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Is There a Triplet State in Photosynthesis?

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FROM spectrophotometric (and other) work *in vivo*, and studies on the photochemistry of chlorophyll in solutions, this pigment molecule has long been thought to have a significant role in the primary events of photosyntnesis[1]. Chlorophyll has been shown to be an efficient photocatalyst, acting as a mediator in various *in vitro* oxidation-reduction reactions, often involving the storage of incoming light. In 1952, Duysens[2] observed a reversible bleaching of the long-wave absorption band of the bacteriochlorophyll found in photosynthetic bacteria; only a very small fraction of the total pigment was involved. Similarly, in green plants Kok[3] discovered a light-induced absorbance decrease centred at about 700 nm due to a small fraction of a special form of chlorophyll *a* (Chl *a*) labelled P700; this was later shown to be due to the oxidation of P700 (ref. 4). Recently, Döring *et al.*[5] and Govindjee *et al.*[6] have shown a second light-induced absorbance change centred at 682 nm (P682). These two changes (P700 and P682) are suggested to originate in separate energy traps (or reaction centres) of the two pigment systems of photosynthesis[7]. The light energy absorbed by various pigments *in vivo* must be transferred efficiently to the reaction centre chlorophyll molecules for photosynthesis to take place[8]. When the photochemistry of photosynthetic systems is blocked or poisoned, it is noted that the fluorescence of chlorophyll increases, suggesting a relationship between photochemistry and Chl fluorescence[9,10]. This relationship holds good when singlet states are involved in photochemistry[11,12].

Chlorophyll molecules can exist in the excited electronic state either in a singlet or triplet configuration. In 1936, Gaffron and Wohl[13] postulated that a metastable state of chlorophyll acts in some step of photosensitized reactions. The fact that many organic molecules can indeed be excited to a metastable level with a high yield was shown by Porter[14], using intense flashes of light. The absorption spectrum of the lowest triplet state of chlorophyll was recorded by Livingston[15] and Linschitz[16] in O_2-free organic solvents. In the present communication, some of the studies of triplet states *in vitro* and in preparations closer to the living state are reviewed. The existence of a triplet state *in vivo* has not yet been convincingly demonstrated. (The techniques of difference and flash spectroscopy, delayed light emission and electron paramagenetic resonance have been used for these studies.) Beyond being an assemblage of pertinent information, this article attempts to present a picture consistent with the available data. One of the aims of this article is to encourage investigators to reinvestigate the role of Chl triplets *in vivo*.

Characteristics of the Triplet State

Excited electronic states are due to the promotion of an electron from the ground state distribution, changing the electron density configuration to one of higher energy. The excited singlet state is generally short-lived (10^{-8}-10^{-9} sec) with the valence electrons having opposite spins. [Lifetimes of Chl singlet excited states *in vitro* and *in vivo*, first measured by Brody and Rabinowitch[17], were 15 and 1 nsec respectively[18]; and excited singlet (S*) to ground state (G) transition leads to fluorescence ($S^* \longrightarrow G + h\nu'$).] An excited triplet state is longer-lived (milliseconds or more) with the two

electrons having parallel spins. Since the singlet and triplet states of molecules are of different multiplicity (i.e. spin quantum numbers $S = 0$ and 1, and therefore $2S+1 = 1$ and 3 respectively), the transitions between them are strictly forbidden. But interactions (mixing) of the electron spin and orbital parts of the total angular momentum allow such transitions [e.g. intersystem crossing ($S^* \rightleftarrows T$, where T = triplet) and phosphorescence ($T \longrightarrow G + h\nu''$, where $h\nu''$ = phosphorescence)]. [We note here that the energy gap between S* and G is usually higher than between T and G, and thus phosphorescence is at a longer wavelength than fluorescence ($E = hc/\lambda$, where E is energy; h, Planck's constant; c, velocity of light; and λ, the wavelength of light.] Triplet states acquire a small component of singlet character and singlets likewise become partially triplet in nature. The spin-orbit coupling depends inversely on the energy gap (ΔE) between the two states (S* and T), and on the symmetries of the triplet and singlet states with respect to each other. Spin-orbit coupling, and hence the probability of populating the triplet state, also increases with atomic number; for example, substituting heavy atoms into aromatic molecules or just into the solvent environment can often augment the singlet-triplet mixing (internal and external heavy atom effect respectively[19]). Heavy atom impurity quenching, in conjunction with flash photolysis, has been used to determine triplet quantum yields[20].

