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Biochemistry of xanthophyll-dependent non-photochemical fluorescence quenching in isolated chloroplasts

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University of Hawai‘i, 1992
BIOCHEMISTRY OF XANTHOPHYLL-DEPENDENT
NON-PHOTOCHEMICAL FLUORESCENCE QUENCHING
IN ISOLATED CHLOROPLASTS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BOTANICAL SCIENCES

(PLANT PHYSIOLOGY)

BY

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ABSTRACT

Higher plants possess several mechanisms that protect the photosynthetic apparatus against damage from excess-light. One mechanism dissipates supersaturating light levels thermally or nonradiatively. Although studies with intact leaves and isolated chloroplasts have shown that the nonradiative dissipation or NRD phenomenon is related to the light-induced transthyakoid pH-gradient (ΔpH) and zeaxanthin formed by violaxanthin de-epoxidation in the xanthophyll-cycle, the biochemical relationship remains unclear. The research plan of this dissertation was based on the premises that the biochemical relationship can be characterized in isolated chloroplasts and that the results of in vitro studies may explain the NRD mechanism in intact leaves. The experimental approach was to probe the NRD mechanism using various mediators, inhibitors, and uncouplers of light-driven electron-transport, the xanthophyll-cycle, and NRD, as well as dark ATP-induced proton pumping. Chloroplasts were isolated from *Pisum sativum* L. cv. Oregon and *Lactuca sativa* L. cv. Romaine. The results suggest that all NRD relates to a common mechanism, occurs in the photosystem II pigment bed, and depends on lumen proton concentration. The data further suggest that the violaxanthin de-epoxidation products, antheraxanthin and zeaxanthin, contribute equally to NRD and that no NRD occurs in their absence. These results contrast earlier studies, which suggest that NRD actually comprises of two mechanisms, one zeaxanthin-dependent in the light-harvesting pigment bed and one constitutive in the photosystem II reaction center. The work investigating ATP-induced ΔpH showed that NRD is

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actually a ‘dark-reaction’, only indirectly related to actinic light. These results appear to exclude the previous suggestions that NRD involves light-dependent changes in the redox-state of electron-transport components. The ATP-induced NRD may also explain the mechanism behind dark-sustained NRD observed in leaves under photoinhibitory conditions where CO₂-fixing capacity was limited and ATPase activation and ATP accumulation were possible.
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<td>Anti-A;</td>
<td>Antimycin</td>
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<td>ASC;</td>
<td>Ascorbate</td>
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<td>ATP;</td>
<td>Adenosine 5'-triphosphate</td>
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<td>ββ;</td>
<td>Betacarotene (β,β-carotene)</td>
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<td>βε;</td>
<td>Alphacarotene (β,ε-carotene)</td>
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<td>BSA;</td>
<td>Bovine serum albumin</td>
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<td>Dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone)</td>
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<td>FQR;</td>
<td>Ferredoxin quinone reductase</td>
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<td>HEPES;</td>
<td>N-2-hydroxyethyl piperazine-n-2-ethanesulfonic acid</td>
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<td>HEQ;</td>
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HEX; Hexokinase plus glucose
HPLC; High-performance liquid chromatography
La; Lactucaxanthin (ε,ε-carotene-3,3′-diol)
L; Lutein (β,ε-carotene-3,3′-diol)
MV; Methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride)
N; 9′-cis-neoxanthin
NPQ; Nonphotochemical quenching of chlorophyll fluorescence
Pa; Pheophytin a
PAR; Photosynthetically Active Radiation
Pb; Pheophytin b
PFD; Photon Flux Density
PMS; Phenazine methosulfate (N-methyl dibenzopyrazine methyl sulfate salt)
PQ; Plastoquinone
PSI; Photosystem I
PSII; Photosystem II
QA; Primary electron acceptor of photosystem II
qE; Coefficient for energy-dependent nonphotochemical quenching
qf; Coefficient for photoinhibitory quenching
qN; Coefficient for nonphotochemical quenching
qO; Coefficient for nonphotochemical quenching of F$_0$
SV$_E$; Stern-Volmer type energy-dependent fluorescence quenching
LIST OF ABBREVIATIONS, cont’d

$S_{V_N}$; Stern-Volmer type quenching of $F_M$; $(F_M / F'_M) - 1$

$S_{V_O}$; Stern-Volmer type quenching of $F_O$; $(F_O / F'_O) - 1$

Tris; Tris hydroxymethyl aminomethane

$V$; Violaxanthin $(3S,5R,6S,3'S,5'R,6'S)-5,6,5',6'-$diepoxo-$5,6,5',6'$-$tetrahydro-\beta,\beta$-carotene-$3,3'$-$diol$)

$Z$; Zeaxanthin $(\beta,\beta$-carotene-$3,3'$-$diol$)
Paradoxically, light that is essential for plant life can be deleterious in excess amounts. In apparent response, plants have evolved various protective mechanisms against the harmful effects of excess light. For example, some higher-plants have evolved unique morphological adaptations to avoid or reflect excess light. However, all higher plants appear to possess a complex biochemical adaptive mechanism which allows rapid, fine-tuned adjustments to excessive light. This highly conserved mechanism results in protective "down-regulation" of the primary photochemical yield at photosystem II (PSII) (Krause and Behrend 1986, Krause and Laasch 1987, Weis and Berry 1987). Down-regulation is apparently associated with thermal or nonradiative-dissipation (NRD) of excess light energy in the PSII pigment bed (Rees et al. 1990) or the PSII reaction center (Krause et al. 1988, Weis and Berry 1987) or both thus preventing overexcitation of the reaction centers. According to present knowledge protective NRD is related to at least three light-induced phenomena: I. formation of a proton-gradient (ΔpH) across the thylakoid membrane (Briantais et al. 1979, 1980), II. oxidation or protonation of an antimycin sensitive site (Noctor et al. 1990, Oxborough and Horton 1987, 1988), and III. the synthesis of the carotenoid zeaxanthin via the violaxanthin cycle (Adams et al. 1990, Demmig-Adams et al. 1990, Noctor et al. 1991, Rees et al. 1989). These phenomena may in fact relate to a common mechanism.

Typically, NRD is associated with the 'high-energy' state or ΔpH required for photophosphorylation (Murata and Sugahara 1969). NRD is measured
experimentally as nonphotochemical quenching of chlorophyll fluorescence (Bradbury and Baker 1981, Schreiber et al. 1986). Although several components comprise nonphotochemical quenching, the major component is 'high-energy' nonphotochemical quenching or HEQ.

This dissertation addresses several areas, regarding the biochemical interrelationship between the violaxanthin cycle and HEQ. One is the role of lumen acidification in HEQ. Apparently, HEQ depends on lumen proton concentration (Briantais et al. 1979, 1980) but ΔpH does not always induce HEQ (Barber et al. 1974 a,b, Krause 1974, Oxborough and Horton 1987, 1988). There are apparently other factors consequential to the ΔpH which are involved in quenching. The specific interaction between ΔpH and the antimycin-sensitive change mentioned above is interesting because it apparently is an important structural and or functional factor in HEQ development. However, it is controversial whether the antimycin-site is a redox-component or a pH-sensitive site. Herein, evidence is presented that indicates the antimycin site is a pH-sensitive site which controls all HEQ, apparently independent of changes in the redox-state of any particular electron-transport component.

Demmig-Adams et al. (1990), proposed that there are two different HEQ mechanisms, one zeaxanthin-dependent, occurring in the pigment bed and the other, independent of zeaxanthin, occurring in the reaction center. The data in this dissertation suggests that antheraxanthin and zeaxanthin quench with equal efficiencies and all observed HEQ can be accounted for if antheraxanthin is
considered. Linear models are developed which, based on linear and multiple regression analysis, closely predict independently measured HEQ. The models show that although the product of (zeaxanthin)(lumen [H\(^{+}\)]) and lumen [H\(^{+}\)], when considered as independent variables in a multiple-regression equation, closely predict HEQ, that considering the product of (antheraxanthin plus zeaxanthin)(lumen [H\(^{+}\)]) allows for equally close prediction of HEQ in a simple linear-regression equation.

It is also determined that xanthophyll HEQ can be induced in vitro and sustained in the dark by ATP-induced \(\Delta pH\) (Petrack and Lipman 1961) similar to the early report of dark ATP-induced de-epoxidation (Hager 1969). The ATP-induced quenching implies that xanthophyll-HEQ may be induced and sustained in the dark in vivo under conditions where ATPase activation and ATP-accumulation in a preliminary light period are possible, such as any photoinhibitory stress which limits \(\text{CO}_2\)-fixation.
CHAPTER 1

LITERATURE REVIEW
VIOLAXANTHIN CYCLE

There is a cyclic pattern of xanthophyll conversion in higher plants known as the violaxanthin cycle (Yamamoto et al. 1962). The existence of this cycle is also documented in green and brown algae (Hager 1975). Figure 1.1 shows the structures of the violaxanthin cycle components. The main component of the cycle is usually the di-epoxide violaxanthin. In the light violaxanthin can be converted in a sequential de-epoxidation reaction to zeaxanthin via the monoepoxide intermediate antheraxanthin. In the dark the cycle reverses so most of the violaxanthin-cycle pool components are converted back to violaxanthin. Both the de-epoxidation and epoxidation reactions are enzyme-mediated.

Role(s) of Light and the Transmembrane Model. Biochemical control of the cycle is complex and integrally related to photosynthetic processes. The de-epoxidation reaction is considered a light-induced reaction, yet both de-epoxidation and epoxidation are dark reactions only indirectly dependent on actinic light (Yamamoto 1979). Both reactions are enzyme-mediated and the entire cycle is believed to reflect a transthylakoid membrane system (Siefermann and Yamamoto 1975b,c). The proposed transmembrane arrangement of the cycle implies that the component pigments are freely mobile in the lipid matrix of the membrane (Siefermann and Yamamoto 1975b). Evidence for this model is based on the different pH-optima of the de-epoxidation and epoxidation reactions (for a summary see Fig. 1.2).
Figure 1.1 Violaxanthin cycle components (Yamamoto et al. 1962).
Early studies indicated that conditions conducive to ATP-synthesis and $\Delta p\text{H}$ formation were similar to those which stimulated de-epoxidation (Hager 1969). However, it later became apparent that these processes were only indirectly linked by their mutual dependency on the $\Delta p\text{H}$. This indirect linkage was shown by the use of the sulfhydryl reagent dithiothreitol (DTT) which inhibits *in vitro* de-epoxidation (Yamamoto and Kamite 1972) but does not inhibit $\Delta p\text{H}$ (Sokolove and Marsho 1976), ATP-synthesis, or ATP-hydrolysis (Petrack and Lipman 1961). DTT also inhibits *in vivo* de-epoxidation without affecting light-limiting rates of photosynthesis (Bilger and Björkman 1990).

