Chapter 1

# Milestones in photosynthesis research

*Govindjee*

## Introduction

The task of writing a chapter on milestones in photosynthesis research is difficult because there are so many milestones that I may not be able to do justice to them all. Thus, at the very outset, I beg forgiveness for incompleteness and I urge the readers not to consider this chapter as a record of the history of photosynthesis. Further, this chapter will not present milestones (including the ones marking one tenths of a mile) in a linear chronological manner, but according to specified topics. For an earlier thought-provoking article on the conceptual development in photosynthesis, see Myers (1974), for historical development, see Huzisige and Ke (1993), and for an introductory overview on photosynthesis, see Whitmarsh and Govindjee (1995). An overview on the milestones in the area of chlorophyll *a* fluorescence has been presented earlier (see Govindjee, 1995; also see Govindjee *et al.,* 1986, and Chapter 25, this volume, by Strasser, Srivastava and Tsimilli-Michael), thus fluorescence will not be covered here unless I consider it pertinent to my discussion.

*A tribute*: This chapter is written in honour of my dear friend, a trusted colleague and a world leader in the field of Photosynthesis, Dr. Prafullachandra Vishnu Sane. I refer here only to three of his contributions. (1) A thought-provoking new model of the distribution of the two photosystems in the thylakoid membrane system was presented elegantly by him in collaboration with Rod Park in their classic review (Park and Sane, 1971; also see Sane, 1977). (2) His pioneering research, in collaboration with V. G. Tatake and T. S. Desai, in devising methods to record the most highly resolved thermoluminescence bands from the photosynthetic material, and for assigning these bands not only to photosystem II, but also to photosystem I, have been reviewed by him (see Sane and Rutherford, 1986). (3) I would also like to mention his discovery of heat-induced "state changes" in photosynthetic systems (see Sane *et al.,* 1984). For a profile of P. V. Sane, see S. K. Sinha, this volume.

The primary source of energy for nearly all life on Earth is the Sun. As early as 1845, Robert Mayer, who provided us with the Law of Conservation of Energy, had already recognized that plants convert light energy into chemical energy on a massive scale. Photosynthesis is the physico-chemical process by which oxygenic (plants, algae, cyanobacteria and prochlorophytes) and anoxygenic (photosynthetic bacteria) organisms convert light energy into redox chemical energy on a global scale. Each year $4 \times 10^{18}$ kilojoules of free energy is stored in reduced carbon by this process. In terms of carbon, each year about $10^{11}$ metric tons of $CO_2$ is converted into organic matter by photosynthesis.

According to Woese *et al.* (1990) the living organisms can be divided into three groups: *archea*, *bacteria* and *eukarya*. *Archea* do not engage in true photosynthesis although a bacteriorhodopsin-containing organism *Halobacterium salinarium* (formerly *H. halobium*) can convert light energy into adenosine tri-phosphate (ATP). Photosynthetic bacteria and cyanobacteria, mentioned above, are clearly *bacteria* as the name implies, whereas plants and algae are *eukarya*. Oxygenic photosynthesis provides us with both food and oxygen, and anoxygenic photosynthesis only with food, needed for the survival of almost all living organisms except certain bacteria (two examples are *Methanococccus janaschii* and *Methanobacterium autotrophicum*). In addition, ancient photosynthesis is still providing us with fossil fuel; at the rate we are using it, it is not going to last forever. We need to understand the basics and the historical development of photosynthesis since it is the only process that can provide us with food, fuel and oxygen needed for the ever-increasing population, and since it is the only major process that may utilize the global increases in $CO_2$ we are experiencing (see Chapter 19 by Reddy and Gnanam, this volume). It is now obvious that by the year 3000, we expect the world's population to be 13 billion, but at the same time the available land for food production per person is decreasing at an alarming rate caused by our follies (increased deforestation and fossil-fuel burning) (Sinha and Swaminathan, 1991; Kendall *et al.,* 1997; Swaminathan, 1998). Drastic measures are required to overcome this impending crisis that is already on our doorstep!

Within eight minutes of its origin, sunlight reaches the photosynthetic organisms on the Earth; almost a billion chlorophyll molecules in a single chloroplast function to capture this energy within femtoseconds ($10^{-15}$ s). (Note just for fun: there are as many femtoseconds in a second as there are seconds in 31 million years.) In what follows, I present a historical and a conceptual perspective on the milestones and breakthroughs in photosynthesis research dealing with the conversion of light energy into chemical energy in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ATP, which leads to the production of food from $CO_2$ (see a general review by Whitmarsh and Govindjee, 1995). The steps for the latter were first fully deciphered by M. Calvin, A. Benson and J. Bassham at the University of California at Berkeley, for which Calvin was rewarded with a Nobel prize in Chemistry in 1961. This aspect of photosynthesis will not be included in my chapter (see e.g., Bassham *et al.,* 1954; Calvin, 1989 for the Calvin cycle; Cleland *et al.,* 1998 for the current understanding of the mechanism of the function of its key enzyme Rubisco and the various chapters in this volume for the newer developments in the area of carbon metabolism and regulation). Later, through the work of Karpilov (1960), Kortsckak *et al.* (1965), and Hatch and Slack (1966), it was discovered that in certain plants (such as sugarcane), the first product of $CO_2$ fixation is a 4-C organic acid (e.g., oxaloacetic acid) in contrast to what Calvin and co-workers had found in the green alga *Chlorella* where the first stable product of $CO_2$ fixation was a 3-C organic acid phosphoglyceric acid. This $C_4$ pathway is an appendage to the Calvin cycle ($C_3$ pathway) in plants such as sugarcane and pineapple. The discovery of the $C_4$ pathway has been elegantly presented by Hatch (1992). An important consideration about photosynthesis must be mentioned here: almost complete photosynthesis, i.e., $CO_2$ fixation, occurs within the chloroplast as first convincingly shown by Arnon (1955).

## Chlorophylls

The most visible colour on our Earth is green, a colour quite pleasing to our eyes. Nature evolved chlorophylls to capture sunlight; their structures are such that they can absorb blue and red light, and transmit green light giving leaves the colour green; nature also evolved rhodopsin in our eyes for us to see efficiently this green light. An early major landmark in photosynthesis research was the elucidation of the physical and chemical properties of the chlorophyll molecule, a tetrapyrrole that has conjugated (alternating) single and double bonds giving it the spectroscopic properties of absorbing blue and red light, and a unique chemical structure allowing it to have different binding properties to different set of amino acids in various proteins to make it into light absorbing antenna, as well as reaction centre chlorophylls labelled as P680 or P700 (P for pigment trap, the numbers indicating the wavelength maxima of the first singlet excited state, in nanometers). Only $P680^+/P680$ was destined to have the redox potential so positive ($Em_{,7}$: $\sim$ +1.1 V) that it can oxidize water to molecular oxygen (average $Em_{,7}$: +0.8 V), whereas $P700^+/P700$ ($Em_{,7}$: +0.4 V) is only able to oxidize intermediates such as the hemes in cytochromes or copper in plastocyanin.

The elucidation of the chemical properties and the structure of chlorophylls was rewarded with Nobel prizes in Chemistry to Richard Wilstätter (in 1915) and to Hans Fischer (in 1930), whereas its total synthesis was recognized with a Nobel prize, also in Chemistry, to R. Woodward (in 1965). It was Duysens (1952) who had first used the term "P" for pigment representing a few reaction centre chlorophyll *a* or bacteriochlorophyll molecules in his Ph.D. thesis at the State University of Utrecht. The term P870 for bacterial reaction centre was then coined. Kok (1956, 1957), who was then at the Wageningen Agriculture University's 'ship-shaped' building, discovered P700, the reaction centre chlorophyll of what we now call photosystem I (PSI, see a later section), whereas it was H.T. Witt and co-workers in Berlin (see Döring *et al.,* 1969) who discovered P680, the reaction centre chlorophyll of what we now call photosystem II, PSII. I note that my mentor and second advisor Eugene Rabinowitch had long suspected its presence, and Rabinowitch and Govindjee (1965) had named it as such prior to its discovery.

Although P680 and P700 are "special pairs" of chlorophyll *a* molecules, they are entirely different mainly because of their binding to different but specific amino acids in PSII and PSI reaction centre proteins. There are other chlorophylls too: Chl *b*, Chl *c* and Chl *d*. In spite of my first advisor Robert Emerson's early speculative and tentative ideas, Chl *b* and Chl *c* are only antenna pigments, i.e., they function only as light-harvesters in plants (as well as in green algae and prochlorophytes) and brown algae (as well as in diatoms and dinoflagellates), respectively. The recognition of Chl *d* as the major antenna pigment in *Acaryochloris marina*, a prochloron-like prokaryote was discovered by Miyashita *et al*. (1996). Chlorophyll *d* has now been shown to be the reaction centre I Chl in the organism (Hu *et al*., 1998). In addition, the possible role of zinc chlorophylls versus magnesium chlorophylls is also being actively investigated by S. Itoh and co-workers (personal communication, also see Chapter 2, this volume, by Itoh and Iwaki). If confirmed, the latter two would break the stereotypic knowledge we had thus far regarding the uniqueness of Chl *a* as the only reaction centre chlorophyll of oxygenic photosynthesis.