Most electronic transitions in the molecules involved in photochemistry are either $\pi\pi^*$ or $n\pi^*$ transitions, due to the excitation of an electron from a π-ring system or a localized non-bonding n orbital to a π^* antibonding orbital respectively. The $\pi\pi^*$ promotion gives strong absorption bands, resulting in part from the symmetrical overlap between the π and π^* orbitals; triplet $\pi\pi^*$ excited states have lifetimes of about 10^{-3} sec. The $n\pi^*$ is less probable, since the spatial overlap between the localized n orbital (e.g. on an O or N atom of chlorophyll) and the delocalized π^* orbital is small; the triplet $n\pi^*$ intrinsic lifetime is 10^{-1}–10^{-2} sec. The long lifetime and high polarization (large electron displacement) of the $n\pi^*$ state makes it a likely candidate for being a reactive partner in electron transfer reactions. Intermolecular quenching is also more likely for this transition, however, and will tend to reduce the actual lifetime[21].

Chlorophyll Triplet in Solution

The kinetics of chlorophyll triplet decay in solution can be approximated by

$$-\frac{d(CT)}{dt} = k_1(CT)+k_2(CT)^2+k_3(CT)(C)+k_4(CT)(Q) \quad \ldots(1)$$

where CT, C and Q are the chlorophyll triplet, ground state and external quencher concentrations respectively, with their associated rate constants. In benzene or pyridine[22], $k_1 \simeq 10^3$ sec^{-1}, $k_2 \simeq 10^9$ L mole^{-1} sec^{-1} and $k_3 \simeq 10^7$ L mole^{-1} sec^{-1}. In the rigid, glassy state, k_1 is very small; phosphorescence is observed. With higher concentrations, k_2 becomes more important. With respect to k_3, the lifetime of the triplet for aggregates of pigment may be much smaller than what is measured in dilute solutions. At concentrations of chlorophyll greater than about 0·5 μM, the bimolecular mode of quenching is observed to dominate. However, as Livingston[23] points out, if low intensity light is used, this decay mechanism will not be significant, due to low triplet concentrations; this condition may hold good for much or all of *in situ* photochemistry. As the concentration exceeds 500 μM, k_3 and hence self-quenching increases[23,24].

Porter[25] measured k_4 to be about 10^{10} L mole^{-1} sec^{-1} in fluid solvents. This implies that there is a mechanism for the protection of the excited triplet state from oxygen, since it is detected in solution, although the data do not ensure that such a process is important for photosynthesis *in vivo*. An oxygen concentration of only 1 μM will decrease the triplet lifetime in benzene by a factor of two. Fujimori and Livingston[26] recorded the effects of various added substances on the triplet decay rate of chlorophyll in benzene. The oxidizing agents *p*-quinone and oxygen, as well as carotenes, some carotenoids and *m*-dinitrobenzene are efficient quenchers, with $k_4 \simeq 10^9 M^{-1}$ sec^{-1}. Quenching by transition group elements was observed by Linschitz and Pekkarinen[27]. But since Mn^{2+} has little quenching action in comparison with the other transition metal elements, one would conclude that the k_4 effect is not related to the magnetic moment of an ion. On the other hand, if the hydration of the ion was increased, k_4 decreased without any change in the magnetic moment. One might suggest that *in vivo* suppression of the chlorophyll triplet state is due to some factor other than the environment of the water side of photosystem II (Mn^{2+}-enzyme complex); Cheniae[28] has reviewed the role of Mn^{2+} in O_2 evolution by photosynthesis. Care must be taken, however, in applying *in vitro* results to photosynthesis.

If the triplet state of chlorophyll is significant in the photosynthetic processes of living organisms and is to be well understood, it seems important to acquire a knowledge of the characteristics of the chlorophyll triplet in more controlled and well-defined conditions. In 1948, Calvin and Dorough[29] reported phosphorescence at 865 nm from chlorophyll *b* in highly viscous solutions at low temperature; this was later confirmed by Becker and Kasha[30]. The quantum yield for this luminescence was low ($<10^{-3}$); the half-time of decay was about 3×10^{-2} sec. Using pheophorbide *a* (a derivative of Chl, without Mg^{2+} and phytol tail), the 'heavy atom effect' was observed by replacing the Mg^{2+} with copper; a quenching of the strong fluorescence and an intensification of the phosphorescence band at 867 nm was observed[31].

Chlorophyll efficiently sensitizes various oxidation-reduction reactions in many different solvents[32,33]. Auto-oxidations involving the following components are observed (Krasnovsky reaction[33]): (hydrazine, cysteine, glutathione, thiourea, ascorbate, etc.) $\xrightarrow{\text{Chl}}$ (viologens, flavins, NAD, etc.). Livingston[23] notes "Comparison of the quantum yield of chlorophyll-sensitized auto-oxidations with the quantum yield of fluorescence and its quenching demonstrates unequivocally that a long-lived excited state is an important intermediate in these reactions". Now experimental data have accumulated showing that the photochemistry of 'monomeric' Chl and dyes

in solution may be considered mainly as the chemistry of triplet excited molecules[34].