The de-epoxidation reaction is ascorbate-dependent and affected by light in at least two ways (Yamamoto 1979). First, the de-epoxidase enzyme is active only at low lumen pH with optimal activity at pH 5.2 *in vitro* (Hager 1969, Yamamoto et al. 1972). This is the approximate pH of the thylakoid lumen under coupled light-driven electron-transport (Hager 1969). Violaxanthin de-epoxidation is stimulated under high-light (Yamamoto et al. 1962) or even in low light when the capacity for CO$_2$-fixation is limited by removal of electron acceptors (Yamamoto et al. 1962, Sapozhnikov et al. 1972, Siefermann 1972).

In addition to activation of the de-epoxidase enzyme, the final extent of de-epoxidation is affected by spectral light quality. For a given rate of electron-transport the final extent of de-epoxidation is attained faster with 670 nm light for PSII activation, than with 710 nm light for PSI activation (Siefermann and Yamamoto 1974a). Based on these and similar experiments (Siefermann and Yamamoto 1975a)
Figure 1.2. Transmembrane model of violaxanthin-cycle in higher plants. Adapted from Hager (1980).
it appears that the level of de-epoxidation is partly controlled by the redox state of an intersystem electron transport component, perhaps plastoquinone. This control of the final extent of de-epoxidation is termed violaxanthin availability and believed to reflect conformational changes in the internal surface of the thylakoid membrane (Siefermann and Yamamoto 1974a, 1975a).

The reverse epoxidation reaction, can be stimulated by low-levels of actinic light (Westerhoff 1974). The epoxidation reaction is optimal at pH 7.5, requires molecular oxygen, and uses NADPH for reducing potential (Perz 1970, Siefermann and Yamamoto 1975b,c). Thus, photosynthesis via O₂ evolution and NADP reduction, may stimulate epoxidation. Yet, sufficient levels of molecular O₂ and NADPH are apparently available in the darkness to facilitate epoxidation (Hager 1975).

The isolated de-epoxidase enzyme requires solubilization of violaxanthin in the chloroplast lipid monogalactosyl diglyceride for activity (Yamamoto et al. 1974, Yamamoto and Higashi 1978). Monogalactosyl diglyceride apparently allows for the suspended pigment to interact with the enzyme active site. Monogalactosyl diglyceride is also a component of the enzyme active site (Yamamoto and Higashi 1978). The de-epoxidase active site is apparently stereospecific for 3-hydroxy, 5,6-epoxy carotenoids which are in a 3S, 5R, 6S configuration. Pigments with 9-cis configurations such as violoxanthin and neoxanthin are inactive. Thus, it was concluded that the active site is a narrow well like cavity which favors an all-trans configuration of the polynene chain. The non-epoxide end of a mono-epoxide also
affects the de-epoxidation rate. For instance, antheraxanthin is de-epoxidized at over 5 times the rate of violaxanthin. The presence of polar substitutions such as epoxide groups may slow de-epoxidation. This would imply that the de-epoxidation step between violaxanthin and antheraxanthin is rate limiting in zeaxanthin formation.

Knowledge of the molecular properties of the epoxidase is scant due to the fact that it has not yet been isolated. Siefermann and Yamamoto (1975b,c) discovered then characterized the NADPH and O$_2$-dependency of \textit{in vitro} epoxidation. They reported that the epoxidase activity is stimulated by bovine serum albumin which protected the system from inhibition by fatty acids. Based on epoxidation inhibition by copper chelate salicylaldoxim (Perz 1970) and the requirement of NADPH and O$_2$, it was deduced that the epoxidase is a metal-containing, mixed-function oxygenase.

\textbf{Adaptive Capacities (High/Low Light).} Several physiological and ecological surveys indicate that the violaxanthin-cycle adapts to different growth-light regimes (for a review see Table III, Demmig-Adams (1990)). This capacity seems to involve biosynthetic control of the total pool-size of the xanthophyll-cycle pigments. In general, plants adapted to high-light or so called 'sun-plants' possess a larger total xanthophyll pool size. The opposite is true for 'shade-plants' adapted to low light regimes. In addition, 'shade-plants' can increase the xanthophyll pool size when grown under high-light and \textit{vice versa} for 'sun-plants'. The function of this adaptability is believed to be related to the capacity of the xanthophyll-cycle pigment
zeaxanthin to dissipate excess light-energy. An inverse correlation was drawn between the adapted xanthophyll pool size and the possible accumulation of excess energy which may induce photoinhibitory damage (Thayer and Björkman 1990). The phenomenon of photoinhibition with respect to light adaptability will be discussed in detail below.
HIGH-ENERGY DEPENDENT NONPHOTOCHEMICAL FLUORESCENCE QUENCHING

Decreases of PSII chlorophyll fluorescence at room-temperature in algae and chloroplasts on illumination comprise of two main types of quenching. Fluorescence is quenched photochemically by the oxidized primary electron acceptor of PSII, $Q_A^-$. Photochemical quenching is fast and can be separated kinetically and chemically from a slower type of nonphotochemical quenching that is apparently due to nonradiative dissipation at or before PSII. Addition of DCMU to protoplasts which had developed quenching in the light leads to a rapid reversal of photochemical quenching by inhibiting oxidation of PSII (Horton and Hague 1988). Nonphotochemical quenching relaxes more slowly on addition of DCMU. The photo- and nonphotochemical components can also be separated by use of the so-called ‘light-doubling’ technique developed by Bradbury and Baker (1981). The technique is based on use of an intense flash of light on top of impinging actinic light to transiently and fully reduce $Q_A^-$, thus closing all PSII traps. Any quenching of fluorescence remaining during the flash is then attributed to nonphotochemical quenching.

Early studies of chlorophyll fluorescence quenching in algae and isolated chloroplasts showed that nonphotochemical quenching, is stimulated under conditions conducive to ATP-formation (Murata and Sugahara 1969). High-energy dependent chlorophyll-fluorescence quenching or HEQ is independent of electron-transport through PSII ($Q_A^-$) as evidenced by studies with DCMU poisoned chloroplasts and
algae. Inhibiting oxidation of the primary electron acceptor of PSII or $Q_A$ with DCMU prevents ATP-synthesis and HEQ development (Murata and Sugahara 1969) in the presence of phenazine methosulfate (PMS), which itself mediates PSI cyclic electron-flow. Further, uncouplers of $\Delta pH$, such as nigericin, gramicidin or NH$_4$Cl, inhibit HEQ.

HEQ is linearly correlated with the intrathylakoid proton concentration, measured as quenching of 9-aminoacridine fluorescence (Briantais et al. 1979, 1980). Other studies indicated that HEQ is not due to the protons but rather to cation-dependent conformational changes induced by the lumen acidification (Barber et al. 1974 a,b, Krause 1974). Lumen acidification causes an efflux of Mg$^{2+}$ ions from the lumen into the stromal compartment of the thylakoids (Barber et al. 1974 a,b, Hind et al. 1974, Krause 1974). Evidence for a role of Mg$^{2+}$ in HEQ was based on studies involving osmotically-shocked chloroplasts which had lost a significant portion of their variable fluorescence ($F_V$) along with their ability to undergo HEQ induction (Barber et al. 1974 a,b, Krause 1974). Shocked chloroplasts retain the ability to develop a $\Delta pH$ (Krause 1974). However, recovery of $F_V$ and the capacity to undergo HEQ require addition of Mg$^{2+}$ to the reaction medium followed by a dark $\Delta pH$ relaxation period. Apparently, the $\Delta pH$ must relax in order for Mg$^{2+}$ to re-enter the lumen.

In the presence of A23187, Mg$^{2+}$ increased $F_V$ even when added in the light (Barber et al. 1974 a,b, Telfer and Barber 1978). A23187 did not increase $F_V$ unless Mg$^{2+}$ was present. In all the evidence appeared clear that lumen acidification
induced Mg$^{2+}$ efflux from the lumen which then caused quenching of the variable fluorescence.

The loss of Mg$^{2+}$-dependent PSII variable fluorescence is not due to the same mechanism as NRD and resultant HEQ. The most compelling evidence that HEQ and the "Mg$^{2+}$ effect" were different is based on comparative low-temperature analysis of quenching under energized and uncoupled conditions in the presence and absence of Mg$^{2+}$ (Briantais et al. 1979, Krause et al. 1983b). Apparently, loss of external Mg$^{2+}$ in osmotically shocked chloroplasts causes energy-transfer from PSII to PSI which lowers the PSII fluorescence yield. In contrast, HEQ is not associated with any PSII to PSI transfer (Briantais et al. 1979, 1980, Krause et al. 1983b). Recovery of fluorescence in shocked chloroplasts is primarily due to interaction of the Mg$^{2+}$ with the external surface of the thylakoid membrane and reversal of the PSII to PSI energy transfer.

In contrast, HEQ is believed to involve an exchange of protons for Mg$^{2+}$ at the inner thylakoid surface. Evidence supporting a role of internal Mg$^{2+}$ in HEQ is based on the observation that the Mg$^{2+}$ induced energy spillover is reversed at a lower concentration of Mg$^{2+}$ than was HEQ (Briantais et al. 1979). Presumably, the exchange of protons for Mg$^{2+}$ at the inner thylakoid surface is associated with membrane structural changes which lead to non-radiative dissipation of energy. Evidence for Mg$^{2+}$ dependent conformational changes was based primarily on ΔpH-dependent light scattering changes in thylakoids (Krause 1974). However, the precise relationship of these changes to HEQ remains unclear.
Evidence that HEQ is not due to ΔpH alone was shown with antimycin (Noctor et al. 1990, Oxborough and Horton 1987, 1988). Antimycin, when added prior to HEQ induction, inhibits HEQ without affecting ΔpH induction. Adding antimycin after HEQ induction does not reverse HEQ unless the ΔpH is reversed. Antimycin lowers the ratio of HEQ to lumen $[H^+]$ (Noctor et al. 1990). Apparently, antimycin inhibits the development of a ΔpH-induced change which, once developed, cannot be reversed until the ΔpH is reversed. Antimycin also inhibits ferredoxin-mediated ATP-synthesis, possibly by inhibiting the oxidation of a ferredoxin quinone-reductase (Moss and Bendall 1984). The ferredoxin-quinone reductase has been proposed to play a role in HEQ development (Oxborough and Horton 1988).

