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An evaluation of the potential triggers of photoinactivation of photosystem II in the context of a Stern–Volmer model for downregulation and the reversible radical pair equilibrium model

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Photoinactivation of photosystem II (PS II) is a light-dependent process that frequently leads to breakdown and replacement of the D1 polypeptide. Photoinhibition occurs when the rate of photoinactivation is greater than the rate at which D1 is replaced and results in a decrease in the maximum efficiency of PS II photochemistry. Downregulation, which increases non-radiative decay within PS II, also decreases the maximum efficiency of PS II photochemistry and plays an important role in protecting against photoinhibition by reducing the yield of photoinactivation. The yield of photoinactivation has been shown to be relatively insensitive to photosynthetically active photon flux density (PPFD). Formation of the P680 radical ($P680^+$), through charge separation at PS II, generation of triplet-state P680 ($^3P680^*$), through intersystem crossing and charge recombination, and double reduction of the primary stable electron acceptor of PS II (the plastoquinone, Q_A) are all potentially critical steps in the triggering of photoinactivation. In this paper, these processes are assessed using fluorescence data from attached leaves of higher plant species, in the context of a Stern–Volmer model for downregulation and the reversible radical pair equilibrium model. It is shown that the yield of $P680^+$ is very sensitive to PPFD and that downregulation has very little effect on its production. Consequently, it is unlikely to be the trigger for photoinactivation. The yields of $^3P680^*$ generated through charge recombination or intersystem crossing are both less sensitive to PPFD than the yield of $P680^+$ and are both decreased by downregulation. The yield of doubly reduced Q_A increases with incident photon flux density at low levels, but is relatively insensitive at moderate to high levels, and is greatly decreased by downregulation. Consequently, $^3P680^*$ and doubly reduced Q_A are both viable as triggers of photoinactivation.

Keywords: photosynthesis; photoinhibition; chlorophyll fluorescence

1. INTRODUCTION

The well-documented, extreme vulnerability of photosystem II (PS II) to light-induced damage is almost certainly linked to its unique ability to oxidize water. Oxygenic organisms are able to overcome this vulnerability through the rapid turnover of what would appear to be the main target of this damage, the D1 polypeptide of PS II. Prior to breakdown of D1, PS II is 'photoinactivated' through the action of one or more light-dependent mechanisms. In this study, potential triggers of photoinactivation are evaluated, taking into account the various pathways for dissipation of absorbed excitation energy at PS II.

The D1 polypeptide forms one-half of the heterodimeric core of PS II, which binds all of the major redox components involved in charge separation and stabilization (the other half being the D2 polypeptide, which is also replaced in some situations). Charge separation at PS II leads to formation of the radical pair, $P680^+/Phe^-$ (see Appendix A for explanation of abbreviations). $P680^+$ has a redox potential that is high enough (1 V or more), not only to oxidize water, but also the pigment molecules of PS II (chlorophyll *a* and β-carotene) or possibly the D1 protein itself (Anderson *et al.* 1998). Consequently, there is a very real possibility that $P680^+$ is a direct trigger of photoinactivation.

Exciton transfer among chlorophylls within the light-harvesting system associated with PS II can lead to formation of the triplet excited state ($^3Chl^*$) from the singlet excited state ($^1Chl^*$) through intersystem crossing. Although $^3Chl^*$ is not likely to induce damage directly, quenching of $^3Chl^*$ by triplet oxygen ($^3O_2$) can result in formation of singlet excited oxygen ($^1O_2^*$), which has been shown to result in preferential destruction of P680 and D1 breakdown (Shipton & Barber 1991; Vass *et al.* 1992). In aerobic photosynthetic organisms, interaction between singlet ground-state β-carotene (^1Car) and $^3Chl^*$ results in formation of the triplet excited state of β-carotene ($^3Car^*$) and ^1Chl, effectively reducing the lifetime of the

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^{3}Chl* by several orders of magnitude (Siefermann-Harms & Angerhofer 1998, and references therein). The ^{3}Car*, formed through this interaction, then reverts to the singlet ground state through intersystem crossing (^{3}Car*$\rightarrow{}^1$Car). β-carotene provides an additional level of protection against $^1O_2^*$ through direct interaction. This leads to formation of $^3O_2$ and ^{3}Car* and decreases the lifetime of any $^1O_2^*$ that may form through interaction with 'unquenched' ^{3}Chl*.

The distribution of spectral types of chlorophylls *a* and *b* within the light-harvesting system of PS II results in a higher density of excitation energy at and around the reaction centre (Dau & Sauer 1996). A consequence of this is that the yield of ^{3}Chl* formation is highest at the core of the PS II light-harvesting system, which includes P_{680}. Evidence has recently been presented that the macrostructure of the region of the light-harvesting system closest to the reaction centre acts as a barrier to the diffusion of O_2, thereby providing yet another level of protection against $^1O_2^*$ formation where the yield of ^{3}Chl* formation is likely to be highest (Siefermann-Harms & Angerhofer 1998).

As noted above, the oxidizing potential of P_{680}^+ is high enough to cause irreversible oxidation of β-carotene and/or adjacent chlorophylls. Consequently, although there is at least one β-carotene within the core complex of PS II, there is some doubt as to whether or not it could be located close enough to P680 to be able to quench any $^3P_{680}^*$ that may be formed through intersystem crossing or as the result of charge recombination between Phe^- and P_{680}^+ (Barber 1998). Consequently, P_{680}^+ may play an indirect role in triggering photoinactivation, by preventing the quenching of $^3P_{680}^*$ by β-carotene while $^3P_{680}^*$ could play a more direct role, through formation of $^1O_2$ (Hideg *et al.* 1994; Ohad *et al.* 1994). It has been suggested, although not demonstrated, that structural characteristics of the PS II reaction centre may exclude O_2 from the region around P680 (Anderson *et al.* 1998), in the same way that it apparently is excluded from the region of the light-harvesting system closest to the core complex (Siefermann-Harms & Angerhofer 1998). Given the potential lack of protection from β-carotene, this may represent the main mechanism protecting against the formation of $^1O_2^*$ at the reaction centre.

