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WINTER COLLEGE ON LASERS, ATOMIC AND MOLECULAR PHYSICS

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Laser Flash Photolysis - Some Applications to the Chemistry of Biology and Medicine.

(i) General Background and Description of Techniques

(ii) Applications Related to Medicine

(iii) Applications Related to Biology

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Laser Flash Photolysis — Some applications to the chemistry of biology and medicine

3 lectures:

(i) General Background and description of techniques

(ii) Applications related to medicine

(iii) Applications related to biology

General References

1. Suitable for the 3 lectures

'Flash Photolysis and Pulse Radiolysis'

by R.V. Bensasson, E.J. Land & T.G. Truscott

Pergamon Press 1983 (Feb)

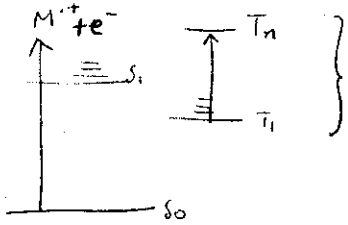
2. Suitable for lecture no. 3 (photosynthesis)

'Photosynthesis: physical methods and chemical patterns'

by R.K. Clayton

Cambridge Univ. Press (IUPAB Biophysics Series) 1980

3. The journal 'Photochemistry and Photobiology' (pub. Pergamon Press)



This $T_1 - T_n$ transition is usually monitored by flash photolysis, as well as short-lived conformational changes.

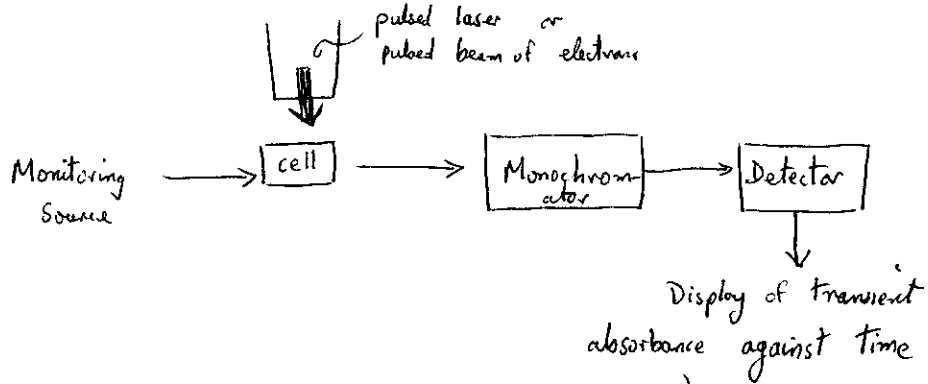
The solvated electron (e^-_{aq} in water) and the radical cations and radical anions ($M + e^- \rightarrow M^{\cdot -}$) can also be monitored by flash photolysis

Although radical ions are often better studied by the related technique of pulse radiolysis.

Together these pulsed radiation techniques in the nano-second time scale are used to determine parameters of transient species (such as triplets, radical ions, and electrons) such as:

1. Lifetimes
2. Rates of reaction with other bi-molecules e.g. O_2
3. Absorption spectra including Extinction coefficients
4. Quantum Yields for transient production (ϕ_T for the triplet; ϕ_i for ionisation etc)

The basic experimental set-up for both laser flash photolysis and pulse radiolysis on the nano-second time scale is



Typical pulsed lasers are Ruby (694 nm)
Frequency doubled to 347 nm

Nd or YAG - Frequency doubled ~ 530 nm
" tripled ~ 353 nm
" quadrupled ~ 265 nm

Typical sources in pulse radiolysis are linear accelerators giving up to 10 MeV — because of the high energy involved the solvent ionisation is important and the lecture will briefly mention the consequences for hydrocarbon, alcohol and water as solvents.

The chemical methods available for resolving the mixture of transients often obtained will be discussed

e.g. Nitrous Oxide is used to remove solvated electrons and consequently to avoid the formation of radical anions ($M^{\cdot -}$)

$$e + N_2O + H_2O \longrightarrow N_2 + OH^{\cdot} + OH^-$$

Quantitative Studies

Typical methods used in laser flash photolysis to determine rates of reaction, transient extinction coefficients and Φ values will be discussed using biological molecules as examples.

For ε_T it is important to remember that laser flash photolysis (and pulse radiolysis) gives difference absorbances (Δ) of the transient which leads to difference extinctions $\Delta\varepsilon = \varepsilon_T - \varepsilon_S$ where ε_S is the extinction coefficient of the ground state.

Two methods of determining $\Delta\varepsilon$ will be given in the lecture (i) Complete Conversion (ii) Energy Transfer

For ϕ_T A comparative method will be used:

$$\phi_x = \phi_s \cdot \frac{OD_T^x/\varepsilon_x}{OD_T^s/\varepsilon_s}$$

where x and S are the unknown and standard

Applications of laser flash photolysis to Medicine.

3 typical examples will be discussed — more details of these and other are given in Reference 1

(i) Treatment of Neo-Natal Jaundice

The yellow pigment BILIRUBIN is the final catabolism product of blood. In very young babies it can cause the dangerous disease Kernicterus.

Current treatment is phototherapy.

Laser flash studies in solution and when bilirubin (BR) is complexed to HSA show ϕ_T ~0

ϕ_i ~0

but photo-isomerisation is quite efficient ϕ_{isom} ~0.2 and very fast - isomers formed in few ps

Information on the binding site of BR in HSA can also be obtained by either laser flash photolysis or pulse radiolysis. This is done by monitoring the effect of the BR on the rate of the radical transformation $Trp^{\cdot} + TyrOH \rightarrow TrpH + TyrO^{\cdot}$ in the protein.

(iii) Porphyrins (Porphyric Disease and Cancer Phototherapy)

Several porphyrins (such as protoporphyrin, PP; and uroporphyrin, up) accumulate to excess in the skin of people suffering from one of the porphyric diseases (enzyme blockages associated with the biosynthesis of blood). These often lead to acute skin photosensitivity — this is often assumed to arise via the porphyrin lowest triplet

e.g. $PP + h\nu \longrightarrow PP(S_1) \xrightarrow{isc} PP(T_1)$

$PP(T_1) + O_2(^3\Sigma_g^-) \longrightarrow PP + O_2(^1\Delta_g)$

$O_2(^1\Delta_g)$ + biosubstrate $\longrightarrow$ skin damage etc.

