor side of PSII, e.g. between H_2O and P680 (the reaction centre Chl *a* of PSII), leads to a slower Chl *a* fluorescence rise and fluorescence remains low. This condition is, however, restored to normal if the cause of the block is removed (see e.g. Mohanty *et al.* 1971*b*; Critchley *et al.* 1982; Metz *et al.* 1989). Fig. 5 shows an example of inhibition on the donor side by chloride depletion.

For application of slow fluorescence changes to CO_2 -fixation reactions, see e.g. Horton (1985) and Lichtenthaler (1988).

The OJIP Transient

A majority of fluorescence transient literature has used the term *OIDP* for the *fast* fluorescence transient, and it has been tacitly assumed that the *OI* phase, measured during transients by all investigators, is equivalent to the photochemical phase *OI*, recorded at high intensity excitation, and with fast measuring instruments (Morin 1964; Delosme 1967). I prefer to call this *I*, I_{Delosme} —the reason will become clear soon. The *I* in the Chl *a* fluorescence transient curve was the name given to the first inflection, observed on linear scale plots from measurements with camera shutter instruments (Munday and Govindjee 1969*a*, 1969*b*). However, using a LED Walz fluorometer, originally developed by Schreiber *et al.* (1986), and extremely high intensity excitation light, Neubauer and Schreiber (1987) and Schreiber and Neubauer (1987) discovered that the *OIDP* should be represented as $OI_1 (D_1) I_2 (D_2) P$ transients since there were two, instead of one, inflection(s) between *O* and *P*. Using a commercial LED Hansatech instrument PEA (Plant Efficiency Analyser), Strasser and Govindjee (1991, 1992) recently observed two inflections between *O* and *P*, and labelled them as *J* and *I*,

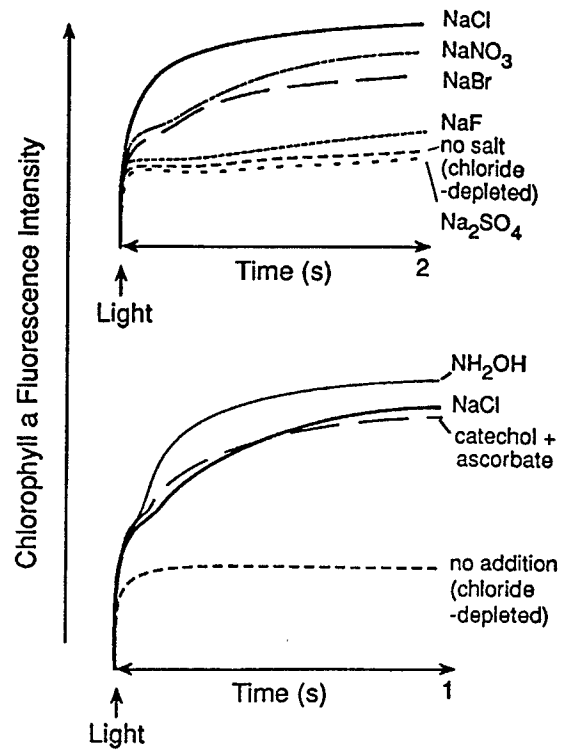


Fig. 5. Chl *a* fluorescence transients in halophyte (*Avicennia germinans*) thylakoids depleted of chloride, and then supplied with various salts or electron donors. Chloride depletion leads to loss of variable fluorescence which is recovered by electron donors (NH_2OH , catechol and ascorbate) or by $NaCl$, $NaNO_3$ and $NaBr$, but not by NaF or Na_2SO_4 . Data from Critchley *et al.* (1982).

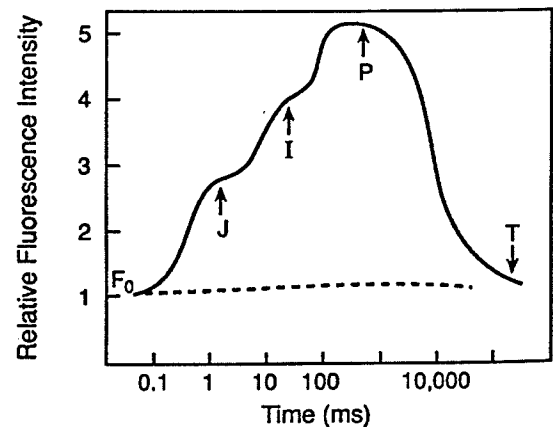


Fig. 6. Chl *a* fluorescence transient of an attached pea leaf, excited with 650 W m^{-2} 650 nm light, plotted on a logarithmic time scale. Two inflections, labelled as *J* and *I*, are observed between the levels *O* and *P*. Data from Strasser and Govindjee (1992).

not I and J , or I_1 and I_2 . This, of course, may cause some confusion in the literature. However, the reason for our nomenclature was: (a) a comparison of the same fluorescence transient plotted on a linear and a logarithmic scale identified the I of the transient, and, the newer inflection was at a time shorter than that of this I ; thus we gave it a new name—that of ' J '; and (b) since we had not proven the identity of these inflections with I_1 and I_2 , we considered it safer to keep different names. The only reason, we think, why these inflections were revealed is very simple: with true ' O ' level (F_0) obtained by careful use of the LED instrument, it was just the use of the logarithmic plot that allowed us to visualise clearly the two inflections on the same plot (see Fig. 6). However, our earlier work on DI mutants of *Chlamydomonas reinhardtii* had neither revealed the two inflections nor the true ' O ' level as the latter two were faster than the opening time of the camera shutter used (Govindjee *et al.* 1991). Measurements of Strasser *et al.* (1995) on the intensity dependence of the quantum yield of fluorescence at O , J , I and P reveal that J behaves like the I of Delosme, and is different from the I of usual fluorescence transients. If we had labelled the transient *OJIP*, it would have been consistent with Delosme (with J being the new inflection) but inconsistent with most of the transient literature. We, therefore, favour the use of *OJIP* with the understanding that J is equivalent of I_{Delosme} . However, OI_1I_2P is another alternative, but is a bit cumbersome due to the use of the subscripts and the implication that I_1 and I_2 may have similar origin. Personally, I like the sound of '*OJIP*'.

Our current understanding of *OJIP* transient rise, that Kautsky and Hirsch could not have observed 60 years ago, is as follows: it reflects, in the first approximation, the successive but overlapping filling-up (i.e. reduction) of the electron acceptor pool of PSII. This pool includes not only the electron carriers in the main pathway (Q_A , Q_B , PQ, where Q_A is the one-electron acceptor-bound PQ, Q_B is the two-electron acceptor-bound PQ, and PQs are mobile PQ molecules), but also Q -400 (non-heme iron). The hypothesis of Duysens and Sweers (1963) that Q_A^- is the determining factor governing the increase in Chl a fluorescence is implicitly accepted here. The inflections represent the heterogeneity of the process. The *OJ* rise is the photochemical phase, the inflection J represents the momentary maximum of $[Q_A^-]$, perhaps, because of limitation in electron acceptance by Q_B , and I reflects the heterogeneity of the PQ pool, fast-reducing and slow-reducing PQ pools, an idea that is really not new (see Lavorel and Etienne 1977). Thus, the *OJIP* transient can be used as a quick monitor of the electron acceptor side reactions, the pool heterogeneity and pool sizes, and the effects of inhibitors and mutations on these processes, as well as on the donor side. Hsu (1993) has confirmed the

earlier conclusion from the Joliot-Delosme laboratory that the fast fluorescence rise is influenced by the S -states. I do not recommend that you throw away your fluorimeters (Holzwarth 1993). However, at the moment, we may not be able to easily obtain any quantitative information on the individual rate constants since the secondary reactions of both photosystem I (PSI) and PSII are slow compared with the single-turnover of the PSII reaction centre leading to the overlapping and complex effects (also see Trissl *et al.* 1993). Thus, we should wait for more sophisticated measurements of parallel transients of individual reactions and components, as well as for the evolution of more sophisticated deconvolution procedures. In the meantime, I recommend the use of kinetics of reactions following single-turnover flashes to monitor individual reactions of PSII.

Two-light-reaction and Two-pigment-system Concept

Kautsky Again

The concept of two light reactions through Chl a fluorescence studies was first considered by Kautsky and U. Franck (1943). They attributed the observed rise and fall of fluorescence to two light reactions succeeding one another almost immediately, one responsible for the rise and the other for the fall. Wassink (1951), however, pointed out that the quenching of fluorescence may have been caused by a side reaction. On the other hand, Rabinowitch (1945) discussed a two-light-reaction scheme (see scheme 7.V, on p. 162) to explain the requirement of eight quanta per O_2 (Fig. 7). And in 1956, Rabinowitch suggested (see p. 1862, par. 2) that one light reaction may transfer electrons from water to cytochrome, and another from cytochrome to a final acceptor. Kautsky *et al.* (1960), based on newer experiments on Chl a fluorescence *in vivo*, reiterated the suggestion that two consecutive light reactions worked in photosynthesis.

We shall now discuss the history of the evolution of the concept of the quencher, Q_A . Kautsky *et al.* (1960) discussed the concept that the oxidised state of a compound, A, a member of the electron transport chain, determined the quenching of fluorescence: when A was oxidised (A_O), Chl fluorescence was quenched, but when A was reduced (A_1), it was not. During Chl fluorescence transient, the rise was due to conversion of A_O to A_1 , whereas the successive decline (presumably P to S ?) was due to oxidation of A_1 by the next member of the chain, B, in its oxidised state (B_O)—the latter was formed from B_1 (reduced state) by another light reaction. The absence of fluorescence decline when the inhibitor phenylurethane was present was explained to be due to a block of reoxidation of A_1 . In their model, A was closer to the O_2 -evolving process, and B to the CO_2 -fixation reactions. Although the above model is quite revealing, it lacked the impact because: (a) it ignored the existence of the

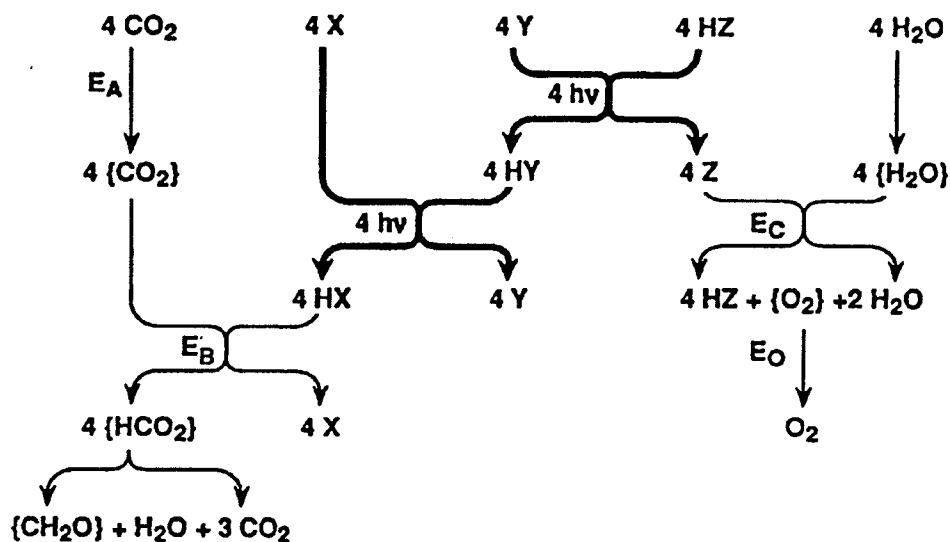


Fig. 7. Oxygenic photosynthesis with oxidation–reduction reactions between three intermediary catalysts (X, Y and Z) utilising two primary photochemical reactions, suggested as one of the several schemes to explain the eight quanta per O_2 measurements. One light reaction (bold lines) functions to transfer an electron (or hydrogen atom) from HZ to Y; the oxidised product Z reacts with H_2O to evolve O_2 (right side). Another light reaction (bold lines) functions to transfer an electron (or hydrogen atom) from HY to X; the reduced product HX reduces CO_2 to carbohydrate (CH_2O) (left side). The E's with subscript indicate that they are enzymatic reactions catalysed by different enzymes. In current language, electron transfer from HZ to Y involves PSII, and that from HY to X involves PSI. Now, Z could be equated with a specific tyrosine residue in PSII, Y with cytochrome *f* and X with NADP^+ or an equivalent intermediate. Original diagram from Rabinowitch (1945).

two-pigment-system concept already evolved by the work of Robert Emerson; (b) it was *not* the correct explanation of the fluorescence decline observed; and (c) it was published in a journal that many scientists did not read. On the other hand, Hill and Bendall (1960) proposed a scheme of two light reactions that had the additional possibility of providing energy for ATP synthesis during a downhill process between the two light reactions (see Duysens 1989, for the historical perspective of the discovery of the two-light-reaction scheme).