Since the phosphorescence of chlorophyll is very weak, the technique of flash-photolysis, especially as developed by Porter[14,35], has proved to be more useful. For example, if a dilute solution (2 μM) of chlorophyll in O_2-free pyridine is exposed to an intense 50 joule pulse of white light lasting only microseconds, almost 90% of the chlorophyll molecules are converted to the lowest triplet state. The lifetime of the metastable state is about 10^{-3} sec[15,22,36]. The difference spectrum of the $\pi\pi^*$ triplet state of chlorophyll has been measured in water by Zieger and Witt[37].

Müller *et al.*[38], in Witt's laboratory, found further evidence for chlorophyll triplets by changing the arrangement of the chlorophylls or inactivation of both light reactions by heating at 65°C. Configurational alteration was achieved by reducing the chlorophyll concentration to 1% of normal, using mutants, with nitrate deficiency or by the separation of molecules with the detergent digitonin. The spectrum obtained was identical to that observed in water by Zieger and Witt[37]. Two negative bands at about 435 and 680 nm and a broad positive band at 470 nm characterize the difference spectrum of this lowest $\pi\pi^*$ triplet state. The lifetime was found to be in the range 10^{-5}-10^{-3} sec, proportional to oxygen concentration and viscosity. Witt and coworkers suggested that (i) energy migration is blocked by changing the chlorophyll arrangement; and (ii) both photosystems are blocked, so that no photochemical utilization of energy occurs. Thus, $\pi\pi^*$ triplets are formed from incident excitation energy and remain in the chlorophyll molecules. Owing to the low chlorophyll concentrations, the photoreactions are not significantly stimulated to be detected.

To produce an experimental situation closer to the *in situ* case, one might try monomolecular films or high concentration solutions to study the chlorophyll triplet transitions. With this in mind, Porter and Strauss[39] prepared both solid solutions of chlorophyll and anaerobic preparations of plant leaves. The former process involved grinding in agate mortar and heating at a few degrees above the melting point of the solvent for about 10 sec; 5 μm thick samples were obtained by pressing between glass slides. The liquid molar extinction coefficient was assumed to be valid for this 'solid state'. The latter arrangement was accomplished by 'encapsulation' of plant leaves in various glassy or crystalline materials (cholesterol, benzhydrol, glucose) to avoid possible oxygen quenching of the chlorophyll triplet; this again required heating, followed by quick chilling.

Using an intense photolysis flash (half-width $\simeq$ 8 μsec for a 50 joule input to the flash tube), chlorophyll *a* and chlorophyll *b* in various solid solvents produced positive transients at about 500 nm, with a half-time of 0·2-3·0 msec. Although intact plant leaves yielded no triplet spectrum, yet if a cationic detergent [dodecyl trimethyl ammonium bromide (DTAB)] was added, followed by drying and encapsulation with glucose, the triplet transient was observed. Almost 15% of the chlorophyll was promoted to the triplet state. With this treatment, a blue shift in the red absorption peak occurs, indicating a structural disruption. Boiling in water gave the same absorption shift as the application of DTAB, but in conjunction with anionic or non-ionic detergents (SDS, Triton-X-100) no transient results. Boiling 8M urea or concentrated LiCl, which are known to denature some chlorophyll-protein complexes, gave no triplet spectra with encapsulation. Further, boiling water yields no changes in fluorescence, whereas the application of DTAB provides a 2-3 factor increase. In slightly different experiments, wheat seedlings grown in darkness and subjected to one day of light produced a pale green species, but no triplet. If the cationic detergent was applied, however, the same results as for 'normal' leaves under the same condition were obtained.

It is known that in $10^{-6}M$ chlorophyll solution the fluorescence and triplet yields are about 25 and 70% respectively, but *in vivo* the quantum yield of fluorescence is about 2%. Thus, if the decay processes in operation *in situ* are the same as those observed *in vitro*, based on the ratio of the fluorescence and triplet yields in solution, one would expect a triplet yield of 5-6%. Since this is not observed with intact chloroplasts and the triplet yield is essentially zero, Porter and Strauss[39] suggest that the triplet state is more effectively quenched than the singlet state. (The situation for solid chlorophyll solutions was found to be opposite.) In support of this, it can be noted that triplets do have longer lifetimes, and that the triplet exciton migration is 10 times more efficient than that for singlets in some organic crystals. There may be a blocking by cationic detergents of triplet exciton transfer by "preferential orientation of cationic and hydrocarbon groups with the chlorophyll molecules, weakening bonds between chlorophylls or between chlorophyll and the structural protein framework"[39]. An increase in fluorescence with detergents is also taken as evidence of the reduced quenching of the singlet state as a retarding of the migration of singlet excitons occurs.