The importance of the chlorophylls has been recognized by at least two books, edited by Vernon and Seely (1966) and by Scheer (1991). And finally, the recent discovery of a new type of bacteriochlorophyll, labelled as bacteriochlorophyll *g* (BChl *g*) (see Gest and Favinger, 1983; Brockman and Lipinski, 1983; Amesz, 1996; Figure 1.1) may have completed the discovery of all the various types of chlorophylls present in nature. BChl *g* resembles BChl *b* in having an ethylidene group on C8, which in the presence of oxygen and light, isomerizes to give a vinyl group. Further, BChl *g* has a second vinyl group on C3; the product of its isomerization is a molecule which is very similar to Chl *a*, the pigment of plants and green algae. Thus, heliobacteria acquire a possibly new evolutionary significance.

The key character of Chl *a* is that its bound forms, P680 and P700, are photoenzymes converting light into chemical redox energy within a few picoseconds, acting as the world's most efficient solar battery:

$$\text{P680} + \text{Pheophytin} + \text{photon (or exciton)} \longrightarrow \text{P680}^{+} + \text{Pheophytin}^{-} \qquad (1.1)$$

(uphill electron transfer overcoming about 1.7 electron volts of energy barrier by a red photon).

$$\text{P700} + \text{Ao} + \text{photon (or exciton)} \longrightarrow \text{P700}^{+} + \text{Ao}^{-} \qquad (1.2)$$

(uphill electron transfer overcoming about 1.1 electron volts of energy barrier by a far-red photon; Ao is a chlorophyll monomer bound at a specific site in photosystem I).

## Photosynthetic unit: antenna and reaction centres

The terms antenna and reaction centre have already been alluded to above (also see Chapter 3, this volume, by Gómez and Chitnis). The concept is simple: a large number of light harvesting molecules function to capture light energy and act as if they are "antennae"; this captured light energy, in the form of excitation energy (or excitons) is transferred to a few pigment molecules that serve as reaction centre pigment molecules where primary photochemistry takes place. By primary photochemistry, we mean conversion of light (or excitation) energy into redox chemical energy that is then stored to do useful work. The birth of this concept, i.e., of a photosynthetic unit, that includes the two components, the antenna and the reaction centre, took place in 1932 in the Kirchhoff Laboratory of Biological Sciences at the California Institute of Technology in Pasadena, CA. There were two players: Assistant Professor of Biophysics Robert Emerson (see Rabinowitch, 1961) and an undergraduate student William Arnold. Emerson and Arnold (1932a,b) discovered that under the most optimal condition of photosynthesis (single turn-over brief and saturating flashes of light, with optimal dark times between them), a maximum of only one oxygen molecule was evolved per about 2,400 chlorophyll molecules present in the green alga *Chlorella* (Figure 1.2) although the maximum quantum yield of oxygen evolution (i.e., the number of $O_2$ evolved per quantum absorbed) must have been very high, 1/8 to 1/10 in today's numbers). Thus, the existence of a unit (and a photoenzyme) was suggested. It was, however, Gaffron and Wohl (1936) who provided the correct and complete interpretation of the highly elegant and sophisticated experiments of Emerson and Arnold: light absorbed by most

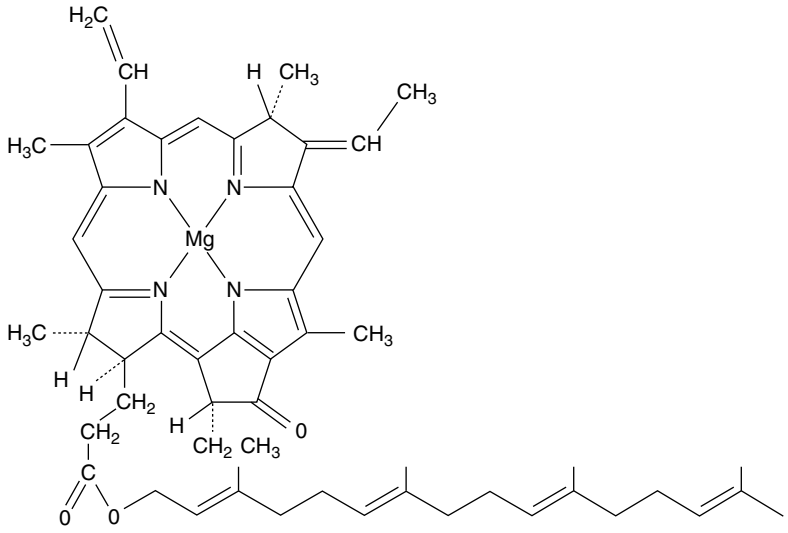


*Figure 1.1* Chemical structure of bacteriochlorophyll *g* found in heliobacteria (after Brockman and Lipinski, 1983).

of the chlorophyll molecules in the "photosynthetic unit" is transferred to the "photo-enzyme" for chemistry. The 1932 experiments were unique in another sense: they were the first experiments to use repetitive flash technique increasing the signal/noise ratio allowing precise measurements of small quantities of oxygen evolution by manometric methods. It is of historical interest to mention here that both the techniques of manometry and the green alga *Chlorella*, used in this work, were introduced by Emerson's own Professor Otto Warburg, who had received the Nobel prize in Physiology and/or Medicine in 1931 for his studies on biochemistry. The 1932 papers of Emerson and Arnold are now classical papers and continue to be regularly cited even 68 years after publication. This milestone discovery was recently discussed in an elegant paper by Myers (1994), in a special issue of *Photosynthesis Research*, edited by Govindjee *et al.* (1996), and in a recent book by Wild and Ball (1997). The Emerson–Arnold "photosynthetic unit" has a functional definition: about 2,400 chlorophyll molecules cooperate to evolve one oxygen molecule (and, thus, reduce one $CO_2$ molecule). As noted above, a photosynthetic unit includes antenna complexes (the light harvesters) and the reaction centres. Exciton migration occurs in the femto-to-picosecond range, among the photosynthetic pigments located in the protein complexes, the antenna proteins (see Hoff and Amesz, 1995; Hoff and Deisenhofer, 1997). The first kinetic evidence for the excitation energy transfer was obtained from time-resolved ultrafast fluorescence spectroscopy experiments initiated by Brody and Rabinowitch (1957) at the University of Illinois at Urbana-Champaign, IL. A wonderful example of excitation energy transfer was shown by Yamazaki *et al.* (1984) in red algae where one can literally watch the excitons move from phycoerythrin to phycocyanin, then to allophycocyanin, and finally to chlorophyll *a* in a cascade-like manner.

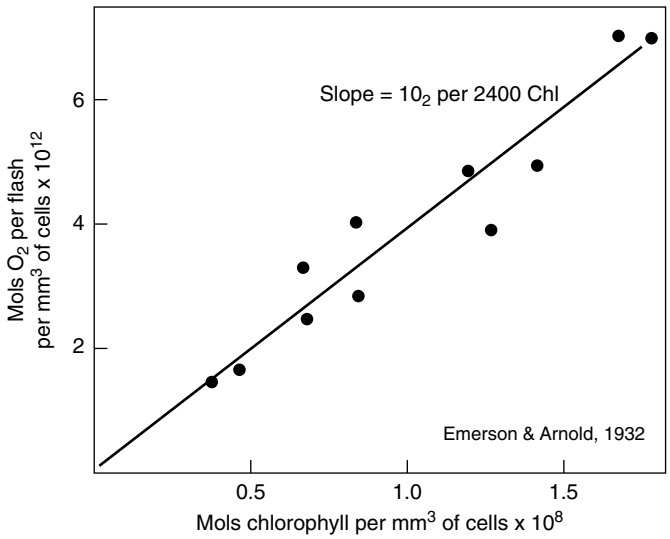


*Figure 1.2* A plot of moles of oxygen evolution per cubic mm of *Chlorella* cells per light flash (strong and brief) versus moles of chlorophyll present per cubic mm of cells. Chlorophyll concentration was varied by growing *Chlorella* cells in several different experimental conditions. Data of Emerson and Arnold (1932a, b). The slope of this curve gave the magic number of one molecule of oxygen per 2,400 chlorophyll molecules present and, thus, the concept of "photosynthetic unit" originated.

Major breakthroughs in the understanding of the antenna structure at the atomic level have been through the availability of X-ray and electron diffraction crystallography. Kühlbrandt *et al.* (1994) have provided the atomic level structure of the largest antenna of photosystem II, the light-harvesting complex II *b*; here one can see where the Chl *a* and Chl *b* molecules are anchored. On the other hand, McDermott *et al.* (1995) and Walz and Ghosh (1997) have provided the atomic level structures of two light-harvesting antenna complexes of anoxygenic photosynthetic bacteria; and Hofmann *et al.* (1996) have provided the atomic level structure of the peridinin-chlorophyll protein complex of the dinoflagellate *Amphidinium carterae*. These provide the most fascinating view of the grand design of the architecture of the photosynthetic apparatus responsible for the capture and trapping of the excitation energy. These newer structures had been preceded by the structure of the chlorobium protein complex (the so-called Fenna–Mathews–Olson protein, see Fenna and Mathews, 1976; Mathews and Fenna, 1980; Tronrud *et al.,* 1986), of phycoerythrin (Fiener and Huber, 1993) and of phycocyanin (Schirmer *et al.,* 1986). Thus, we are now able to ask meaningful questions about the detailed physico-chemical reactions in the antenna and finally begin to understand the molecular mechanism of excitation energy transfer in photosynthesis, an area that had been dominated in the past mainly by speculative and theoretical arguments.