Double reduction of the plastoquinone Q_A has also been proposed as a potential trigger for photoinactivation (Van Wijk *et al.* 1992; Vass *et al.* 1992). The idea is that double reduction of Q_A results in formation of hydroplastoquinone (Q_AH_2), which is released from its binding site. This loss of Q_A could lead to a substantial increase in the yield of $^3P_{680}^*$ through charge recombination. The possibility that double reduction of Q_A could be the trigger for photoinactivation has been strongly contested on the grounds that target theory reveals photoinactivation to be a single-photon event (Sinclair *et al.* 1996; Anderson *et al.* 1998).

Photoinactivation of PS II has been shown to follow the reciprocity law in isolated thylakoids of *Spinacea oleracia*, in cells of *Anacystis nidulans* (Jones & Kok 1966) and *Synechocystis* 6803 (Nagy *et al.* 1995), and in leaf discs from a number of higher plant species (Park *et al.* 1995; Anderson *et al.* 1998). Consequently, photoinactivation, *in vivo*, appears to be proportional to the number of photons absorbed. For example, 5 h exposure to 500 μmol $m^{-2}s^{-1}$ would be expected to result in the same number of PS II units becoming photoinactivated as 1 h exposure to 2500 μmol $m^{-2}s^{-1}$. Anderson *et al.* (1998) have suggested that this 'light-dose' response of photoinactivation implies the existence of a single trigger for photoinactivation. Clearly, the yield of this putative trigger (the probability of an absorbed photon inducing formation of the trigger) must be largely independent of incident photosynthetically active photon flux density (PPFD).

There are two processes operating at PS II that are very likely to play important roles in the regulation of photoinactivation: changes in the capacity for stable charge separation (photochemistry) and the effective rate constant for one or more non-radiative processes that compete with photochemistry (downregulation). Changes in the capacity for photochemistry and downregulation at PS II can be monitored through measurement of chlorophyll fluorescence (reviewed by Krause & Weis 1991; Dau 1994). Under *in vivo* conditions, at incident light levels ranging from darkness to full sunlight, the combination of photochemistry and downregulation normally result in a 'quenching' of chlorophyll fluorescence to a steady-state yield within a narrow range of between *ca.* 2 and 4% (Havaux *et al.* 1991; Genty *et al.* 1992; Laisk *et al.* 1997). Downregulation has been shown to protect against photoinhibition (Krause & Behrend 1986; Oxborough & Horton 1988), presumably by decreasing the yield of the trigger(s) of photoinactivation.

Charge separation at PS II is stabilized through the transfer of an electron from Phe^- to the first stable acceptor, Q_A. Further stable charge separation at PS II can only occur when $P680^+$ and Q_A^- have returned to the ground state. Because oxidation of Q_A^- is roughly four orders of magnitude slower than reduction of P_{680}^+ (Robinson & Crofts 1983; Meyer *et al.* 1989; Crofts *et al.* 1993; Dau 1994), PS II centres are described as being 'open' (capable of stable charge separation) when Q_A is in the ground state and as 'closed' (not capable of stable charge separation) when Q_A is carrying a single negative charge. If all PS II centres were isolated from each other, the fluorescence yield above F_o or F_o' would be directly proportional to the fraction of closed centres ($1-[Q_A]$). In reality, connectivity among centres results in a curvilinear relationship between $1-[Q_A]$ and the fluorescence yield above F_o or F_o' (Joliot & Joliot 1964). The effect of connectivity on fluorescence parameters is discussed in more detail below (§ 2(b)).

Evidence from a large number of empirical observations suggest that downregulation is dominated by Stern–Volmer quenching (Lavergne & Trissl 1995). Within the Stern–Volmer model, fluorescence and quencher concentration are linked through the Stern–Volmer equation, which simply states that the reciprocal of fluorescence yield is proportional to quencher concentration. In reality, there is little evidence that fluorescence yield is actually modulated by quencher concentration and it should be noted that a change in the effective rate constant for a quencher within the pigment bed would have exactly the same effect as a change in quencher concentration.

The widely used fluorescence parameter, $(F_m/F_m')-1$, is derived from the Stern–Volmer equation and can be used

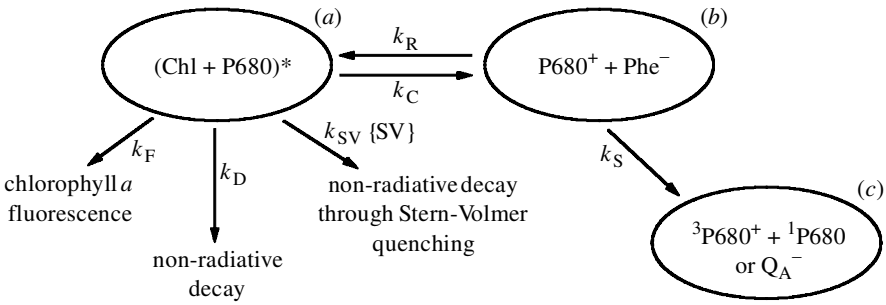

Figure 1. A schematic showing the different ways in which de-excitation can occur at PS II. (*a*) represents the equilibrated excited state within the pigment bed. k_C is the apparent rate constant for charge separation that leads to formation of (*b*) the radical pair. k_R is the rate constant for charge recombination, leading to re-formation of the equilibrated excited state, (*a*). k_S is the rate constant for charge stabilization, which is the sum of electron transfer to Q_A and charge recombination leading to formation of $^3P^+_{680}$ or $^1P_{680}$. k_F is the rate constant for fluorescence emission. k_D is the rate constant for non-radiative decay that occurs in the dark-adapted state. k_{SV} is the rate constant for non-radiative decay that results from accumulation of one or more Stern–Volmer quenchers within PS II. [SV] is the concentration of Stern–Volmer quencher.

to follow changes in apparent quencher concentration (Bilger & Björkman 1991). Dominance of downregulation by Stern–Volmer quenching provides a plausible explanation for the linearity that has often been observed between the fluorescence parameter F'_q/F'_m (which is often written as $\Delta F/F'_m$ in the literature) and the quantum yield of CO_2 assimilation (ϕ_{CO_2}). This relationship was first described by Genty *et al.* (1989) and has been verified since by data from a number of other groups (e.g. Di Marco *et al.* 1990; Krall & Edwards 1990; Edwards & Baker 1993); indeed this relationship provides perhaps the strongest empirical evidence that downregulation is dominated by Stern–Volmer quenching (Lavergne & Trissl 1995).