Cancer phototherapy uses the fact that hematoporphyrin (Hp), or more probably some derivative or aggregate of Hp, accumulates preferentially in some malignant tumours. Red light (often from a laser) is then used to destroy the tumours possibly by a mechanism analogous to that for PP

The triplet state spectra and other parameters will be discussed in the lecture. Recent studies have concerned the interaction of $Hp(T_1)$ with oxygen. Three processes occur

$$Hp(T_1) + O_2(^3\Sigma_g^-) \begin{cases} \rightarrow Hp + O_2(^1\Delta_g) \\ \rightarrow Hp^{\bullet+} + O_2^{\bullet-} \\ \rightarrow Hp + O_2(^3\Sigma_g^-) \end{cases}$$

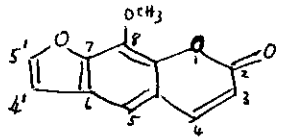
depending on the environment. Laser flash photolysis has estimated the relative yields of these. The detailed reasons for the amount of each reaction are not well understood but clearly could be related to the photo-therapeutic process.

(iv) Psoralen Photochemotherapy (PUVA) Treatment of Psoriasis

This disease is characterised by overactive DNA synthesis and cell division in the skin leading to maturation of the cells in 3 or 4 days instead of ~28 days as in normal skin.

Psoralen phototherapy (called PUVA) involves oral or topical application of a psoralen drug followed by UVA light irradiation (UVA - 320—400 nm). The treatment has to be repeated every few months.

The triplet state parameters of several psoralens including the most important 8—methoxy psoralen will be given in the lecture



The interaction of psoralen triplet states and amino acids such as tryptophan and nucleic acid bases such as thymine are of importance. The complementary techniques of laser flash photolysis and pulse radiolysis imply charge transfer processes :-

Psoralen (T_1) + Tryptophan $\longrightarrow$ Psoralen$^{\bullet-}$ + Tryptophan$^{\bullet+}$.

Lecture 3 Photosynthesis and Vision

Laser flash photolysis results on a pigments important in both process e.g. carotenoids (C_{40} in photosynthesis and C_{20} in vision) will be described.

The use of nanosecond and picosecond techniques to study the early stages of vision will be briefly discussed in terms of isomerisation and proton translocation in the visual pigments such as rhodopsin.

More detail will be given on applications of laser flash photolysis to understanding the light reactions of green plant and bacterial photosynthesis. The following description of these application are taken from Reference 1.

7.1. INTRODUCTION

Photosynthesis is the process by which green plants and photosynthetic bacteria use solar light energy for the biosynthesis of cell components such as carbohydrates which are used as an energy source for all living systems. It is usual to consider the overall process, which may be represented as

$$CO_2 + 2H_2A \xrightarrow{\text{light}} (CH_2O) + A_2 + H_2O,$$
(carbohydrate)

where H_2A symbolizes a hydrogen donor such as H_2O or H_2S. This overall reaction occurs in two phases: the light reactions in which solar energy is captured and converted into the chemical energy associated with adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADPH) in green plants (NADH in photosynthetic bacteria), and the dark reactions in which these energy-rich species, ATP and NADPH (or NADH), are used to reduce carbon dioxide to carbohydrate, the Calvin cycle. At the same time ATP and NADPH are converted back to ADP and $NADP^+$ (NAD^+ in photosynthetic bacteria).

The overall photosynthetic apparatus occurs in a membrane system – the thylakoid membrane in green plants and the cytoplasmic membrane in photosynthetic bacteria. Early work (Emerson and Arnold, 1932) based on repeated flash irradiation of algae showed that the maximum number of O_2 molecules evolved per flash was very small compared to the number of chlorophyll molecules, this result being consistent with the bulk of the pigments (such as chlorophylls, carotenoids and phycobilins) being an antenna system which absorb the solar energy and transfer the excitation energy to a special type of pigment in the reaction centres, where the photochemistry of the process takes place. Similar conclusions for bacterial photosynthesis were inferred from the small changes in the near IR (~ 870 nm) absorption spectra following continuous irradiation of whole cells (for example, see Duysens, 1954).

Whilst the orientation of this chapter to examples of the application of flash photolysis to photosynthetic systems does not do justice to the many other techniques which have given important information (such as ESR, electron nuclear double resonance (ENDOR), fluorescence and circular dichroism), it does show, however, that the improvements in the time resolution achieved in the flash photolysis techniques together with the improvements in biochemical techniques in recent years have led to a further unravelling of the complex molecular processes in photosynthesis and particularly in bacterial photosynthesis. This chapter will emphasize the application of flash photolysis to bacterial systems rather than green plant photosynthesis. Bacterial photosynthesis is inherently simpler than green plant

photosynthesis because bacteria contain only one light-reaction or photosystem (PS), whereas green plants contain two photosystems (PSI and PSII), this relative simplicity of bacteria making them attractive systems to study. In addition, standard biochemical procedures have allowed the reaction centres and antenna complexes of bacterial systems to be completely separated, thus leading to a detailed understanding of the reaction centre processes in bacteria. No such complete separation is yet available for green plants and consequently knowledge of the primary processes in green plants is much less detailed than in bacteria. Nonetheless, despite concentrating mainly on bacterial photosynthesis, a few examples of the use of flash photlysis in the study of photosynthesis in green plants will also be given.

7.2. BACTERIA

7.2.1. General Background

The photosynthetic unit of a typical non-sulphur photosynthetic bacterium such as *Rhodopseudomonas sphaeroides (Rps. spb.), Rhodospirillum (R) rubrum, Rps. viridis* and *Cbromatium vinosum* consists of an antenna system, of 40–200 bacteriochlorophyll molecules (normally bacteriochlorophyll *a*) per reaction centre and various somewhat smaller amounts of carotenoids. These photosynthetic units may be disrupted into reaction centres (a typical spectrum is given in Fig. 7.1) and antenna complexes by treatment with detergents (e.g. lauryl dimethylamine-*N*-oxide, LDAO), each of which may be separated and purified.

The reaction centres usually contain three polypeptides of molecular weights near 26, 32 and 37 X 10^3, four molecules of bacteriochlorophyll, two molecules of bacteriopheophytin and non-ubiquinone (or, in *Chromatium vinosum* menaquinone) complex, and, in reaction centres from carotenoid containing strains, one molecule of a specific carotenoid. Both resonance Raman spectroscopy (Lutz *et al.*, 1976, 1978) and the visible absorption spectra of carotenoidless reaction centres from *R. rubrum* (G9), when compared to the spectra with carotenoids added (Boucher *et al.*, 1977), imply that the reaction centre carotenoid has a cis configuration.