The conversion of light energy into chemical energy occurs in the world's most efficient solar battery, in the picosecond time range, in the reaction centre molecules. This conversion leads to the formation of the primary charge-separated state, $P^+A^-$, from PA, where P is the special reaction centre molecule, as already mentioned, and A is the primary electron acceptor. Examples of this reaction were already given under the section on chlorophylls. This is the only true light reaction of photosynthesis; all

others can, in principle, occur in darkness. They are the only ones that are "uphill" because the free energy is positive due to the unfavourable difference in redox potentials of $P/P^+$ and $A/A^-$. These primary reactants and those involved in charge stabilization are located in the reaction centre complexes. The X-ray diffraction crystal structure of the reaction centre complex of the anoxygenic photosynthetic bacterium *Rhodopsuedomonas viridis* was the first one to be published, and was rewarded with the 1988 Nobel prize in Chemistry to H. Michel, J. Deisenhofer and R. Huber (see Deisenhofer *et al.,* 1984, 1985, 1995; Feher *et al.,* 1989; Feher, 1998). Recently, Rhee *et al.* (1997) have provided a crude structure of P680–containing Photosystem II reaction centre complex at 8 Å resolution; there are no details available. However, knowledge-based atomic level models of photosystem II are now available (see e.g., Xiong *et al.,* 1996, 1998a,b; see Figure 1.3 and its legend (see plate section); also see a partial model by Coleman *et al.,* 1997). Just as femtosecond-to-picosecond absorption spectroscopy has provided information on the primary charge separation in anoxygenic photosynthetic reaction centre (see e.g., Hoff and Deisenhofer, 1997; Zinth *et al.,* 1998), information on photosystem II is also now available (see Greenfield and Wasielewski, 1996; Groot *et al.,* 1997). Greenfield *et al.* (1997) have shown that at ambient temperatures two time constants of charge separation can be measured: (1) approximately 8 ps, an upper limit for charge separation, due in part to equilibration of excitons among the core reaction centre chlorophylls (the "red" pool, R); and (2) approximately 50 ps, due in part to equilibration of excitons among the accessory chlorophylls (the "blue" pool, B).

$$\text{Chl } a \text{ (B)}^* \text{ Chl } a \text{ (R) P680 Pheo} \xrightarrow{50\text{ ps}} \text{Chl } a \text{ (B) Chl } a \text{ (R)}^* \text{ P680 Pheo} \qquad (1.3)$$

$$\text{Chl } a \text{ (B) Chl } a \text{ (R)}^* \text{ P680 Pheo} \xrightarrow{8\text{ps}} \text{Chl } a \text{ (B) Chl } a \text{ (R) P680}^* \text{ Pheo} \qquad (1.4)$$

$$\text{Chl } a \text{ (B) Chl } a \text{ (R) P680}^* \text{ Pheo} \xrightarrow{3\text{ps}} \text{Chl } a \text{ (B) Chl } a \text{ (R) P680}^+ \text{ Pheo}^- \qquad (1.5)$$

We believe that the actual charge separation time is closer to 3 ps, the same as in anoxygenic photosynthetic bacteria. (In the equations shown above, * represents the molecule with an exciton or a photon.) All of the experiments on the primary photochemistry of photosystem II have been possible because of the success of Nanba and Satoh (1987) in isolating the simplest, although incomplete, reaction centre protein of photosystem II.

In contrast to PSII, a 6 Å resolution structure of P700-containing PSI reaction centre was published by the research group of H.T. Witt of Berlin (Krauss *et al.,* 1993); a 4 Å resolution structure of the same complex is also available (Krauss *et al.,* 1996). In addition to differences in the details, e.g., PSI being a Fe-S containing reaction centre, the PSI reaction centre complex contains also the core chlorophylls. I consider this work to be one of the major milestones in the history of photosynthesis.

## The two pigment systems and the two light reactions

Electron transfers occur in the pico-to-millisecond time range; they involve, in oxygenic photosynthesis, three major protein complexes: photosystem II (water-plastoquinone oxido-reductase); Cytochrome *b6/f* complex (plastoquinol-plastocyanin oxido-reductase); and photosystem I (plastocyanin-ferredoxin oxido-reductase). A fourth,

ATP synthase, is required for ATP synthesis (see Figure 1.4 and its legend). An enigma, discovered by Emerson and Lewis (1943), was that the quantum yield of oxygen evolution declines suddenly beyond 680 nm, when chlorophyll *a* is still absorbing light; this so-called "red-drop" phenomenon led to the current concept of the two light reactions and two pigment systems when Emerson *et al.* (1957) discovered the enhancement effect of short-wavelength light on the yield of photosynthesis by far-red light. This two-light reaction and two-pigment system concept is based on solid ground: from the early ideas of Rabinowitch (1945, 1956) and of Hill and Bendall (1960). In the experiments on the so-called Emerson enhancement in photosynthesis (see Figures 1.5 and 1.6 and their legends), or in the Hill reaction, yield in far-red light (light I) by simultaneous exposure to shorter wavelength light (light II), is enhanced (Emerson *et al.,* 1957; also see Emerson, 1958; Emerson and Rabinowitch, 1960; Govindjee and Rabinowitch, 1960; R. Govindjee *et al.,* 1961, 1962, 1964; and a review by Myers, 1971). Further, crucial experiments on the antagonistic effect of light I and II on the redox state of P700 (Kok, 1959; see Figure 1.7 and its legend), on the chlorophyll *a* fluorescence yield (Govindjee *et al.,* 1960; Butler, 1962; Duysens and Sweers, 1963), and, most importantly, on the redox state of cytochrome *f* (Duysens *et al.,* 1961; Duysens, 1989) established the two-light reaction scheme of photosynthesis. In fact, the existence of the series scheme of photosynthesis is best proven by light I (absorbed in photosystem I) oxidizing cytochrome *f* and light II (absorbed in photosystem II) reducing the oxidized cytochrome *f* (see Figure 1.8 and its legend). However, without the fast absorption spectroscopic work on the new intermediate X-320 (now known to be equivalent to the so-called $Q_A$, the first bound plastoquinone molecule), the history of the discovery of the two light reactions would be incomplete (see Witt *et al.,* 1961; Witt, 1991). Figure 1.9 shows the current model of the so-called Z scheme of electron transfer in oxygenic photosynthesis, whereas the legend of Figure 1.9 describes the current scheme. (The reader should refer to Figure 1.4 for the location of the components described in this figure.)

### *Electron transport: general*

The mechanism by which electrons are transferred from one molecule to another has now been explained by Marcus (1993) who was awarded the 1992 Nobel prize in Chemistry for it. A brief discussion follows. When a system such as e.g., DA is converted into $D^+A^-$, i.e., an electron is transferred from D to A, the energy curves of the two states DA and $D^+A^-$ are shifted with respect to each other on the axis of the reaction path (see Figure 1.10). Marcus defined a new term called the reorganizational energy ($\lambda$) by the length of the vertical line from the energy curve of the DA state to where the line hits the $D^+A^-$ curve. The rate constant of the reaction is given by:

$$k = A \exp ( - \Delta G^*/k_B T) \tag{1.6}$$

where $A$ depends on the nature of the electron transfer reaction, $k_B$ is the Boltzmann constant, $T$ is the absolute temperature in Kelvin, and $\Delta G^*$ is the activation free energy for the reaction, and,

$$\Delta G^* = \lambda/4 \, (1 + \Delta G^o/\lambda)^2 \tag{1.7}$$

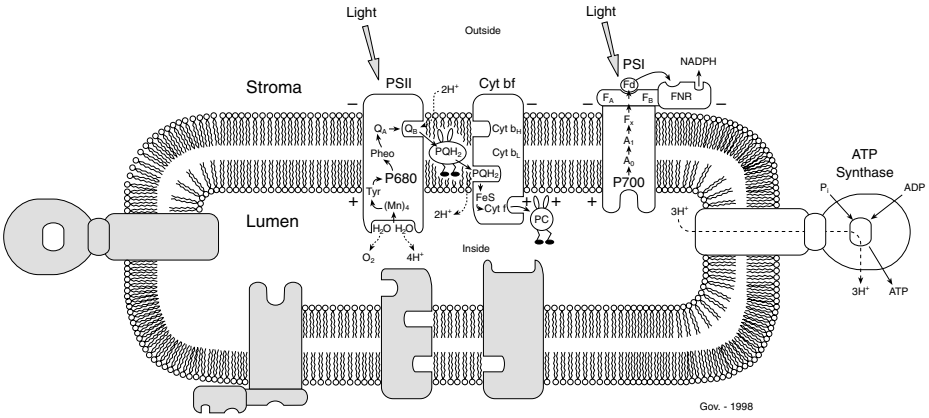


*Figure 1.4* Four major protein complexes are used for the production of the reducing power NADPH and ATP, both needed for the fixation of $CO_2$ and the production of glucose. Photosystem II (PSII, which oxidizes water to oxygen, reduces a plastoquinone molecule, and releases protons in the interior of the thylakoid membrane; it is also-called water-plastoquinone oxido-reductase); cytochrome *b/f* (Cyt bf) complex (which oxidizes reduced plastoquinone, reduces a copper protein plastocyanin (PC), and releases protons in the interior of the thylakoid membrane; it is also-called plastoquinol-plastocyanin oxido-reductase); photosystem I (PSI, which oxidizes reduced plastocyanin and reduces $NADP^+$, the nicotinamide adenine dinucleotide phosphate, to NADPH; it is also-called plastocyanin-ferredoxin oxido-reductase); and ATP synthase (which uses the membrane potential and the proton gradient to produce ATP from ADP and inorganic phosphate). The membrane potential and electron transport are produced when photosynthesis is simultaneously powered by light absorbed in both photosystems I and II that leads to electron transfer from the inner side of the thylakoid membrane to the outer side of the membrane; this makes one side of the membrane more negative than the other. The names of the electron carriers except for the reaction centre chlorophylls P680 (in photosystem II) and P700 (in photosystem I) may be ignored at the moment. Diagram modified from Whitmarsh and Govindjee (1995).