Govindjee *et al.* (1960) discovered that far-red light (absorbed in Emerson's longwave system, later known as system I, Duysens *et al.* 1961) quenched the high Chl *a* fluorescence (excited by blue or 670 nm light, Emerson's short-wave system, system II) in *Chlorella* cells. This antagonistic effect of light I and II on Chl *a* fluorescence yield was considered the fluorescence evidence for the two-light-reaction–two-pigment-system concept of photosynthesis. Butler (1962) demonstrated the same phenomenon in anaerobic leaf with red (650 nm, system II) and far-red (>720 nm, system I) beams. However, it was Duysens and Sweers (1963) who provided the current explanation: light II, absorbed in PSII, reduces a quencher of Chl *a* fluorescence, labelled as Q , and light I, absorbed in PSI, oxidises Q^- back to Q . The herbicide DCMU was shown to block the reoxidation of Q^- , but not the reduction

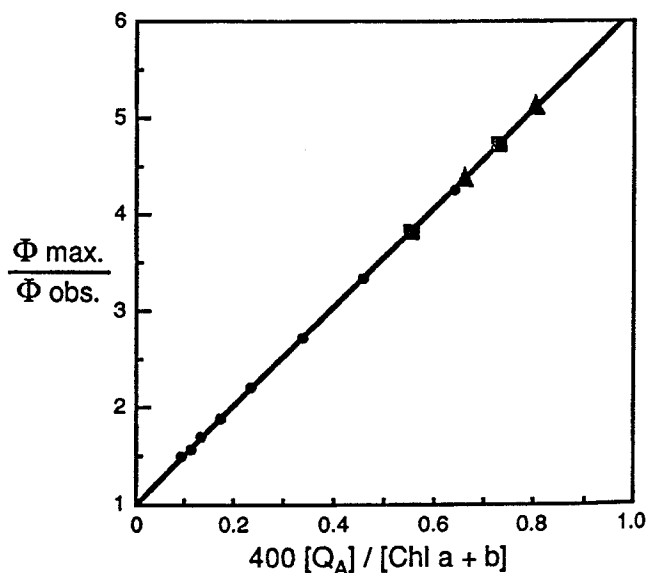


Fig. 8. Chl *a* fluorescence changes in Tris-washed thylakoids measured as a ratio of F_{maximum} (labelled as Φ_{max}) to F_{observed} (labelled as Φ_{obs}), plotted as a function of Q_A ($400[Q_A]/[\text{Chl } a + \text{Chl } b]$). $[Q_A^-]$ was measured by absorbance changes at 320 nm due to the formation of plastoquinone. Changes were induced by a series of non-saturating flashes; different symbols refer to different experiments. These data suggest a linear relationship between Chl *a* fluorescence parameters and $[Q_A]$. Data from Van Gorkom *et al.* (1978).

of Q . Today, this Q is known as Q_A , and is shown to be a PQ molecule (see Van Gorkom *et al.* 1978) (Fig. 8). The antagonistic effect of light I and II on Chl a fluorescence yield is still a useful tool to investigate the site of an inhibitor between Q_A and P700, the reaction centre Chl a of PSI, as recently shown for bicarbonate-reversible formate inhibition (Govindjee *et al.* 1993a).

The Two-pigment Systems

Different spectral varieties of Chl a (see French 1971) are present in different pigment-protein complexes of both PSI and PSII. As earlier mentioned, most of the Chl a fluorescence (approx. 90%) at room temperature originates in PSII complexes, PSI complexes being weakly fluorescent. Further, it is only the PSII fluorescence that varies with changes in photochemistry, i.e. the variable Chl a fluorescence belongs strictly to PSII. Why is PSI weakly fluorescent, and why there is no variable fluorescence in it are important questions that have not been systematically dealt with yet. Among several others, the following speculations can be made:

- The reaction centre Chl a of PSI, the P700, is a deeper energy trap than the trap of PSII, the P680, and, thus, its photochemistry may not be 'trap-limited', i.e. energy transfer to it is more irreversible than to P680; the antenna fluorescence of PSI does not compete with PSI chemistry.
- The immediate electron acceptor or donor to P700 is not a chemical quencher of Chl a and, thus, variations in Chl a fluorescence do not occur.
- The physico-chemical nature of antenna Chl a of PSI, that absorb, on the average, longer wavelength of light than shorter wavelength, is such that k_h predominates over k_f . We know that the lifetime of PSI Chl a

fluorescence is shorter than that of PSII Chl fluorescence, i.e. it traps energy faster than PSII (Holzwarth 1991). Of course, this means a lower quantum yield of fluorescence as $\phi_f = \tau/\tau_0$.

Room temperature

Although there is a heterogeneity in Chl a fluorescence at room temperature because of the existence of two photosystems (PSI and PSII), the major fluorescence band at 683–685 nm and its vibrational satellite at 720–735 nm originate mostly in the PSII antenna complexes (Table 2). I am unable to state as to exactly what proportion comes from which of the PSII complexes. I suspect that most of the variable Chl a fluorescence originates in CP43 and CP47 Chl a protein complexes, with CP47 being responsible for a small 693–695 nm emission when PSII reaction centres are closed either by strong light or by DCMU. The existence of a 693–695 nm emission at room temperature in different organisms was shown by Krey and Govindjee (1964, 1966), Papageorgiou and Govindjee (1967, 1968a, 1968b) and Govindjee and Briantais (1972). On the other hand, a PSI emission, that may be from an 'ordered' Chl a , is centered around 705–715 nm (Lavorel 1963; Wong and Govindjee 1979; Goedheer 1981). Fig. 9 summarises these concepts.

Low temperature

At 77K, Chl a *in vivo* produces, at least, four emission bands: F685, F695, F720 and F740 in addition to the long wavelength shoulders due to the various vibrational satellite bands. Brody (1958) discovered that cooling *Chlorella* cells to 77K leads to the formation of a new broad emission band at about 725 nm. It was shown by Govindjee and Yang (1966) and Cho and Govindjee (1970a) to be composed of, at least, two bands (Table 2). Mar *et al.* (1972) showed that

Table 2. Chlorophyll a fluorescence bands in oxygenic thylakoids

Emission range – peak or shoulder (nm)	Name	Possible origin of Chl a	Comments
<i>At room temperature</i>			
683–687	F685	PSII core: CP43, CP47??	Major band
693–698	F695	PSII core: CP47?	Minor shoulder: with high $[Q_A^-]$ (1964)
705–712	F710	PSI core or antenna	Minor: F_0/F_m spectrum (1963)
720–760	F740	PSII + PSI	Broad vibrational satellite bands
<i>At low temperatures (4–120K)</i>			
<i>Upon cooling</i>			
679–682	F680	LHCII (1979)	Minor: appears at 4K
683–687	F685	PSII core: CP43 (1984)	Major: increases below 20K (1966)
693–700	F695	PSII core: CP47 (1984)	Major: appears below 120K (1966); decreases below 20K
705–715	F710	???	
715–730	F720	PSI reaction centre complex (1980)	Major: includes vibrational satellite bands
730–750	F740	LHCI (1980)	Major: includes vibrational satellite bands

it could also be distinguished from F685 as it had a longer lifetime of fluorescence. Although Litvin and Krasnovsky (1958) had observed the existence of a band at 695 nm in etiolated leaf (originating in a chlorophyll precursor), it was not until 1963 that the existence of F695 was discovered and generally recognised to originate in PSII (Bergeron 1963; Brody and Brody 1963; Govindjee 1963; Kok 1963). Although it was recognised independently in three laboratories that F685 and F695 belong to PSII and F720 and F740 to PSI (Boardman *et al.* 1966; Cederstrand and Govindjee 1966; Govindjee and Yang 1966; Murata *et al.* 1966b), earlier assignments to particular protein complexes were in error. Contrary to earlier beliefs, F685 and F695 *do not* belong to light-harvesting complex II (LHCII) and reaction centre II, respectively. Although their complete assignment is still not fully established, most of F685 and F695 belong to Chl *a* in core PSII complexes (Gasanov *et al.* 1979; Rijgersberg *et al.* 1979), and F720 and F740 to PSI core (reaction centre I containing intrinsic antenna Chls) and light-harvesting complex I (LHCI), respectively (Mullet *et al.* 1980a, 1980b). Although Nakatani *et al.* (1984) turned out to be in error in assigning the function of reaction centre II to CP47, they are correct in assigning F685 to originate in CP43 Chl *a* and F695 to Chl *a* in CP47. PSI band F720 originates in a Chl *a* complex absorbing at 695 nm (Das and Govindjee 1967) and F740 in a Chl *a* complex absorbing at 705 nm (Butler 1961). On the other hand, a band at 680 nm (F680) appears at 4K only when LHCII is present (Rijgersberg *et al.* 1979). Thus, F680 belongs to Chl *a* from LHCII; it cannot be normally observed due to highly efficient transfer from it to other complexes (see Fig. 9). In addition, Shubin *et al.* (1991) have observed a new emission band in a cyanobacterium *Spirulina platensis* at 758 nm (F758), at 77K, which originates in a Chl complex with an absorption band at 735 nm (Chl₇₃₅⁷⁵⁸). Interestingly, this complex transfers its excitation energy to the oxidised form of the reaction centre of PSI, P700⁺, and thus, quenching F758 during photo-oxidation of P700.

Photosynthetic Unit and Excitation Energy Transfer

Photosynthetic Unit

Gaffron and Wohl (1936a, 1936b) interpreted the results of Emerson and Arnold (1932a, 1932b) stating that a collection of 2500 Chl molecules somehow cooperates to evolve, with high quantum efficiency, one molecule of O₂, as follows: light energy, absorbed anywhere in this *unit*, the *photosynthetic unit*, migrates by excitation energy transfer to the *photoenzyme* where several excitons cooperate to initiate photosynthesis. This is in contrast to diffusible chemicals being formed at each site, and then diffusing to the *photoenzyme*. This concept of a photosynthetic unit composed of many pigments serving a photoenzyme has been conceptually supported by the discovery of excitation energy transfer and of the reaction centre chlorophylls P700 (Kok 1956) and P680 (Döring *et al.* 1967) and the many pigment-protein complexes that contain only antenna or bulk pigments.

Robinson (1967) coined the terms *lake* versus *puddles* for the organisation of antenna and reaction centre chromophores. In the lake model, also called the statistical or the matrix model, the exciton may freely visit territories surrounding reaction centres. In contrast, in the isolated puddles, the separated units, or the restricted model, the exciton can visit only its own reaction centre. However, the situation may be 'in-between', i.e. there may be some probability of energy exchange between the different puddles. Looking at the existence of various pigment-protein complexes, it is quite likely that a 'pebble-mosaic' model (Sauer 1975) is the real picture. It remains a challenge to provide a complete mathematical and physical model for exciton migration in photosynthesis.