**HEQ in Relation to Quantum Yield of PSII.** The quantum yield of photosynthetic $O_2$-evolution or $CO_2$-fixation is normally high under low light, and as photosynthetic capacity becomes saturated the quantum yield normally decreases. With increasing light intensity the redox state of $Q_A$, indicated by a decrease in photochemical fluorescence quenching or $q_Q$ (Schreiber et al., 1986), increases. Accordingly, as the quantum yield of PSII decreases the probability of absorbing excess light energy increases. The oxidation of $Q_A$ and quantum yield of PSII are under ‘photosynthetic’ feedback control. As ΔpH increases, the oxidation of plastoquinone and forward electron flow are restricted thus plastoquinone and $Q_A$ become reduced (Weis et al. 1987, 1990).
There is a close negative correlation between the quantum yield and HEQ (Krause and Laasch 1987, Krause et al. 1988), yet HEQ and quantum yield reduction are apparently independent parallel responses to the ΔpH. Oxborough and Horton (1988) showed that neither the quantum yield of PSII nor ΔpH are significantly affected by antimycin at any given light intensity whereas HEQ is completely inhibited. That HEQ is linearly related to lumen [H+] (Briantais et al. 1979, 1980) serves as further evidence that HEQ is an effect and not the cause of photosynthetic control. In no known case has it been clearly shown that the reduction of quantum yield of PSII photochemistry associated with HEQ is directly related to the NRD which causes HEQ. Unfortunately the ΔpH and HEQ are virtually inseparable under most cases except with antimycin. Nevertheless, it seems reasonable to conclude that the purpose of HEQ is the harmless thermal dissipation of absorbed energy that is in excess due to the decrease in quantum yield caused by photosynthetic control (Weis et al. 1990). Apparently, this thermal dissipation protects the photosynthetic apparatus against over-excitation and photoinhibitory damage (Krause and Behrend 1986, Krause et al. 1988, Oxborough and Horton 1988).

**Proposed role of Zeaxanthin in HEQ.** The formation of zeaxanthin is correlated with HEQ capacity in both leaves (Adams et al. 1990, Demmig-Adams et al. 1990) and chloroplasts (Demmig-Adams et al. 1990, Noctor et al. 1991, Rees et al. 1989). Demmig-Adams (1990) concluded that there were actually two HEQ
components. She proposed that a DTT-insensitive, zeaxanthin-independent or constitutive HEQ component with fast kinetics occurs in the PSII reaction center while a slow component correlated with zeaxanthin quenches in the PSII-antennae. This proposal was based on interpretations of the fluorescence signals according to the PSII fluorescence quenching model of Butler and Kitajima (1975). This model predicts that quenching in the PSII reaction center quenches the maximal ($F_M$) but not the dark level fluorescence ($F_D$) level yield. Quenching in the PSII antenna quenches both $F_M$ and $F_D$. A more detailed analysis of this model is presented below in the discussion of photoinhibition.

Several possible explanations for the apparent correlation between quenching and zeaxanthin have been proposed. Demmig-Adams et al. (1990) proposed that zeaxanthin actively quenches fluorescence, perhaps by creating alternate quenching centers in the light-harvesting pigment bed which compete with the PSII reaction centers for excitation energy. Other researchers proposed that zeaxanthin does not actually quench fluorescence, but rather affects the conformation and or aggregation state of the light-harvesting chlorophyll pigment-protein complexes thus stimulating HEQ (Noctor et al. 1991, Rees et al. 1989, Ruban et al. 1991). In any case, the presence of zeaxanthin does not obligate quenching unless a $\Delta \rho H$ is present or the zeaxanthin is formed under conditions conducive to photoinhibition. In the latter case, high-light stress (Bilger and Björkman 1990, Demmig-Adams et al. 1989, Demmig et al. 1987, Demmig et al. 1988), water stress (Demmig et al. 1988), and temperature stress (Bilger and Björkman 1991, Demmig-Adams et al. 1989) are
believed to somehow allow the ΔpH-induced conformational change which causes HEQ to be sustained after reversal of the ΔpH. Exactly how this conformational change remains induced in the absence of a ΔpH is still unknown. This slowly relaxing zeaxanthin-quenching is believed to have a dark-sustained, long-term protective function in vivo under conditions which limit CO₂-fixation (Demmig-Adams 1990).

Recent in vitro evidence suggests that during illumination under conditions of inhibited CO₂-fixation, linear electron-transport, ΔpH (Asada and Takahashi 1987), and zeaxanthin formation can be facilitated by a Mehler-Peroxidase (MP) reaction (Neubauer and Yamamoto 1992). This MP reaction dissipates light energy at PSII both photochemically through electron-flow and non-photochemically through HEQ. The reaction is O₂-dependent. The O₂-dependency can be circumvented by adding exogenous H₂O₂ which stimulates linear electron-flow. Neubauer and Yamamoto (1992) suggest that in vivo ‘pseudocyclic’ electron flow may allow electron-flow and ΔpH to continue under CO₂-limiting conditions where H₂O₂ forms via the Mehler-Reaction. The MP-reaction may have an additional protective function in detoxifying potentially destructive H₂O₂ (Asada and Takahashi 1987).
PHOTOINHIBITION OF PHOTOSYNTHESIS

**Damage or Protection?** Photoinhibition is usually defined symptomatically as a decreased photon yield or quantum yield of photosynthesis (Björkman 1987), because the mechanism is poorly understood. In general, there appear to be two types of photoinhibition, protective and destructive. Protective photoinhibition is believed to be due to nonradiative energy-dissipation in the pigment bed. This may protect the reaction centers from damage due to overexcitation (Björkman 1987). Destructive photoinhibition is believed to be due to overexcitation of the reaction centers that results in reaction center inactivation (Cleland et al. 1986, Guenther and Melis 1990, Kyle 1987). Conditions which stimulate photoinhibitory responses are almost any stress that lowers a plant's capacity to dissipate absorbed light energy through photosynthesis, i.e. high-light, water-deficit, salinity or combinations of these and other stress factors (for a general review, see Powles 1984). In both cases a common symptom is a decreased photon or quantum yield of photosynthesis (Björkman 1987). Additionally, in neither type of photoinhibition is there presumed to be any net loss of chlorophyll or carotenoid pigment due to photobleaching (Powles 1984).

**Quantum Yield, Fluorescence Quenching, and Photoinhibition.** Quantum yield depression is usually measured with gas exchange, i.e. CO₂ uptake or O₂ evolution or as *in vitro* measurements of quantum yields of electron-transport with isolated chloroplasts. Non-invasive chlorophyll fluorescence measuring techniques
have been applied extensively and in many cases have been used to differentiate between the two types of photoinhibition. The basic premise behind use of fluorescence parameters is that photoinhibition reflects an increased amount of absorbed light-energy being diverted away from photosynthesis. Nonphotochemical chlorophyll fluorescence quenching at both room temperature (Krause and Behrend 1986) and at 77K (Björkman 1987) is believed to reflect energy dissipation and thus be a good indicator of photoinhibition. However, interpretation of fluorescence measurements is complex, especially in vivo where a myriad of factors affect photosynthetic and nonphotochemical energy dissipation.

The most widely applied model of fluorescence quenching in photoinhibition studies is the bipartite model proposed by Butler and Kitajima (1975). The model assumes that PSII photochemistry, non-radiative dissipation of energy in the PSII reaction centers, non-radiative dissipation of energy in the PSII antenna chlorophylls, transfer of energy from PSII to PSI, and radiative dissipation of energy as PSII chlorophyll fluorescence are competing first order processes. As mentioned earlier, the basis for interpretation of both protective and damaging photoinhibition is a decrease in the quantum yield of photosynthesis. According to the Butler-Kitajima model, the quantum yield of PSII photochemistry is defined by the equation:

\[
\phi_{PC} = \frac{k_p}{k_p + k_d + k_D + k_T + k_F}
\]  

(1)
where \( k_p \) is the rate constant for photochemistry, \( k_d \) is the rate constant for nonradiative dissipation in the PSII reaction center, \( k_D \) is the rate constant for nonradiative dissipation in the PSII antennae complexes, \( k_T \) is the rate constant of energy transfer form PSII to PSI, and \( k_F \) is the rate constant for fluorescence. The definition for the quantum yield of the dark level of fluorescence (\( F_0 \)) with all PSII traps open is defined as,

\[
\phi_{F_0} = \frac{k_F}{k_F + k_p + k_T + k_D}
\]

(2)

where \( k_F \) is the rate constant for PSII fluorescence. Because all \( F_0 \) emanates from the bulk chlorophyll or antennae complexes no dissipation occurs in the reaction center so \( k_d \) is not a factor in the \( \phi_{F_0} \). The quantum yield of maximal fluorescence \( F_M \) with all PSII traps closed (thus in the absence of any photochemistry or \( k_p \)) is defined by the following equation:

\[
\phi_{F_M} = \frac{k_F}{k_F + k_d + k_T + k_D}
\]

(3)

It follows that \( \phi_{PC} \) can be calculated from \( F_M \) and \( F_0 \) based on equations 1, 2, and 3 where:

\[
\phi_{PC} = \frac{F_M - F_0}{F_M} = \frac{F_V}{F_M}
\]

(4)
Since $F_V = F_M - F_O$, anything that quenches $F_M$ or increases $F_O$ lowers $F_V/F_M$. Therefore it follows that non-radiative dissipation in the pigment bed ($k_D$) quenches both $F_M$ and $F_O$ proportionally, with $F_M$ being quenched to a larger extent than $F_O$. Dissipation in the PSII reaction center ($k_d$) quenches $F_M$ but not $F_O$ and damage to PSII or a decrease in photochemistry ($k_p$) increases $F_O$.

Although, this simple model seems ideal for interpretation of the two types of PSII-associated non-radiative dissipation, namely $k_D$ and $k_d$, it requires the assumption of several criteria for $F_O$. First, $F_O$ is defined as the fluorescence level when all PSII traps are open, i.e. $Q_A$ is completely oxidized, and all $\Delta pH$ and HEQ is relaxed (van Kooten and Snel 1990). The $F_O$ signal in the presence of $\Delta pH$ or HEQ is defined as $F'_O$ and further assumes that $Q_A$ is completely oxidized. In vivo, it can be difficult to oxidize $Q_A$ in the presence of a $\Delta pH$, due to the inhibited oxidation of plastoquinone as well as the possibility for reverse electron-flow (Avron and Schreiber 1977). Importantly, selective illumination of PSI with far-red light will stimulate plastoquinone and $Q_A$ oxidation. In vitro, $Q_A$ is readily oxidized by the use of powerful electron acceptors and or far-red light.

Evidence for a protective role of zeaxanthin under photoinhibitory conditions was gained in in vivo studies with DTT-infused leaves (Bilger and Björkman 1990, Winter and König 1989). Inhibition of zeaxanthin formation led to destructive photoinhibition. Destructive photoinhibition is normally seen as an increased $F_O$ and decreased $F_V$. While it seems reasonable to attribute the increase in $F_O$ to PSII damage or a decrease in $k_p$, in most cases the mechanism of the $F_V$ quenching during
destructive photoinhibition has not been confirmed. Kyle (1987) suggested that quenching of $F_v$ during destructive photoinhibition could be by any of three mechanisms: 1) formation of a futile cycle around PSII possibly mediated by Cytochrome $b_{559}$ or $Q_A$ donation to $P_{680}^+$, thus being equivalent to a photochemical dissipation, 2) inhibition of the donor side of PSII thus inhibiting reduction of $Q_A$ or 3) simultaneous pigment bed quenching and PSII damage thus quenching $F_M$ and increasing $F_O$. Several researchers report evidence of a futile PSII cycle possibly mediated by Cytochrome $b_{559}$ (Falkowski et al. 1988, Horton and Lee 1985, Schreiber and Rienits 1987) but the role of such a cycle *in vivo* has not been confirmed. Based on the observation that $F_v$ cannot be recovered by artificial electron-donation to PSII it is concluded that inhibition of the donor side of PSII does not cause $F_v$ quenching (Critchley 1981). In sum, it seems most likely that both pigment-bed quenching ($k_D$-increase) and reaction center damage ($k_P$-decrease) overlap so that $F_M$ is quenched and $F_O$ increases (Björkman 1987).