where $\Delta G^o$ is the standard free energy for the reaction, and $\lambda$ is the reorganizational energy, as mentioned above. A major prediction of the Marcus equation (1.7) is that as $\Delta G^o$ is varied from zero to some negative value, $\Delta G^*$ decreases and becomes zero at $\Delta G^o = -\lambda$, then increases when $\Delta G^o$ is made still more negative, i.e., $-\Delta G^o > \lambda$. The initial decrease of $\Delta G^*$ with increasingly negative $\Delta G^o$ was as expected, but what the Marcus equation explained was the rate of electron flow in what he had called the "inverted region": increases in $\Delta G^*$ when $\Delta G^o$ was made increasingly negative. This prediction was made in 1960, but was confirmed experimentally only in 1984 by Miller *et al* (1984) in colloboration with G. L. Closs and co-workers. Marcus (1993) has extended his theory to explain the characteristics of electron transfer in the bacterial reaction centre. It is interesting to point out that electron transfers during the primary charge separation, discussed earlier, do not slow down at cryogenic temperatures; in fact, they become temperature independent. Such a phenomenon had been discovered by DeVault and Chance (1966) (also see DeVault, 1989): it was suggested that electrons can "tunnel" through barriers as if they squeeze through with certain probability. Further, if the energetics and other entropic parameters are constant, electron transfer rates can be predicted from the distances of the acceptor from the donor molecules. Moser *et al.*

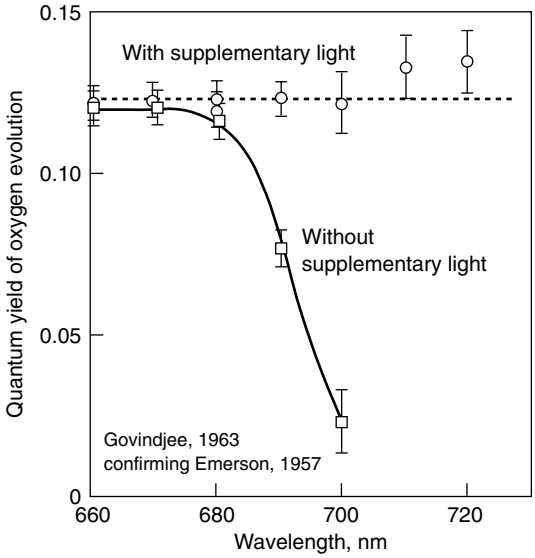


*Figure 1.5* The "red drop" and the Emerson enhancement effect, discovered by Emerson and Lewis (1943) and Emerson *et al.* (1957), respectively. The red drop was interpreted to mean that light absorbed beyond 680 nm was not sufficient to run efficient photosynthesis, whereas the Emerson enhancement effect was interpreted to mean that photosynthesis required two light reactions and two photosystems, the far red light activates only one system and the simultaneous excitation by red or other shorter wavelength light allowed excitation of both the systems (now called I and II) and thus efficient photosynthesis. In this graph, the maximum quantum yield of oxygen evolution, measured at low light intensities, in *Chlorella* cells is plotted as a function of the wavelength of light in the red region of the spectrum showing the red drop (without supplementary light). It is also shown that the low quantum yield of oxygen evolution is replaced by high yield when supplementary light is added even in the red drop region (the Emerson enhancement effect). Note that the maximum quantum yield is 0.12 (or 1 oxygen molecule per minimum of 8 photons absorbed), as observed by Robert Emerson and co-workers, but not 0.25 or higher as claimed by O. Warburg. The figure is from the unpublished data of Govindjee (1962–1963), as corrected fully for absorbed photons at all wavelengths.

(1992) showed that if the acceptor is 1.4 Å further away, the transfer rate decreases by an order of magnitude. This, known as the "Dutton ruler", does not explain why electrons flow only on one side of the bacterial reaction centre molecule, not on the other. Future prospects of understanding the mechanism of electron transfer are increasing as we begin to have atomic structures of the various intermediates. For example, we now have access to atomic level structures of the hydrophilic parts of cytochrome f (Martinez *et al.*, 1994); of plastocyanin (Collyer *et al.*, 1990, Redinbo *et al.*, 1993); and of the Cyt b/c complex of beef-heart mitochondria (Xia *et al.*, 1997) that may have similarities to the Cyt b6/f complex of plants. Zhang *et al.* (1998) have described a model of electron transfer in which movement of domains occurs in cytochrome b/c1 complex; I expect it to have application to electron transfer in Cyt *b6/f* complex. However, in spite of the available structure of the reaction centres of anoxygenic photosynthetic bacteria, which have tremendous similarities in their two halves, we still do not fully understand why the electrons flow mostly on one, rather

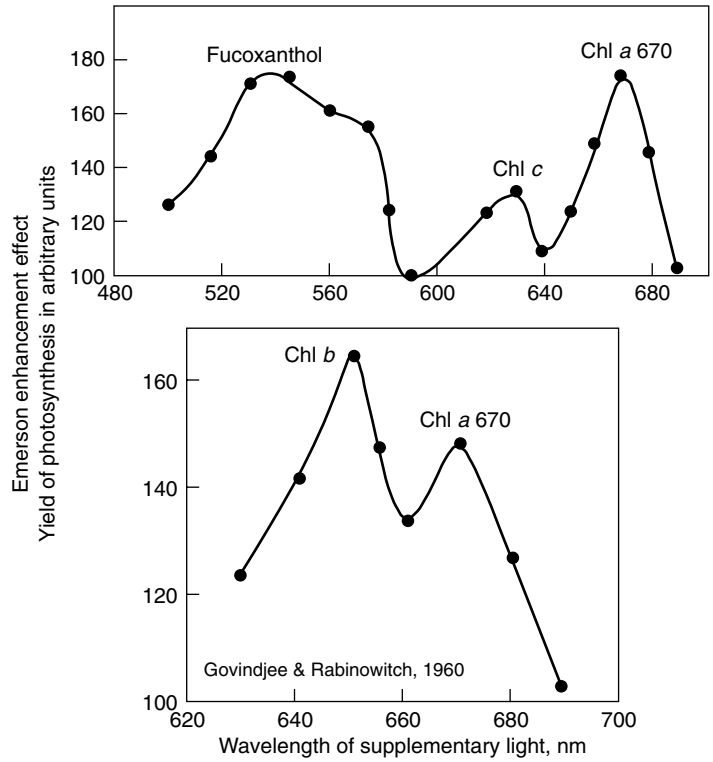


*Figure 1.6* Action spectra of the Emerson enhancement effect in the green alga *Chlorella pyrenoidosa* (bottom) and the diatom *Navicula minima* (top). In these experiments, one beam of light (now called light I, absorbed in pigment system I) was a band of far-red light (700–720 nm), and the other beam was of shorter wavelengths (light II, absorbed in the pigment system II) and varied. The enhancement was calculated from the difference between the rate of oxygen evolution in both the beams (given simultaneously) minus that in the far-red band, and this difference was divided by the rate in the far-red beam alone; the numbers obtained were then plotted in arbitrary units. Note that these spectra showed that Chl *a* (with a peak at 670 nm) was in the same system as Chl *b* (in *Chlorella*) or as fucoxanthol and Chl *c* (in *Navicula*). These data did not support the speculative, unpublished, ideas of Emerson that one system was run by Chl *a* and the other by Chl *b* (or other accessory pigments), but it supported instead the idea that both systems had Chl *a* as sensitizers, but of different absorption characteristics. Data of Govindjee and Rabinowitch (1960).

than on the other side of the molecule. Here, combinations of molecular biology, physics and physical chemistry have begun to play important roles in the understanding of this process. We are still waiting for the final understanding.