F'_q/F'_m provides an estimate of the operating efficiency of photochemistry at PS II and is actually the product of two other fluorescence parameters, F'_v/F'_m and F'_q/F'_v. F'_v/F'_m provides an estimate of what the maximum efficiency of photochemistry at PS II would be, in the light-adapted state, if all centres were open ($[Q_A] = 1$). F'_q/F'_v is a factor that relates the maximum and operating efficiencies of photochemistry at PS II and is mathematically the same as the widely used 'coefficient of photochemical quenching' (q_P). Use of the term q_P has been avoided here because, in the context of a Stern–Volmer model for downregulation, F'_q/F'_v is a factor (rather than a coefficient). Also, q_P has been widely used either as a proxy for $[Q_A]$ or as a semi-quantitative indicator of $[Q_A]$, neither of which is reasonable within a model where downregulation is dominated by Stern–Volmer quenching and there is also a high level of connectivity among PS II centres (see § 2).

A number of different models (reviewed by Dau 1994) have been proposed to relate the de-excitation processes that operate within PS II (Butler & Kitajima 1975; Butler 1978; Schatz *et al.* 1988; Laible *et al.* 1994; Dau & Sauer 1996). An increasing amount of evidence, mostly from picosecond fluorescence studies (Schatz *et al.* 1988; McCauley *et al.* 1989; Roelofs *et al.* 1992; Dau & Sauer 1996), strongly supports the reversible radical pair (RRP) equilibrium model, originally proposed by Schatz *et al.* (1988). The 'equilibrium' part of the RRP equilibrium model refers to a rapid equilibration of excited states that is established among the chlorophylls within the PS II complex, including P680, within a much shorter timeframe (*ca.* 15 ps), than the mean lifetime of an exciton (> 1 ns) (Schatz *et al.* 1988; Dau & Sauer 1996). The 'RRP' part of the model refers to the possibility that charge recombination between P^+_{680} and Phe^- can result in re-formation of the singlet excited state of P680 ($^1P^*_{680}$) and subsequent re-equilibration of the excitation among the chlorophylls within the PS II complex.

Data from picosecond fluorescence studies also indicate that charge separation between P680 and Phe is strongly inhibited at closed PS II centres (Schatz *et al.* 1988; McCauley *et al.* 1989; Roelofs *et al.* 1992), a view that is also supported by the electron paramagnetic resonance (EPR) and flash spectroscopic measurements of Van Meighem *et al.* (1995). Since the fraction of closed PS II centres increases at moderate to high light levels, the lower rate of charge separation at closed centres must be taken into account when considering likely mechanisms for the triggering of photoinactivation, in the context of a light-dose response for this process.

2. MATERIAL AND METHODS

(a) *Rate constants*

The method of analysis used in this study is based on probabilities, expressed in terms of rate constants, for each of the possible de-excitation pathways. As such, it is essentially the same as the approach used by Butler & Kitajima (1975) to describe their 'bipartite' model, which is homologous to the RRP model (Dau 1994). Derivations of the equations used are presented in Appendix B. A schematic of the model used is shown in figure 1.

The data presented here are derived from calculations that use rate constants calculated for α-centres within isolated membranes by Roelofs *et al.* (1992). The *in vivo* values of these rate constants may be somewhat different. However, they would have to be markedly different for any of the conclusions reached in this paper to be invalidated. A complete set of the values used is given in table 1.

An arbitrary value of $1000\,\mu s^{-1}$ was selected for the value of k_{SV}. As noted in § 1, the 'apparent' Stern–Volmer quenching could actually result from a change in the rate constant, rather than quencher concentration. Consequently, it would have been

Table 1. *Values of the rate constants used in this study*

(The values of k_C, k_S, k_R and k_D are those calculated for α-centres by Roelofs *et al.* (1992). The values of k_F and k_D were selected to give a fluorescence yield of 2% at F_o and the observed value of F'_v/F_m. The value of k_{SV} is arbitrary.)

process	term	value (μs^{-1})	1/value (ps)
charge separation (open centres)	$k_{C(o)}$	3030	330
charge separation (closed centres)	$k_{C(c)}$	476	2100
charge stabilization (open centres)	$k_{S(o)}$	2325	430
charge stabilization (closed centres)	$k_{S(c)}$	1000	1000
reversal of radical pair (open centres)	$k_{R(o)}$	303	3300
reversal of radical pair (closed centres)	$k_{R(c)}$	345	2900
emission as chlorophyll fluorescence	k_F	61	16 000
non-radiative decay (light independent)	k_D	333	3000
non-radiative decay (light dependent)	k_{SV}	1000	1000

equally valid to assign a constant arbitrary value to [SV] and vary the value of k_{SV}.

With each data set, the values of k_F and k_D were selected, through iteration, to give a fluorescence yield of 2% at the dark-adapted F_o and the observed value of F_v/F_m. The value of F'_o, which is required for calculation of F'_v/F'_m and F'_q/F'_v, was calculated using the method of Oxborough & Baker (1997), using the values of F_o and F_m measured at the first saturating pulse. At each data point, values of [SV] and [Q_A] were selected, through iteration, to match with the calculated values of F'_v/F'_m and F'_q/F'_v.

(b) *Connectivity among PS II centres*

It has long been appreciated that the level of connectivity among PS II centres affects the relationship between $1-[Q_A]$ (the proportion of closed PS II centres) and ϕ_F (Joliot & Joliot 1964; Butler & Kitajima 1975; Lavergne & Trissl 1995). In the 'separate package' model, where energy transfer among PS II units is not possible, the relationship between $1-[Q_A]$ and ϕ_F is linear. With increasing connectivity, the relationship becomes increasingly curvilinear with ϕ_F being lower than $1-[Q_A]$ at intermediate values for [Q_A] (Joliot & Joliot 1964). If energy transfer among PS II units is unrestricted (a 'lake' model) Q_A behaves as a Stern–Volmer quencher (Shinkarev & Govindjee 1993). Although the equations used here only consider the lake model for energy transfer, changing the level of connectivity within the model would not affect any of the relationships examined in this study.

(c) *Experimental*

(i) *Growing of plants*

Chamber-grown plants of maize (*Zea maize* cv. LG 20.80), bean (*Phaseolus vulgaris*) and commelina (*Commelina communis*) were used for the experiments described here. Growing conditions for all three species were the same as described for maize in Oxborough & Baker (1997).