**Damage and Repair of the $Q_B$-binding Protein.** Overexcitation of the reaction centers first inhibits quinone reduction then apparently damages the 32kD $Q_B$-binding protein which mediates electron transport between $Q_A$ and plastoquinone (Baker 1991, Kyle 1987). There are two hypothetical mechanisms for damage of the $Q_B$-binding protein and both are believed to occur at the quinone binding site. One involves a semiquinone radical and the other action of molecular oxygen ($O_2$). The $O_2$ is believed to competitively inhibit the quinone binding site. In both cases, namely
semiquinone or $O_2$, reduced $Q_A$ may lead to formation of hydroxyl radicals which could oxidize important histidine residues in the $Q_B$-binding pocket. To date $Q_B$-protein damage has been observed only under \textit{in vitro} conditions and any \textit{in vivo} physiological significance has not been ascertained (Baker 1991).

The basis of the slow reversal of destructive photoinhibition associated with $Q_B$ protein damage is presumably the slow turnover of the protein (Guenther and Melis 1990). Repair of photodamaged reaction centers is proposed to involve a cycle wherein damaged reaction centers, migrate to the stromal lamellae where the damaged D1 proteins are degraded and newly synthesized proteins are inserted. The reaction centers in the non-appressed region are termed beta-centers (PSII$_B$) or $Q_B$-nonreducing centers. Once repaired, the now $Q_B$-reducing or PSII$_\alpha$ centers, return to service in appressed regions of the thylakoid.

\textbf{Adaptation to Avoid Photoinhibition.} Early on a parallel was noted on between a plants' adaptation to a given light regime and its susceptibility to both protective and destructive photoinhibition. For reviews of adaptation to light and photoinhibition, see Anderson and Osmond (1987), Björkman (1987), and Powles (1984). In general, plants phenotypically adapted to high light regimes are less susceptible to photoinhibitory damage. In contrast, low light adapted plants are more prone to photoinhibitory damage. In addition to the differential susceptibilities to photoinhibition, sun and shade plants also possess different capacities to adapt to incident light environments. Heliophilic plants can normally adapt to growth in a low
light regime. In contrast, umbrophilic plants cannot readily adapt to growth in a high light regime, and are prone to chronic photoinhibition. Thayer and Björkman (1990) noted that this pattern of adaptability to photoinhibition is similar to the adaptability of plants with regard to the violaxanthin-cycle pool components. This does not mean that adaptation to light is solely centered around the violaxanthin-cycle pigments. Light-regimes also have profound effects on levels of light-harvesting pigment-protein complexes, synthesis of electron-transport components, ribulose-bisphosphate carboxylase, and ATPase (Anderson and Osmond 1987). Nevertheless, there is a general trend of adaption to high-light which seems to center around the capacity to dissipate the excess light (Thayer and Björkman 1990). To this end, the violaxanthin appears to play a major role in a plants adaptation to light conditions.
ATP AND PSII FLUORESCENCE

Based on several different observations it is generally believed that ATP does not play a significant role in fluorescence quenching in vivo under high-energy conditions or under dark-adapted conditions (Foyer et al. 1990, Horton 1990). Several reports indicate that addition of ATP to coupled chloroplasts in the light has virtually no effect on the fluorescence quenching (Bennet et al. 1980, Horton and Black 1981, Krause and Behrend 1983). Recently, it was proposed that in vivo ATP levels are regulated by modulation of the ATP-consuming enzymes of the Benson-Calvin cycle such that formation of ATP does not exceed the capacity of CO₂-fixation (Foyer et al. 1990, Horton 1990). Dark-sustained accumulation of ATP is not believed to occur to any significant extent.

The role of ATP in protection against photoinhibition is also controversial. The same early in vitro studies which discounted ATP in HEQ concluded that, in uncoupled chloroplasts with reduced plastoquinone, ATP phosphorylated the light-harvesting chlorophyll pigment-protein complexes (LHCP's) (Bennet et al. 1980, Horton and Black 1981, Krause and Behrend 1983). This LHC phosphorylation increases the flow of energy from PSII to photosystem I (PSI) in a so-called state 1-state 2 transition. Fₘ and F₀ fluorescence are quenched in proportion, however, this quenching is maximally only 20 to 30 % of the dark-adapted Fᵥ (Horton and Black 1981) and thus much smaller than HEQ which can quench over 100 % of the Fᵥ (Bilger and Björkman 1990). In fact, Krause et al. (1983) reported that HEQ probably masks the ATP-dependent quenching. The LHC phosphorylation and
subsequent diversion of energy from PSII is suggested to protect against photoinhibition (Horton and Lee 1985). However, other researchers found that photoinhibitory treatments led to a de-phosphorylation of the LHC and that phosphorylation of the LHC is not correlated with the fluorescence transients associated with reversible protective photoinhibition (Demmig et al. 1987a).

Despite the fact that ATP-hydrolysis and ΔpH-formation are well defined phenomenon in vitro it is generally believed that they are not factors in dark-sustained fluorescence transients in vivo (Foyer et al. 1990, Horton 1990). In contrast to HEQ, ATP-hydrolysis ΔpH can drive reverse electron-flow and increase $F_V$ in isolated chloroplasts (Avron and Schreiber 1977). This $F_V$-increase is due to a reduction of $Q_A$ due to the reverse electron-flow driven by ΔpH from ATP-hydrolysis. Reverse electron-flow and fluorescence increases were stimulated by the presence of artificial electron donors and carriers such as DTT and PMS respectively (Schreiber 1980, Schreiber 1984). Accordingly, the $F_V$ rises were inhibited by agents which oxidized the electron-transport chain, and reversed by uncouplers which dissipated the ATP-induced ΔpH. Because normally no such dark-sustained $F_V$ increases are observed in vivo, their possible in vivo significance is unclear and it may be assumed that such inhibition of forward electron-flow would increase the possibility of destructive photoinhibition.
HYPOTHESES

1). There are two kinetically distinct HEQ components with differential sensitivities to inhibitors of violaxanthin de-epoxidation.

2). Antimycin inhibits both HEQ components by inhibiting a ΔpH-dependent change.

3). Dark-sustained ATP-induced ΔpH can induce xanthophyll-dependent HEQ of both $F_M$ and $F_O$ similar to light-induced ΔpH.

4). Antheraxanthin accounts for any HEQ which is not due to zeaxanthin. Xanthophyll-dependent HEQ can be described with a simple linear equation assuming the product of (lumen $[H^+])$(antheraxanthin plus zeaxanthin) determines HEQ.
EXPERIMENTAL METHODS AND DESIGN

Chloroplast Isolation. Chloroplasts of *Pisum sativum* L. cv. Oregon or cv. Manoa or *Lactuca sativa* L. cv. Romaine and were isolated according to Horton and Black (1981) with modifications as described below. The pea plants were dark-adapted at room-temperature for at least 12 h before harvesting to reduce the background levels of zeaxanthin and antheraxanthin. The market lettuce was obtained locally and the heads were stored in darkness at 4°C for at least 12 h to reduce the background levels of zeaxanthin and antheraxanthin. Prior to chloroplast isolation the lettuce leaves were washed, deveined, and chilled on ice under darkness for at least 1 h; the pea leaves were also chilled in the dark on ice for at least 1 h. All chloroplast isolation was at 4°C and under dim laboratory light. Pea leaves or lettuce leaves (12 to 15 g) were ground, using three 3 to 5 s bursts, in a chilled Waring Microblendor with 50 ml grinding buffer containing 0.33 M glucose, 50 mM Na₂HPO₄, 50 mM KH₂PO₄, 25 mM KCl, 5 mM MgCl₂, 0.2 % Na-ascorbate, and 0.1 % BSA, adjusted to pH 6.5 with NaOH. The resulting brie was filtered through 37 μm nylon mesh and centrifuged at 1500g for 2 min. The pellet was rinsed once with 50 ml of rinsing buffer containing 0.33 M glucose, 5 mM MgCl₂, 26 mM Sorbitol, and 4 mM HEPES, adjusted to pH 7.6 with NaOH. The pellet was suspended in approximately 1.5 ml resuspension buffer containing 0.33 M sorbitol, 4 mM EDTA, 5 mM MgCl₂, 2 mM MnCl₂, 100 mM HEPES, and 0.1 % BSA, adjusted to pH 7.6 with NaOH. The reaction mixture of Horton and Black (1981) contained 0.1 M sucrose, 10 mM NaCl, 5 mM MgCl₂, and 10 mM tricine, adjusted to pH 7.8 with
NaOH and was, unless otherwise stated, the reaction mixture used. Broken chloroplasts were obtained by diluting the chloroplast suspension in distilled water in a 1 to 10 volume/volume ratio for 15 to 20 s. After osmotic-shock, the reaction was brought to a final volume of 3 ml with reaction medium. Chlorophyll determination was according to Vernon (1960). The final total chlorophyll concentration was 30 μg ml⁻¹ for all reactions, unless otherwise stated.

Simultaneous Measurement of Absorbance and Room-temperature Chlorophyll Fluorescence. Room-temperature chlorophyll-fluorescence induction (Schreiber et al. 1986) and the 505-540 nm absorbance change associated with zeaxanthin formation (Yamamoto et al. 1972) were measured simultaneously with a PAM 101 Chlorophyll Fluorometer (Heinz Walz, Effeltrich, FRG) and a DW-2000 UV-VIS spectrophotometer (SLM-Aminco), respectively, see Fig. 1.3. A Unitron microscope lamp and KL-1500 flash unit (Heinz Walz, Effeltrich, FRG) provided actinic light and saturating flashes, respectively. Both light sources were filtered through Corning CS2-58 (red) and CS1-75 (infrared) filters. Actinic light and saturating flash intensities were measured at the terminal surface of the fiber optic probe. A Corning CS4-96 filter protected the DW2000 photomultiplier tube from the actinic light and saturating flashes. Opal glass, a quartz diffuser plate and a beam scrambler between the cuvette and the photomultiplier tube reduced light-scattering effects. All reactions were at 20°C, unless otherwise stated, and stirred continuously.
Figure 1.3. Simultaneous acquisition of absorbance and room-temperature chlorophyll-fluorescence. The PAM fiber-optic probe is placed in the sample compartment of the DW2000 UV-Vis spectrophotometer.
All quantitative fluorescence data interpretation relating to de-epoxidation and lumen acidification was according to a Stern-Volmer (SV)-type analysis (Bilger and Björkman 1990), unless otherwise stated. The SV-type expressions are directly proportional to fluorescence quencher concentrations and SV-type calculations do not require knowing, assuming, or calculating $F_O$ under conditions of quenching where $Q_A$ is assumedly not oxidized. The SV-type measurements along with the simultaneous absorbance acquisitions allowed us to determine the possible concentration dependent relationships between violaxanthin de-epoxidation and lumen acidification. The $\Delta A_{505.540}$ (Yamamoto et al. 1972) and $\Delta A_{520}$ (Siefermann-Harms 1978) readings can, based on the Beer-Lambert relationship, be used as relative indicators of the concentrations of zeaxanthin and lumen protons relative to chlorophyll.