### *Electron transport: acceptor side of photosystem II*

A major advancement took place in the understanding of the functioning of the electron transfer pathway when Velthuys and Amesz (1974) and Bouges-Bocquet (1973) independently showed that there is a two-electron gate on the acceptor side of PSII (see Figure 1.11 and its legend ); a similar gate was discovered later in anoxygenic photosynthetic

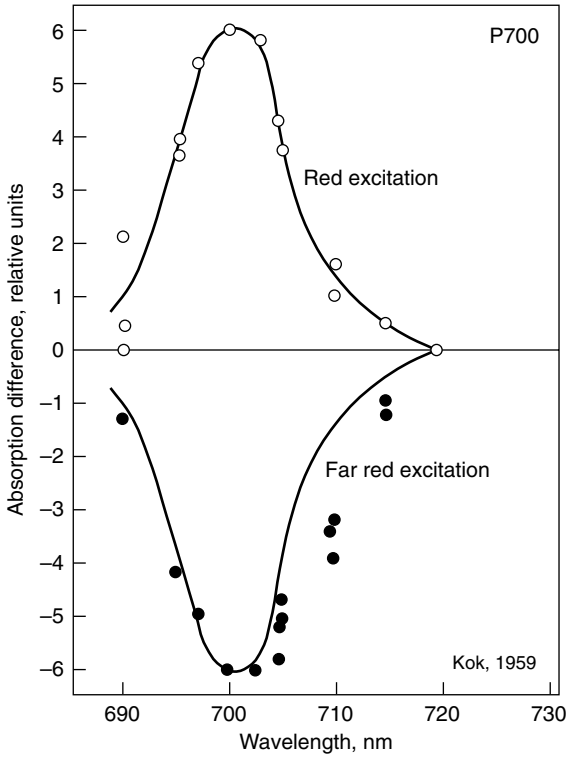


*Figure 1.7* Difference absorption spectra of the reaction centre Chl *a* P700 in the 690 to 725 nm region as excited by orange-red light (exciting photosystem II) and as excited by far-red light (exciting photosystem I) in the cyanobacterium *Anacystis nidulans*. It is observed that the far-red light oxidized P700 to $P700^{+}$ causing a bleaching of the absorption band, whereas the orange-red light restored it. B. Kok was the first person to discuss the relevance of his experiments to Emerson's discovery of the enhancement effect where the concept of two light reactions was proposed. He, however, had assumed that there was only one reaction centre, the P700. Note that in the special issue of *Plant Physiology* where Kok's paper was published (Robert Emerson Memorial issue), neither Robert Hill nor Louis N.M. Duysens presented any data related to two light reactions and two pigment systems, or even discussed any such concept, although they had papers on other topics in that issue of the journal. Data of Kok (1959).

bacteria, independently by Vermeglio (1977) and by Wraight (1977). Electrons from the reaction centres are transferred first to a bound quinone molecule $Q_A$ which is, surprisingly, a one-electron acceptor. The reduced $Q_A$ transfers its electron to a loosely bound quinone $Q_B$; the reduced $Q_B$ is tightly bound and has a long lifetime; it waits for a second electron until it can be doubly reduced to $Q_B^{2-}$. The latter then becomes a quinol ($Q_BH_2$) after grabbing two protons ultimately coming from the side of the membrane it is located on. The electron flow at the $Q_BH_2$ site can be written as (see a review by Crofts and Wraight, 1983):

$$Q_AQ_B + \text{light} \longrightarrow Q_A^- Q_B \xleftarrow{\text{- - -}} Q_A Q_B^- \qquad (1.8)$$

$Q_AQ_B$ + light →

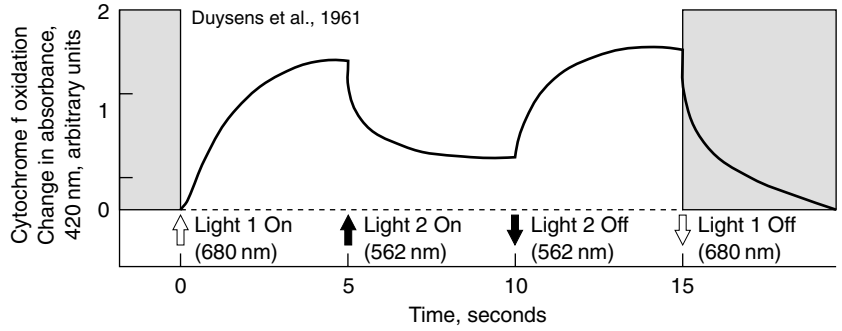


*Figure 1.8* The crux of the series scheme of photosynthesis (the so-called "Z" scheme) is in the classical experiments of Duysens *et al.* (1961): Light I (light absorbed in pigment system I) oxidizes an intermediate between the two systems, and simultaneous exposure with light II (light absorbed in pigment system II) reduces the oxidized intermediate. This idea was described by Rabinowitch (1956, p.1862) for cytochromes, and was elegantly demonstrated in these experiments where Duysens *et al*. showed that red light (absorbed in pigment system I (chlorophyll *a*) of red alga *Porphyridium cruentum*) oxidized cytochrome *f*, and further addition of green light (absorbed in pigment system II (phycoerythrin) of this red alga) reduced the oxidized cytochrome *f* proving the series scheme of photosynthesis requiring two light reactions.

$$Q_BQ_B^- + \text{light} \longrightarrow Q_A^-Q_B^- \rightleftharpoons Q_AQ_B^{2-} \tag{1.9}$$

$Q_AQ_B + \text{light} \rightarrow$

$$Q_A\,Q_B^{2-} + 2H^+ \longrightarrow Q_A\,Q_BH_2 \tag{1.10}$$

$$Q_A\,Q_BH_2 + PQ \longrightarrow Q_A\,Q_B + PQH_2. \tag{1.11}$$

Although it is not certain, especially in plants, whether the protonation steps occur before the electron flow or *vice versa*, the above steps are referred to as the "two-electron gate".

### *The bicarbonate effect*

I have had a personal interest in this two-electron gate. Over the past twenty-seven years or more, we have been trying to understand the role of bicarbonate in the reactions on the two photosystems, a phenomenon discovered by Warburg and Krippahl (1958). Warburg (1964) took this phenomenon to suggest that oxygen in photosynthesis originated in $CO_2$ not in water, contradicting the existing idea that oxygen originated in water. He argued that (1) the $^{18}O$ experiments of Ruben *et al*. (1941) could not be trusted because of equilibration with both water and $CO_2$; (2) the hypothesis of C. B. van Niel was only from comparative biochemistry between oxygenic and anoxygenic organisms, and thus had no definitive value; and (3) the requirement of bicarbonate in the Hill reaction showed that it cannot be taken to prove that $CO_2$ was not the source of oxygen in photosynthesis. I note here parenthetically that the discovery of the Hill reaction (see Hill and Scarisbrick, 1940), i.e., reduction of an artificially added electron acceptor and evolution of oxygen by chloroplasts, was itself a major finding that allowed

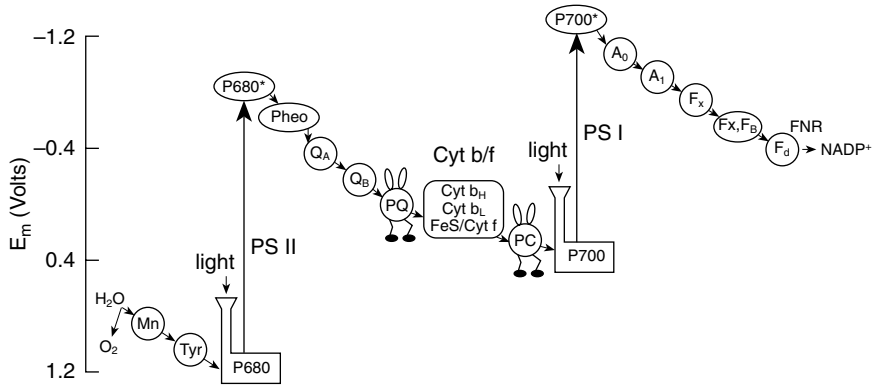


*Figure 1.9* The electron transport pathway of plant photosynthesis (see Figure 1.4 to physically locate these intermediates in the thylakoid membrane). Photosynthesis starts by simultaneous excitation of special reaction centre chlorophylls labelled as P680 and P700. Excited P680 (P680*) and P700 (P700*) have excess energy provided by light. An electron transfers out of P700* to $A_O$ (another special chlorophyll *a* molecule) producing oxidized P700 ($P700^+$) and reduced $A_O$ ($A_O^-$). At about the same time, an electron transfers out of P680* to a pheophytin (Pheo) molecule producing oxidized P680 ($P680^+$) and reduced Pheo ($Pheo^-$). These are the only steps where light energy is used to produce oxidation-reduction energy. The rest of the reactions are downhill energywise. $P700^+$ recovers to P700 receiving an electron originating in $Pheo^-$ that is passed on to $P700^+$ via the following intermediates *in a bucket brigade manner*: from $Pheo^-$ to $Q_A$ (a bound plastoquinone) to $Q_B$ (another bound plastoquinone) to PQ (freely *mobile* plastoquinone) to an iron sulphur protein (FeS) to a cytochrome (Cyt *f*) to a freely mobile plastocyanin (PC), and then to $P700^+$. On the other hand, an electron on $A_O^-$ is passed on, ultimately, to the $NADP^+$ via several other intermediates ($A_1$, a phylloquinone, $F_x$, $F_A$ and $F_B$, three iron-sulphur proteins, and $F_d$, ferredoxin). The missing electron on $P680^+$ is recovered, *ultimately*, from water molecules ($H_2O$) via an amino acid tyrosine (D1-Y-161, see Figure 1.3(b); $Y_z$) and a manganese complex (Mn). Four such reactions (utilizing a total of 8 photons (see Figure 1.5), 4 in PSII, and 4 in PSI) are required to oxidize $H_2O$ to $10_2$ and to reduce 2 $NADP^+$ to 2NADPH. The left side of the diagram shows an energy scale in terms of oxidation reduction potential ($E_m$) at pH 7. (At pH 7, the standard hydrogen electrode has an $E_m$ of – 0.4 volts.) Intermediates high up in the diagram have a lower (or more negative $E_m$) and can easily *reduce* (i.e., add an electron to) any intermediate below them in the diagram by a downhill energywise process. Energy is, however, needed to transfer electrons from intermediates on the lower part of the diagram to those above them. This is why "light" is needed to transfer electrons from P680 to pheophytin and from P700 to $A_o$. The diagram is a modified version of that by Whitmarsh and Govindjee (1995).