However, the emphasis placed on a triplet exciton process in this interpretation is questionable in the light of current evidence on energy transfer in photosynthetic units. The effect of treating living material with heat, even for modest time intervals, would also seem to require attention; the melting point of cholesterol is about 150°C. Thermal rearrangement may contribute to the expression of the triplet, but the detergent results do indicate a fairly specific effect as well. Light-harvesting chlorophylls of the 'photosynthetic unit' appear to transfer excitation energy by a singlet mechanism, possibly an exciton process, which allows the energy to span large areas. But in any case, there is always a certain probability of conversion to the triplet state in the bulk molecules. Evidently, there is also an extremely rapid transfer of chlorophyll triplet energy to the protective carotenoid population, 'draining off' harmful photooxidative energy. The spatial configuration and molecular relation of the chlorophylls to the juxtaposed carotenoids ensures a high rate of intersystem crossing. Cationic detergents may destroy this communication, thus allowing an artificially high yield (15%) of chlorophyll triplets.

Since the triplet yield is 15% in detergent-treated samples, Porter indicates that the origin must be in the bulk chlorophyll, for the concentration of

energy sinks (reaction centres) is only about 0·4% of the total chlorophyll content. On the other hand, Müller *et al.*[38] find yields of 0·1% in chloroplasts with other treatments; this suggests participation of the energy sinks. Porter and Strauss[39] feel that heating, chlorophyll depletion and digitonin treatment in the experiments of Witt and coworkers may prevent quenching of the triplets by the sinks, i.e. energy transfer from the sink to acceptors is blocked, but without disrupting energy flow in the bulk chlorophyll. Thus, the data of the research groups of Porter and Witt may not be incompatible; the difference in triplet yields may be due to the state of the framework of the antennae and specialized chlorophylls.

Flash Photolysis *in vivo*

All the experiments in the preceding section may be criticized, because they significantly alter the *in vivo* situation to enable observation of the chlorophyll triplet. In order to unequivocally demonstrate the functional existence of a metastable energy level, one would like to have data for flash-illuminated chloroplasts. Rosenberg *et al.*[40], using the spectrographic apparatus of Livingston, were able to instantaneously record on a spectrographic plate the entire absorption spectrum of the chlorophyll triplet in benzene solutions; the yield was 70% when the photolytic flash (half-time about 10 μsec) and spectroscopic flashes were separated by only about 35 μsec. But with well-washed sugar beet chloroplasts or chlorella suspensions in air, nitrogen or water vapour environments at below 20°C, none was observed within the empirical limit of 5% absorption. Two explanations were considered: (i) All chlorophylls are identical *in vivo*, but the lifetime is much shorter than for the *in vitro* situation. The half-time of some reaction or process 'draining away' the metastable energy would necessarily be less than 10 μsec, based on their experiments (Indeed Mathis[41] found the time of transfer to carotenoids to be 24 nsec). In fact, Rosenberg *et al.*[40] had suggested that the chlorophyll metastable state may react with a non-diffusable substance closely bound to the chlorophyll, such as a protein moiety or carotenoid. (ii) Their second alternative assumes that most chlorophylls do not undergo intersystem crossing, presumably due to the environmental situation of the bulk molecules. Rather, absorbed light energy is funnelled by resonance processes to a trapping centre, either a different pigment or a specially located chlorophyll. At the trap, the molecular arrangement affords singlet to triplet conversion. Low concentrations of such reaction centres would explain the negative results of this study. Other workers have also obtained the same type of results for normal plants[38,42,43]. We see no reason why factors involved in both (i) and (ii) above are not responsible for the observed effects.

In Witt's laboratory, chlorella and spinach chloroplasts in their normal physiological condition were examined[38]. The light-induced absorption change obtained includes a decrease at 430 nm and an increase at 520 nm. This is also seen in lyophilized or frozen preparations; the half-time of decay is about 20 μsec and is independent of temperature. Paramagnetic gases such as O_2 or NO will completely quench the changes, but they are fully restored by non-paramagnetic gases. This suppression involves a decrease in amplitude, rather than a change in the decay half-time. It was proposed that a metastable state ($n\pi^*$ triplet?) is statically quenched in this process. Witt distinguishes this signal (type 1) from the triplet chlorophyll (type 0) observed in the denatured material. It is suggested that type 0 triplet chlorophyll is a direct precursor of type 1 substance (possibly chlorophyll *a* in a reduced metastable state).