Both ESR and ENDOR spectroscopy show that of the four bacteriochlorophyll molecules in the reaction centre, two interact with each other as a 'special pair' (called P870 due to the characteristic absorption peak near 870 nm, see Fig. 7.1) and that this 'dimer' is involved in the initial electron ejection of the photochemical process (McElroy *et al.*, 1972 and Feher *et al.*, 1975). Oxidized reaction centres of

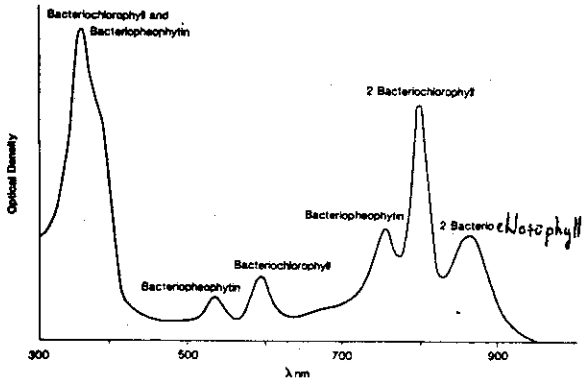


Fig. 7.1. Absorption spectrum of Bacterial Reaction Centres in *Rps. spb.*

Rps. viridis which contain bacteriochlorophyll *b* do not show similar ESR and ENDOR spectral characteristics†. Nevertheless, the special pair is still thought to exist in bacteriochlorophyll *b*-containing reaction centres with the spectroscopic data being accounted for by assuming that these 'dimers' have a twisted structure (Davies *et al.*, 1979).

The 'primary' photochemical process following excitation of P870 to an excited singlet state P870* is the ejection of an electron which reduces ubiquinone (Q) via various intermediate species (I), that is

$$P870^*(I)Q \rightarrow P870^{\cdot+}(I^{\cdot-})Q \rightarrow P870^{\cdot+}(I)Q^{\cdot-}$$

The non-haem ferrous iron contained in the reaction centre is thought to be involved not in this primary step but in secondary electron transfer processes, as discussed later. Laser flash photolysis, carried out with increasingly improving resolution times, has gradually unravelled the various early processes prior to the electron being transferred to the ubiquinone; this is discussed in detail in the next section. In addition, we will consider as examples of the use of flash photolysis the re-reduction of P870* (by cytochrome *c*) and the secondary electron transfer processes from $Q^{\cdot-}$.

The position of these processes in the tentative overall electron transport scheme is indicated in Fig. 7.2. This simplified scheme shows P870 functioning as an electron donor with a sequence of acceptors, with reduction of cytochrome c_2 leading to the re-reduction of $P870^{\cdot+}$ to P870. The second quinone (Q_b) is protonated on reduction from Q_A (see 7.2.2.2) and then reduces a quinone in the 'pool' which spans and transfers protons across the membrane for ATP production (the possible involvement of cytochrome *b* in this secondary transfer is discussed later). This overall scheme can be compared with the equivalent but more complex electron transport (the 'Z' scheme) thought to operate in higher plants – this is shown in Fig. 7.8.

The light harvesting antenna pigments exist as pigment–protein complexes and several types can be distinguished by their absorption spectra in the 800–900 nm region. Thus, for example, *Rps. spb.* strain 2.4.1 contains two types called B870 and B800–850 (the numbers being based on their absorption maxima). One of these, B800–850, is rather easy to isolate, and so far detailed information is available for this complex both from *Rps. spb.* and *Rps. capsulata* (Cogdell and Crofts, 1978; Austin, 1976; Shiozawa *et al.*, 1980).

The pigment content of various strains of B800–850 show a strict stoichiometric ratio of 3 : 1 between the bacteriochlorophylls and the carotenoids. However, the carbtenoid content of the complex reflects the carotenoid content of the parent chromatophore membrane (Cogdell, 1978). For example, the G1C strain antenna complex contains 100% neurosporene as carotenoid, while the 2.4.1 strain antenna complex contains only 0.06% neurosporene together with 91% spheroidene and 8.94% spheroidenone. Singlet–singlet energy transfer from carotenoid to bacteriochlorophyll (the light harvesting role of carotenoids)

$$^1Car^* + BChl \rightarrow Car + {}^1BChl^*$$

has been studied both in the intact photosynthetic membranes (Goedheer, 1959) and more recently in

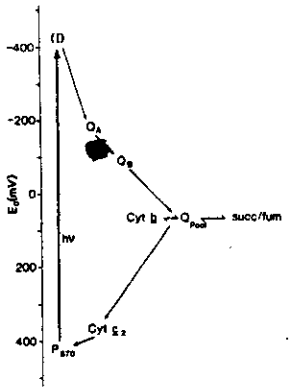


Fig. 7.2. Typical Scheme for Electron Transport in Photosynthetic Bacteria.

† A $\sqrt{2}$ narrowing of the ESR line and a 50% decrease in the ENDOR splitting is expected for a symmetrical cation dimer compared to the corresponding monomer.

B800–850 antenna complexes themselves (Cogdell *et al.*, 1981). Triplet energy transfer from bacteriochlorophyll to carotenoid

$$^3\text{BChl} + \text{Car} \rightarrow \text{BChl} + {}^3\text{Car}$$

(the protective role of carotenoids) has been studied using flash techniques both with intact chromatophores (Monger *et al.*, 1976; King and DeVault, 1976 and Renger and Wolff, 1977) and isolated antenna complexes (Cogdell *et al.*, 1981).

7.2.2. Reaction Centres

As examples of the application of flash photolysis to the study of bacterial reaction centres we shall consider two main areas: the 'primary' photochemical process in which P870 transfers an electron, via I, to a quinone and the 'secondary' electron transfer processes in which the electron is transferred to a second quinone.

7.2.2.1. PRIMARY PROCESSES

Early applications of flash photolysis to chromatophores gave useful information on reaction centre processes. Thus, for example, Parson (1968) reported a transient absorption decrease at a monitoring wavelength ~ 880 nm following flash excitation of *Chromatium* chromatophores which was interpreted as the oxidation of P870 and also showed that this process was complete in < 0.5 μs. The depletion was followed by an absorption increase ($t_{\frac{1}{2}}$ ~ 2 μs) as $P870^+$ was reduced back to P870. Transient measurements at 422 nm implied that cytochrome 422^+ became oxidised at the same rate. These results confirmed that P870 oxidation is the primary photochemical reaction of bacteria and also indicated a direct electron transfer from cytochrome c-422 to $P870^+$.