a tremendous number of biochemical studies that ensued from that time; it opened the field to look under the "hood" of the car, so to say. The arguments of Warburg, mentioned above, fascinated me. I presented them to a photosynthesis course I was teaching in the early 1970s; it was attended, among others, by Prasanna Mohanty and Alan Stemler, my Ph.D. students. Alan jumped at the idea of working on the exciting project of attempting to understand the role of bicarbonate in the Hill reaction. Our first paper (Stemler and Govindjee, 1973) suggested that bicarbonate may function on the water side of photosynthesis. However, very soon we discovered a major effect on the acceptor side of photosystem II. We located a unique role of bicarbonate on the electron acceptor side, specifically at the two-electron gate (Wydrzynski and Govindjee, 1975; Govindjee *et al.*, 1976; Jursinic *et al.*, 1976; Khanna *et al.*, 1977, 1981; Blubaugh and Govindjee, 1988; Eaton-Rye and Govindjee, 1988; Xu *et al.*, 1991; Govindjee,

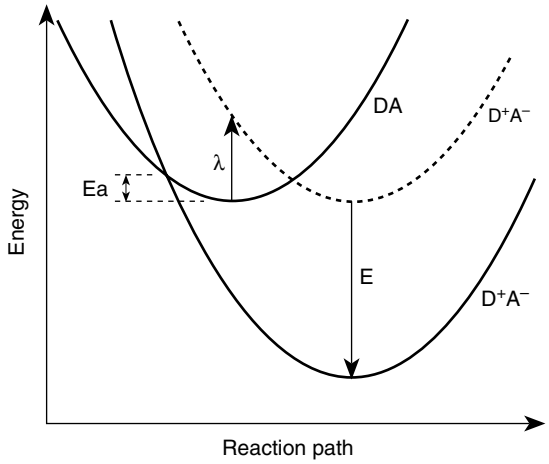


*Figure 1.10* The theory of Marcus (1993). The ordinate is energy in relative units; the abscissa is simply the reaction path. The U-shaped diagrams are energy diagrams of two states (reactants: DA and products $D^+A^-$) of a couple; Ea refers to the activation energy and is represented by $\Delta G^*$, E is represented by $\Delta G°$ in the text , and $\lambda$ is the new term, the reorganizational energy, invented by Marcus. It refers to the reorganization of charges when the electron moves out of the neighbourhood of D to that of A.

1993; Govindjee and van Rensen, 1993). Bicarbonate seems to be involved not so much in the electron flow *per se*, but in the protonation steps (also see van Rensen *et al.,* 1988). Interestingly, this effect is absent in anoxygenic photosynthetic bacteria (see e.g., Shopes *et al.,*1988; Wang *et al.,* 1992). Differences between photosynthetic bacteria and photosystem II are, we believe, due to a preponderance of positively charged amino acids in the $Q_B$ binding region of PSII over that in photosynthetic bacteria (Xiong *et al.,* 1996; 1998a). This may be responsible for different protonation channels in anoxygenic photosynthetic bacteria and in PSII. This bicarbonate site seems close to (Khanna *et al.,* 1981), but not identical to that where certain herbicides bind and act (Velthuys, 1981; Wraight, 1981). Terbutryn inhibits and kills plants by displacing $Q_{B,}$ and bicarbonate seems to be involved in the protonation of the reduced form of $Q_B$. One of the binding sites of bicarbonate has been shown to be on the non-heme iron (see e.g., Diner *et al.,* 1991) and the other near an arginine residue, D1-R-257 (Xiong *et al.,* 1998b). It is not yet clear how this acceptor side binding (Figure 1.12, see plate section) would provide another major bicarbonate action on the oxygen evolving complex expounded by O. Warburg, H. Metzner and A. Stemler (Stemler, 1982), and now being investigated in great detail by Klimov and co-workers (Allakhverdiev *et al.,* 1997; Klimov *et al.,* 1997; Hulsebosch *et al.,* 1998; Yruela *et al.,* 1998). It calls for further research (see Stemler, 1998; van Renson *et al.,* 1999).

### *Oxygen evolution*

The answer to the question of the minimum number of quanta needed to evolve one oxygen molecule was solved in favour of 8 to 10 (Emerson and Lewis, 1943; Emerson,

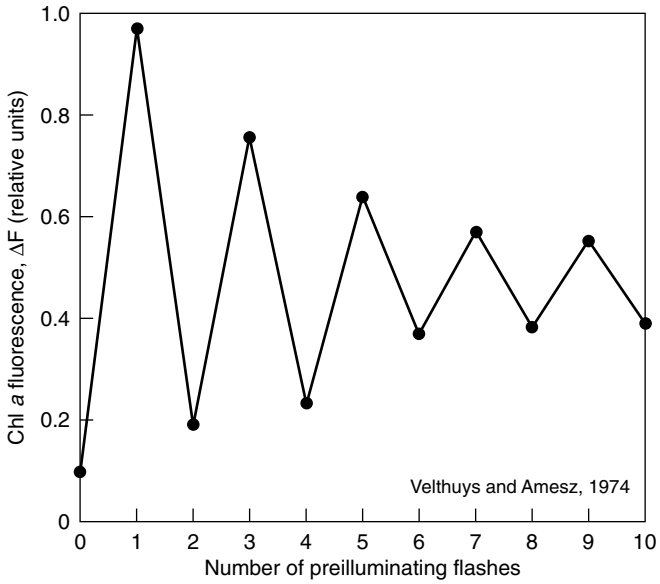


*Figure 1.11* Chlorophyll *a* fluorescence changes ($\Delta F$) observed after pre-illumination of dark-adapted chloroplasts by a series of light flashes and subsequent addition of the herbicide diuron. In these experiments, the oxygen clock (water oxidation) was blocked artificially by TRIS treatment and artificial electron donors were added to oxidize Yz or P680 to run the photosystem II. A periodicity of 2 was observed when Chl *a* fluorescence yield was measured in this ingenious manner: it was high after flash 1 and 3, etc. This observation was taken to mean that there is a "two-electron gate" on the acceptor side of photosystem II (see text): $Q_A$ is followed by $Q_B$, where $Q_A$ is a one-electron acceptor, and $Q_B$ is a two-electron acceptor. After pre-flash 1, one has reduced $Q_A^-$; this is followed by electron transfer to $Q_B$ forming $Q_A\ Q_B^-$; addition of diuron (in dark) does not remove $Q_B^-$, but an equilibration between $Q_A^-\ Q_B$ and $Q_A\ Q_B^-$ leads to enough $Q_A^-$. However, after two flashes, $Q_A\ Q_B^{2-}$ is made, the latter exchanging with a plastoquinone molecule leading to the formation of $Q_A\ Q_B$ ; thus, even after diuron addition (in dark), that displaces $Q_B$, only $Q_A$ is present. Duysens and Sweers (1963) had suggested that Chl *a* fluorescence yield is high when reduced $Q_A$ was present and low when oxidized $Q_A$ was present. Thus, after 1 preflash, Chl *a* fluorescence is high, and after 2 preflashes, it is low. Data of Velthuys and Amesz (1974) are shown. Independent and equally ingenious, but different, experiments of Bouges-Bocquet (1973) had provided the same conclusion, i.e., of the existence of a "two-electron" gate between PSII and PSI. (Velthuys and Amesz had called the intermediate "R" and Bouges-Bocquet had called it "B"; now everyone uses $Q_B$ for it.)

1958; R. Govindjee *et al.*, 1968; Govindjee, 1999) rather than of 3 to 4 (Warburg and Negelein, 1923). This is in agreement with the scheme that two light reactions are needed for oxygen evolution and $NADP^+$ reduction. A major breakthrough in the oxygen evolution steps was the discovery by Joliot *et al.* (1969) that oxygen/flash as a function of flash number oscillates with a period of 4 indicating accumulation of 4 positive charges on some intermediate before water is oxidized to molecular oxygen (Figure 1.13). A theory evolved by Kok *et al.* (1970) became the major framework (see Renger and Govindjee, 1993, for alternate models, see Mar and Govindjee, 1972), where the system started mostly in the $S_1$ state in darkness, and oxygen release took place when $S_4$ was converted to $S_0$ (S representing the redox state of the tetranuclear Mn complex):