The standard nomenclature for Stern-Volmer (SV) treatments of chlorophyll fluorescence used throughout the following chapters are defined as follows: $SV_N = (F_M / F'_M) - 1$ and $SV_O = (F_O / F'_O) - 1$ for non-photochemical quenching of $F_M$ and $F_O$, respectively. The $SV_N$ and $SV_O$ calculations and nomenclature substitute for the quenching coefficient expressions $q_N$ and $q_O$ developed by Bilger and Schreiber (1986). The fluorescence-intensity indicators are defined by van Kooten and Snel (1990).

**HPLC Chloroplast Pigment Analysis.** Unless stated otherwise HPLC pigment analysis was according to Chapter 4.
CHAPTER 2

ZEAXANThIN-FORMATION IN qE-INHIBITED CHLOROPLASTS

INTRODUCTION

Non-photochemical fluorescence quenching, $q_E$, appears to have several components (Demmig and Winter 1988). A slowly relaxing component has been correlated with zeaxanthin formation (Demmig-Adams et al. 1989). Development of $q_E$ and zeaxanthin formation are both dependent on formation of a trans-thylakoid $\Delta p\text{H}$ (Oxborough and Horton 1986), the relationship between $q_E$ and zeaxanthin was investigated with antimycin. We show herein that ascorbate, which is required for zeaxanthin formation in isolated chloroplasts (Yamamoto 1979), stimulated induction of a $q_E$ component possibly related to zeaxanthin-formation. However, antimycin inhibited this $q_E$ without inhibiting zeaxanthin formation.
MATERIALS AND METHODS

Osmotically shocked chloroplasts were isolated from *Pisum sativum* L. cv. Manoa seedlings according to Chapter 1. Chloroplast concentration for all reactions was equivalent to 15 μg total chlorophyll ml\(^{-1}\) unless otherwise stated.

Absorbance and room-temperature chlorophyll fluorescence induction were measured simultaneously also according to Chapter 1 except actinic light intensity was 350 μmol photons m\(^{-2}\) s\(^{-1}\) unless stated otherwise. Saturating flash intensity was approximately 1600 μmol photons m\(^{-2}\) s\(^{-1}\). Calculation of fluorescence values was according to Schreiber et al. (1986). HPLC pigment analysis was according to Siefermann-Harms (1988). Xanthophyll concentrations were expressed relative to the total area of (violaxanthin + antheraxanthin + zeaxanthin).
RESULTS AND DISCUSSION

Figure 2.1 shows typical (A) $q_E$ induction and (B) 505 nm change under MV-mediated linear electron transport in the presence and absence of ascorbate. Ascorbate enhanced $q_E$ above a large no-ascorbate 'basal' level and stimulated zeaxanthin formation as indicated by a large irreversible 505 nm absorbance change. The 505 nm change without ascorbate was small, reversible, and presumed to be from light-scattering and not de-epoxidation; pigment analysis confirmed that no de-epoxidation occurred under these conditions. Figure 2.1 also shows that in the presence of ascorbate the kinetics of de-epoxidation and total $q_E$ differed significantly.

Figure 2.2 shows the effects of ascorbate concentration on the final extent of $q_E$ and xanthophyll composition after illumination under (A) linear and (B) cyclic electron-transport conditions. In the absence of ascorbate the basal $q_E$ was about 0.30 but no de-epoxidation was evident under either electron-transport condition. In the presence of ascorbate, the extent of the additional ascorbate-stimulated $q_E$ and de-epoxidation appeared to correlate under both conditions, although more closely under linear electron-transport. Development of $q_E$ and changes in the violaxanthin cycle pigments leveled off at 5 mM and 15 mM ascorbate for linear and cyclic conditions, respectively.
Figure 2.1. (A) $q_E$ induction curves for osmotically-shocked pea chloroplasts in the absence (○) and presence (●) of ascorbate, under linear electron-transport. (B) 505-nm change kinetics acquired simultaneously with fluorescence induction curves in (A). Reagent concentrations were 30 μg total chlorophyll ml$^{-1}$, 60 mM ascorbate when present and 0.1 mM MV.
Figure 2.2. The effects of ascorbate concentration on extent of de-epoxidation and $q_E$ development after 9 min illumination under (A) linear and (B) cyclic electron-transport. Abbreviations are (V) violaxanthin, (Z) zeaxanthin, and (A) antheraxanthin.
Figure 2.3 shows that antimycin inhibited the development of ascorbate-enhanced \( q_E \) under linear (A) or cyclic (B) electron-transport conditions. Although the inhibition was strong, it was incomplete. In the presence of antimycin, a small amount of \( q_E \) developed slowly. The slow kinetics and resistance to antimycin suggest that resistant \( q_E \) has a different origin than antimycin-sensitive \( q_E \). At 1 \( \mu M \) antimycin, resistant \( q_E \) was also resistant to complete uncoupling by nigericin, thus confirming that the antimycin resistant \( q_E \) is not directly related to antimycin sensitive/nigericin sensitive \( q_E \). Addition of antimycin during illumination led to slight reversals of previously developed \( q_E \) for both the linear and cyclic systems. The nature of these reversals is unclear; they could be a direct effect of antimycin on the \( q_E \)-inhibiting target site.

Table 2.1 summarizes experiments on the relationship between \( q_E \) and zeaxanthin formation under various conditions. Experiment 1 shows that under MV-mediated linear electron-transport, antimycin inhibited both basal and ascorbate-enhanced \( q_E \) but had no effect on ascorbate-enhanced de-epoxidation. Also no zeaxanthin was present or formed in the absence of ascorbate so involvement of zeaxanthin in basal \( q_E \) can be excluded. The possibility that the antimycin-resistant \( q_E \) is related to zeaxanthin-formation is unlikely inasmuch as the levels observed were similar in the presence and absence of de-epoxidation. Experiment 2 shows that DBMIB inhibited both \( q_E \) and zeaxanthin-formation. The addition of PMS restored zeaxanthin formation completely and \( q_E \) to about the basal level under linear electron flow in Experiment 1. Experiment 3 shows that while antimycin inhibited \( q_E \)
development under PMS-mediated cyclic electron-transport, zeaxanthin formation was not inhibited. Because violaxanthin de-epoxidase requires an acidic lumen for activity (Yamamoto 1979), it can serve as an endogenous pH probe. Formation of zeaxanthin in the presence of antimycin in this experiment and in Exp. 1 indicates that antimycin does not inhibit ΔpH formation. This agrees with Oxborough and Horton (1987).

Overall these experiments suggest that qE induction comprises three components, namely, (I) the basal component that is independent of ascorbate and zeaxanthin, (II) the ascorbate induced component possibly related to zeaxanthin and, (III) a small component that is apparently resistant to antimycin. All three components can be mediated by linear or cyclic electron transport. Since antimycin inhibits component (II) but not zeaxanthin formation, ΔpH plus zeaxanthin formation do not obligate qE development.

Since qE develops under both MV-mediated and PMS-cyclic mediated electron-transport, qE appears to be a PSI activity that is expressed as PSII fluorescence quenching. Oxborough and Horton (1986) have suggested that the antimycin sensitive site could be a cyclic electron-transport component that is independent of the linear electron-transport pathway. Cytochrome b563, b559, and a ‘ferredoxin quinone reductase’ (FQR) were considered as possibilities. Although these electron-transport components are not directly involved in the PMS-ascorbate mediated cyclic electron path, their involvement in redox control of qE cannot be ruled out since their redox states may be indirectly affected under these conditions.
Figure 2.3. The effects of 1 \( \mu \text{M} \) antimycin on \( q_E \) when added prior to and during illumination under (A) linear and (B) cyclic electron-transport conditions.
The mechanism of the putative zeaxanthin-mediated quenching is as yet unexplained. Perhaps the ascorbate-enhanced q_E develops as a consequence of zeaxanthin forming a pigment-pigment or pigment-protein complex which interacts with PSII. Antimycin's inhibitory effect on ascorbate-enhanced q_E may be explained as a direct or indirect block on zeaxanthin complex formation. Zeaxanthin formation and q_E development could also be completely independent phenomena that coincidently respond to the redox state of the electron transport components. Indeed, the availability of violaxanthin for de-epoxidation has been related to a redox component near plastoquinone (Yamamoto 1979).

The mechanism of basal and ascorbate-enhanced q_E may be similar based on their mutual sensitivity to antimycin. If the ascorbate-enhanced component is due to zeaxanthin, perhaps basal q_E is mediated by another carotenoid in chloroplasts. While only zeaxanthin can as yet be excluded, lutein or \( \beta,\beta \)-carotene appear to be likely candidates for basal q_E from their molecular structure and location in the photosynthetic apparatus. Both lutein and \( \beta,\beta \)-carotene are associated with PSI and PSII pigment-protein complexes (Siefermann-Harms 1985). However \( \beta,\beta \)-carotene is the primary carotenoid in the reaction center of PSII (Searle and Wessels 1978, Siefermann-Harms 1985) and may be an alternative source of zeaxanthin aside from violaxanthin (Demmig 1988). Lutein is the \( \alpha \)-carotene analog of zeaxanthin and differs structurally from zeaxanthin only in the presence of one double bond. It is the major xanthophyll in chloroplasts and is primarily associated with the light harvesting proteins, especially the Chl \( a/b \)-Lutein proteins (Siefermann-Harms 1985). Thus q_E
development could be a consequence of β,β-carotene or lutein acting sequentially and additively with zeaxanthin at PSII to increase rates of non-radiative dissipation of energy. This activity requires a ΔpH and possibly redox or conformational changes.
Table 2.1. Effects of ascorbate, antimycin, DBMIB, and PMS on extent of de-epoxidation and $q_E$.