Whether there is a directed or a random exciton migration must depend on many factors including the relative energy levels of the donors and the acceptors. The directed model (the *funnel* model) seems to be appropriate for heterogeneous energy transfer in phycobilisomes, or even when one deals with transfer from shortwave to long

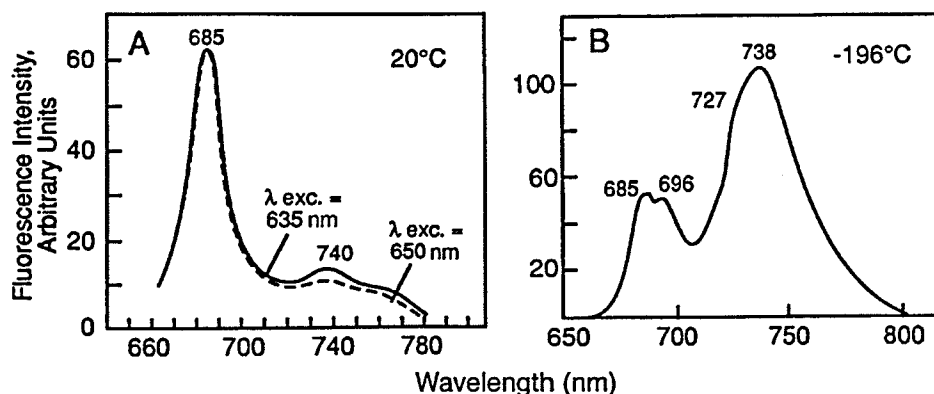


Fig. 9. Chl *a* fluorescence spectrum of spinach thylakoids at room temperature (A) and at 77K (B). Presented by Govindjee at the 10th International Botanical Congress, Edinburgh 1964. Data from Govindjee and Yang (1966).

wavelength forms of Chl *a* (Govindjee *et al.* 1967; Seely 1973). However, a random migration is more appropriate for homogeneous energy transfer among isoenergetic pigment molecules (see Discussion in Pearlstein 1982).

Butler and Strasser (1977), Strasser and Butler (1977), Butler (1978) and Strasser (1978) have discussed various *bipartite* or *tripartite* and *grouping* models of organisation of pigments. These concepts have been extensively used in the literature, but will not be discussed here.

Excitation Energy Transfer

A detailed and mechanistic picture of excitation energy (exciton) transfer is only possible when the detailed distances and orientations of the chromophores are known. A major breakthrough in this direction has been the recent electron diffraction picture of LHClI by Kuhlbrandt *et al.* (1994). It shows the detailed arrangement of individual Chl *a* and Chl *b* molecules, their orientation and distances (approx. 5 Å edge-to-edge). From Förster's resonance theory (Förster 1948), one can calculate excitation energy transfer from one molecule to another—the rate of this transfer is dependent upon three crucial parameters:

- (a) $1/R^6$, where R is the distance between the donor and the acceptor molecules;
- (b) $(\kappa)^2$, where κ (orientation factor) = $\cos\alpha - 3\cos\beta_1\cos\beta_2$, with α and β being angles between the acceptor and donor dipoles and between the vector that connects the dipoles and each dipole, respectively; and
- (c) the overlap of energy levels, as calculated by the overlap integral between the fluorescence spectrum of the donor and the absorption spectrum of the acceptor molecule (see e.g. Van Grondelle and Amesz 1986).

In Förster's theory, excitation energy transfer from a donor to an acceptor occurs after there is a relaxation by internal conversion within the donor molecule. The overlap integral between donor and acceptor molecules is temperature dependent. Thus, temperature dependence of energy transfer had been predicted. Cho *et al.* (1966) and Cho and Govindjee (1970a) observed changes in emission spectra of *Chlorella* cells as they decreased temperatures down to 4K; similar changes were observed in cyanobacterium *Anacystis* (Cho and Govindjee 1970b). Although other interpretations are possible, these results were taken to support the Förster theory for energy transfer from phycobilins to Chl *a* and for transfer from Chl *a* fluorescing at 685 nm to that fluorescing at 695 nm.

Excitation energy migration (homogeneous energy transfer) studies between Chl *a* molecules were pioneered by Arnold and Meek (1956) through observation of depolarisation of Chl fluorescence. These investigations were later pursued in my laboratory by Ted Mar and Daniel Wong (see Mar and Govindjee 1972; Wong and Govindjee 1981) and Whitmarsh and Levine (1974). A decrease in the

polarisation of Chl fluorescence by closure of PSII reaction centres was taken as evidence of increased energy migration. However, due to a lack of detailed knowledge of the orientation of dipoles, and due to a possible lack of coherence of excitons even after one or two transfers, conclusions from such studies have been rather limited, and extraction of quantitative information about energy migration rather difficult (see e.g. Knox 1975).

The existence of excitation energy transfer (heterogeneous energy transfer), however, has been convincingly shown by the technique of steady-state sensitised fluorescence, from fucoxanthol to Chl *a* (Dutton *et al.* 1943), from phycobilins to Chl *a* (Duysens 1952; French and Young 1952) and from Chl *b* to Chl *a* (Duysens 1952). Excitation in the donor molecule shows a quenching of the donor fluorescence and a stimulation or enhancement of the acceptor fluorescence.

Müller (1874) had already commented on the lower fluorescence intensity of leaves over that in solution, implying the use of the absorbed energy in a leaf for photosynthesis. This concept has been quantitatively emphasised when it was noted that the quantum yield of Chl *a* fluorescence *in vivo* is 0.03–0.06 in contrast to 0.25–0.30 *in vitro* (Latimer *et al.* 1956)—the majority of the absorbed energy *in vivo* must be used in photosynthesis. Since the quantum yield of fluorescence (Φ_f) is directly proportional to the lifetime of fluorescence (τ), and since the latter can also provide unique information on the primary photochemical events of photosynthesis, a major advancement was made when Brody and Rabinowitch (1957) and Dmetrievsky *et al.* (1957), independently, and by independent methods (direct flash and phase shift), measured the lifetime of Chl *a* fluorescence *in vivo*. Even in the very first paper, Brody and Rabinowitch (1957) showed that there was a delay in observing Chl *a* fluorescence when phycoerythrin was excited, showing that energy transfer takes a finite time when it moves from phycobilins to Chl *a*. Tomita and Rabinowitch (1962) calculated this time to be about 300 ps and the efficiency of energy transfer to be 80–90%. The time of energy transfer from Chl *b* to Chl *a* was too fast to be resolved, but the efficiency of transfer was confirmed to be 100%, as earlier found by Duysens (1952) in steady-state measurements.

Indeed, when ultrashort (femtoseconds to picoseconds) flashes of light are used to excite donor molecules, one can measure precise times for the movement of excitation energy from the donor to the acceptor molecule: as the donor fluorescence subsides, the acceptor fluorescence appears. A beautiful cascade has now been observed in the red algae where one can follow precisely the excitation energy transfer by this technique, from phycoerythrin to phycocyanin to allophycocyanin (see e.g. Yamazaki *et al.* 1984). These events occur in picosecond time scale (see Fig. 10).

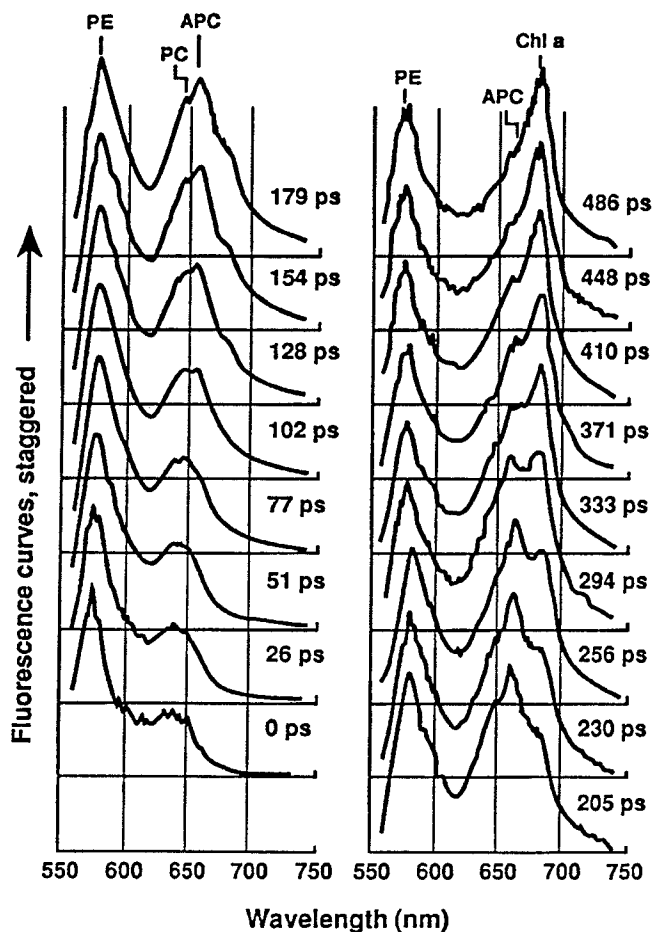


Fig. 10. Cascade of excitation energy transfer in picosecond time domain from PE (phycoerythrin) to PC (phycocyanin) to APC (allophycocyanin) to Chl *a* in the red alga *Porphyridium cruentum* after excitation with a 6 ps 540 nm flash. The vertical lines mark the maximum of fluorescence of various pigments. The time sequence starts at the bottom of the left, and, then again at the bottom of the right graph. Data from Yamazaki *et al.* (1984).

Probe of Photosystem II

Chlorophyll *a* fluorescence measurements have been most useful in probing PSII reactions since, as mentioned twice, most Chl *a* fluorescence at room temperature is from PSII.

Basic Relationship

Ignoring extraneous quenching processes, assuming that all energy transfer in PSII leads to photochemistry, and omitting terms for quenching by $P680^+$ (see later discussion) and those originating in *S*-state transitions, Eqn (1) can be simplified to:

$$\Phi_f = \frac{k_f}{k_f + k_h + k_i + k_q + k_p} \quad (2)$$

where it is assumed that we are only considering PSII, and $[Q_A^-]$ is the concentration of electron acceptor of PSII.

At low light intensities, when most $Q_A = Q_A^-$ (assume it is 1), i.e. its concentration is maximal, Chl *a* fluorescence yield is minimal:

$$\Phi_f^{\min} (\text{at } F_0) = \frac{k_f}{k_f + k_h + k'_p} \quad (3)$$

On the other hand, at strong light intensities, when all $Q_A = Q_A^-$, $[Q_A^-] = 0$, Chl *a* fluorescence yield is maximal:

$$\Phi_f^{\max} (\text{at } F_{\max}) = \frac{k_f}{k_f + k_h} \quad (4)$$

Dividing (3) by (4):

$$\frac{\Phi_f^{\min}}{\Phi_f^{\max}} = \frac{k_f}{k_f + k_h + k'_p} \times \frac{k_f + k_h}{k_f} = \frac{k_f + k_h}{k_f + k_h + k'_p}$$

Adding and subtracting k'_p in the numerator:

$$= \frac{k_f + k_h + k'_p - k'_p}{k_f + k_h + k'_p} = 1 - \frac{k'_p}{k_f + k_h + k'_p} = 1 - \Phi_p^{\max}$$

Thus,

$$\Phi_p^{\max} = 1 - \frac{\Phi_f^{\min}}{\Phi_f^{\max}} = \frac{\Phi_f^{\max} - \Phi_f^{\min}}{\Phi_f^{\max}} = \frac{\Delta\Phi_f^{\max}}{\Phi_f^{\max}} \quad (5)$$

which is equivalent to $F_{\text{variable}}^{\max} / F_{\text{max}}$. This is the origin of the well-known relationship between measured fluorescence parameters and the quantum yield of photochemistry of PSII. This relationship has the implicit assumption that not only are all the postulates given above true, but none of the rate constants k_f , k_h and k'_p change between F_0 and F_{max} , i.e. the restrictions are indeed severe.