(ii) *Measurement of chlorophyll fluorescence*

All fluorescence measurements were made using a Hansatech FMS2 fluorometer (Hansatech Instruments Ltd, Norfolk, UK). Data were acquired using Hansatech FluorChart software running under Windows 98© on a notebook computer. Plants were dark adapted in a growth chamber for at least 1 h at 22 °C before measurements were made at the same temperature. After an initial measurement of F_v/F_m, saturation pulses of 900 ms duration were applied at 5 min intervals throughout the light curve. Additional measurements of F_v/F_m were made at points during the light curve, after 15 min dark adaptation. The internal halogen lamp of the FMS was used as the light source in all cases. Measurements were always made from the lowest light intensity to the highest. The light intensity was increased when the fluorescence yield at F'_m was within 0.8% of the previous value and within 1.2% at F, with a minimum of three pulses at each light intensity. A sample trace is shown in figure 2. Although calculated values of F'_o were used for all of the data presented, F'_o was also measured during 5 s far-red light treatment, 45 s after each saturating pulse.

3. RESULTS

All of the data presented are derived from a single light curve of commelina (the full fluorescence curve is shown in figure 2). Identical treatments with more than 20 other plants (maize, bean and commelina) produced very similar data sets. For the data presented, F'_o was always calculated using the values of F_o and F_m from the initial F_v/F_m pulse and the value of F'_m at the point of calculation (Oxborough & Baker 1997). Basing the analysis on values of F'_o that were calculated using the nearest appropriate values of F_o and F_m or using measured values of F'_o did not produce data that were significantly different from those presented.

(a) P^+_{680} *as a potential trigger of photoinactivation*

Anderson *et al.* (1998) have recently suggested that the reciprocity observed between light dosage and photoinactivation (Jones & Kok 1966; Park *et al.* 1995) can be accommodated within a model in which photoinactivation is triggered by the presence of P^+_{680}. This possibility is considered here.

Data from a number of fluorescence studies provide a range of values for the rate constants of each of the pathways within the RRP equilibrium model (e.g. Schatz *et al.* 1988; Roelofs *et al.* 1992; Dau & Sauer 1996). As noted in the introduction, all of these studies indicate that the rate constant for charge separation at open PS II centres ($k_{C(o)}$ in the terminology used here) is much higher than for charge separation at closed centres ($k_{C(c)}$); a result that is in agreement with the EPR and flash spectroscopic measurements of Van Mieghem *et al.* (1995). In the absence of downregulation, the proportion of centres in the open state ([Q_A]) would be expected to decrease with increasing PPFD. However, the relationship between incident PPFD and [Q_A] is complicated by the increase in downregulation with incident PPFD.

In assessing the likelihood that P^+_{680} is a trigger of photoinactivation, it is important to consider the

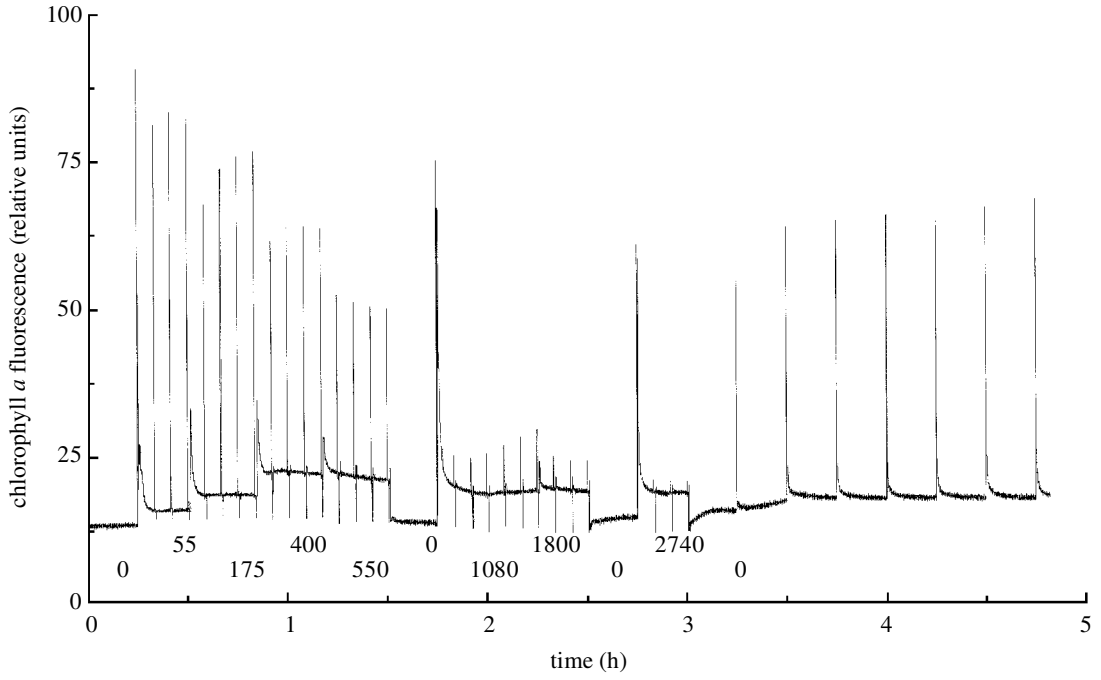

Figure 2. Fluorescence trace from an attached commelina leaf, from which all of the data in figures 3 and 4 are derived. The numbers within the frame of the graph show the incident PPFD (μmol m^{-2} s^{-1}) at different points throughout the curve. Growing and measuring conditions are detailed in § 2.

integrated lifetime of this radical, which is given by the product of yield and lifetime. The integrated lifetime of P^+_{680} formed through charge separation is dependent on the way in which it is taken back to the ground state, which, in turn, is very largely dependent on whether the centre is open or closed.

At open centres, charge separation is most frequently followed by charge stabilization (see § 1), which involves the transfer of an electron from Z to P^+_{680}. In the remaining cases, P^+_{680} at open centres is taken back to the ground state through charge recombination. Conversely, P^+_{680} at closed centres is nearly always taken back to the ground state through charge recombination.

The time constant for electron transfer from Z to P^+_{680} is between 20 and 300 ns, depending on the current S-state of the oxygen-evolving complex, with a mean value of *ca.* 90 ns (Deprez *et al.* 1983; Meyer *et al.* 1989). Assessing the lifetime of P^+_{680} in situations where it is taken back to the ground state through charge recombination is complicated by the fact that charge recombination can result in formation of $^1P^*_{680}$, $^3P^*_{680}$ or $^1P_{680}$. The time constants for formation of $^1P^*_{680}$ through charge recombination at open and closed centres are given by $1/k_{R(o)}$ and $1/k_{R(c)}$, which provide values of 3.3 and 2.9 ns, respectively. Charge recombination giving rise to formation of $^3P^*_{680}$ and $^1P_{680}$ cannot be distinguished from charge stabilization, since all three pathways for de-excitation give rise to non-fluorescence states.