<table>
<thead>
<tr>
<th>EXPT.</th>
<th>CONDITION</th>
<th>ANTI-A</th>
<th>$q_E$</th>
<th>V</th>
<th>A</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control, No Light</td>
<td>---</td>
<td>0.96</td>
<td>0.04</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No Asc, MV</td>
<td>-</td>
<td>0.49</td>
<td>0.97</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>No Asc, MV</td>
<td>+</td>
<td>0.15</td>
<td>0.96</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Asc, MV</td>
<td>-</td>
<td>0.75</td>
<td>0.69</td>
<td>0.14</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Asc, MV</td>
<td>+</td>
<td>0.21</td>
<td>0.66</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>2.</td>
<td>Control, No Light</td>
<td>---</td>
<td>0.97</td>
<td>0.03</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asc, DBMIB</td>
<td>-</td>
<td>0.05</td>
<td>0.97</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Asc, DBMIB, PMS</td>
<td>-</td>
<td>0.44</td>
<td>0.70</td>
<td>0.13</td>
<td>0.17</td>
</tr>
<tr>
<td>3.</td>
<td>Control, No Light</td>
<td>---</td>
<td>0.96</td>
<td>0.04</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asc, DBMIB, PMS</td>
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<td>0.61</td>
<td>0.16</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Asc, DBMIB, PMS</td>
<td>+</td>
<td>0.14</td>
<td>0.63</td>
<td>0.15</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Ascorbate concentration was 60 mM in Expts. 1 and 2 and 30 mM in Expt. 3. Illumination was for 9 min in Expts. 1 and 2 and 500 μmol photons m$^{-2}$ s$^{-1}$ for 5 minutes in Expt. 2. When present final reagent concentrations were 1 μM antimycin (Anti-A), 2 μM DBMIB, 50 μM MV, and 1 μM PMS. Abbreviations: A, antheraxanthin; V, violaxanthin; Z, zeaxanthin.
CHAPTER 3

ZEAXANTHIN FORMATION AND ENERGY-DEPENDENT FLUORESCENCE QUENCHING IN PEA CHLOROPLASTS UNDER ARTIFICIALLY MEDIATED LINEAR AND CYCLIC ELECTRON TRANSPORT

The contents of this chapter are presented as published. See ref. Gilmore AM and Yamamoto HY (1991) Zeaxanthin formation and energy-dependent fluorescence quenching in pea chloroplasts under artificially mediated linear and cyclic electron transport. Plant Physiol 96:635-643
ABSTRACT

Artificially mediated linear (methylviologen) and cyclic (phenazine methosulfate) electron transport induced zeaxanthin-dependent and independent (constitutive) non-photochemical quenching in osmotically-shocked chloroplasts of Pisum sativum L. cv. Oregon. Non-photochemical quenching treated as Stern-Volmer quenching (SV_N) and calculated as (**F_M / F'_M**) - 1 allowed quantitation of the zeaxanthin dependency. Reversal of quenching by nigericin and electron-transport inhibitors showed that both were ΔpH-dependent SV_N, namely SV_E. Under light-induced saturating ΔpH, constitutive-SV_E reached steady state in about one minute whereas zeaxanthin-SV_E continued to develop for several minutes in parallel with the slow kinetics of violaxanthin de-epoxidation. SV_E and relative zeaxanthin concentration showed high linear correlations at steady state and during induction. Furthermore, F_O quenching treated as Stern-Volmer quenching (SV_O) and calculated as (**F_O / F'_O**) - 1 also showed high correlation with zeaxanthin and consequently with SV_E. These results support the view that zeaxanthin increases SV_E in a concentration-dependent manner and that zeaxanthin-dependent SV_E occurs in the pigment bed. Preforming zeaxanthin increased the rate and extent of SV_E indicating that slow events other than the amount of zeaxanthin also affect final zeaxanthin-SV_E expression. The redox state of Q_A did not appear to determine SV_E. Antimycin, when added while chloroplasts were in a dark-adapted or non-energized state, inhibited both zeaxanthin-SV_E and constitutive-SV_E induced by linear and cyclic electron transport. Neither neutral red uptake (ΔpH) nor zeaxanthin formation were
affected by antimycin. These results suggest zeaxanthin-$SV_E$ and constitutive-$SV_E$ are mechanistically similar and the antimycin target is an activity or component common to both electron-transport systems.
INTRODUCTION

Light energy that is not used photosynthetically is dissipated radiatively as fluorescence or non-radiatively as heat. Non-radiative energy dissipation at PSII is thought to serve a protective function against excess light and appears to be under photosynthetic control (Bilger and Björkman 1990, Krause and Behrend 1986, Weis and Berry 1987). Non-radiative dissipation of excitation energy at PSII is seen experimentally as a component of the non-photochemical fluorescence-quenching coefficient, $q_N$ (Schreiber et al. 1986). The light-doubling technique (Bradbury and Baker 1981) and advances in instrumentation (Schreiber et al. 1986) have enabled resolution of various $q_N$ components, see Krause (1988) for a review. The major component is $\Delta pH$ or energy-dependent fluorescence quenching, $q_E$ (Krause et al. 1982) which also appears to depend on the redox state of a membrane component (Oxborough and Horton 1988). Recently, $q_E$ has been related to "down regulation" of photochemistry at PSII (Weis and Berry 1987).

The mechanism for $q_E$ is unclear. Exchange of protons for Mg$^{2+}$ (Mills and Barber 1975), conversion of PSII from fluorescent to non-fluorescent forms (Weis and Berry 1987), and zeaxanthin formation have been implicated (Demmig-Adams et al. 1990). Zeaxanthin is formed from violaxanthin (Yamamoto et al. 1962) by action of violaxanthin de-epoxidase whose activity in part requires an acidified lumen (Hager 1969). Depending on treatment, zeaxanthin formation results in increased irreversible or reversible $q_N$ (Demmig-Adams et al. 1990, Demmig et al. 1988). Zeaxanthin-dependent $q_N$ is believed to play a role in photoprotection (Bilger and
Demmig et al. (1988) suggested that irreversible zeaxanthin-dependent \( q_N \) is related to photoinhibition and reversible zeaxanthin-dependent \( q_N \) is believed to be related to \( q_E \) (Demmig-Adams et al. 1990, Chapter 2).

Whether \( q_E \) is comprised of more than one component is controversial. The results of several laboratories support the view that zeaxanthin-dependent \( q_E \) adds to an underlying zeaxanthin-independent \( q_N \) (Bilger and Björkman 1990, Demmig-Adams et al. 1990, Chapter 2). Other researchers conclude instead that zeaxanthin sensitizes \( q_E \) to \( \Delta pH \) and that under saturating \( \Delta pH \), zeaxanthin does not increase total \( q_N \) (Noctor et al. 1990). Both \( q_N \) (Bilger and Schreiber 1986) and zeaxanthin-dependent \( q_E \) (Bilger and Björkman 1990, Demmig-Adams et al. 1990) have been correlated with \( F_O \) quenching \( (q_O) \) which suggests, according to the Butler-Kitajima model (Butler and Kitajima 1975), that quenching occurs in the pigment-bed. However, where zeaxanthin dependent and constitutive \( q_N \) are thought to be separate activities, zeaxanthin-independent quenching is proposed to quench in the reaction center (Adams et al. 1990, Demmig-Adams et al. 1990).

Here the relationships between non-photochemical quenching and zeaxanthin formation in osmotically-shocked pea chloroplasts under artificial electron transport are reported. We used Stern-Volmer data treatment of non-photochemical quenching, (Bilger and Björkman 1990), because it allowed quantitation of concentration-dependent quenching. Zeaxanthin formation directly increased energy-dependent quenching above an underlying zeaxanthin-independent quenching at saturating \( \Delta pH \). Expressed as Stern-Volmer quenching, zeaxanthin-dependent
quenching of $F_M$ and $F_O$ was proportional, consistent with pigment-bed quenching. We also show that zeaxanthin-dependent and independent non-photochemical quenching, the latter hereafter referred to as constitutive quenching, have several common properties, suggesting that these two types of energy-dependent quenching are mechanistically related.
MATERIALS AND METHODS

Chloroplast Isolation and Simultaneous Absorbance and Fluorescence Acquisition was according to Chapter 1. Zeaxanthin concentrations were determined relative to Chl a by a new HPLC method described in Chapter 4.

Stern-Volmer Type (SV-type) Analysis. In a conventional SV plot, absolute quencher concentration is plotted against the SV expression, the resulting slope being the SV-quenching constant. Here, the plots of zeaxanthin concentration and $SV_N$ are SV-type rather than classical SV plots since zeaxanthin concentration is expressed relative to Chl a. The absolute concentration of zeaxanthin in the membrane cannot be readily determined. Zeaxanthin-dependent $SV_N$ can be resolved therefore as $\Delta SV_N$, calculated as $SV_N$ with zeaxanthin formed *minus* $SV_N$ with zeaxanthin formation completely inhibited (≥1 mM DTT) unless otherwise stated. $\Delta SV_O$ can be resolved in a similar way as $\Delta SV_N$. Photochemical quenching, $q_P$, (Schreiber et al. 1986) was estimated using the initial $F_O$ instead of $F'_O$ for computations since the latter value during actinic illumination was unknown.
RESULTS

Effects of Methylviologen on Photochemical and Non-photochemical Fluorescence Quenching. MV was used to support linear electron transport quenched $F_M$ in dark-adapted, non-energized chloroplasts (Fig. 3.1). Even saturating flashes of over 20,000 $\mu$mol photons m$^{-2}$ s$^{-1}$ did not restore $F_M$ to levels before MV addition. Vernotte et al. (1979) reported a similar effect which they attributed to non-photochemical quenching of PSII by oxidized PQ. In the presence of ascorbate, inhibiting electron flow with DBMIB restored the original $F_M$ but with DCMU $F_M$ was only partly restored (Fig. 3.1 A and B). Uncoupling with nigericin further decreased $F_M$ (Fig. 3.1B). These effects are consistent with quenching of $F_M$ by oxidized PQ and highlight the potential of MV and uncouplers to confound $F_M$.

To account for MV quenching, a saturating pulse was delivered before and after MV addition. When PQ oxidation was not inhibited with DBMIB or when linear flow was uninterrupted by DCMU the latter $F_M$ was used; when DBMIB or DCMU were present, the former $F_M$ was used for calculations. Since DCMU and reduced DBMIB abolish $\Delta$pH, these inhibitors also enabled qualitative resolution of $SV_E$ from $\Delta$pH-independent $SV_N$. However, only data employing DBMIB are strictly quantitative because, as previously mentioned, DCMU does not fully restore $F_M$. 