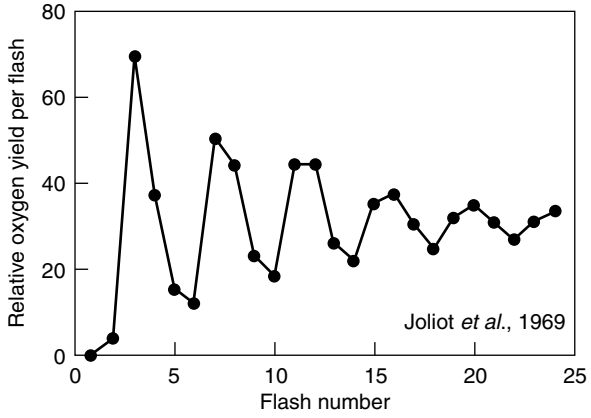


*Figure 1.13* The yield of oxygen from photosynthetic membranes exposed to a series of (brief and strong) flashes oscillates with a four-point periodicity. It is highest after the third flash and peaks again four flashes later, but the variation in the amplitude gradually decreases as the number of flashes increases. The occurrence of the peaks every 4th flash is explained by the four-step cycle of the water-oxidizing clock. The main spring of the clock needs to be wound up four times: wind, wind, wind, and wind, and then it ticks once. What winds it is the light. The water oxidizing clock is a cyclic mechanism that supplies electrons to the oxidized P680, $P680^+$. After one flash of light, the $P680^+$, thus formed, picks up an electron from a maganese complex (4 Mn atoms) oxidizing it once. After a second flash, a second oxidation occurs at this Mn complex; after a third flash, a third oxidation occurs; and after a fourth flash, a fourth oxidation occurs, i.e., the Mn complex accumulates 4 extra positive (+) charges. This oxidized complex then has enough oxidizing power to oxidize $2H_2O$ into $O_2$, to release 4 protons ($H^+$) into the thylakoid lumen, and to get itself reduced with 4 electrons (from $2H_2O$) returning to the original state. This is the oxygen clock. To match this theory with the above data, it is necessary to propose that in darkness, the Mn complex starts already one step ahead, i.e., first time around, the 3rd flash does the trick, and then every 4th flash. This is the oxygen clock. (Data of Joliot *et al.,* 1969, redrawn by Govindjee and Coleman, *Scientific American*, February, 1990)

$$S_1 + \text{light flash } \#1; \text{ or } \#5 \rightarrow S_2 \tag{1.12}$$

$$S_2 + \text{light flash } \#2; \text{ or } \#6 \rightarrow S_3 \tag{1.13}$$

$$S_3 + \text{light flash } \#3; \text{ or } \#7 \rightarrow S_4 \tag{1.14}$$

$$S_4 + 2H_2O \rightarrow S_0 + O_2 + 4H^+ \tag{1.15}$$

$$S_0 + \text{light flash } \#4; \text{ or } \#8 \rightarrow S_1 \tag{1.16}$$

The elegant measurements of Dismukes and Siderer (1980) on multiline EPR (electron paramagnetic resonance) signals of Mn, and those of M. Klein and co-workers (see Yachandra *et al.*, 1996; Roelofs *et al.,* 1996; also see Chapter 7, this volume, by Åhrling and Styring) on EXAFS (Extended X-ray Absorption Fine Structure) and XAS (X-ray Absorption Spectroscopy) led to the identification of the S-state intermediates with mixed valence Mn complex (a tetranuclear Mn/PSII). The $S_0$ is suggested to be Mn (II), Mn (III), Mn (IV), Mn (IV) (alternatively, Mn (III), Mn (III), Mn (III), Mn (IV)); $S_1$ to

be Mn (III), Mn (III), Mn (IV), Mn (IV); and both $S_2$ and $S_3$ as Mn (III), Mn (IV), Mn (IV), Mn (IV), Mn (IV). The mechanism of water oxidation to dioxygen is not yet known; however, there are theories as to how this occurs. I refer the readers to the papers of Renger (1997) and Babcock and co-workers (Hoganson and Babcock, 1997; Tommos and Babcock, 1998) to obtain a glimpse of the current and two different thoughts on the mechanism of oxygen evolution. The recent finding of Messinger *et al.* (1997a) that $S_0$ is also paramagnetic will further enhance our ability to understand the transitions of the S states, presented above. In addition, the possibility of showing that the S states can be reduced to the S (–3) state lends credence to the current picture of the valence state of the Mn in the water oxidase (see Messinger *et al.,* 1997b). On the personal side, I want to mention that Coleman and Govindjee (1987) were the first to suggest that the Mn atoms of the Mn cluster were bound to the reaction centre proteins D1 and D2; and Kambara and Govindjee (1985) and Padhye *et al.* (1986) were the first to suggest that an organic radical, specifically a histidine, may be the charge accumulator in addition to Mn during some of the S-state transitions, an idea that is gaining new ground from the work of R. Pace and co-workers in Australia. The role of histidine is being actively investigated in several laboratories; first, it seemed evidence was obtained, then, it was challenged; now, I am waiting for the final judgement before citing any paper on this topic. Following the early pioneering work of W. F. J. Vermaas and of B. Diner and co-workers, Bowlby *et al.* (1996) have reported that Glutamic 65 and Histidine 337 on D1 protein may be ligands to Mn; and, Aspartic 103 and Glutamic 104, also on D1, may be ligands to calcium. On the other hand, T. Wydrzynski and co-workers (Messinger *et al.,* 1995) are providing new information, using elegant mass spectroscopic methods, on the exchangeable water molecules at the site of water oxidation. Evidence for the proximity of a calcium to the Mn cluster, discussed above, has now been implied from X-ray absorption spectroscopy (Latimer *et al.,* 1995). Oxygen, the by-product of water oxidation, is released through the operation of the "oxygen clock" that involves 4 Mn atoms/PSII (see a detailed earlier review by Debus, 1992).

### *Thermoluminescence*

A major tool to study the S-states was shown to be thermoluminescence, a phenomenon discovered by W. Arnold (see, for example, a paper by Rutherford *et al.*, 1984 and reviews by Sane and Rutherford, 1986; Vass and Govindjee, 1996). Thermoluminescence ($T_L$) is light emission from PSII that originates mainly from illuminated samples when they are heated in darkness. If one shines light on a photosynthetic material, and freezes it to 77 K, all the separated charges are frozen, and heating the sample in darkness leads to thermoluminescence bands originating from the back reactions (i.e., recombination of charges) of, e.g., $S_2$ with $Q_B^-$ or $S_2$ with $Q_A^-$, etc. P. V. Sane, in collaboration with V. G. Tatake and T. S. Desai, was responsible for placing India on the world map of pioneering research in this area of thermoluminescence. The design of the instrument at the Bhabha Atomic Research Centre (BARC), Mumbai, India, where this work was done, provided a very high signal/noise ratio. Several (6 or 7) $T_L$ bands were fully separated from each other and these bands, through separation of pigment systems I and II and through the ingenious use of inhibitors and electron carriers, were related to specific reactions in PSII and even PSI (see Sane and Rutherford,

1986; and Vass and Govindjee, 1996). It was because calculations, by Tatake *et al.* (1981), of the activation energies of the various $T_L$ bands did not match the Randall–Wilkins theory that I approached Don DeVault for help; this led to the formulation by DeVault *et al.* (1983) of the most appropriate theory for $T_L$ in plants. Without the leadership provided by P. V. Sane, researches on $T_L$ at BARC would have been in directions different from those on the photosynthetic systems. The relationship of the S-states to the $T_L$ was, however, pioneered by Inoue and Shibata (1978) when they discovered the period-4 oscillation in $T_L$ and related it to oxygen evolution steps (see reviews by Vass and Inoue, 1992; Inoue, 1996). I refer the readers to the cited reviews to discover for themselves the discoveries of A. W. Rutherford, and of J.-M. Ducruet at Saclay, France, G. Renger in Berlin, Germany and the research groups of S. Demeter, I. Vass and G. Horvath in Szeged, Hungary, without whose work, $T_L$ would not be where it is today. Currently, the $T_L$ method is being used to monitor and characterize site-directed mutants both on the acceptor (Mäenepää *et al.,* 1995) as well as on the donor side of PSII (Kramer *et al.,* 1994).

## Photophosphorylation

ATP is the energy currency of life. Production of glucose (food) from $CO_2$ requires both NADPH and ATP. Below, we will discuss the breakthroughs that led to the understanding of ATP synthesis: the chemiosmotic hypothesis and the alternate binding site hypothesis (for details of energy transduction and ATP generation by ATP synthase, see Chapter 4, this volume, by Berzborn).