At open centres, the yield of $^3P^*_{680}$ and $^1P_{680}$ through charge recombination is unlikely to be very significant, because the yield of competing charge stabilization is known to be very high. At closed centres, formation of $^3P^*_{680}$ and $^1P_{680}$ through charge recombination almost certainly represent the main post-charge separation, non-fluorescence pathways for de-excitation, since the only alternative pathways that have been established are electron transfer to Q_A^- (Van Wijk *et al.* 1992; Vass *et al.* 1992) and to cytochrome b_{559} (Poulson *et al.* 1995), neither of which is thought capable of supporting significant rates of electron flow.

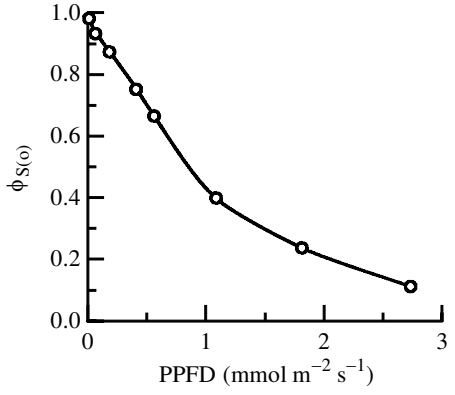

Figure 3. The relationship between incident PPFD and the yield of charge stabilization at open PS II centres ($\phi_{S(o)}$), derived from the fluorescence trace in figure 2, over a range of PPFDs between 0 and 2740 μmol m^{-2} s^{-1}. Details of the rate constants and method used to calculate values of $\phi_{S(o)}$ are given in § 2.

The overall time constant for formation of $^3P^*_{680}$ and $^1P_{680}$ through charge recombination, plus charge stabilization at open centres, is given by $1/k_{S(o)}$, which has a value of 0.43 ns. At closed centres, the equivalent time constant is given by $1/k_{S(c)}$, which has a value of 1 ns.

Clearly, the lifetime of P^+_{680} is very much longer at centres that have undergone charge stabilization than at centres where charge recombination has occurred (by a factor of approximately 30). Consequently, the integrated lifetime of P^+_{680} at centres that undergo charge

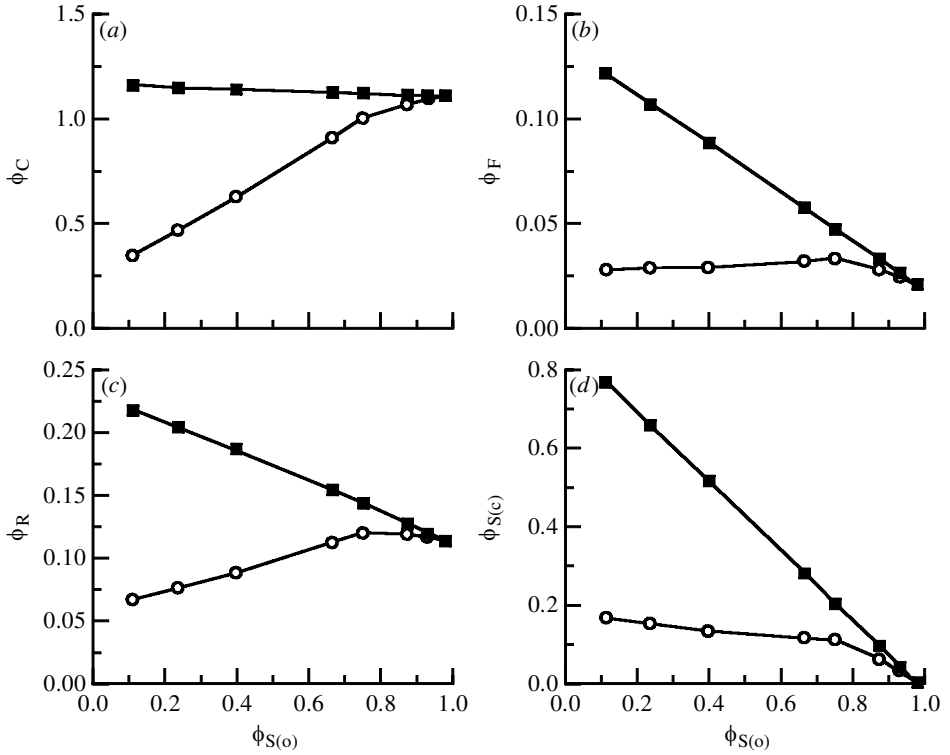

Figure 4. Data illustrating the effect of downregulation of the relationships between the yield of charge stabilization at open PS II centres ($\phi_{S(o)}$), and (*a*) the yield of charge separation (ϕ_C), (*b*) the yield of chlorophyll fluorescence (ϕ_F), (*c*) the yield of charge recombination that leads to formation of $^1P^*_{680}$ (ϕ_R), and (*d*) the yield of charge stabilization at closed PS II centres ($\phi_{S(c)}$). The open circles show the relationships at the observed level of downregulation, while the closed squares show what the relationships would be in the absence of downregulation.

recombination will only be significant if the yield of charge separation (ϕ_C) is very high, relative to the yield of charge stabilization at open centres ($\phi_{S(o)}$).

Anderson *et al.* (1998) have suggested that repeated generation of P^+_{680} at closed centres (through repeated generation of the radical pair) increases the probability that an 'inadvertent oxidation causes photodamage'. Although the RRP model allows for the yield of P^+_{680} to exceed a value of 1, none of the data analysed within this study gave rise to values for the ratio of ϕ_C to $\phi_{S(o)}$ of more than 3. Since the lifetime of P^+_{680} is some 30 times longer at centres that have undergone charge stabilization, rather than charge recombination, even a ratio as high as 3 for ϕ_C to $\phi_{S(o)}$ means that the integrated lifetime of P^+_{680} is almost entirely defined by charge stabilization at open centres. The data in figure 3 show the range of values for $\phi_{S(o)}$ from the fluorescence trace in figure 2. The large range of these values, from 0.98 in the dark-adapted state to 0.11 at 2740 $\mu mol\, m^{-2}\, s^{-1}$, is not easy to reconcile with the light-dose response of photoinactivation.