Some of the first important details concerning the primary photochemical electron transfer reactions in reaction centre preparations themselves using nanosecond flash photolysis arose from the work of Parson and co-workers (for example, Parson *et al.*, 1975 and Cogdell *et al.*, 1975). Using purified reaction centres both from strains which lack carotenoids (*Rps. spb.* R26 and *R. rubrum* G9) and also from those containing carotenoids (*Rps. spb. 2.4.1* and *Ga* and *R. rubrum S1*) which were reduced with sodium dithionite to block the photochemical electron transfer reactions, Parson and co-workers detected various transient species following nanosecond laser excitation.

Thus for reaction centres from the carotenoidless mutants an immediate absorption increase was monitored at 420 nm, followed by a relaxation with $t_{\frac{1}{2}}$ ~ 20 ns at room temperature. Figure 7.3 shows

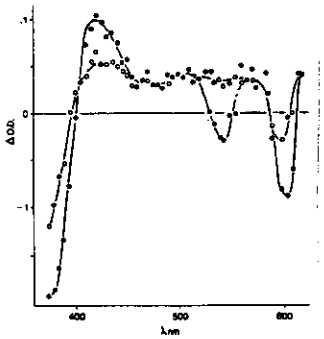


Fig. 7.3. Spectra of Flash-induced optical density changes in Reaction Centres from *R. rubrum G9*: ●, initial optical density change ($P870 \rightarrow P^F$); ○, optical density change ~ 200 ns after the flash ($P870 \rightarrow P^R$) (taken from Cogdell *et al.*, 1975.

the absorption spectrum (for G9) of this initial change, the transient species formed being termed P^F. This figure also shows the spectrum of the longer-lived species to which P^F relaxes. This is called P^R, assigned to bacteriochlorophyll triplet on spectral considerations (see Fig. 2.1.6), the lifetime of P^R obtained being ~ 6 μs at room temperature. The assignment of P^F (the lifetime of ~ 10 ns rules out the fluorescent excited single state) awaited elucidation by picosecond flash studies. Parson *et al.*, (1975) also measured the quantum yields of P^F and P^R

† *Chromatium* contains at least 3 different *c-type* cytochromes which can be distinguished by, for example, their absorption spectra.

formation by monitoring the transient absorbance (at 425 nm for P^R and 420 nm for P^F) as a function of flash intensity and obtained values of near unity for P^F but only ~ 0.2 for P^R. These data led Parson *et al.* to conclude that P^F might be an intermediate in the photochemical electron transfer reaction while P^R (the bacteriochlorophyll triplet) was of less interest and simply a side product associated with the experimental conditions. The extension of these studies to carotenoid-containing strains (Cogdell *et al.*, 1975) again led to the observation of the immediate (time resolution ~ 6 ns) formation of P^F so that this work generalized the conclusion that P^F was an intermediate in the photochemical electron transfer reaction. However no evidence for P^R formation was found, and instead for these carotenoid containing strains, P^F decays rapidly to produce a new longer-lived state which is decidedly different from state P^R. The difference spectra for these new states are given in Fig. 7.4. The spectrum obtained from *Rps. spb. 2.4.1* shows a peak at ~ 545 nm (Fig. 7.4a), while the spectra for *Rps. spb. Ga* and *R. rubrum S1* are similar but shifted to shorter and longer wavelengths respectively. These transients were identified as carotenoid

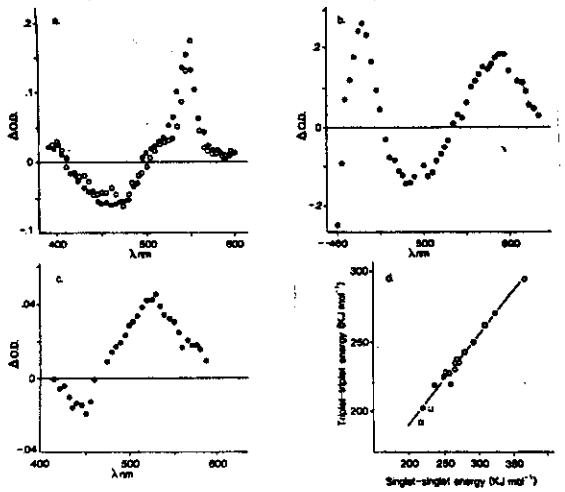


Fig. 7.4. Flash-induced difference spectra for the formation of carotenoid triplet states in reaction centres from three carotenoid-containing strains. (a) ●, Reaction centres of *Rps. spb. 2.4.1* at room temperature; ○, reaction centres of *Rps. spb. 2.4.1* at 77 K. (b) Reaction centres of *R. rubrum S1* at room temperature. (c) Reaction centres of *Rps. spb Ga* at room temperature. (d) Energy of the long wavelength triplet–triplet absorption bands vs the energy of the long wavelength singlet–singlet absorption band. ○ and □ data for carotenoids and various polyenes in solution, taken from Truscott *et al.* (1973) and Mathis and Kleo (1973). ●, data for carotenoids in reaction centres; triplet–triplet energies were calculated from the flash-induced difference spectra of (a)–(c), and singlet–singlet energies were calculated from the absorption spectra of the corresponding reaction centre preparations (taken from Cogdell *et al.*, 1975).

triplets firstly because they were seen only in reaction centres which contain carotenoid and secondly because of the similarity with the spectra of triplet states of other carotenoids, such as β-carotene, in organic solvents (Chessin *et al.*, 1966; Truscott *et al.*, 1973; Mathis and Kleo, 1973 and Bensasson *et al.*, 1976a).

As noted in chapter 3, Truscott *et al.* (1973) and Mathis and Kleo (1973) reported a linear relationship between the energy of the first singlet-singlet transition and the first triplet-triplet transition for a series of carotenoids. A similar trend was found by Cogdell *et al.* (1975) for the three reaction centre carotenoids studied and Fig. 7.4d shows the fit of these experimental points. Finally, Cogdell *et al.* reported the decay rates for the reaction centre carotenoid triplets to be 2–6 μs, that is in a similar range to that established for carotenoids in organic solvents. It was originally suggested that carotenoid triplets in reaction centres were formed directly from P^F but subsequent flash data at lower temperatures (Parson and Monger, 1976) showed that the carotenoid