After indications in several laboratories that algal cells produce ATP in light, Arnon *et al.* (1954) and Frenkel (1954) discovered photophosphorylation in chloroplasts of plants, and chromatophores of anoxygenic photosynthetic bacteria, respectively. This was a major breakthrough and it took many years to recognize that *only* in some anoxygenic photosynthetic bacteria, almost all light energy is first converted into ATP energy and then this energy is used for the reversed electron flow to produce the reducing power in the form of NADH. In oxygenic organisms, however, this is not the case as correctly expressed by Rabinowitch (1956). As we know today, ATP synthesis follows electron transport steps that first store energy temporarily by creating a proton motive force; this energy would be otherwise lost if not used for ATP formation (see the scheme of Hill and Bendall, 1960). P. Mitchell (1961), who received the Nobel prize in Chemistry in 1978, provided the theory that a proton motive force (that is a sum of a pH gradient and a membrane potential) is the energy source of ATP synthesis. Membranes are normally impermeable to protons; and, protons are transferred from one side of the membrane to the other by virtue of the alternate electron and hydrogen atom transfers due to specific location of the electron and hydrogen atom carriers and the energetics of the light-induced electron transfers in photosynthesis. An early breakthrough was the independent experiments of Shen and Shen (1962) and of Hind and Jagendorf (1963) showing that light forms some entity ($X_E$) that can be used later in darkness to produce ATP. Hind and Jagendorf showed that $X_E$ is a pH gradient that drives ATP synthesis. Jagendorf and Uribe (1966) showed that ATP can be synthesized from pH gradient created by acid–base transition in total darkness. These experiments, along with those of the others, provided major evidence for the chemiosmotic hypothesis of Mitchell (see a personal perspective of Jagendorf, 1998). In photosystem II, the

water oxidation complex that liberates protons is on the inner side (the lumen) of the thylakoid membranes. Further, plastoquinone is reduced to plastoquinol on the outer side (stroma side) of the thylakoid membrane; the plastoquinol (a hydrogen atom carrier) moves towards Rieske iron sulphur and cytochrome *f* (both are electron, not hydrogen atom carriers) that are located towards the lumen side. Here, plastoquinol delivers its electrons to the Rieske iron sulphur centre and the cytochrome *f*, leaving the protons to be released into the lumen. This arrangement thus allows natural proton translocation from the stroma to the lumen side as the electron transport takes place in PSII. This adds to the pH gradient to be used for ATP synthesis. The equivalence of pH gradient and membrane potential in synthesizing ATP was shown by the elegant experiments of Gräber *et al.* (1984) and Hangarter and Good (1982) when they varied one keeping the other constant and showing that it was the sum of the two (proton gradient and membrane potential) that was important for initiating ATP synthesis.

The understanding of the mechanism by which ATP synthesis takes place at the ATP synthase using the proton motive force has been influenced by the following three breakthroughs. (1) The binding change hypothesis of P. Boyer and co-workers (see Boyer *et al.,* 1973; and a review by Boyer, 1997; also see Figure 1.14 and its legend) that suggests that at one time (a) ADP and $P_i$ (inorganic phosphate) bind weakly at one site of the $\alpha$-$\beta$ pair of the F1 part of the ATP synthase enzyme; (b) bound ATP is formed from bound ADP and $P_i$, without the use of energy at a second $\alpha$-$\beta$ pair; and (c) the pH gradient energy is converted into rotational energy, mainly of the $\gamma$ subunit that extends up to the third $\alpha$-$\beta$ pair, that is used to flip off the ATP free from the third $\alpha$-$\beta$ binding site. These three sites alternate in time. (2) The rotation feature of the Boyer model has been elegantly demonstrated directly by fluorescence microscopy and by photoselection and other experiments (see Capaldi, 1994; Duncan *et al.,* 1995; Sabbert *et al.,* 1996, 1997; Junge *et al.,* 1997). (3) The atomic resolution structure (Abrahams *et al.,* 1994) of beef-heart mitochondrial F1 showed that the $\gamma$ subunit indeed looks through the $\alpha$-$\beta$ pairs; and in agreement with the Boyer model, the structure shows one $\alpha$-$\beta$ pair site empty (as if ATP was released); another with bound ADP and Pi; and the third with an equivalent of bound ATP. I consider it highly likely that the plant, algal and cyanobacterial ATP synthase will be basically similar to that of the cow. However, differences in details must be expected and explored.

## Concluding personal remarks

The mechanism of photosynthesis has been probed by several means. Its face has been changed by the experiments of many investigators. I restrict my personal list to deal mainly with oxygen evolution. It includes, among others, the experiments of my first mentor Robert Emerson (Emerson and Arnold, 1932a, b). Using the repetitive flash method and Warburg's manometry, they led to the concept of the "photosynthetic unit" where excitation energy, absorbed by hundreds of antenna pigment molecules, is transferred to the reaction centre chlorophyll molecules, the "photoenzyme" or the "energy trap" for chemistry. Next, was the discovery of the "red drop" (Emerson and Lewis, 1943) in the quantum yield action spectrum of oxygen evolution in the green alga *Chlorella pyrenoidosa*, and the enhancement effect of certain wavelengths of light on the yield of oxygen evolution in the red drop region (Emerson *et al.,* 1957), discovered by the use of an excellent monochromator and state-of-the-art manometry.

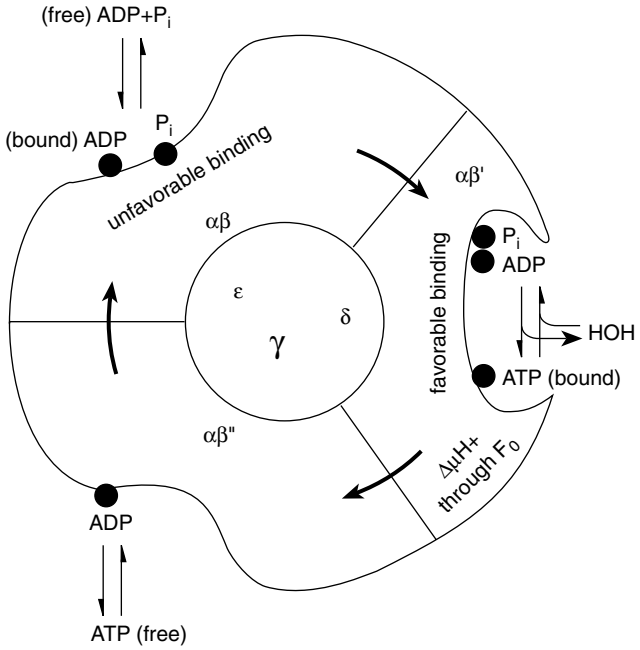


*Figure 1.14* The enzyme ATP synthase consists of a membrane portion and an exposed portion (see Figure 1.4). The exposed portion, which looks like a door knob, has five subunits. $3\alpha$, $3\beta$, $1\gamma$, $1\delta$, and $1\varepsilon$). The $3\alpha$, $3\beta$ are paired as $\alpha$-$\beta$ pairs and these subunits are the catalytic sites of the enzyme. The $\gamma$ subunit sort of connects the exposed part ($F_1$) to the membrane part ($F_o$). The diagram shows a model of the top of the ATP synthase according to Paul Boyer of UCLA, California. There are three alternate binding sites. At one site ADP and $P_i$ bind; at another site ADP and $P_i$ produce bound ATP; and at the third site bound ATP is released. In this model, most energy is used to release bound ATP. Each of the three sites perform all three steps, but at different times. Thus, the activity "rotates" on the $\alpha$-$\beta$ pairs. The energy of the proton gradient is converted, in this model, to conformational energy of the $\gamma$ protein that rotates and transfers the energy to the $\alpha$-$\beta$ pairs for the simultaneous binding of ADP + $P_i$ and the release of ATP. (Support for such a scheme has been found in a paper by Abrahams *et al.* (1994) on the structure of beef-heart mitochondrial ATPase, obtained by X-ray crystallography.)

(I was fortunate to use this during my Ph.D. thesis under Emerson and Eugene Rabinowitch.) It lead to the concept of the two-light reaction and the two-pigment system mechanism of electron transport. In contrast to Emerson's ideas, where one system was sensitized by accessory pigments and the other by chlorophyll *a*, I showed that both the light reactions are sensitized by chlorophyll *a*, although of different spectral variety (Govindjee and Rabinowitch, 1960). The two-light reaction scheme became well known due to the working hypothesis of Hill and Bendall (1960) and became a scientific fact only through the experiments of Duysens *et al.* (1961) on the antagonistic effect of light absorbed in pigment system I and II on the redox state of cytochrome *f*. The important concept that there is an "oxygen clock", where four positive charges must accumulate before water can be oxidized to oxygen was enshrined before us by (a) the experiments of Joliot *et al.* (1969) on the periodicity of 4 in the plots of the amount of oxygen evolved per flash as a function of flash number, and (b) the so-called

"S-states" model of charge accumulation enunciated by Kok *et al.* (1970). Such a periodicity of 4, reflecting indirectly the S-states, has also been observed in chlorophyll *a* fluorescence (Delosme, 1971; Joliot and Joliot, 1971), and recently, Shinkarev *et al.* (1997) have even managed to obtain the kinetics of the last step of oxygen evolution from analysis of the data on the decay of chlorophyll *a* fluorescence in single flashes of light. Thermoluminescence, discovered by W. Arnold and H. Sherwood (see Arnold, 1991), and exploited both by P. V. Sane (Sane and Rutherford, 1986) as well as myself (DeVault *et al.,* 1983; Vass and Govindjee, 1996), has uniquely probed the characteristics of the S-states of oxygen evolution (Inoue and Shibata, 1978; Inoue, 1996). The most elegant probes for showing that Kok's S-states are manganese are the low temperature EPR (Dismukes and Siderer, 1980); and EXAFS spectroscopy (see Yachandra *et al.*, 1996).

I do want to emphasize two elegant conceptual works in another area, that of phosphorylation: (a) the chemiosmotic theory of P. Mitchell in which proton motive force across a membrane provides energy for ATP synthesis (Mitchell, 1961); and (b) the elegant theory of Paul Boyer as to how this comes about by conversion of electrochemical energy to conformational energy (Boyer, 1997).

Finally, I wish to mention that mimicking photosynthesis *in vitro* has been a dream of many, and the recent success of Steinberg-Yfrach *et al.* (1998) in making "lots of ATP" in artificial liposome membranes, energized by a synthetic system (carotene-porphyrin-quinone), is highly commendable. It would have made my second mentor Eugene Rabinowitch quite happy.

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