More recently, Mathis[41] has done experiments with intact chloroplasts. A Q-switched ruby laser could populate the triplet state of carotenoids (half-time 6 μsec, λ_{max} 515 nm, ϵ_{max} 2×10^5). The rapid response flash spectrophotometer also recorded an absorption transient, which was accredited to the triplet state of chlorophyll. When the chloroplast structure was altered by the action of detergents or non-polar solvents, a longer-lived chlorophyll *a* triplet was observed in the anaerobic state. Mathis indicates that the light curves he measured can be explained on the basis of a scheme where a chlorophyll *a* triplet is depopulated by triplet-triplet annihilation and by triplet energy transfer to carotenoids (half-time of transfer determined to be 24 nsec). These two means of deactivation are presumed to compete with each other.

Detection by Electron Paramagnetic Resonance (EPR)

Since its discovery[44] in 1945, EPR spectroscopy has enjoyed wide application to biological research, including photosynthesis[45]. Substances may exhibit a net electronic magnetic moment due to a variety of 'modes' of an unpaired electron: (i) conduction electron of a semiconduction process or a metal; (ii) physically trapped electron in some lattice structure; (iii) fission of a paired electron bond; (iv) transition element ions; (v) triplet electronic states of a molecule or atom. It is conceivable that any of these processes could contribute to the production of an EPR signal in connection with the mechanism of photosynthesis, although closer inspection may easily rule out some. One hopes that the main features of a single EPR line (intensity, g-value, line shape, multiple line structure) will provide enough information to distinguish the source of a given signal. But to gain the maximum amount of information from EPR spectra, well-defined and well-ordered systems are required; photosynthetic preparations may fulfil this to only a small degree. One advantage of the EPR method is that microwave radiation is not known to disturb the plant or living material being studied; also, spectra taken with an EPR apparatus do not suffer from interference due to overlapping pigments[45]. In detecting long-lived radicals, the EPR method is highly sensitive, with the ability to detect[46] about 1 μM in 0·1 cm^3; but a distinct disadvantage is the frequently encountered low signal-to-noise ratio, since water will absorb microwaves. More information on EPR spectra and interpretation is provided by Androes[47].

The Russian investigators Rikhireva *et al.*[48] reported in 1965 the existence of an EPR signal due to a chlorophyll *b* triplet in frozen, illuminated ethanol solutions. In further experiments, ethanol-pyridine solutions of chlorophyll *a*, chlorophyll *b* and pheophytin *a*+*b*, films of chlorophyll *a*+*b*, pea-leaf chloroplasts in buffer, and greening etiolated

maize leaves with different preillumination times were examined with an EPR spectrometer. Triplet EPR signals were recorded for the pigment solutions with the relative intensities 1, 0·5, 0·1 for chlorophyll *b*, chlorophyll *a* and pheophytin *a*+*b* respectively; no such signals were seen for films of chlorophyll *a*+*b*. Using two distinctive buffer mixtures (50% glycerol, 0·125*M* KCl, 0·02*M* Tris-HCl and 0·35*M* NaCl, 0·02*M* EDTA, 0·4*M* K phosphate; both buffers *p*H 7·5 at 77°K), no photo-induced triplet could be observed in the chloroplast suspensions, unless there was preincubation with 50% pyridine. In relieving the effect of pigment aggregation, pyridine seemed to allow the expression of the triplet excited state of 'monomeric chlorophyll'. To pursue the point, leaves in the early stages of greening (when there is mostly monomeric chlorophyll) were examined. One might suspect that deactivation of the triplet state due to energy transfer might be minimal, but no triplet spectra were obtained. Ethanol solutions of these pigments (first extracted with acetone) did reveal the presence of chlorophyll triplets. The conclusion is drawn that *in vivo* preparations lack sufficient steady state concentrations of triplet pigment molecules to be detected. Processes that might contribute to such deactivation might include triplet-triplet energy transfer and photochemical processes of electron transfer.

In 1972, Leigh and Dutton[49] and Dutton *et al.*[50] reported on other *in situ* studies of bacteriochlorophyll and chlorophyll triplets. If the primary electron acceptor in various bacterial chromatophores or sub-chromatophores is pre-reduced at 4°K before illumination with laser pulses, a $g \simeq 2$ signal (corresponding to oxidized bacteriochlorophyll) is not seen, but is replaced by a different EPR spectrum. Based on the observed redox potential dependence and kinetics of this new signal, a triplet state is implicated. It is suggested that this represents an early light-activated intermediate in the primary processes of photosynthesis. The results of a less extensive investigation[49] by these workers on green plant photosynthesis were in agreement with this proposal, but this work has not been published in detail, to our knowledge.

Chlorophyll Triplets and Delayed Light Emission

Delayed light emission (DLE) in photosynthetic cells is luminescence having the spectral characteristics identical to those of fluorescence, but with a much longer lifetime (1 msec to several seconds). While it is beyond the scope of this article to review DLE work in detail, it is important to consider the role of luminescence in plants as based on measurements made under various conditions (temperature, inhibitors, other chemicals and mutants). To whatever extent the mechanism of DLE depends on a triplet molecule, it must be consistent with the remaining data presented.