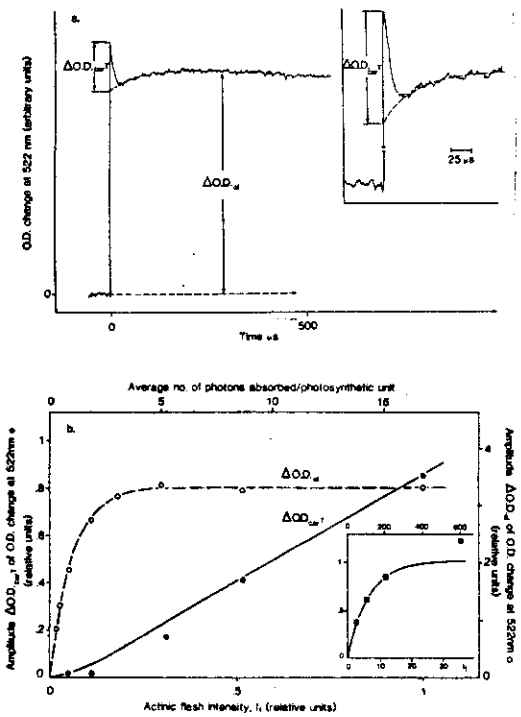


Fig. 7.5. (a) Kinetics of the flash-induced optical density change at 522 nm in chromatophores of *Rps. spb. strain 2.4.1.* Inset: 4-fold magnification of the rapid formation of carotenoid triplet. (b) Dependency of $\Delta OD_{car}T$ and ΔOD_{el} of the 522 nm optical density change on the flash intensity (taken from Renger and Wolff, 1977).

triplets were formed via P^R and that their role was thus an energy sink for deactivation of reaction centre bacteriochlorophyll triplet.

Similar conclusions for the role of carotenoids as a 'triplet valve' in bacteria were reached by Kung and DeVault (1976) and Renger and Wolff (1977) who studied chromatophores of *Rps. spb.* rather than reaction centres themselves. These workers reported the effect of laser flash intensity on the yield of the carotenoid triplet (monitored immediately after the laser flash) and compared this with the slowly formed ($t_{½}$ ~ 100–150 μs) transient absorption known to be due to the electrochromic effect on the carotenoid ground state absorption (caused by the electron transfer from cytochrome *c* to $P870^{\cdot+}$). This latter measurement is an indication of the primary electron transfer effect and is therefore expected to show the same light saturation effects as bacterial photosynthesis itself. Figure 7.5a shows a typical oscillogram obtained by Renger and Wolff illustrating both the rapid formation of the carotenoid triplet ($\Delta OD_{car}T$) and the long-lived transient due to the electrochromic effect (ΔOD_{el}). Figure 7.5b shows the dependence of the amplitudes of these transients on the laser flash intensity indicating that the absorption changes due to the valve reaction ($\Delta OD_{car}T$) are observed only when the reaction centre becomes saturated, that is, closed.

The first picosecond flash photolysis studies of reaction centres were reported by Netzel *et al.* (1973) who excited the 530 nm bands of the reaction centre bacteriophaeophytin and detected a bleaching of the P870 band in < 10 ps. While this result could not be unambiguously linked to $P870^{\cdot+}$ formation, the work did open the door to the study of the picosecond reaction in bacterial photosynthesis.

Subsequent to the work of Parson *et al.* (1975) which noted the formation of the P^F state following nanosecond studies of reduced reaction centres as described above, picosecond studies, for example, Dutton *et al.* (1975), Rockley *et al.* (1975) and Kaufmann *et al.* (1975), were undertaken to further characterise P^F and to establish whether the formation of P^F was an artifact of the reducing conditions employed by Parson *et al.* Clearly a role for P^F in bacterial photosynthesis requires information on whether P^F is formed under conditions that permit the electron transfer reaction to occur. Using picosecond flash photolysis it was found that P^F was formed in < 20 ps after the flash and decays with $t_{½}$ ~ 100–200 ps (compared with 30 ns for the closed, i.e. reduced, reaction centres as noted above). As the P^F transient decayed the radical cation of the bacteriochlorophyll complex was revealed. The most important aspects of the spectrum of P^F are the negative bands at ~545 nm and ~800 nm associated with the simultaneous bleaching of bacteriophaeophytin and bacteriochlorophyll (see Fig. 7.1) and the formation of a band at 680 nm due to the radical anion of bacteriophaeophytin (see 2.1.6 and Fajer *et al.* (1975) together with the positive band, reported by Dutton *et al.* (1975) at 1250 nm, this band at 1250 nm being characteristic of the radical cation of the bacteriochlorophyll complex. So that, at this time P^F was identified as a biradical of the type:

$$^{\cdot}(BChl)_2^{\cdot+} \ldots\ldots BPheo^{\cdot-}$$

the radical anion species $BPheo^{\cdot-}$ is often called $I^{\cdot-}$ so that $P^F \equiv (BChl)_2^{\cdot+} \ldots I^{\cdot-}$. The decay of P^F in the open or unblocked reaction centres is assumed to be due to the further transfer of the electron from $BPheo^{\cdot-}$ to ubiquinone, as shown by the disappearance of the 545 nm negative band, the 1250 nm band being common to both P^F and the dimer radical cation. Picosecond studies on species other than *Rps spb* such as *Rps viridis* and *Chromatium vinosum* (Netzel *et al.*, 1977) imply that this electron transport process is a more general phenomena.

Photoreductive trapping experiments on subchromatophore preparations from various species in which the intermediate acceptor is stabilised using both a low redox potential to reduce the quinone and a bound fast reacting cytochrome which can transfer an electron to the oxidised dimer in $(BChl)_2^{\cdot+} \ldots I^{\cdot-}$, have also been used to study both the optical and ESR properties of $I^{\cdot-}$ (see, for example, Shuvalov and Klimov, 1976; Tiede *et al.*, 1976 and Okamura *et al.*, 1979). Such data have been interpreted as showing that there is not intermediate (e.g. bacteriochlorophyll$^{\cdot-}$) between P870 and bacteriophaeophytin. This conclusion is not unambiguous however because the reducing conditions employed, i.e. reduced bacteriophaeophytin, could preclude the transfer from P870 to bacteriochlorophyll.