Shinkarev and Govindjee (1993) have re-emphasised Eqn (1) in which the rate constant kq cannot be ignored under many experimental conditions. It should, we believe, include the terms $kq[P680^+]$, and perhaps, $kq[Pheo^-]$ unless they are proven to be zero, and kp should include the concentration of the pair $P680Q_A$, not just Q_A , as was already done by Duysens (1979). Extending the concept to include a possible quenching by O_2 (see Discussion in Papageorgiou 1975), we may rewrite Eqn (1) as:

$$\Phi_f = \frac{k_f}{k_f + k_h + k_i + k_q[O_2] + kq[P680^+] + kq[Pheo^-] + k'_p[P680Q_A]} \quad (6)$$

If, however, we must deal with high intensity flashes, where the possibility of triplet formation exists, one has to include it as another quenching process (Sonneveld *et al.* 1979).

The concept of fluorescence yield as a monitor of only one process is obviously wrong and the early concepts of Kautsky and Hirsch (1931) and MacAlister and Myers (1940) about the inverse relationship between fluorescence and photosynthesis are specific but important cases when most of the other rate constants remain constant except for the major one that governs photosynthesis. Genty *et al.* (1989) have provided the most significant correlation between the measured quantum yield of photosynthesis and that calculated from fluorescence measurements (Fig. 11).

Connectivity Between Units

In the *lake* model, excitons migrate freely (random walk). If they encounter a closed reaction centre Chl *a*, they can just go to another centre (Knox 1975; Pearlstein 1982). Such a model predicts a linear relationship between lifetime of fluorescence, τ , and quantum yield of fluorescence, Φ_f as the traps are closed, by varying the intensity of excitation or by addition of an inhibitor. Briantais *et al.* (1972) introduced a τ versus Φ_f diagram, and showed a proportionality between the two quantities throughout the entire range of measurements in *Chlorella* cells (Fig. 12). This result and the earlier results of Tumerman and Sorokin (1967) were taken to support the *lake* model. It did not support the strict 'isolated puddles' model, where exciton can visit *only* its own reaction centre, because fluorescence would have to be dealt with as a sum of fluorescence from open and closed units, leading to a significant nonlinearity in the τ - Φ_f curve.

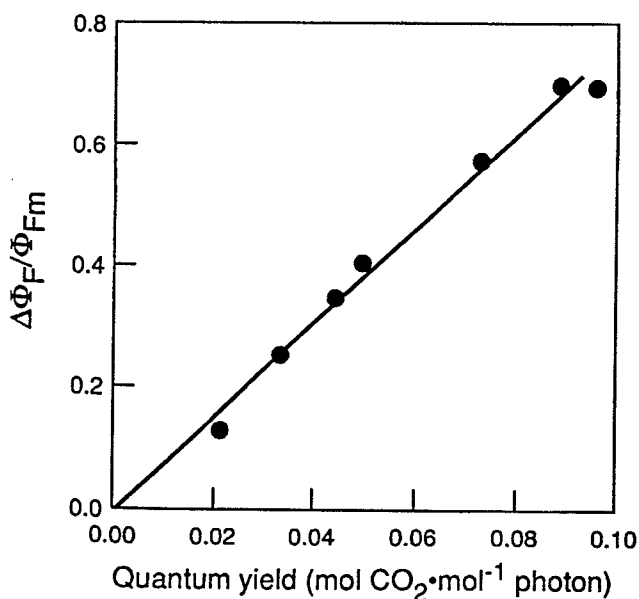


Fig. 11. Relationship between the photochemical yield of PSII, calculated from variable/maximum ($\Delta\Phi_f/\Phi_{Fm}$) Chl *a* fluorescence yield, and the quantum yield of CO_2 fixation in a barley leaf. Note the linear relationship. Data from Genty *et al.* (1989).

In reality, however, the picture may be 'in-between', i.e. there may be a certain probability of exciton migration from one unit to another, as if there were interconnected puddles or a pond. Joliot and Joliot (1964) had derived a relationship:

$$\frac{F_{(t)} - F_o}{F_{\max} - F_o} = \frac{(1-p)q}{1-pq}, \quad (7)$$

where, $F_{(t)}$ is the Chl *a* fluorescence yield at time *t*, F_o is the fluorescence yield when all Q_A is in the oxidised state, F_{\max} is the maximum fluorescence yield when all Q_A is in the reduced state, *p* is a parameter related to the probability of intersystem energy transfer, and *q* is the fraction of closed reaction centres, counted in the coin of Q_A . Here $q = 1$, when Q_A^- is maximum. Joliot's calculated the parameter *p*, that depended solely on the variable Chl fluorescence. The calculated values of *p* have hovered around 0.5 in most cases.

Both Paillotin (1976, 1978) and Strasser (1978) have independently pointed out difficulties with this concept and have suggested modifications. As the centres close, the proportion of open centres decrease. Many scientists, including ourselves (Xu *et al.* 1989), have used Eqn (7) without questioning its validity. Paillotin (1976) suggested

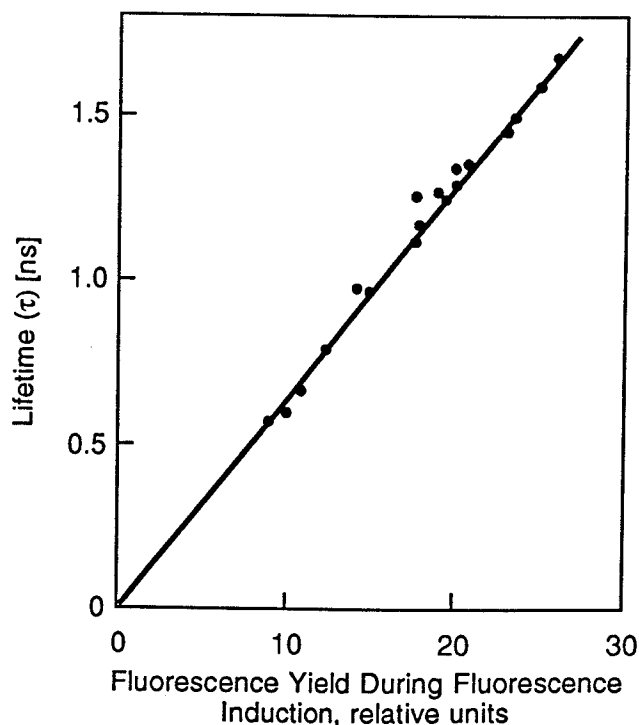


Fig. 12. τ (lifetime) versus Φ_f (yield) diagram for Chl *a* fluorescence of the green alga *Chlorella*. Φ_f was changed by varying the flow rate of algae suspension. The frequency of modulation for τ measurement was 102.207 MHz. Wavelength of excitation was 632.8 nm. Note the linear relationship of τ with changes in Φ_f suggesting the 'lake' model for the organisation of pigments. Data from Briantais *et al.* (1972).

using a physical connection parameter P that depends only upon exciton migration from a closed to an open reaction centre; he relates it to Joliot's p as follows:

$$p = P(1 - F_o/F_{\max}) = P \times F_{\text{variable}}/F_{\max} \quad (8)$$

On the other hand, Strasser (1978) has proposed that the probability of exciton migration in Joliot's equation be corrected by the ratio of F_{variable}/F_o . For a relationship between the three equations, see Strasser *et al.* (1992).

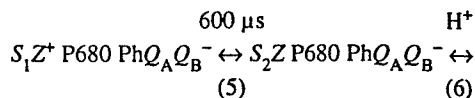
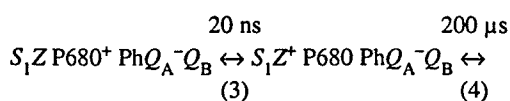
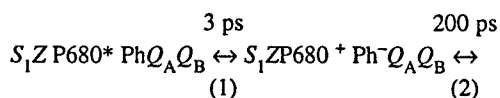
In a rather effective manner, Trissl *et al.* (1993) have challenged many concepts and provided reasons for further caution in blindly using fluorescence induction measurements to make quantitative calculations. We need to look seriously at this paper and the follow-up paper (Trissl and Lavergne 1995) in which a parameter J , related to P , is explicitly given by a number of rate constants. I shall now discuss where Chl *a* fluorescence measurements have been highly useful in probing PSII reactions, but first I will present a detailed schematic of PSII reactions that is needed to appreciate the experiments.

The Photosystem II Reactions

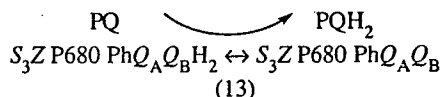
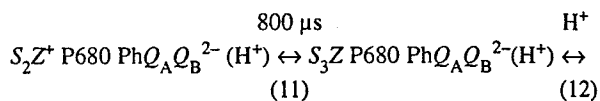
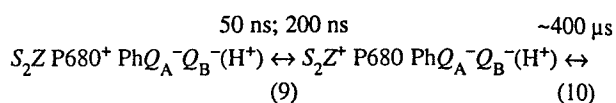
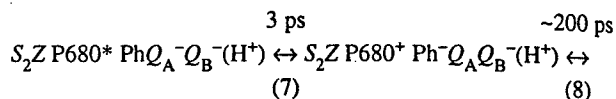
Most of the PSII Chl *a* fluorescence in PSII preparations and in thylakoids that we measure is from antenna Chl *a* molecules, not reaction centre Chl *a* molecules. The variable Chl *a* fluorescence is created either from exciton equilibration between the antenna and the reaction centre Chl *a*, or from exciton/radical pair equilibration (see Renger 1992, for literature and discussion of PSII chemistry). It had been generally believed that all the PSII fluorescence was prompt fluorescence. Klimov *et al.* (1977) suggested that all of the variable Chl *a* fluorescence of PSII was recombinational luminescence from the back reaction of $P680^+$ with Pheo^- . Although there hasn't been a general acceptance of this concept (see Van Gorkom 1986), the exciton/radical pair equilibration recombination model (see Holzwarth 1991) seems capable of accommodating it. A good part of fluorescence from the *isolated* PSII reaction centre, however, originates in recombination of $P680^+$ with Pheo^- (see e.g. Govindjee *et al.* 1990a).

Upon excitation of the reaction centre Chl *a* P680 of PSII, the following reactions occur (Eqn 9(1-13)).

Eqn (9):



Q_B^- has a long lifetime; its neighborhood gets protonated; after another excitation, the reaction continues:



Here, S_n represents the redox states of the O_2 -evolving Mn complex, Ph is pheophytin, and other symbols have been defined earlier. Due to the imprecise knowledge of the exact times listed above, it is not clear which reactions control the relaxation of the S -states.

Analysis of primary photochemical reactions by lifetime of fluorescence measurements has led Van Mieghem *et al.* (1992) and Govindjee *et al.* (1993b) to conclude that PSII charge separation (Eqn 9(1)) is decreased if Q_A^- is present, but is increased if doubly reduced Q_A, Q_A^{2-} , is present. The prior redox state of the donor side may also have effects on the reactions presented above.