Parker and Joyce[51], in 1966, looked at the effects of light intensity, gaseous atmosphere (O_2, CO_2) and different wavelengths of excitation on DLE transients. The origin of the luminescence is suggested to be either: (i) Parker and Hatchard's[52] P or E type DLE due to chlorophyll triplets, or (ii) 'chemiluminescence' arising from a recombination of primary products. P type DLE is ascribed to triplet-triplet annihilation, whereas E type involves thermal activation of molecules from the triplet to singlet states. To explain the possible oxygen quenching of the triplet *in situ*, they suggest that the organized chlorophyll in chloroplasts may behave like concentrated solutions in rigid glass; the triplet molecules are then shielded from collisional quenching. In analogy to phenanthrene in low temperature rigid media[53], the non-exponential decay of DLE, which they observed in leaves, may be due to triplet-triplet annihilation, assuming a non-random distribution of triplet molecules. The 'recorded' lifetime of the triplet was greater than 100 msec, which in turn was very much greater than that for solutions at low temperatures. On the other hand, it was considered that the non-exponential decay of DLE may just "reflect the presence of a variety of reaction centres having different degrees of access to substrate". The 'substrate' would quench the triplet. The chlorophyll singlet might be repopulated thermally, so that the reaction centre itself would not be a chlorophyll triplet.

Investigating the prolonged luminescence of plant leaves, Shuvalov and Litvin[54] separated DLE into five major components, based on duration and differential sensitivity to temperature and chemicals. Their component III (half-time 1·7 sec), sensitive to oxygen, quinone and diurone, has a thermoluminescence maximum at −15°C, and maxima in the emission spectrum at 685 and 740 nm. In the excitation spectrum there are peaks at 650, 660 and 675 nm, with a decline in yield beyond 680 nm. On this basis, it is postulated that component III represents a triplet energy trap (0·35 eV deep) of photosystem II, involving chlorophyll molecules. The energy stored in this trap could be used for the photolysis of water.

In recent models[55–57], the possibility of a triplet state participating in the processes responsible for DLE has been pursued. (It should be noted that other models, e.g. electron-hole recombination, have not been eliminated[58].) A scheme can be outlined for photosystem II, which is generally held responsible for the observed phenomena; the steps presented below are a composite of the proposals found in the earlier models:

$$\left.\begin{array}{l}
\text{(i)}\ \ \text{Bulk Chl} \xrightarrow{h\nu} \text{Chl S}^* \\
\text{(ii)}\ \ \text{Chl S} \xrightarrow[\text{migration}]{\text{singlet}} \\
\qquad\qquad \text{Trap Chl} \begin{cases} \xrightarrow{k_c} \text{Z}^+\text{ Chl Q}^- \xrightarrow{\text{chemistry}} \text{ETC} \\ \xrightarrow{} \text{Radiation-less or Int. Conv.} \\ \xrightarrow{k_i} \text{Z(Chl T)Q (ISC)} \end{cases} \\
\text{(iii)}\ \ \text{Z}^+\text{ Chl Q}^- \xrightarrow{k_b} \text{Z(Chl T)Q} \\
\text{(iv)}\ \ 2\text{Z(Chl T)Q} \xrightarrow{k_f} \text{Z(Chl S)Q} + \text{Z Chl Q} \\
\text{(v)}\ \ \text{Z(Chl S)Q} \longrightarrow \text{Z Chl Q} + h\nu'\text{(DLE)}
\end{array}\right\} \quad \ldots(2)$$

where ISC stands for intersystem crossing; ETC, electron transport chain, Chl S*, excited singlet chlorophyll; Chl T, triplet chlorophyll; Z and Q, primary electron donor and acceptor of photosystem II; and k_c, k_i, k_b and k_f are the rate constants for chemical deactivation of the trap, intersystem crossing, chemical DLE reaction and

bimolecular triplet fusion respectively. If the trap captures only singlet excitons, triplets may escape and either decay or form an excited singlet by annihilation[56]. In organic crystals, the DLE produced by triplet fusion is sensitive to an externally applied magnetic field. At low light intensity I, where DLE is proportional to I^2 (chemical back reactions are minimal), a decline in the magnitude of DLE was anticipated due to the effect of a magnetic field on chlorophyll *a in vivo*; none was observed[56]. But from the expression for the DLE rate, $L = 0{\cdot}5\ k_f.\ \phi.(T)$, where ϕ is the total fluorescence efficiency; the triplet exciton density (T) was estimated to be 2×10^{-7} at 1 msec after the actinic light was extinguished. Stacy *et al.*[56] point out that this concentration would not be expected to produce a measureable absorption change[39] and probably does not produce a sufficiently large DLE effect to be detected by the magnetic field measurements.