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Figure 3.1. The effects of MV on $F_M$ in osmotically-shocked pea chloroplasts. Saturating flashes were delivered to dark-adapted chloroplasts exposed only to the low level 1.6 kHz PAM illumination. MV-induced quenching of $F_M$ (A) was further enhanced by nigericin (B). DBMIB restored $F_M$ in both cases. Final concentrations were 60 mM ascorbate (present from the beginning of the experiment), 0.1 mM MV, 2 μM NIG, and 2 μM DBMIB.
Ascorbate is required if DBMIB is used to restore maximum fluorescence because oxidized DBMIB quenches fluorescence. Ascorbate is also necessary for zeaxanthin formation in broken chloroplasts (Yamamoto et al. 1972). Preliminary experiments showed that ascorbate affected the dynamic relationship between photochemical electron transport, zeaxanthin formation and SV$_E$. We therefore characterized these effects. Figure 3.2 shows the effects of ascorbate and MV on 1-qp (Panel A), SV$_N$ (Panel B), and zeaxanthin formation (Panel C) in osmotically-shocked pea chloroplasts. The zero time for A and B represents the time from beginning of actinic illumination in C (ON). In the absence of MV or ascorbate, 1-qp was high, SV$_N$ was low, little if any SV$_E$ was observed, and no zeaxanthin was formed (confirmed by HPLC analysis). The addition of MV reduced 1-qp markedly and stimulated a low level of constitutive-SV$_E$, but again no zeaxanthin was formed. Ascorbate and ascorbate plus MV stimulated SV$_E$ and zeaxanthin formation. Ascorbate, however, lowered 1-qp relative to the no-additions treatment and increased 1-qp relative to the MV-only treatment. In the former case, addition of ascorbate stimulated electron flow whereas in the latter case electron flow through QA was reduced. The reason for these apparently opposite effects of ascorbate on 1-qp is not clear. Perhaps in the absence of a more suitable electron acceptor, the small amount of dehydroascorbate that is a contaminant in our ascorbate solutions served as an electron acceptor, mediating QA oxidation. Siefermann and Yamamoto (1974b) reported dehydroascorbate could be reduced photosynthetically and thus support de-epoxidation of zeaxanthin in light but not if an alternative (competitive)
electron acceptor such as MV was present. In the present study when MV was present, ascorbate somehow either reduced electron flow or served as an alternative electron donor. Regardless of mechanism, MV kept $Q_A$ relatively oxidized as indicated by the $1-q_p$ values even with ascorbate present. These results show that maximal $S_{VE}$ development required zeaxanthin formation but neither $S_{VE}$ nor zeaxanthin formation required complete reduction of $Q_A$.

**Correlation of Zeaxanthin and $S_{VE}$ at Steady-State and During Induction.** Chapter 2 showed that the kinetics and extent of zeaxanthin formation qualitatively correlated with $q_E$ under both linear and PSI-cyclic electron transport. Here we quantitated the steady-state and kinetic relationships between zeaxanthin-formation and $S_{VN}$ under artificially mediated electron-transport. In Figure 3.3 steady-state $S_{VE}$ and zeaxanthin formation were varied with increasing DTT concentrations. DTT inhibits de-epoxidase activity (Yamamoto and Kamite 1972) but has no effect on the quantum yield of photosynthesis, electron-transport rates (Bilger et al. 1989), or $\Delta pH$ (Sokolove and Marsho 1976). Figure 3.3A shows $S_{VN}$ development at various DTT concentrations under PMS-mediated cyclic electron transport. Similar experiments were also done under MV-mediated electron transport (development data not shown). Figure 3.3B shows zeaxanthin concentration at 13 min plotted against the corresponding $\Delta S_{VN}$. According to classic SV quenching, the relationship between fluorescence-quencher concentration and quenching is directly proportional to the expression $(F_M/F_{M}^*)^{-1}$.

The SV-type quenching for the two types of electron
transport closely fit the same line ($R^2=0.992$), suggesting that induction of zeaxanthin-$SV_E$ by both electron transport systems are similar if not identical. The linear relationship indicates a concentration-dependent quenching mechanism. The $SV$-type quenching constant was $0.031 \; SV_N \; \text{units mmol}^{-1} \; \text{zeaxanthin mol}^{-1} \; \text{Chl} \; a$.

The quantitative relationship of $SV_N$ and zeaxanthin during induction was examined by continuously monitoring 505 nm absorbance changes to follow zeaxanthin formation. Figure 3.4 shows $SV_N$ kinetics (A) and corresponding 505 nm kinetics (B) under MV-mediated electron transport. The zero time in Fig. 3.4A corresponds to the beginning of actinic illumination (ON) in Fig. 3.4B. Without DTT, the development of $SV_N$ and 505 nm change were high. Zeaxanthin forms irreversibly under these conditions and the irreversible part of the 505 nm change (Fig 3.4B) corresponds to zeaxanthin formation. Small absorbance changes at 505 nm observed with DTT present were apparently unrelated to zeaxanthin formation; HPLC pigment analyses of samples at the end of the treatments confirmed no zeaxanthin had formed. In the absence of DTT, the final pigment concentration was $37.1 \; \text{mmol zeaxanthin mol}^{-1} \; \text{Chl} \; a$. DBMIB-reversible $SV_N$ that developed in the presence of DTT is constitutive-$SV_E$ (Fig. 3.4A). Likewise, the difference between the DBMIB-reversible $SV_N$ (DTT absent minus present) is zeaxanthin-$SV_E$. Constitutive-$SV_E$ reached steady state in about one minute whereas zeaxanthin-$SV_E$ developed more slowly and appeared to correlate with zeaxanthin.
Figure 3.2. Effects of ascorbate and MV on (A) 1-qP, (B) SVN, and (C) 505 nm kinetics of zeaxanthin formation. Final zeaxanthin levels determined by HPLC were 37.1 and 36.7 mmol mol\(^{-1}\) Chl \(\alpha\) for the (ASC + MV) and ASC reactions respectively. No zeaxanthin was formed in the MV only or NO ADDITIONS reactions. Final DBMIB concentration was 2 \(\mu\)M.
Figure 3.3. Relationship between zeaxanthin concentration and $\Delta S_{VN}$ at steady state under linear and cyclic electron transport. Zeaxanthin levels were varied with DTT. Panel (A) shows $S_{VN}$ development under cyclic conditions (2 $\mu$M DBMIB, 1 $\mu$M PMS, 60 mM ascorbate). Panel (B) shows the $S_{V}$ plot of zeaxanthin concentration and $\Delta S_{VN}$ at steady state (13 minutes) using data from experiment in (A) for cyclic conditions (hollow symbols) and a similar experiment (development data not shown) for linear conditions (60 mM ascorbate, 0.1 mM MV; solid symbols). The final zeaxanthin levels determined by HPLC were plotted (SV-type) against $\Delta S_{VN}$, the difference between $S_{VN}$ when zeaxanthin was formed ($< 1$ mM DTT) minus $S_{VN}$ when it was totally inhibited (1 mM DTT). The equation for the regression line is $y=0.031x+0.001$. 

57
A

- 0.00mM
- 0.05mM
- 0.25mM
- 0.40mM
- 1.00mM

NIG

DTT

MIN

SV_N

0.0 0.2 0.4 0.6 0.8 1.0

0 2 4 6 8 10 12 14

B

R^2 = 0.992

\[ \Delta SV_N \]

ZEAXANTHIN, mmol mol^{-1} Chl

0 4 8 12 16 20

0.0 0.1 0.2 0.3 0.4 0.5 0.6
Figure 3.4. Kinetics of $SV_N$ (A) and 505 nm changes (B) under linear electron transport. The final amounts of zeaxanthin formed were 37.1 and 0.0 mmol zeaxanthin mol$^{-1}$ Chl $a$ for the (MV + ASC) and DTT reactions respectively. Final DBMIB and DTT concentrations were 2 µM and 2 mM, respectively. Linear conditions were as described in Figure 3.3.
Figure 3.5 shows a SV-type plot of the data derived from Fig. 3.4. Zeaxanthin-SV$_E$ was calculated as $\Delta SV_N$, i.e. $SV_N$ with zeaxanthin formed minus $SV_N$ with zeaxanthin formation inhibited with DTT. The quantity of zeaxanthin at each corresponding point of fluorescence quenching in Figure 3.4A was estimated from the 505 nm absorbance change (Fig. 3.4B) calibrated for zeaxanthin concentration by HPLC analysis. The SV-type treatment shows a linear relationship ($R^2=0.997$) between relative zeaxanthin concentration and $\Delta SV_N$ during the course of zeaxanthin formation. The SV-type quenching constant was 0.026 $\Delta SV_N$ units per mmol zeaxanthin mol$^{-1}$ Chl $a$.

Although the correlation in Figure 3.5 is high, close examination of the data shows that zeaxanthin-SV$_E$ did not correlate with zeaxanthin until after about 5 mmol zeaxanthin mol$^{-1}$ Chl $a$ had formed. This amount formed during the first minute of illumination. While there could be a lag between zeaxanthin formation and expressed quenching, the data during the first minute is inconclusive because $\Delta p$H induction, constitutive-SV$_E$ (Fig. 3.5A) and light-scattering changes obscure the results during this period (Fig. 3.5B). After one minute zeaxanthin-SV$_E$ development clearly followed zeaxanthin formation. This direct relationship between zeaxanthin and SV$_E$ seen kinetically was in the absence of DTT, in contrast with the previous experiment where steady-state zeaxanthin levels were varied with DTT.
Figure 3.5. Relationship $\Delta S_{V_N}$ and zeaxanthin concentration during induction. $\Delta S_{V_N}$ was calculated from the data in Fig. 3.4 as the difference between $S_{V_N}$ in the (MV+ASC) reaction minus $S_{V_N}$ in the DTT reaction at corresponding times. The 505 nm absorbance change due to zeaxanthin was calculated as $\Delta A_{505}$, absorbance of the (MV+ASC) reaction minus absorbance of DTT reaction at corresponding times to $\Delta N_{P_Q}$. The $\Delta A_{505}$ change was calibrated for zeaxanthin by HPLC. The equation for the regression line is $y=0.026x-0.033$. 
To further investigate the temporal relationship between zeaxanthin formation and $SV_E$ development we examined the effects of pre-forming zeaxanthin (Fig. 3.6). Zeaxanthin was formed by an initial light treatment and its further development inhibited with DTT. After a three-minute dark relaxation period, the chloroplasts were given a second light treatment. Chloroplasts were also treated with DTT from the outset to determine the contribution of constitutive-$SV_E$. Figure 3.6A shows that during the first minute of the initial light treatment, the kinetics of $SV_N$ with DTT present and absent were superimposed. In the second light treatment (Fig. 3.6B) $SV_N$ during the first minute developed faster with zeaxanthin already formed than with zeaxanthin formation inhibited. As before, subtracting $SV_N$ with $\geq 1$ mM DTT present from total $SV_N$ gave $\Delta SV_N$ or zeaxanthin-$SV_E$ (Fig. 3.6B). The curves clearly show zeaxanthin-$SV_E$ developed faster and to a higher extent when zeaxanthin was preformed (Fig. 3.6B). The higher $SV_N$ extent during the second light treatment, even with no further zeaxanthin formed, suggests other slow changes in addition to the amount of zeaxanthin present affect total zeaxanthin-$SV_E$ expression.