Z to P680⁺ reaction

In the nanosecond to sub-microsecond time scale, Chl *a* fluorescence yield rise, after a brief (\leq ns) actinic flash, measures the electron flow from Z to P680⁺ (steps (3) and (9) in Eqn 9). This rise was discovered by Mauzerall (1972) and explained by Butler (1972) to be due to the removal of the quencher P680⁺ (Fig. 13) Sonneveld *et al.* (1979) have elegantly measured this reaction, after correcting for quenching due to triplets, and showed that this reaction was faster (approx. 20 ns) during transition of S_0 and S_1 , and slower and more complex during transitions of S_2 and S_3 (for a description of S -states, see Kok *et al.* 1970). This fluorescence rise can be observed even at longer times due to the equilibria reactions between $S_n \leftrightarrow Z \leftrightarrow P680$ (see e.g. Kramer *et al.* 1990).

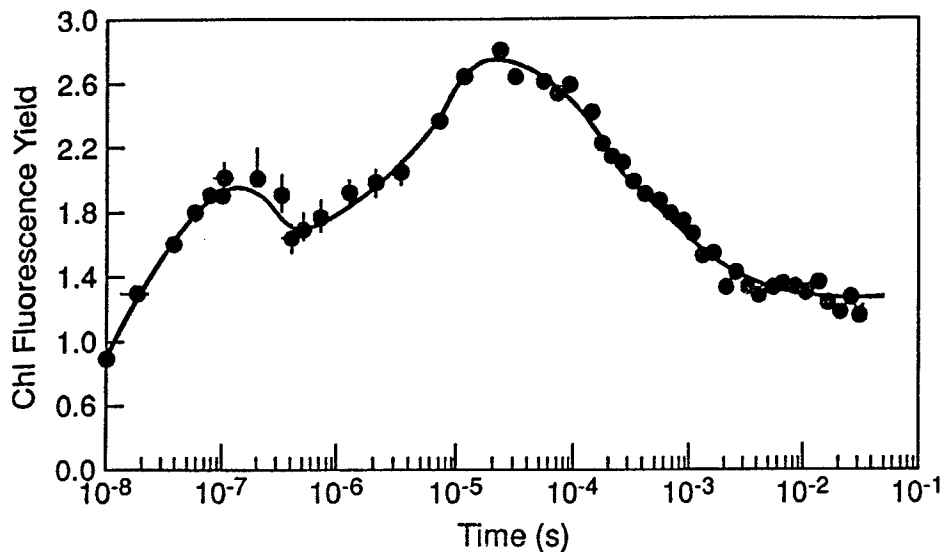


Fig. 13. Chl *a* fluorescence yield changes in the dark-adapted cells of the green alga *Chlorella* after a saturating nanosecond laser flash. The rise near 20 ns was ascribed by Butler (1972) to the reduction of the quencher P680⁺ by Z; the rise in the microsecond range is due to the disappearance of the carotenoid triplets (see Sonneveld *et al.* 1979); the decrease in fluorescence yield in the 10 μ s to millisecond range is due to the electron transfer from Q_A^- to Q_B (Forbush and Kok 1968). Data from Mauzerall (1972).

Q_A^- to PQ reactions

In the microsecond to millisecond time scale, Chl *a* fluorescence yield decay, after a brief flash, measures the electron transfer from Q_A^- to Q_B (steps (4) and (10) in Eqn 9). These measurements were presented, in a preliminary fashion, by Forbush and Kok (1968) who used a 1 ms (approx.) saturating flash to induce a single turnover of PSII reaction centre; they observed a fast decay phase of $t_{1/2} \sim 0.6$ ms, which they correctly attributed to re-oxidation of Q_A^- ; about 18 flashes were needed to fill completely the secondary acceptor PQ pool then called the A pool. They also remarked at the heterogeneity of this A pool. Although Mauzerall (1972) showed the microsecond to millisecond fluorescence decay (Fig. 13), the first detailed and reliable measurements on this decay was by Zankel (1973) who observed a phase of $t_{1/2} \sim 200$ μ s and another of 1 ms, and related them to the equilibria between $Q^- \leftrightarrow A$ and $Q^- \leftrightarrow A' \leftrightarrow A''$ (Q being Q_A , and A and A' being the two fractions of the A pool, the fast and the slow reducing pool).

Further, Zankel (1973) was the first one to observe that the Chl *a* fluorescence decay after first flash was faster than after the second flash (Fig. 14) as was rediscovered by Bowes and Crofts (1980). Instead of explaining this result on the basis of electron transfer on the acceptor side of PSII, Zankel related it incorrectly to the donor side since, I believe, he had just confirmed in the same paper the period 4 oscillation in the 35 μ s fluorescence signal, originally discovered by Delosme (1971) and related to the S-states of the O_2 -evolution steps.

The two-electron gate

The concept of the two-electron gate was elegantly demonstrated in an experiment, that I consider to be a major breakthrough, by Velthuys and Ames (1974). In these

experiments, the possible oscillations due to the donor side (the S-state cycling related to O_2 -evolution steps) were eliminated by alkaline Tris-washing, and an external electron donor was added to run PSII. A series of preflashes were given and then the herbicide DCMU was injected and Chl *a* fluorescence yield monitored. There was an obvious binary oscillation in the Chl *a* fluorescence yield: high after the first and all uneven preflashes, and low after the second and all even preflashes (Fig. 15). This work provided, for the first time, information on how one electron acceptor, Q_A (then called Q), communicates with the two-electron-acceptor PQ molecule. The authors assumed that a carrier they had called R (now called Q_B) exchanges electrons one

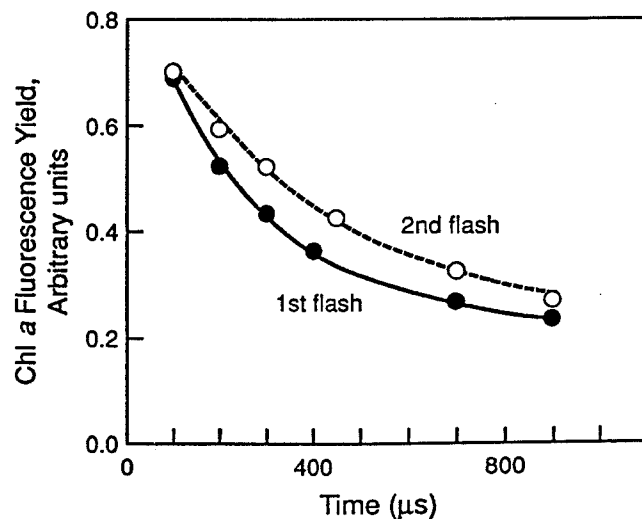


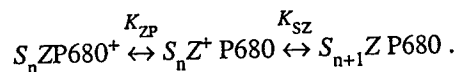
Fig. 14. Decay of variable part of Chl *a* fluorescence yield as a function of time after flash 1 and 2, spaced 2 s apart. Sample: dark-adapted spinach chloroplasts at 22°C. Note that the decay is faster after the first than after the second flash. Data from Zankel (1973).

by one with Q_A , but two by two with PQ. This is the meaning of, what we call today, the *two-electron gate*. Bowes and Crofts (1980) explained their results, in which Chl *a* fluorescence yield decays faster after the first than after the second flash, in terms of a slower electron flow from Q_A^- to Q_B^- (step (10)) than from Q_A^- to Q_B (step (4)) possibly because of electrostatic repulsion from Q_B^- .

The existence of a 'two-electron gate' somewhere between PSII and electron acceptance by methyl viologen from PSI was shown by Bouges-Bocquet (1973) in a paper that was submitted within a week or so of that by Velthuys and Amesz (1974). Bouges-Bocquet had called the carrier *B*, and shares the credit of independent discovery of the two-electron gate.

Kinetics of *S*-states

As mentioned earlier, electron transfer from Z to P680⁺ can be measured through Chl *a* fluorescence rise in the nanosecond to sub-microsecond range after an actinic flash. However, this does not take into account the equilibria between the *S*-states and Z, and Z and P680. Ignoring the acceptor side, this can be written as:



There are two possibilities of how *S*-states can control Chl *a* fluorescence yield: (a) a greater positive charge on the *S*-states can slow the positive charge transfer from Z to S_n and,

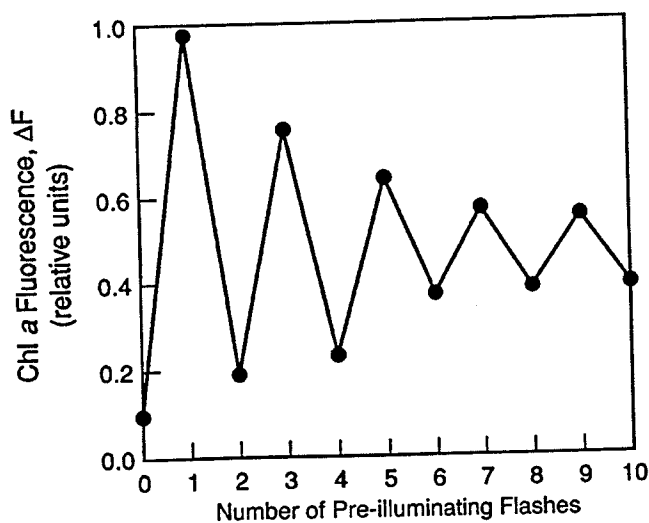


Fig. 15. Chl *a* fluorescence yield changes (ΔF) observed after a series of pre-illumination flashes, followed by DCMU addition. The units on the ordinate are equivalent to $F - F_0 / F_0$ (or $(\phi_F - \phi_{F_0}) / \phi_{F_0}$). The period 4 oscillation involved in O_2 evolution was abolished by alkaline Tris treatment, but PSII was operative due to the addition of an artificial electron donor. The binary oscillation, observed in this experiment, led to the concept of the *two-electron gate*. Data from Velthuys and Amesz (1974).

thus, the latter can slow the positive charge transfer from P680⁺ to Z, leading to a higher concentration of the natural quencher [P680⁺]; and (b) a direct influence of *S*-states on the Chl *a* fluorescence yield. I have no idea as to how this will function. There also exists the possibility of O_2 , *per se*, released during S_4 to S_0 transition to cause quenching of Chl *a* fluorescence (Shinkarev *et al.* 1994). Shinkarev *et al.* (1994) have measured the kinetics of the difference between the inverse of the fluorescence yield after the first flash (S_1 to S_2 transition, no O_2 evolution) and that after the third flash ($S_3 \rightarrow S_4 \rightarrow S_0$ transition, O_2 evolution; see Kok *et al.* 1970; Renger 1993). Analysis of this data shows that a quencher is produced with a lag of approximately 1 ms and a rise half-time of about 2 ms (Fig. 16). The amplitude of

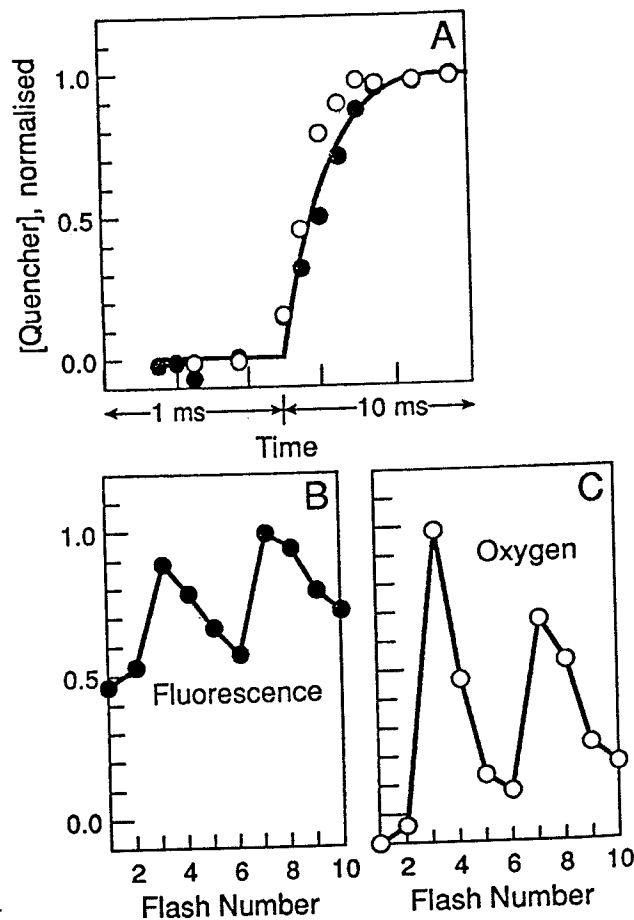


Fig. 16. (A) Kinetics of a quencher (or quenchers) of Chl *a* fluorescence evaluated from the difference of inverse of fluorescence yield after flash 3 from that after flash 1 as a function of time. Different symbols: different methods of calculation. (B) Flash number dependence of quenching of Chl *a* fluorescence determined from the difference of reciprocal of fluorescence yield at 2 and 8 ms after flash. (C) Flash number dependence of O_2 evolution, measured by the Joliot electrode. Sample: spinach thylakoids. Data from Shinkarev *et al.* (1994).

this quencher oscillates with a period of 4 in synchrony with O_2 evolution, but there are serious quantitative differences. In the same way, there are inconsistencies with the H^+ release patterns (Lavergne and Junge 1993). It is still tantalising to consider the possibility that this phase is a monitor of the kinetics of the $S_4 \rightarrow S_0$ O_2 -evolving step. Whether it could be O_2 itself (for arguments regarding O_2 as a quencher of Chl fluorescence, see Papageorgiou 1975) is a valid question to ask. Since fluorescence can be measured in intact leaves, Chl *a* fluorescence kinetics could become an excellent probe for monitoring crucial steps of PSII *in situ*.