In an important contribution to our understanding of the primary process Shuvalov *et al.* (1978) and Akhmanov *et al.* (1980) used an excitation wavelength of 880 nm (15 ps resolution time) so that the irradiation only excites P870. This work showed that

the difference absorption spectrum obtained for *R. Rubrum* immediately after the flash corresponded to the formation of $P870^{\cdot+}$ (such as bleaching of the 870 nm band) as well as a developing of the wide 660 nm band and a bleaching of the bacteriochlorophyll bands at ~ 600 nm and 800 nm. In particular it should be noted that bleaching at 545 nm which corresponds to bacteriophaeophytin bands had a short time delay with respect to bleaching of the 600 nm band. In agreement with the results of Rockley *et al.* (1975) the bacteriophaeophytin radical anion is oxidised by ubiquinone in about 250 ps. Thus the electron transfer from P870 to ubiquinone was seen, at this stage, to involve at least two intermediate steps. In the work of Akhmanov *et al.* (1980) the serious artifact (see, for example, Campillo *et al.*, 1977) which can arise in using picosecond excitation was noted in that the state formed by electron transfer between bacteriochlorophyll and bacteriophaeophytin has some absorption at the excitation wavelength (880 nm) so that further photons can be absorbed which could greatly obscure the kinetics of the primary process. Using equipment in which the number of photons in each picosecond pulse could be reduced to ~ < 1 photon per reaction centre, it was found that one-photon absorption resulted in charge separation with the formation of $P870^{\cdot+}$ – Bacteriochlorophyll$^{\cdot-}$ in < 10 ps and then a fast electron exchange (≤ 10 ps) between bacteriochlorophyll$^{\cdot-}$ and bacteriophaeophytin occurs. The early processes can thus be written as:

$$
\begin{array}{lllll}
P870 & - BChl & - BPhe & - Q \\
 & \downarrow & < 10\ ps & \\
P870^{\cdot+} & - BChl^{\cdot-} & - BPhe & - Q \\
 & \downarrow & \sim 30\ ps & \\
P870^{\cdot+} & - BChl & - BPhe^{\cdot-} & - Q \\
 & \downarrow & \sim 250\ ps & \\
P870^{\cdot+} & - BChl & - BPhe & - Q^{\cdot-}
\end{array}
$$

Recent sub-picosecond studies (Holten *et al.*, 1980) have been interpreted as showing that electron transfer to bacteriochlorophyll is complete within ~ 1 ps and that the movement of electron density from this species to bacteriophaeophytin takes ~ 4 ps (or possibly somewhat longer under the conditions used by Akhmanov *et al.* (1980)).

† Footnote on page 172.

Schenck *et al.* (1981) excited reaction centres from *Rps viridis* with 7 ps flashes (600 nm) and, when Q was oxidised, detected a transient state in which the electron had moved from P870 to an acceptor complex involving bacteriophaeophytin and bacteriochlorophyll complex (P^F). This state decayed in ~ 200 ps as $Q^{\cdot-}$ is produced. If Q and one or both of the bacteriophaeophytins were photochemically reduced before excitation no indication of electron transfer from P870 to bacteriochlorophyll could be detected but a different transient ($t_{1/2}$ ~ 340 ps) was observed. This state was shown not to be the excited singlet of P870 and, it was speculated, that it could be a triplet state, a charge-transfer state of P870, or another singlet state that is non-fluorescent.

Very recently Shuvalov and Parson (1981) have reported the temperature dependence of the transient absorbance changes for *Rps sph* reaction centres (blocked) and interpreted their spectral results to mean that P^F is an equilibrium mixture of two radical-pairs $^1\{P870^{\cdot+} \ldots \text{bacteriochlorophyll}^{\cdot-}\}$ and $^1\{P870^{\cdot+} \ldots \text{bacteriophaeophytin}^{\cdot-}\}$. The electron in the acceptor complex is mainly on bacteriopnaeophytin at 77 K but shared with bacteriochlorophyll at 293 K.

The temperature dependence of the absorbance changes was used to estimate an energy difference between these two radical-pairs such that $^1\{P870^{\cdot+} \ldots \text{bacteriochlorophyll}^{\cdot-}\}$ lies ~ 0.025 eV above $^1\{P870^{\cdot+} \ldots \text{bacteriophaeophytin}^{\cdot-}\}$. Also, the energy gap between these radical-pairs depends upon the charge on Q_A which is consistent with the idea that Q_A interacts more strongly with bacteriophaeophytin$^{\cdot-}$ than bacteriochlorophyll$^{\cdot-}$.

Clearly research in this field will continue to benefit from the use of laser studies with still faster sub-picosecond resolution time.

7.2.3. Light-Harvesting (Antenna) Complexes

While the structure and function of bacterial reaction centres is now quite well understood, the so-called antenna complexes have been relatively neglected and there are few photochemical studies on isolated pigment-protein complexes themselves. We have noted earlier the flash photolysis studies of Monger *et al.* (1976), Kung and DeVault (1976) and Renger and Wolff (1977) in which triplet energy transfer from bacteriochlorophyll to carotenoid was studied in intact chromatophores. Singlet-singlet energy transfer from carotenoid to bacteriochlorophyll in intact membranes was reported by Goedheer (1959) using fluorescence techniques. Recently, similar studies have been reported by Cogdell *et al.* (1981) using the B800–850 complex isolated from *Rps sph*. The type of carotenoid present in the complexes studied was varied by growing the cells under differing oxygen concentrations (Goodwin, 1956; Cogdell and Crofts, 1978) and using different carotenoid mutants. The fluorescence studies on the isolated complexes showed that singlet–singlet energy transfer from carotenoid to bacteriochlorophyll was efficient (75–100%) and is rather insensitive to carotenoid type over the range tested (spheroidene, spheroidenone, neurosporene, methoxyneurosporene and chloroxanthin). The simplest explanation of these results is that the major factor controlling the energy transfer in the B800–850 complexes is the geometry of the system.

Laser flash photolysis (20 ns, 694 nm) of isolated antenna complexes resulted in a strong transient absorption change decaying in a few microseconds. The difference spectrum reported, for example, for strain G1C containing neurosporene as shown in Fig. 7.7a was similar to that previously found for the carotenoid triplet state in intact photosynthetic membranes (Monger *et al.*, 1976). In addition, the transient lifetimes and oxygen quenching rates were comparable in both systems for all the isolated antenna complexes studied by Cogdell *et al.* so that these strong transients were confirmed as representing the carotenoid triplet state. The variation of the absorbance of the carotenoid triplet with laser intensity is shown in Fig. 7.7b for a typical antenna complex. Cogdell *et al.* (1981) used the comparative technique described earlier (1.5.3) to obtain the carotenoid triplet yields and these were shown to be low. However it was noted that the actual value did not depend upon the carotenoid type but rather varied depending on the amount of detergent present in the sample, and this was correlated with the yield of bacteriochlorophyll triplets. Thus detergent conditions which lead to high carotenoid triplet yield also lead to high bacteriochlorophyll fluorescence. The overall low yield of carotenoid triplets in the antenna complexes studied arise from a low yield of bacteriochlorophyll triplet rather than from inefficient triplet