Transfer of Triplet Energy in the Photosynthetic Unit

The triplet level of chlorophyll may be implicated in a primary reaction involving a specialized complex, but the transfer of energy in the chlorophyll aggregate is a separate question. It has been suggested that the lack of chlorophyll triplet absorption may be due to the migration of triplet excitation[59]. One might expect the same sort of transfer ability for a triplet as a singlet mechanism, due to its longer lifetime. Thus, Wolf[60] considers this process to be a good candidate for storage of energy and its transfer in photosynthetic systems. Excitation energy may be transferred by means of a singlet dipole exchange interaction, a 'hopping' exciton model. However, if the hopping time, t_H, is short enough, an exciton may 'scan' an entire crystal, the energy being distributed effectively to various possible traps. In some organic crystals, t_H has been measured to be about $2{\cdot}5 \times 10^{-12}$ sec. Wolf[60] suggests that the diffusion length in naphthalene and anthracene crystals may be 100-1000 times greater for a triplet as opposed to singlet migration. The existence of triplet excitons in molecular crystals has been demonstrated by others[61,62].

If chlorophyll *a*-naphthacene (1 μM) in toluene is flashed through a red filter which limits the direct photooxidation of naphthacene, the chlorophyll triplet lifetime is observed to decrease. In the process, new absorption bands develop, similar to those seen with the direct photooxidation of naphthacene. In this manner, triplet-triplet energy transfer between chlorophyll and naphthacene molecules has been implicated[63].

But there is really no clear reason to believe that the chloroplast substructure is compact and ordered enough to be considered a crystal *per se*. Rabinowitch[64] has done some interesting calculations in this regard. Resonance migration in the metastable state necessitates the occurrence of spin forbidden singlet-triplet and triplet-singlet transitions. The improbability can, however, be superseded if the energy-exchanging pair are "close enough for their interaction energy to be not small compared to the singlet-triplet separation energy, (thus) considered as a single system as far as spin conservation is concerned"[64]. Rabinowitch estimates that the area which a chlorophyll molecule occupies in the substructure interfaces is significantly more than that expected for a crystalline monolayer of chlorophylls. Also, the shift in absorption peaks when going from dilute solutions to the *in vivo* situation (10-15 nm) does not correspond to the shift observed for three-dimensional chlorophyll microcrystals or crystalline monolayers; the chlorophyll is believed to be in a more 'amorphous' state. The chlorophylls *in situ*, then, do not seem to be packed tight enough for the above singlet-triplet selection rule to be exchanged for the single spin system case.

Further indication that a singlet transfer process, rather than triplet migration, operates in the photosynthetic unit comes from chlorophyll fluorescence data. When the primary photochemical reaction centre chlorophylls become saturated, a doubling of the fluorescence yield is observed*, due to radiative decay of the chlorophyll singlet level[65-67]. Clayton[69] notes that "such variations in the fluorescence should not be expected if the primary singlet energy is converted locally (in the light-harvesting aggregate) to triplet and then transferred to reaction centres". And as observed above, the triplet density in the bulk is so insubstantial as to preclude a triplet transfer process.

It has already been noted how the participation of the carotenoids in conjunction with triplet events in photosynthesis is likely. More specific empirical data strengthen this. Using a repetitive ultrashort flash spectrophotometer (resolution of 0·01% absorbance in 10^{-7} sec), it was found[70] that air-saturated suspensions of spinach chloroplasts show light-induced absorbance changes with a half-time of less than 500 nsec and with a decay time of about 3 μsec, depending upon the O_2 content. The difference spectrum has negative changes at 430, 460 and 490 nm and a positive peak at 520 nm. As indicated by Mathis and Galmiche[71] and Zieger *et al.*[42], a metastable state is involved. Since no simultaneous absorption changes are observed in the red region, the metastable state is concluded to be due to carotenoids. These spectral changes are excited by red flashes, absorbed only in the chlorophylls, which implies that energy is transferred from chlorophyll to carotenoids; it is speculated that triplet-triplet transfer is responsible.