Understanding the Site of Bicarbonate in Photosystem II

Photosystem II, but not the purple or green bacterial or PSI, reaction centre shows a bicarbonate-reversible formate or NO inhibition of electron transfer from Q_A^- to the PQ pool (for reviews, see Govindjee and Van Rensen 1978, 1993; Blubaugh and Govindjee 1988; Diner *et al.* 1991; Govindjee 1991, 1993; Van Rensen 1992). Using Chl *a* fluorescence transient measurements, Wydrzynski and Govindjee (1975) were the first to demonstrate that a major bicarbonate effect was on the electron acceptor side of PSII: the effect of bicarbonate depletion was more like a block by DCMU than by a block on the donor side of PSII (Fig. 17).

Using Chl *a* fluorescence yield changes, after a series of repetitive flashes, Jursinic *et al.* (1976) concluded that electron flow out of Q_A^- to the PQ pool (steps (4), (10) and (13)) is reversibly affected by bicarbonate, but electron flow from Z to P680⁺ (steps (3) and (9), etc.) is not (Fig. 18). Soon thereafter, Govindjee *et al.* (1976) showed that 160 ms after a series of individual 3 μ s flashes, spaced 30 ms, the Chl *a* fluorescence intensity in bicarbonate-depleted thylakoids was high after flash 3 and beyond. Further, the binary oscillations, due to the existence of the two-electron gate, discovered by Velthuys and Ames (1974), were abolished. These results, obtained with thylakoids thoroughly depleted of bicarbonate, suggested that the protonation and the exchange of Q_B^{2-} by the PQ pool (steps (12) and (13)) is drastically, but reversibly slowed down since addition of bicarbonate reversed all the effects (Fig. 19).

As noted above, Z to P680⁺ reaction was shown by Jursinic *et al.* (1976) to be unaffected by bicarbonate-reversible formate. Govindjee *et al.* (1989) confirmed this result and showed that this result was independent of the S-states. However, this does not contradict (or disprove) the existence of a small, although statistically significant, effect on the donor side of PSII (Jursinic and Dennenberg 1990; Stemler and Jursinic 1993). In fact, under other experimental conditions (e.g. at low pH), a bicarbonate-reversible formate effect prior to Q_A has been observed also in our laboratory (El-Shintinawy and Govindjee 1989;

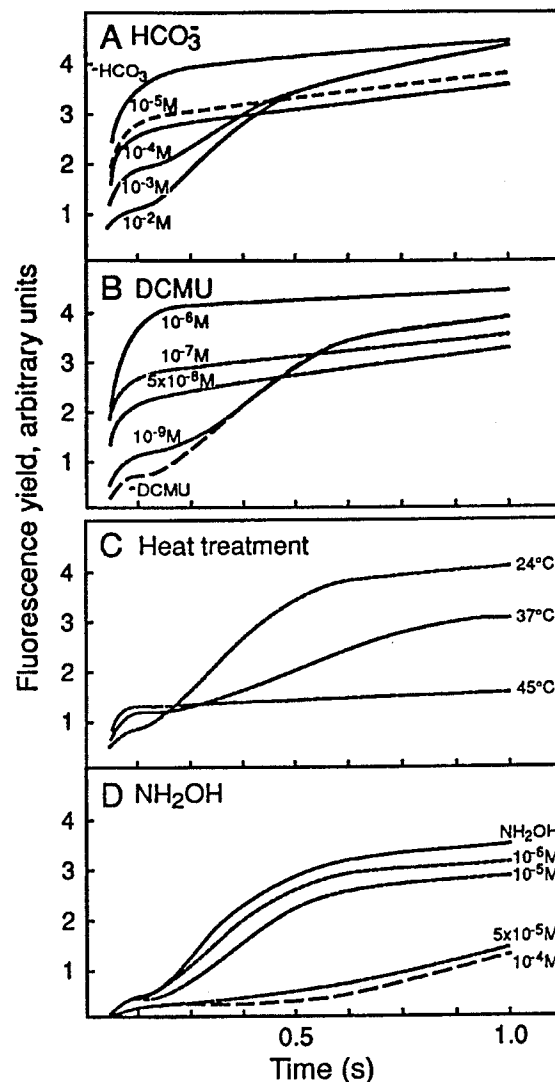


Fig. 17. Comparison of concentration dependence of variable Chl *a* fluorescence on bicarbonate with various System II inhibitory treatments. (A) HCO_3^- -depleted thylakoid sample to which various $NaHCO_3$ concentrations were added. (B) Normal thylakoids at various DCMU concentrations. (C) Normal thylakoids heat-treated for 1 min at different temperatures. (D) Normal thylakoids at various NH_2OH concentrations. Preincubation time was 5 min in dark. In other experiments, it was shown that the bicarbonate effect persists in Tris-washed thylakoids with artificial H-donors (hydroquinone, $MnCl_2$, NH_2OH or diphenyl carbazide). These experiments established the role of bicarbonate on the acceptor side of PSII. Data from Wydrzynski and Govindjee (1975).

El-Shintinawy *et al.* 1990; Xu 1992, and citations therein). Further research is needed to investigate the significance of these observations.

On the other hand, there is indeed a major bicarbonate effect between Q_A and PQ, as discussed above. Eaton-Rye and Govindjee (1988a, 1988b) and Xu *et al.* (1991) showed a drastic bicarbonate-reversible slowing down, by formate, of electron transfer from Q_A^- to Q_B after the second and

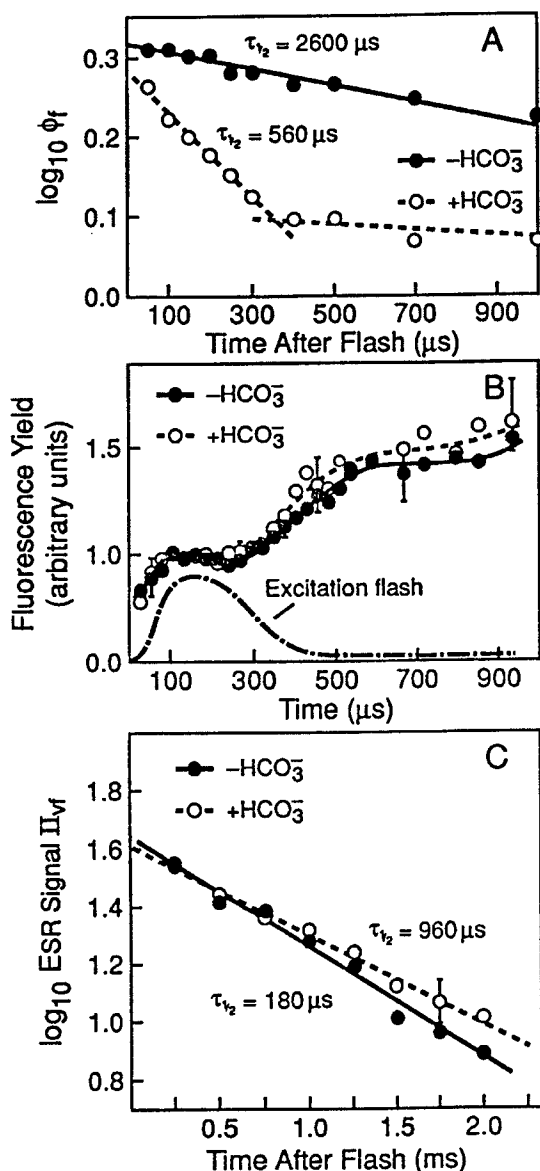


Fig. 18. (A) Semilog plot of the decay of Chl *a* fluorescence yield for Q_A^- to Q_B or Q_B^- reaction with and without 10 mM bicarbonate. (B) The rise in Chl *a* fluorescence yield (Z to $P680^+$ reaction) during and after an excitation flash with and without bicarbonate, normalised at 3 μs ; a trace of the excitation flash is also shown. (C) Semilog plot of the ESR signal II_{vf} (S_n to Z^+ reaction) with and without bicarbonate. All experiments were made with spinach thylakoids. These experiments showed the effect of bicarbonate on Q^- reoxidation and the lack of effect between the *S*-states and $P680$. Data from Jursinic *et al.* (1976).

subsequent flashes, but not after the first flash (Fig. 20). This has been interpreted to suggest that it is not so much the electron flow (Eqn 9 (4)), but protonation of the site near Q_B^- (Eqn 9 (6)) that is inhibited by bicarbonate-reversible formate. A similar result was obtained by Diner and Petrouleas (1990) for the bicarbonate-reversible NO effect.

A role of HCO_3^- in protonation reactions has also been suggested from proton measurements by Van Rensen *et al.* (1988). Allakhverdiev *et al.* (1994) have suggested that such reactions may add an entropic factor to variable thermal emission, not detected by fluorescence.

The hypothesis of Blubaugh and Govindjee (1988) is that one of the functions of bicarbonate, suggested to be bound on (or H-bonded to) a particular arginine (D1-R269 and/or R257) and, perhaps, stabilised by other arginines, is to deliver a H^+ to a particular histidine to stabilise the negative charge on Q_B^- formed after the flash. In the absence of HCO_3^- , this is much slowed and, thus, electron transfer after the second and succeeding flashes is slowed (Xu *et al.* 1991). The importance of D2-R251 and D2-R233, but not D2-R139, for stabilisation of HCO_3^- was shown by Cao *et al.* (1991) (see Govindjee 1993) through the use of site-directed *Synechocystis* sp. PCC 6803 mutants D2-R251S, D2-R233Q and D2-R139H. However, we suspect that D1-R269 is one of the most crucial amino acids for the HCO_3^- effect (Fig. 21). Xiong *et al.* (1994) have constructed, in *Chlamydomonas reinhardtii*, a D1-R269G mutant. Interestingly, this mutant exhibits normal levels of D1 protein in dark-grown cells, does not show formate-induced Q_A^- - Fe^{2+} EPR signal, has a very slow electron transfer rate beyond Q_A^- , and has a very low affinity for the herbicide terbutryn (Fig. 22). It remains to be seen if it has the non-heme iron or not, or if only the HCO_3^- binding site is lost. We consider it likely that D1-R269 may be one of the major binding sites of HCO_3^- in addition to the non-heme iron (Diner and Petrouleas 1990).