Since P^+_{680} is formed through charge separation at both open and closed centres, the most important relationship for assessing the effect of downregulation on the integrated lifetime of P^+_{680} is that between the yield of charge stabilization at open centres ($\phi_{S(o)}$) and the total yield of charge separation (at open plus closed centres), which is given by ϕ_C. This relationship is illustrated by the data in figure 4*a*, which are derived from the fluorescence trace in figure 2. The open circles show the relationship when [SV] and [Q_A] are adjusted to give the observed values of F'_v/F'_m and F'_q/F'_v while the closed squares show what the relationship would be if there were no downregulation ([SV] = 0) and the same values of $\phi_{S(o)}$ were defined by the value of [Q_A] alone. Although the removal of downregulation increases the ratio of ϕ_C to $\phi_{S(o)}$ from less than 2 to over 10, at high incident PPFD, the much longer lifetime of P^+_{680} at open centres where charge stabilization has occurred still leaves the integrated lifetime of P^+_{680} largely defined by $\phi_{S(o)}$. Consequently, downregulation does not significantly increase $\phi_{S(o)}$, relative to the integrated yield of P^+_{680}, as would be expected if P^+_{680} were a significant trigger of photoinactivation.

(b) $^3P^*_{680}$ *as a potential trigger of photoinactivation*

(i) $^3P^*_{680}$ *formed through intersystem crossing*

As noted in § 1, there are two ways in which $^3P_{680}$ can be formed; directly from $^1P^*_{680}$, through intersystem crossing, and through charge recombination between P^+_{680} and Phe^-. Both pathways are considered here.

Despite the protection afforded by the presence of β-carotene and the putative O_2 barrier, formation of $^3Chl^*$ within the light-harvesting system of PS II can still result in the $^1O_2$-induced photodestruction of chlorophylls, with an apparent yield of between 10^{-5} and 10^{-6} (Krasnovsky 1994). If P_{680} is simply considered as one of a number of chlorophylls within the pigment bed, with no more and no less susceptibility to $^1O_2$-induced photodestruction than any other, then an overall yield of between 10^5 and 10^6 would be at least an order of magnitude too low to be considered as a potential trigger for photoinactivation.

However, there are reasons for thinking that the yield of $^1O_2$-induced photodestruction of P_{680} may be significantly higher than the average for chlorophylls within the pigment bed as a whole.

First, the distribution of excitation energy within the pigment bed, although not strictly a Bolzmann distribution, is still largely dependent on the spectral characteristics of the chlorophylls present (Laible *et al.* 1994; Dau & Sauer 1996). Consequently, the rapid equilibration of excitation energy within the pigment bed results in a higher density of excitation energy on the longer wavelength chlorophylls, which includes P_{680}. Second, the potential lack of a proximal β-carotene could increase the lifetime of $^3P^*_{680}$ by orders of magnitude, thereby increasing the yield and lifetime of $^1O_2$.

There is no obvious reason why a centre being open or closed should have any impact on either the probability of an exciton 'visiting' P_{680} or the yield of $^3P^*_{680}$ that is formed through intersystem crossing. Consequently, the yield of $^3P^*_{680}$ formation through intersystem crossing is largely dependent on the lifetime of an exciton, which is proportional to the yield of fluorescence (ϕ_F). Since ϕ_F changes very little with PPFD (see figure 4*b*), the concept of photoinactivation being triggered by formation of $^3P^*_{680}$ through intersystem crossing fits well with the light-dose response of photoinactivation.

One issue that needs to be addressed is the process of charge separation and recombination that results in reformation of $^1P^*_{680}$, since this accounts for a fraction of the lifetime of an exciton. The relationship between $\phi_{S(o)}$ and the yield of charge recombination (ϕ_R), derived from the trace in figure 2, is shown in figure 4*c*. These data show that ϕ_R varies by less than a factor of 2 and has a maximum value (at the lowest PPFDs) of slightly over 0.1. The time taken for reformation of $^1P^*_{680}$ through charge separation and recombination is in the same range as the mean lifetime of an exciton within the pigment bed (Roelofs *et al.* 1992) and, consequently, does not represent a strong argument against $^3P^*_{680}$ being a trigger for photoinactivation.

The effect of downregulation on the yield of $^3P^*_{680}$ formation through intersystem crossing can be assessed from the data in figure 4*b*,*c*. As with the data in figure 4*a*, the impact of downregulation was assessed by setting [SV] to zero in the modelled data, and achieving the observed value of F'_q/F'_m by adjusting $[Q_A]$. It is clear, from these data, that downregulation decreases both ϕ_F and ϕ_R in roughly equal proportion and would, therefore, be expected to decrease the yield of $^3P^*_{680}$ formation through intersystem crossing.

(ii) $^3P_{680}$ *formed through charge recombination*

As noted above (§ 3(a)), the formation of $^3P^*_{680}$ through charge recombination is one of three processes that contribute to charge stabilization at open and closed centres, the other two being formation of $^1P_{680}$ through charge recombination and charge stabilization, resulting from electron transfer to Q_A. Consequently, the yield of $^3P^*_{680}$ through charge recombination cannot be calculated directly.

If it is assumed that the probability of charge recombination leading to the formation of $^3P^*_{680}$ (rather than $^1P_{680}$ or $^1P^*_{680}$) is the same at open and closed centres, then the yield of $^3P^*_{680}$ would obviously be proportional to the yield of $^1P^*_{680}$, given by ϕ_R, which can be calculated. The relationship between $\phi_{S(o)}$ and ϕ_R for the trace in figure 2 is demonstrated by the data in figure 4*c*, which show a 44% decrease in ϕ_R between the highest and lowest values. Although strict compatibility with the light-dose response would require a stable yield for ϕ_R with changing $\phi_{S(o)}$, the relatively narrow range observed is not incompatible with $^3P^*_{680}$ formed through charge recombination being a trigger for photoinactivation.

As already noted, the data in figure 4*c* show that downregulation decreases ϕ_R, with the largest effect being seen at the highest PPFD values (lowest values of $\phi_{S(o)}$). Consequently, downregulation would be expected to decrease the yields of $^3P^*_{680}$ formed through either intersystem crossing or charge recombination.