*Franck and Rosenberg[68] discuss a theory of photosynthesis based on such observations. In low light intensity, the two photo acts of photosynthesis proceed once through the reaction centre in singlet state and then through the triplet state. Both reactions lead to a net electron transfer operating in series. In the case of the reaction centre operating through singlet state, all fluorescence is quenched because of efficient photochemistry. In the case of the reaction centre operating through triplet state, fluorescence originates before triplets are formed and so photochemistry does not compete with that fluorescence. At high light intensities, when photosynthesis is saturated (low yield) photochemistry no longer quenches fluorescence in reaction centres operating through singlets, and so the fluorescence yield doubles. This ingenious hypothesis of Franck has not been confirmed. However, if the reaction centre I of photosynthesis operates via the triplet state, fluorescence of system I will not compete with photochemistry, and no change in yield with increasing light intensity will be found, as is perhaps the case[66]. And if the reaction centre II of photosynthesis operates via singlet states, an increase in the yield will be found upon increasing the light intensity, as is perhaps the case too[66]. However, if Franck's theory is correct, fluorescence emission spectra at low light intensities should be that of pigment system I, but that does not seem to be the case. Franck's theories and their implications require further studies.

All the above observations are maintained even with the addition of DCMU (which blocks photosystem II) or when done at −160°C, ruling out the participation of a chemical electron transfer intermediate. As photosynthesis is saturated with higher light intensities, more antennae chlorophyll are excited than used for photosynthesis; 'superfluous' excited antennae chlorophylls transfer energy to a carotenoid 'energy trap', occurring in less than 500 nsec. The decay of the changes within 3 μsec suggested to Wolff and Witt[70] that it is a reflection of a 'valve reaction'.

Mathis[72] also agrees that the excitation of chlorophyll *a in vivo* produces a triplet state, followed by rapid transfer to a carotenoid, where in turn its triplet is realized. He suggests that these events may either aid in the protection of chlorophyll *a* against photooxidation[73] or serve more directly in the primary photochemistry of photosynthesis.

For some time, it has been known that the bacteriochlorophyll (BChl) of the carotenoid-less mutants of the purple bacterium *R. spheroides* suffer destruction if aerobic samples are illuminated[74]; no photochemistry is observed. As seen above, carotenes and caroteroids effectively quench the chlorophyll triplet state. Crounse *et al.*[75] have determined that a minimum number (about nine) of conjugated double bonds in carotenoids are necessary to give protection against the photooxidation of BChl. These results suggest that the protective action of neighbouring interdigitated carotenoids is more than a simple screening, since a critical 'trapping energy' level is required.

The curent state of research does not allow a conclusive role in photosynthesis to be assigned to the triplet state of chlorophyll *a*, but there exist boundaries within which meaningful future evaluation can be done. On the absorption of a quantum of light in the bulk chlorophyll aggregate, singlet excitation energy is transferred by resonance processes to a specialized trap chlorophyll molecule. Formation of the triplet state has been shown to be associated with the environmental configuration of a molecule; en route to a reaction centre, then, there exists a certain limited probability that a chlorophyll singlet will undergo intersystem crossing to the triplet state. To avoid the destructive effects of photooxidation, the triplet energy is quickly funnelled to a carotenoid of a lower energy band. This is presumably by triplet-triplet transfer, as the close spatial arrangement of the interspersed carotenoid population encourages this, while limiting the inter-chlorophyll transfer in the bulk to a singlet mechanism. Rapid decay of the antenna chlorophyll triplet is due to a large non-collisional k_4 effect, with the primary quencher a caroteniod, rather than bimolecular deactivation (k_2) or self-quenching (k_3).

Singlet excitation energy arriving at and trapped by a specialized chlorophyll complex may either result in a separated oxidized-reduced species (utilized in ordinary photochemical electron transport), or interconvert directly to a chlorophyll triplet. A chemical back reaction involving this oxidized donor-reduced acceptor pair may also contribute to the chlorophyll triplet population; annihilation of any two of these 'excitons' results in the production of a singlet and a quantum of delayed light. The molecular configuration and chemical situation of the reaction centre seem to favour not only the capture of singlet energy, but the formation and expression of a small population of chlorophyll triplets. Exactly what this local geometrical situation is and a more complete view of the relation of observed triplet phenomena to *in vivo* photosynthesis remain to be determined on the basis of forthcoming experiments. More precise instruments than what have been used thus far are needed to detect triplets *in vivo*.

Summary

The conversion of light energy into chemical free energy is the essential consequence of the process of photosynthesis that takes place in green plants; all life on earth depends directly or indirectly on it. In photosynthesis, light energy absorbed by various pigments is transferred to the 'reaction centres' involving 'singlet' excitation states. The possible participation of the metastable triplet state in the events involving the specialized reaction centre chlorophyll molecules is discussed. The triplet energy in chlorophylls of the light-harvesting pigment bed is potentially photodestructive, but could be channelled to a protective lower energy carotenoid reservoir by triplet-triplet communication. Efforts to observe a metastable state both *in vitro* and *in vivo* by flash spectroscopy, electron paramagnetic resonance and delayed light emission are reviewed and recent reports on chlorophyll and carotencid triplets are discussed. The relation of the observed triplet phenomena to *in vivo* photosynthesis is still questionable.

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