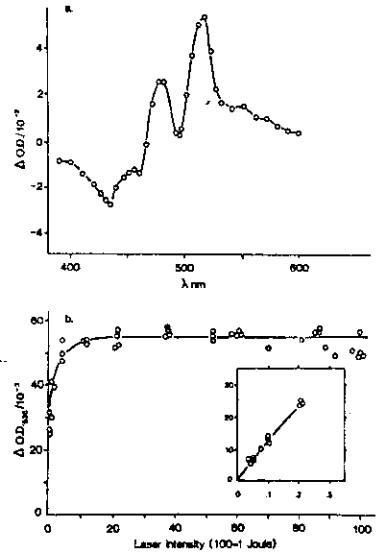


Fig. 7.7. (a) Difference spectrum for the formation of a carotenoid triplet state of the B800–850 light-harvesting pigment-protein complex from *Rps sph G1C*. (b) Variation of the size of the carotenoid triplet with laser intensity for B800–850 from *Rps sph strain 2.4.1* grown anaerobically (taken from Cogdell *et al.*, 1981).

energy transfer. The rate of the triplet transfer is very fast and also relatively independent of the type of carotenoid present. In summary, Cogdell *et al.* (1981) have shown that energy transfer between the carotenoid and bacteriochlorophyll in B800–850 complexes, both at the singlet and triplet energy levels, is rather independent of the carotenoid type present

and to be controlled mainly by the pigment-protein interactions. This is in contrast to the singlet energy transfer in reaction centres isolated from *R. Rubrum* (Boucher *et al.*, 1977) in which it was shown that the fluorescence energy transfer was very dependent on the carotenoid present.

7.2.4. Cytochrome *c* Reactions

As noted above the oxidation of *c-type* cytochromes were detected relatively early in the detailed study of bacterial photosynthesis (for example, see Duysens, 1954). Subsequently Parson and coworkers (for example, Parson, 1967 and Parson and Case, 1970) showed from kinetic studies following laser flash photolysis that the reduction of $P870^{+}$ was concomitant with the oxidation of *c*-type cytochromes.

Grondelle *et al.* (1976) used flash photolysis to study the function of three cytochromes in whole cells of *R. Rubrum*. These were *c-420* (or c_2), cytochrome *b* and *c-428*. The kinetics of the $P870^{+}$ reduction indicated that there is only one *c-420* cytochrome per two reaction centres and is thus present in low concentrations compared to other photosynthetic bacteria (see, for example, Prince and Olsen, 1976). Also, detailed kinetic studies led Grondelle *et al.* to conclude that there were two types of reaction centres in *R. Rubrum*, one associated with *c-420* (95%) and one with *c-428* (5%). It was further concluded that the latter type (*c-428* reaction centre) differs from the *c-420* centre in that it is associated with ~ 10–12 times more antenna bacteriochlorophyll. Grondelle *et al.* speculated that the physiological explanation for such an arrangement is that *R. Rubrum* converts energy with optimal efficiency at very low light intensity by means of *c-428* and at high light intensity by *c-420*. These workers also reported that the oxidised form of *c-420* itself was reduced by cytochrome *b* with $t_{½}$ ~ 7 ms.

Prince and Durton (1975) have also reported that a *b-type* cytochrome reduced cytochrome c_2 in *Rps sph* after cytochrome *b* itself has been reduced via ubiquinone.

In recent flash photolysis studies Bowyer *et al.* (1979) and Bowyer and Crofts (1980) have studied cytochrome c_2-reaction centre coupling in chromatophores of *Rps capsulata* and *Chromatium vinosum*. Their results clearly indicated that, unlike earlier conclusions based on redox titrations which implied two cytochrome c_2 molecules attached to each reaction centre, there is only one molecule per reaction centre in *Rps spb Ga* and *Rps capsulata BY761* and < 1 per reaction centre in a carotenoidless mutant of *Rps capsulata*. These results can be compared with the situation noted above for *R. Rubrum* (Grondelle *et al.*, 1976) showing one cytochrome c_2 per two reaction centres. Also in agreement with Grondelle *et al.*, the multiple flash studies of Bowyer *et al.* imply that the cytochrome c_2 is mobile between the oxidised reaction centres.

Of particular interest is the finding of Bowyer *et al.* (1979) that the extent and kinetics of cytochrome c_2 photo-oxidised and the P870 re-reduced did not match in the presence of the cyclic electron transfer blocking agent antimycin. These results are interpreted in terms of an Fe–S protein (called J) which re-reduces part of the photo-oxidised cytochrome c_2.

7.3. GREEN PLANTS AND ALGAE

In this section we will briefly consider the overall electron transport chain in the green plant photosynthetic process and then comment upon one or two typical examples of the application of flash photolysis.

7.3.1. General Background

The photosynthetic unit may be considered as an antenna system containing about 300 chlorophyll molecules per reaction centre together with accessory pigments such as carotenoids and phycobilins. The reaction centres contain a number of proteins, electron carriers and specialised chlorophyll species (P680 in system II and P700 in system I). Both P680 and P700 may be dimers and are the reaction centre traps receiving the excitation energy from the light harvesting antenna pigments.

Hill (1937) first demonstrated that illumination of green plant extracts in the presence of artificial electron acceptors such as ferricyanide leads to the evolution of oxygen and the reduction of the electron acceptor. This process is often called the 'Hill Reaction' and the acceptor a 'Hill Reagent'. In normal photosynthesis it is the endogenous electron acceptor $NADP^{+}$ which becomes reduced to NADPH. The overall electron transport scheme from water splitting

coupling oxygen (PSII) to the reduction of $NADP^{+}$ (PSI) is often represented as the 'so-called' Z scheme. A typical example is given in Fig. 7.8, however some of the steps are still controversial.

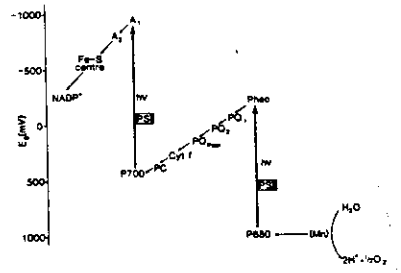


Fig. 7.8. Typical Chloroplast Electron Transfer (Z) Scheme showing the co-operation of the two Photosystems in the Transference of Electrons from water to $NADP^{+}$.