(c) *Double reduction of Q_A as a potential trigger of photoinactivation*

The possibility that double reduction of Q_A could be a trigger for photoinactivation (Van Wijk *et al.* 1992; Vass *et al.* 1992) has been strongly contested on the grounds that the application of target theory reveals that photoinactivation is a single-photon event (Sinclair *et al.* 1996; Anderson *et al.* 1998). Whilst double reduction of Q_A at open PS II centres is clearly a two-photon event, it is perfectly valid to consider closed PS II centres as targets, where double reduction of Q_A is actually a single-photon event (since, by definition, Q_A already carries a single negative charge at these centres). The observation that photoinactivation is a single-photon event can be reconciled with closed centres being the target, providing the probability of a photon-inducing stable charge separation at a closed centre is insensitive to PPFD. The data in figure 4*d* show that the probability of a photon being used to drive stable charge separation at a closed centre increases with light at low PPFD, but remains fairly stable as PPFD increases beyond this range. Sinclair *et al.* (1996) noted that the yield of photoinactivation is low at low photon doses, which included low PPFDs; a result that is consistent with the lower yield at low PPFDs observed here. The data in figure 4*d* also show that, with increasing PPFD (going from right to left along the *x*-axis), downregulation increases the ratio of $\phi_{S(o)}$ to $\phi_{S(c)}$ and therefore provides a high level of protection against double reduction of Q_A; a result that is also consistent with triggering of photoinactivation by this process.

4. DISCUSSION

The objective of this study was to determine how well four potential triggers of photoinactivation (formation of P^+_{680}, formation of $^3P^*_{680}$ through intersystem crossing, formation of $^3P^*_{680}$ through charge recombination and double reduction of Q_A) could be reconciled with the reversible exciton–radical pair equilibrium model of Schatz *et al.* (1988), a Stern–Volmer model for downregulation and the apparent light-dose response of photoinactivation.

It has been argued that the apparent light-dose response of photoinactivation is strong evidence for a single trigger (Anderson *et al.* 1998). Although it would

perhaps be surprising if two or more triggering processes had similar yields (and were, therefore, both significant triggers of the photoinactivation process), the apparent light-dose response of photoinactivation is not, in itself, an argument against this being the case. One criterion that does need to be satisfied is that the yield of any putative trigger should be constant with changing PPFD. Given the strong evidence that downregulation 'protects' against photoinactivation, it seems reasonable to specify a second criterion for any potential trigger of photoinactivation; that its yield is lowered by this process, relative to $\phi_{C(o)}$. When these two criteria are considered together, the formation of $^3P^*_{680}$ through intersystem crossing is perhaps the strongest candidate, followed by formation of $^3P^*_{680}$ through charge recombination and double reduction of Q_A. Formation of P^+_{680} is a poor candidate in the context of both criteria, once the large difference between the lifetimes of P^+_{680} that is taken back to the ground state through electron transfer from Z and through charge recombination with Phe^- is taken into account.

A third criterion that obviously needs to be satisfied is that the yield of any putative trigger should be high enough to account for the observed yield of photoinactivation, estimated at between 10^{-6} and 10^{-7} by Anderson *et al.* (1997). From the information currently available, it would seem that the yield of $^3P^*_{680}$ through intersystem crossing is likely to be between 10^{-3} and 10^{-4}, assuming that its yield is roughly twice that of chlorophyll fluorescence (Durrant *et al.* 1990). Whether or not this is high enough for it to be a serious contender largely depends of the level of protection that is afforded by β-carotene. If this protection is as efficient as that afforded the other chlorophylls within the pigment bed of PS II, then the yield of $^3P^*_{680}$ through intersystem crossing would be orders of magnitude too low, since its lifetime would probably be too short for it to induce significant formation of $^1O_2$. However, the lifetime of $^3P_{680}$ could be increased by orders of magnitude if, as seems perfectly feasible, the level of protection afforded by β-carotene is very low (Barber 1998). A yield for $^3P^*_{680}$ of *ca.* 30% has been observed at closed centres within isolated reaction centre complexes (Durrant *et al.* 1990). Most of this yield was attributed to charge recombination, largely because the yield of fluorescence was only 2% and the yield of $^3P^*_{680}$ through intersystem crossing is likely to be roughly twice this figure. These data strongly suggest that the formation of $^3P^*_{680}$ through charge recombination is likely to be a much more efficient trigger of photoinactivation than formation of $^3P^*_{680}$ through intersystem crossing.

It has been argued previously that double reduction of Q_A is unlikely as a mechanism of photoinactivation because the probability of charge separation occurring at a closed centre is too low (Park *et al.* 1997; Anderson *et al.* 1998). In actual fact, as the data in figure 4*d* clearly illustrate, the yield of charge stabilization at closed centres is relatively high, once PPFD is above *ca.* 200 μmol m^{-2} s^{-1}, at between 0.06 and 0.16. Although the fraction of charge stabilization events that result in double reduction of Q_A is not known, a comparison of the yields of photoinactivation (between 10^{-6} and 10^{-7}) and charge stabilization at closed centres (*ca.* 10^{-1}) clearly shows that it would not have to be very high.

In conclusion, the formation of $^3P^*_{680}$ through intersystem crossing, and through charge recombination and the double reduction of Q_A, are all feasible as triggers for the photoinactivation process in terms of the first two above criteria. That is, their yields are relatively insensitive to PPFD and their yields are lowered, relative to $\phi_{S(o)}$, by downregulation. P^+_{680} clearly fails both of these criteria. Given that the yield of $^3P^*_{680}$ formed through charge recombination is likely to be much higher than the yield of $^3P^*_{680}$ formed through intersystem crossing, it seems unlikely, on current evidence, that $^3P^*_{680}$ formed through the latter pathway makes a significant contribution to the photoinactivation process. The relatively high yield of charge stabilization at closed centres, at all but the lowest PPFDs, means that double reduction of Q_A should also be considered as a viable trigger of photoinactivation.