Chlorophyll *a* fluorescence measurements on several herbicide-resistant D1 mutants of the Q_B -binding niche (between helices IV and V) have revealed that different amino acids have different sensitivities to bicarbonate-reversible formate emphasising the role of a broad binding niche for bicarbonate ions (Govindjee *et al.* 1990b, 1991, 1992; Cao *et al.* 1992; Vernotte, Briantais and Govindjee, unpublished data). The (bi)carbonate binding niche in human lactoferrin (Anderson *et al.* 1989), the only other Fe-(bi)carbonate protein known to us, has served as a partial model for further investigations. Here (bi)carbonate is not only liganded to Fe, but is H-bonded to an arginine and several other amino acids.

Recently, Mäenpää *et al.* (1995) demonstrated that a mutant (CA1) of *Synechocystis* sp. PCC 6803 that lacks certain glutamic acids in the loop between helix IV and V of its D1 protein, shows a high resistance to bicarbonate-reversible formate treatment. Since the mutation is not in the Q_A^- - $\text{Fe}Q_B$ niche, this result may suggest the importance of conformational changes. We are, obviously, far from an understanding of the bicarbonate binding and its function in PSII.

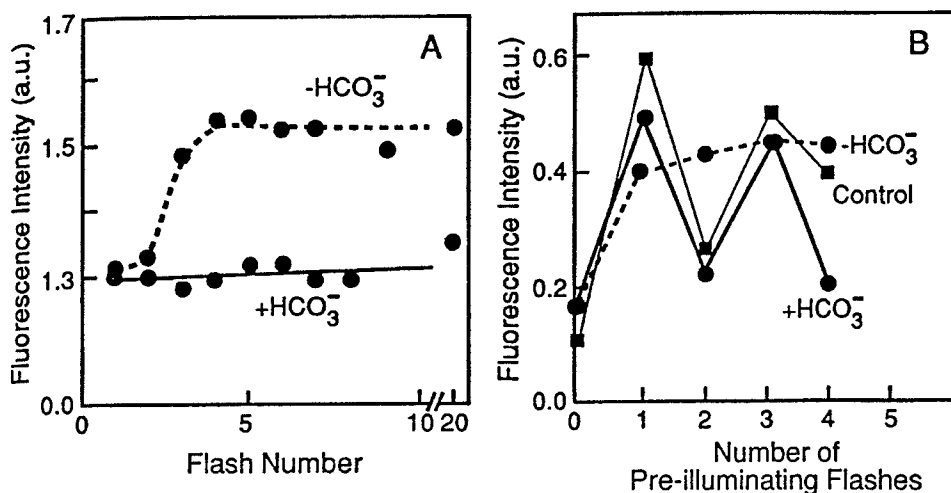


Fig. 19. (A) Chl *a* fluorescence intensity 160 ms after the last of a series of 3 μ s saturating flashes, spaced at 30 ms, as a function of the number of flashes. Sample: spinach thylakoid suspension. (B) DCMU-induced fluorescence increase as a function of the number of pre-illuminating flashes (see Fig. 15). Sample: spinach thylakoid suspension. These experiments showed the role of bicarbonate at the *two-electron gate*. Data from Govindjee *et al.* (1976).

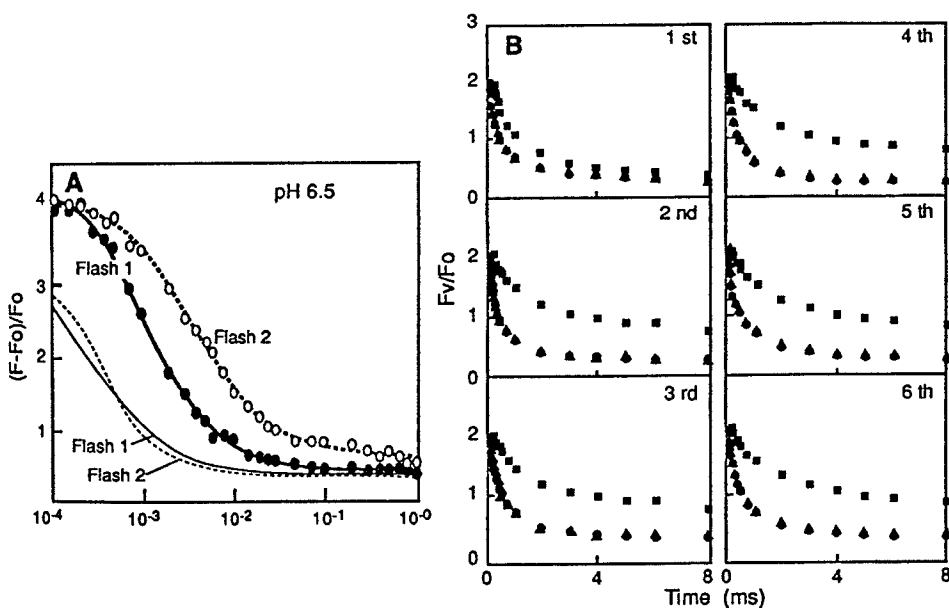


Fig. 20. (A) Decay of variable Chl *a* fluorescence yield with and without bicarbonate (formate-treated) after flash 1 and 2 up to 1 s. Slowing down by bicarbonate-depletion was much more after flash 2 than after flash 1. Data from Eaton-Rye and Govindjee (1988b). (B) The same as above, after direct addition of formate, but plotted for flashes 1-6, and up to 8 ms only. Both these data suggested that bicarbonate may be involved in protonation of Q_B^- . Experiments by Xu and Govindjee, unpublished data; and data from Xu (1992).

Comments on Non-photochemical Quenching

Higher plants and algae can adapt to high light intensities by down-regulating PSII photochemistry (Demmig-Adams and Adams 1992). This down regulation is usually achieved by increasing the heat dissipation in the light-harvesting antenna complexes. The heat dissipation decreases the quantum yield of PSII photochemistry as well as Chl *a* fluorescence and, thus causes non-photochemical quenching (NPQ). Non-photochemical quenching of Chl *a* fluorescence simply implies that de-excitation pathways other than involved in photochemistry increase to quench Chl *a* fluorescence. The most obvious pathways are that by heat loss (k_h), as noted above, and/or by quenching (k_q) with physico-chemical quenchers (e.g. carotenoids, O₂, triplets, etc.). Thus, these include what we may call 'non- Q_A related' changes.

Louisa Yang and I (cited in Govindjee *et al.* 1967) observed a quenching of Chl *a* fluorescence by PMS (phenazine methosulfate) in DCMU-treated thylakoids. The explanation considered then was a change in k_t (rate constant of excitation energy transfer from strongly fluorescent PSII to weakly-fluorescent PSI). We never published this work as the explanation was not substantiated by low temperature fluorescence spectroscopy of the sample. Papageorgiou and Govindjee (1967, 1968a, 1968b) began looking at the effects of uncouplers of photophosphorylation, even in the presence of DCMU, on Chl *a* fluorescence of intact green and blue-green photosynthetic cells. We observed complex changes in both kinetics and emission spectra and it was evident that these changes are also 'non- Q_A -related' as was the experiment of L. Yang. We invoked cyclic changes around PSI as well as

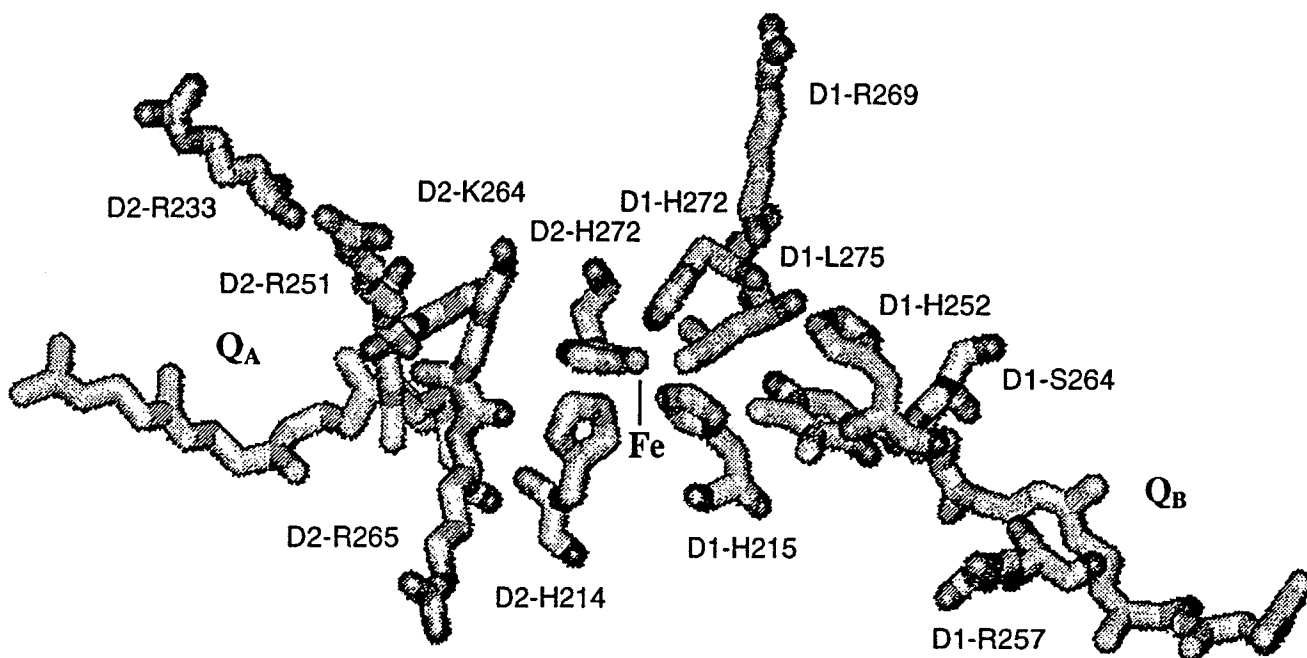


Fig. 21. A theoretical model of the acceptor side (Q_A -Fe- Q_B) of PSII reaction centre of *Synechocystis* sp. PCC 6803, constructed on QUANTA program (version 3.3). The model is based upon the sequence homology of PSII reaction centre proteins D1 and D2 with the bacterial reaction centre subunits L and M. Only the plastoquinones Q_A and Q_B , the non-heme iron and the important amino acid residues related to the bicarbonate effect are shown (unpublished model by Xiong, Subramaniam and Govindjee). Site-directed mutagenesis done by Cao *et al.* (1991) on D2-R233 and D2-R265, by Diner *et al.* (1991) on D2-K264 and D2-R265, and by Xiong *et al.* (1994) on D1-R269 have implicated the roles of these amino acid residues in stabilisation, binding, and functioning of bicarbonate in PSII.

structural changes to explain these results. The non-relationship of slow changes to photosynthesis was also obvious when we observed that the rate of O_2 evolution paralleled the SM fluorescence rise, and remained constant during the MT fluorescence decline (Papageorgiou and Govindjee 1968a, 1968b; Mohanty *et al.* 1971a).

Murata and Sugahara (1969) observed an uncoupler sensitive lowering of Chl *a* fluorescence yield when they added reduced PMS to DCMU-treated spinach chloroplasts. Wraight and Crofts (1970) showed a correlation between the protonation of the interior of the thylakoid, and the lowering of the Chl *a* fluorescence yield. When the quenching of fluorescence by Q_A was optimal at pH 6.5, the 'high energy state' (protonation) quenching was optimal at pH 8.5. Briantais *et al.* (1979, 1980) showed that the slow decline phase of Chl *a* fluorescence (the SMT phase) is correlated with the lumen $[H^+]$ in isolated chloroplasts. This fluorescence lowering cannot be due to direct quenching by H^+ s as they cannot accept electronic excitation energy.