The two photosystems in the scheme may be distinguished in that PSII operates efficiently in red light ($\lambda < 700$ nm) and produces a strong oxidant (P680) sufficiently electropositive to oxidise water and also generates a weak reductant, while PSI operates efficiently in longer wavelength ($\lambda < 720$ nm) light and produces a strong reductant sufficiently electronegative to reduce $NADP^{+}$ and also generates a weak oxidant. The primary electron acceptor of PSII is probably a molecule of pheophytin and electrons then presumably flow via quinones to the plastoquinone (PQ) pool. This pool acts to shuttle protons across the membrane to build up a pH gradient which is subsequently used for ATP synthesis via a chemiosmotic mechanism (cf. bacterial photosynthesis). Subsequent to the PQ pool the electrons are transferred to P700 probably via an iron-sulphur protein (Fe–S), cytochrome *f*, and plastocyanin. The primary acceptor of PSI is not well identified and intermediate acceptors prior to P430 (ferredoxin) may include a chlorophyll monomer molecule, as discussed below.

7.3.2. Applications of Flash Photolysis

Flash photolysis work has been concerned with several aspects of these processes including energy transfer in the accessory pigments (probably via an inductive resonance mechanism), P700 and P680 oxidation reflecting the primary processes in the reaction centre, various steps in the electron transport chain and photophosphorylation. Useful general reviews of these and other applications of flash photolysis are given by Govindjee (1975), Barber (1977), Wolstenholme and Fitzsimons (1979) and, for the study of energy transfer processes in photosynthetic membranes by picosecond fluorescence measurements, Breton and Geacintov (1980).

The precision of the picosecond studies has been significantly increased using a streak camera-vidicon multichannel analyser introduced by Porter and coworkers (see, for example, Tredwell *et al.*, 1978).

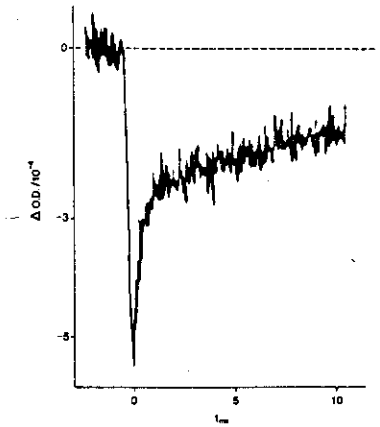


Fig. 7.9. Difference optical density at 690 nm in spinach chloroplasts as a function of time after a 20 μs flash (taken from Döring *et al.*, 1969).

However Breton and Geacintov conclude that the fluorescence decay kinetics obtained with picosecond lasers have, in fact, given relatively little new information from that available from more standard techniques such as phase fluorimetry. Nevertheless picosecond studies have given valuable information on the energy transfer mechanisms of photosynthetic membranes and an interesting example of this has

been the elucidation of the energy transfer pathway in the red alga *Porphyridium cruentum* (Porter *et al.*, 1978 and Searle *et al.*, 1978).

Particularly good examples of the use of fast nanosecond repetitive flash photolysis to improve the signal to noise ratio for weak transients obtained from photosynthetic systems are the studies of Witt and coworkers (see, for example, Witt, 1975). Thus, typically Döring *et al.* (1969) reported the time-course of the transient absorption change at 690 nm following repetitive flash excitation of whole chloroplasts. Figure 7.9 gives a typical oscillogram reported by these workers. This shows a rapid bleaching, followed by a biphasic recovery the fast component ($t_{½} \sim 0.2$ ms) has subsequently been associated with the reduction of oxidised P680 (PSII) and the slower component with the reduction of oxidised P700 (PSI).

Using chloroplast fragments enriched in P700 several workers have recently used both picosecond and picosecond laser flash techniques to study the primary processes associated with PSI. Thus Fenton *et al.* (1979) used an 8 ps excitation pulse (528 nm) and obtained transients interpreted in terms of formation of the primary radical cation $P700^{\cdot+}$ in ≤ 10 ps. Shuvalov *et al.* (1979) using flash photolysis confirmed the existence of two electron carriers (denoted A_1 and A_2) between the primary donor P700 and the primary acceptor P430. The spectra of these two species imply that A_1 is a chlorophyll and A_2 a Fe–S protein. Using a 50 ps pulse (694 nm) Shuvalov *et al.* showed that the charge pair $P700^{\cdot+} \ldots A_1^{\cdot-}$ (now thought to be a monomer) is formed in ≤ 60 ps and the subsequent electron donation to A_2 occurs in 200 ps. There have also been several recent flash photolysis studies of the reduction of $P700^{\cdot+}$ (see, for example, Haehnel *et al.*, 1980 and Olsen *et al.*, 1980). The major conclusion is that $P700^{\cdot+}$ is reduced by plastocyanin (PC) and that there is no extra electron transfer component between PC and $P700^{\cdot+}$ as was implied by earlier studies. So that, if we also assume no intermediate between P700 and A_1 we may write the early processes associated with PSI as

$$\begin{array}{l} PC - P700 - A_1 - A_2 - P430 \rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad \overset{<20\ ps}{\rightarrow} \\ \qquad\qquad\qquad\qquad PC - P700^{\cdot+} - A_1^{\cdot-} - A_2 - P430 \\ \qquad\qquad\qquad\qquad\qquad\qquad \downarrow 200\ ps \\ PC^{\cdot+} - P700 - A_1 - A_2 - P430^{\cdot-} \leftarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad \mu s/ms \\ \qquad\qquad\qquad\qquad PC - P700^{\cdot+} - A_1 - A_2^{\cdot-} - P430 \end{array}$$

7.4 SUMMARY AND COMMENTS

Flash photolysis studies have been most impressive in their success in understanding many aspects of the early processes in bacterial photosynthesis, and the continually improving time resolution available has enabled, and will continue to enable, considerable detail to be established for such primary processes. Furthermore, recent flash photolysis work with enriched particles of PSI and PSII from chloroplasts has begun to establish the details of the early steps in green plants and has also shown the value of studying the inherently simpler bacterial photosynthesis since many of the conclusions from such studies seem capable of extension to the more complex green plant systems.

It is of interest to note the resemblance between the electron transfer kinetics recently deduced in PSI and the rates of electron transport in bacterial photosynthesis. Also, while we have not chosen to discuss in detail the many flash photolysis studies of PSII such comparisons with bacterial photosynthesis are striking. Thus several recent (see, for example, Shuvalov *et al.*, 1980) studies have shown the involvement of a molecule of phaeophytin in the primary process, that is, a scheme such as:

$$\begin{array}{l} P680 - Pheo - PQ \overset{<1\ ns}{\rightarrow} P680^{\cdot+} - \\ \qquad\qquad Pheo^{\cdot-} - PQ \overset{\mu s}{\rightarrow} P680^{\cdot+} - Pheo - PQ^{\cdot-} \end{array}$$

very analogous to the detailed scheme given above for bacterial systems.