APPENDIX A. EXPLANATION OF ABBREVIATIONS

F', chlorophyll fluorescence signal in the light-adapted state; F_m and F'_m, chlorophyll fluorescence signal when all PS II centres are closed in the dark- and light-adapted states, respectively; F_o and F'_o, chlorophyll fluorescence signal when all centres are open in the dark- and light-adapted states, respectively; F'_q, difference between F' and F'_m; F_v/F_m and F'_v/F'_m, fluorescence parameter that provides an estimate of the maximum efficiency of PS II photochemistry (when $[Q_A] = 1$) in the dark- and light-adapted states, respectively; F'_q/F'_m, fluorescence parameter that provides an estimate of the efficiency of PS II photochemistry (the product of F'_v/F'_m and F'_q/F'_v); F'_q/F'_v, fluorescence parameter that quantifies the photochemical capacity of PS II; F'_v and F'_v, variable chlorophyll fluorescence ($F_m - F_o$ or $F'_m - F_o'$); k_C, apparent rate constant for charge separation at PS II; k_D, apparent rate constant for non-radiative decay at PS II in the dark-adapted state; k_F, apparent rate constant for chlorophyll *a* fluorescence; k_R, apparent rate constant for charge recombination at PS II, leading to formation of $^1P^*_{680}$; k_S, apparent rate constant for the sum of stable charge separation and charge recombination, leading to formation of P_{680} or $^3P_{680}$; k_{SV}, apparent rate constant for non-radiative decay by light-induced Stern–Volmer quenchers at PS II; Phe, pheophytin; P_{680}, electronically excitable component of PS II; $^1P_{680}$, P_{680} in the (singlet) ground state; $^1P^*_{680}$, P_{680} in the singlet excited state; $^3P^*_{680}$, P_{680} in the triplet excited state; $[Q_A]$, the concentration of open PS II centres; [SV], the concentration of light-induced Stern–Volmer quenchers associated with PS II; $_{(o)}$ and $_{(c)}$, subscripts applied to rate constants and other terms to signify open or closed PS II centres, respectively (if no subscript is used, the term applies to open plus closed centres); ϕ_x, probability of process 'x' occurring; Φ_x, yield of process 'x'.

APPENDIX B

The probability of a photon being re-emitted as chlorophyll fluorescence, dissipated through non-radiative decay or being used to drive charge separation, can be calculated as the rate constant for a particular process

divided by those for all of the competing processes. For example, the probability of charge separation occurring within dark-adapted material (when all PS II centres are open and [SV] is zero) can be represented by equation (B1):

$$\phi_{C(o)} = \frac{k_{C(o)}}{k_F + k_D + k_{C(o)}}. \quad \text{(B1)}$$

Because charge separation is a reversible process, the probability of charge recombination and subsequent transfer of excitation energy back to the pigment bed must be taken into account when calculating the yield of each pathway for de-excitation. Equation (B2) expresses the probability of charge recombination at open PS II centres, once charge separation has occurred

$$\phi_{R(o)} = \frac{k_{R(o)}}{k_S + k_{R(o)}}. \quad \text{(B2)}$$

Following charge recombination, the exciton is transferred to the pigment complex where, once again, it can be re-emitted as chlorophyll fluorescence, dissipated through non-radiative decay or used to drive charge separation. The reversibility of charge separation must be taken into account when expressing the yield of each pathway for de-excitation. For example, the yield of charge stabilization at open centres, in the dark-adapted state, is expressed by equation (B3*a*):

$$\Phi_{S(o)} = \frac{k_{C(o)} - k_{C(o)} \times \phi_{R(o)}}{k_F + k_D + k_{C(o)}} \times [1 + \phi_{C(o)} \times \phi_{R(o)} + (\phi_{C(o)} \times \phi_{R(o)})^2 + \ldots (\phi_{C(o)} \times \phi_{R(o)})^n]. \quad \text{(B3}a\text{)}$$

This simplifies to equation (B3*b*):

$$\Phi_{S(o)} = \frac{k_{C(o)} - k_{C(o)} \times \phi_{R(o)}}{k_F + k_D + k_{C(o)} - k_{C(o)} \times \phi_{R(o)}}. \quad \text{(B3}b\text{)}$$

In the light-adapted state, the increase in non-radiative decay through an increase in [SV] and the presence of both open and closed PS II centres must be taken into account. For example, the yield of charge stabilization at open PS II centres is expressed by equation (B4):

$$\Phi_{S(o)} = \frac{(k_{C(o)} - k_{C(o)} \times \phi_{R(o)}) \times [Q_A]}{k_F + k_D + k_{SV} \times [SV] + (k_{C(o)} - k_{C(o)} \times \phi_{R(o)}) \times [Q_A] + (k_{C(c)} - k_{C(c)} \times \phi_{R(c)}) \times (1 - [Q_A]))}. \quad \text{(B4)}$$

There are four other yields that are of relevance to this study; the yield of charge separation, the yield of fluorescence, the yield of charge recombination, leading to formation of $^1P^*_{680}$ and the yield of charge stabilization at closed PS II centres. These are given by equations (B5), (B6), (B7) and (B8), respectively.

$$\Phi_C = \frac{k_{C(o)} \times [Q_A] + k_{C(c)} \times (1 - [Q_A])}{k_F + k_D + k_{SV} \times [SV] + (k_{C(o)} - k_{C(o)} \times \phi_{R(o)} \times [Q_A] + (k_{C(c)} - k_{C(c)} \times \phi_{R(c)}) \times (1 - [Q_A]))}. \quad \text{(B5)}$$

$$\Phi_F = \frac{k_F}{k_F + k_D + k_{SV} \times [SV] + (k_{C(o)} - k_{C(o)} \times \phi_{R(o)}) \times [Q_A] + (k_{C(c)} - k_{C(c)} \times \phi_{R(c)}) \times (1 - [Q_A]))}. \quad \text{(B6)}$$

$$\Phi_R = \frac{k_{R(o)} \times [Q_A] + k_{R(c)} \times (1 - [Q_A])}{k_F + k_D + k_{SV} \times [SV] + (k_{C(o)} - k_{C(o)} \times \phi_{R(o)}) \times [Q_A] + (k_{C(c)} - k_{C(c)} \times \phi_{R(c)}) \times (1 - [Q_A])}. \quad \text{(B7)}$$

$$\Phi_{S(c)} = \frac{(k_{C(c)} - k_{C(c)} \times \phi_{R(c)}) \times (1 - [Q_A])}{k_F + k_D + k_{SV} \times [SV] + (k_{C(o)} - k_{C(o)} \times \phi_{R(o)}) \times [Q_A] + (k_{C(c)} - k_{C(c)} \times \phi_{R(c)}) \times (1 - [Q_A])}. \quad \text{(B8)}$$

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