Papageorgiou (1975) has considered the possibility that some of the 'non- Q_A related' or 'high-energy-state, or X_E ' quenching may occur through changes in structure that allow diffusion of quenchers (such as O_2) to the pigment site. Fixation of cells by glutaraldehyde does eliminate quenching of Chl *a* fluorescence by PMS (Mohanty *et al.*

1973). In view of the absence of PMS-induced effects on excitation energy transfer from PSII to PSI, and in view of the fact that fluorescence intensity changes paralleled lifetime of fluorescence changes, Mohanty *et al.* (1973) concluded that these changes were due to increases in rate constant of heat loss, k_h , not k_i , as originally thought. These were the beginnings of the observations on non-photochemical quenching of Chl *a* fluorescence of PSII.

Since the conclusions of Murata and Sugahara (1969), Wraight and Crofts (1970) and Mohanty *et al.* (1973) and of Briantais *et al.* (1979, 1980) on thylakoids and chloroplasts were more understandable than those we had earlier obtained on algal cells, we investigated the effects of salicylanalides, uncouplers of photophosphorylation, on DCMU-treated cyanobacterial cells (Mohanty and Govindjee 1973). We observed that these uncouplers abolished the time-dependent Chl *a* fluorescence increase, a sort of opposite effect to that observed with the PMS-system in thylakoids. Moreover, in both cases uncouplers of photophosphorylation caused drastic changes in 'non- Q_A -related' Chl *a* fluorescence changes. I hope that with the new theoretical and experimental framework available now, these early observations in intact cells can be reinvestigated and finally understood at a molecular level.

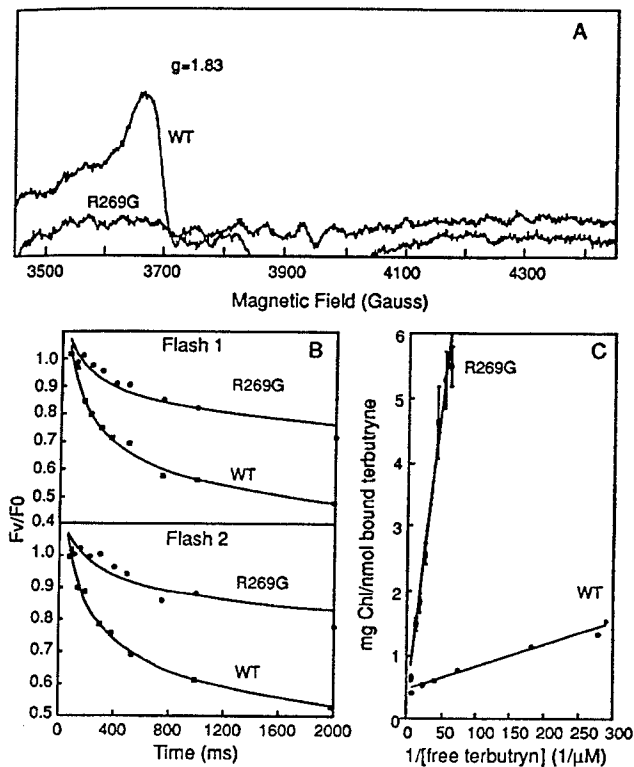
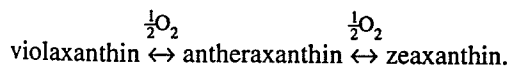


Fig. 22. Characterisation of the dark grown D1-R269G mutant of *Chlamydomonas reinhardtii*. (A) The EPR spectrum showing the absence of formate-induced $g = 1.83$ signal. (B) The normalised fluorescence decay pattern showing the slowed electron transfer from Q_A^- to $Q_B^-(Q_B^-)$. (C) The herbicide binding assay showing an extremely low affinity of terbutryn binding. In addition, the mutant shows inability to evolve oxygen and low levels of D1 protein when grown in light. These data suggest the importance of the amino acid residue D1-R269 in several aspects of PSII including its role in the bicarbonate effect. Preliminary data of Xiong *et al.* (1994).

Seven years before the observations of Murata and Sugahara, Yamamoto *et al.* (1962) discovered what is called the *xanthophyll cycle*:



Harry Yamamoto, who has invested years of research characterising biochemistry of this cycle, concluded that it played an unknown but important regulatory role in photosynthesis (see Yamamoto 1979). It was only recently that Barbara Demmig-Adams and her coworkers suggested that the pigments of the Yamamoto cycle play a role in NPQ of Chl *a* fluorescence by increasing k_h (see e.g. Demmig-Adams *et al.* 1990). There is a general consensus among most of the researchers (for fear of missing any names, I do not list their names) that $[H^+]$ s may not only activate the enzyme violaxanthin de-epoxidase to convert violaxanthin to antheraxanthin and zeaxanthin, but may also affect the

conformational state of the antenna pigment protein complexes such that the quenching of Chl *a* fluorescence by zeaxanthin and antheraxanthin (Gilmore and Yamamoto 1993) is favoured. Quenching processes in fluorescence studies are best analysed by the well-known Stern-Volmer relationships (Stern and Volmer 1919; Papageorgiou 1975; Demmig-Adams *et al.* 1990):

$$\frac{F(\text{control})}{F(\text{with quencher})} - 1 = kp\tau [\text{Quencher}],$$

where F = fluorescence intensity, k = collision rate constant, p = probability of effective collisions and τ = lifetime of fluorescence in the absence of the quencher.

Using the Stern-Volmer relationship, Gilmore and Yamamoto (1993) have obtained a correlation between the Chl *a* fluorescence yield and the combined $[H^+]$ and [zeaxanthin (Z) + antheraxanthin (A)]. Thus, the k_h , proposed earlier, may be equated most simply to $k_q [H^+] [Z + A]$. This does not preclude the existence of other quenching mechanisms. It becomes a matter of knowledge of which mechanism dominates when. However, the role of zeaxanthin in photoprotection *in vivo* has been emphasised by several, including Barry Osmond and coworkers (see Casper *et al.* 1993).

A decrease in fluorescence intensity, even when the number of absorbed quanta is kept constant, need not necessarily mean a decrease in quantum yield of fluorescence if the absorption cross-section of the fluorescent pigment bed decreases. Such a change would lead to what is called *static* quenching and would not reflect changes in rate constants of de-excitation pathways given in Eqn (1). However, if fluorescence intensity changes strictly parallel lifetime of fluorescence changes, we can be sure that these changes are in the quantum yield and, thus, in the rate constants of de-excitation. Gilmore *et al.* (1995) have observed an almost linear relationship (Fig. 23) between Chl *a* fluorescence intensity changes (measured by a Walz fluorometer) and the fraction of a short (approximately 0.4 ns) lifetime Lorentzian component of Chl *a* fluorescence (measured by a multifrequency phase fluorometer) during quenching of Chl *a* fluorescence that was dependent upon $[H^+]$ and [zeaxanthin + antheraxanthin]. Gilmore *et al.* (1995), further, observed that the increase in the fraction of the short lifetime fluorescence component was accompanied by a decrease in the fraction of a 2 ns lifetime Lorentzian component. These data are interpreted in terms of the formation of a complex that has a low quantum yield of fluorescence and, thus, increased rate constant of heat loss within it. For a further discussion of the mechanism of non-photochemical quenching, and of how carotenoids may quench Chl *a* fluorescence, see Crofts and Yerkes (1994) and Frank *et al.* (1994), respectively.

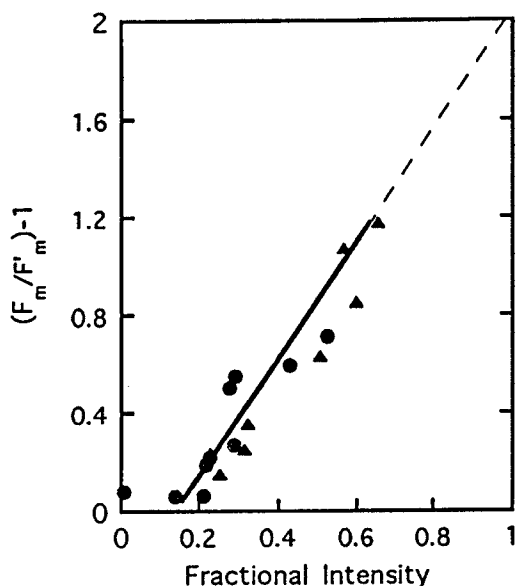


Fig. 23. The relationship between $(F_m/F'_m)-1$ and the fractional intensity of the shorter lifetime of Chl *a* fluorescence centre of a bimodal Lorentzian distribution in thylakoids. The fractional intensity (abscissa) is increased as the concentration of zeaxanthin + antheraxanthin was increased. The short lifetime centres for spinach (\blacktriangle) and lettuce (\bullet) thylakoids, respectively, were 0.42 ± 0.06 and 0.34 ± 0.10 ns. The fractional intensity of the longer lifetime centres that decreased in parallel (not shown) were 1.87 ± 0.08 and 2.14 ± 0.07 ns. F_m is the maximal fluorescence intensity and F'_m is the same in the presence of non-photochemical quenching. Measurements were made in the presence of $10 \mu\text{M}$ DCMU; fluorescence quenching was induced by ATP hydrolysis, as published by Gilmore and Yamamoto (1992). Data from Gilmore *et al.* (1995).

Concluding Remarks

I see no reason to throw away our fluorometers, but to further improve our fluorometers. We should, however, realise that it is essential to make parallel measurements on other parameters (absorption, heat changes, rates of electron transfer, etc.) before conclusions can be drawn from Chl *a* fluorescence measurements. Allakhverdiev *et al.* (1994) have provided parallel measurements on thermal and light emissions in PSII. To this, we should add measurements on lifetime of fluorescence and on absorption changes of key intermediates in order to obtain a thorough understanding of the phenomenon under investigation.

Two major areas that I have not covered in this viewpoint, both dear to me because of my personal involvement in them, are: use of Chl *a* fluorescence as a probe of dynamics of thylakoid membranes (see Barber 1982), and of dynamics of primary photochemical events of PSII (Dau 1994). I ask for forgiveness for not including these topics here.

An interesting future prospect is the possibility of using time-dependent and flash-number-dependent Chl *a* fluorescence yield changes to probe not only the acceptor

side of PSII, as is being currently done in several laboratories, but also the donor side of PSII including the S-state transitions.

In spite of my own intellectual shortcomings and that of Chl *a* fluorescence, we have come a long way since Kautsky's paper 63 years ago. Chl *a* fluorescence has provided new and important information on the composition of the pigment systems, excitation energy transfer, physical changes in pigment-protein complexes, primary photochemistry, kinetics and rates of electron transfer reactions in PSII, the sites of various inhibitors, and activators, and of lesions in newly constructed mutants. The current use of Chl fluorescence as a probe of photosynthesis (Evans and Brown 1994) and stress (Lichtenthaler and Rinderle 1988) is evident from the listing of 599 papers in all of the *Current Contents* series during a period of 2.5 years (1992 to 23 April 1994). The *viewpoint* presented here is only a drop in the *lake* of Chl fluorescence research.

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Page 140, right column, line 23. 'Fig. 9 summarises' should read 'Table 2 summarises'.

Page 143, equation 2. The correct equation is as follows:

$$\Phi_f = \frac{k_f}{k_f + k_n + k_p' [Q_A]}$$

Page 143, right column, line 7. 'Chl *a* fluorescence yield is minimal' should read 'Chl *a* fluorescence yield is maximal'.

Page 151, Fig. 21 caption, line 5. 'and D2-R265, by Diner *et al.*' should read 'and D2-R251, by Diner *et al.*'