As noted earlier, DTT inhibits zeaxanthin formation without inhibiting $\Delta pH$ (Sokolove and Marsho 1976). Since DTT did not inhibit zeaxanthin-$SV_E$ after zeaxanthin was formed, DTT apparently has no observable effect on zeaxanthin-$SV_E$ other than inhibiting violaxanthin de-epoxidase activity.
Figure 3.6. Kinetics of $SV_N$ formation under linear electron transport during sequential light treatments. In (A) DTT was added at the beginning of the reaction (DTT) or at the end of the first light period (ZEA). In (B) $\Delta SV_N$ representing zeaxanthin-dependent $SV_E$ was obtained by subtracting the $SV_N$ in the DTT reaction from the $SV_N$ in the ZEA reaction. The final zeaxanthin levels formed were 17.2 and 0.0 mmol mol$^{-1}$ Chl $a$ for the ZEA and DTT reactions respectively. Final DTT concentration when present was 2 mM.
**Zeaxanthin F₆ Quenching.** According to Butler and Kitajima (1975), non-radiative dissipation of energy in the pigment bed of PSII quenches both Fᵥ and Fₒ proportionally whereas energy dissipation in the reaction center quenches only Fᵥ. Figure 3.7 shows (A) the zeaxanthin-concentration dependency of ΔSV₅ and ΔSVₒ and (B) the data replotted as ΔSV₅ vs. ΔSVₒ at corresponding zeaxanthin concentrations. MV-mediated electron transport was used and the actinic light intensity was saturating for ΔpH. Zeaxanthin formation and the associated fluorescence quenching were poised with DTT at various times. SV₅ was derived as in the previous experiments. SVₒ was measured after quenching had reached steady-state by turning off the actinic light and simultaneously switching the PAM illumination from 100 kHz to 1.6 kHz. The presence of MV assured rapid and complete opening of traps for accurate measurement of F'O but as shown earlier (Fig. 3.1) depressed F'M. F'O held momentarily then relaxed within 90 s to a steady-state Fₒ level which remained unchanged for over 20 minutes.

Both ΔSV₅ and ΔSVₒ were highly correlated with zeaxanthin (Fig 3.7A) and consequently with each other (Fig 3.7B). The SV-type constants for SV₅ and SVₒ were 0.025 and 0.012 SVᵥ units per mmol zeaxanthin mol⁻¹ Chl a, respectively. The former result is similar to the results in Figs. 3.3 and 3.5. The proportional quenching of SV₅ and SVₒ is consistent with zeaxanthin quenching in the pigment bed. No conclusion on the location of constitutive quenching is possible from these experiments since under the saturating ΔpH conditions used, constitutive SV₅ is constant.
Figure 3.7. Relationship between $\Delta SV_O$ and $\Delta SV_N$ and zeaxanthin concentration. Panel (A) shows the direct zeaxanthin-concentration dependency of $\Delta SV_O$ and $\Delta SV_N$. The regression coefficients for the $\Delta SV_O$ and $\Delta SV_N$ lines were $R^2=0.998$ and $R^2=0.997$, respectively. The slope of the regression lines (SV-type) constants were 0.012 and 0.025 for $\Delta SV_O$ and $\Delta SV_N$, respectively. Panel (B) shows a plot of $\Delta SV_O$ against $\Delta SV_N$ at corresponding zeaxanthin concentrations in panel (A). Illumination conditions were 10 min at 350 $\mu$mol photons m$^{-2}$ s$^{-1}$ for all reactions. DTT was added either before illumination or at different points in time during the reaction to inhibit zeaxanthin formation. Final reagent concentrations were 60 mM ascorbate, 50 $\mu$M MV and 1 mM DTT when added.
Antimycin Inhibition of Zeaxanthin-$SV_E$ and Constitutive-$SV_E$. We reported previously that antimycin inhibited $SV_E$ development without affecting zeaxanthin formation under either linear or cyclic electron flow (Chapter 2). The sensitivity of each of the two $SV_E$ components to antimycin, however, was not determined. Figure 3.8 shows that antimycin does indeed inhibit both types of $SV_E$. Under cyclic electron transport, zeaxanthin-$SV_E$ and constitutive-$SV_E$ that developed in the absence of antimycin (Fig 3.8A) were completely inhibited when antimycin was added prior to illumination (Fig. 3.8B). As in our previous report (Chapter 2), antimycin did not inhibit zeaxanthin formation (see legend in Fig. 3.8 for HPLC data). Since zeaxanthin formation requires a $\Delta\mathrm{pH}$ (Hager 1969), the inhibition of $SV_E$ under these conditions ($2\mu M$ antimycin) cannot be ascribed to uncoupling. In preliminary experiments neither zeaxanthin formation nor $\Delta\mathrm{pH}$ measured as neutral-red uptake were affected by $2\mu M$ antimycin. Zeaxanthin formation was $50\%$ inhibited at $50\mu M$ antimycin which is similar to the 9-aminoacridine quenching data of Oxborough and Horton for uncoupling (Oxborough and Horton 1987).

In isolated chloroplasts, a low level of $SV_N$ that is independent of zeaxanthin and $\Delta\mathrm{pH}$ is often present. This component is clearly evident in Fig. 3.8B; nigericin did not reverse this low-level antimycin-resistant $SV_N$. The rate and extent of this $SV_N$ were nearly identical for reactions with and without zeaxanthin formation. The slow induction kinetics of this $\Delta\mathrm{pH}$-independent $SV_N$ suggest it may be the same component Oxborough and Horton (1987) observed after the reversal of $\Delta\mathrm{pH}$ with DCMU. They termed this component $q_R$ and suggested it was probably pho...
tion or $q_L$. The possibility, however, that this type of $SV_N$ is an artifact, reflecting an inherent sensitivity of isolated chloroplasts to damage is not excluded.

Antimycin added prior to illumination completely inhibits $SV_E$ but its addition during illumination has either no effect or only partly reverses the previously formed $SV_E$ (Oxborough and Horton 1987, 1988, Chapter 2). Accordingly, Figure 3.9 shows that antimycin added prior to illumination completely inhibited $SV_E$; antimycin added during illumination only slightly reversed the partly formed $SV_E$. However, after an intervening dark period, $SV_E$ in both antimycin-treated reactions were completely inhibited. Apparently the antimycin site was changed to its sensitive form in the dark. These results and those in Figure 3.8 indicate antimycin inhibits both zeaxanthin-$SV_E$ and constitutive-$SV_E$ by a common mechanism.
Figure 3.8. Effects of antimycin (2 μM) on zeaxanthin-$SV_N$ and constitutive-$SV_N$ induction under cyclic electron transport. (A) Zeaxanthin-$SV_N$ (ZEA) and constitutive-$SV_N$ (DTT) development in the absence of antimycin (-ANTI A). (B) Effect of antimycin added before illumination on $ΔSV_N$. Final zeaxanthin levels formed were 13.9 and 14.8 mmol zeaxanthin mol$^{-1}$ Chl $a$ for the reactions without DTT in A and B respectively. No zeaxanthin formed in the presence of DTT. Final concentrations were 2 μM nigericin and 2 mM DTT. Cyclic electron-transport conditions were as described in Figure 3.3.
Figure 3.9. Effects of dark relaxation on the inhibitory effects of antimycin on $SV_N$ under linear electron transport. Antimycin (2 $\mu$M) was added at 3 minutes in the ANTI reaction, added at the beginning in the PRE reaction and absent in the control reaction. All three reactions were interrupted with a 3 min dark period. Final DCMU and nigericin concentrations were 0.1 mM and 2 $\mu$M, respectively. Linear electron transport conditions were as described in Figure 3.3.
DISCUSSION

**Zeaxanthin Formation and \( SV_E \) in Osmotically-Shocked Chloroplasts.** Use of osmotically-shocked chloroplasts under artificially mediated electron transport coupled with Stern-Volmer treatment of quenching proved highly satisfactory for the quantitative characterization of non-photochemical quenching. Both zeaxanthin-dependent and independent forms of non-photochemical quenching seen in leaves and intact chloroplasts (Adams et al. 1990, Bilger and Björkman 1990, Demmig-Adams et al. 1990) were also seen in these preparations. The use of various photosynthetic mediators and inhibitors showed unambiguously that both zeaxanthin- and constitutive non-photochemical quenching were energy-dependent quenching and that both could be supported by artificial linear and cyclic electron transport. A few precautions were necessary for accurate quantitation of \( SV_N \). When MV was used as the electron-transport mediator, DBMIB was better than DCMU for the measurement of \( F_M \). The latter gave incomplete recovery of fluorescence quenching, presumably due to oxidized PQ. Uncoupling MV-linear electron transport increased the confounding effect of PQ quenching. Use of DTT to vary zeaxanthin formation was especially advantageous; we observed no effect of DTT on the \( SV_E \) mechanism under these conditions other than inhibition of the de-epoxidase enzyme. DTT inhibits ascorbic acid peroxidase in whole chloroplasts (Schreiber and Neubauer 1990) but this activity is rapidly lost in broken chloroplast systems (Asada and Takahashi 1987).
Correlations between zeaxanthin and non-photochemical quenching have now been observed in leaves (Adams et al. 1990, Bilger et al. 1989, Demmig et al. 1988) and whole chloroplasts (Demmig-Adams et al. 1990) under a variety of experimental conditions. Here the correlations between zeaxanthin and $S_{VE}$ were seen under both artificially-mediated linear and cyclic electron transport at steady state and during induction. Furthermore the zeaxanthin-dependent quenching was in addition to an underlying zeaxanthin-independent $S_{VE}$ and observed under saturating $\Delta pH$.

We conclude in agreement with Demmig-Adams et al. (1990) that zeaxanthin quantitatively increases non-photochemical quenching. This contrasts with Noctor et al. (1990) who reported that zeaxanthin increased the $q_E$ to $\Delta pH$ ratio at subsaturating $\Delta pH$ levels and that at saturating $\Delta pH$, the $q_E$ level was equal with or without zeaxanthin. Since saturating $\Delta pH$ conditions were used in the present work, the difference cannot be attributed to this aspect of the experimental protocol. While other possible reasons for the difference can only be speculated, contrasting data treatment, quenching coefficients versus Stern-Volmer, may partly explain the difference. As Demmig-Adams et al. (1990) discussed previously whereas $q_E$ is constrained ($0 < q_E < 1$), SV parameters or the equivalent ($k_D$) are not similarly constrained. Thus at high levels of quenching, differences are less apparent as quenching coefficients than as SV.

Demmig-Adams et al. (1990) and Bilger and Björkman (1990) reported evidence supporting the view that zeaxanthin quenches fluorescence in the pigment bed. Our Stern-Volmer treatment of both $F_O$ and $F_M$ also indicate that zeaxanthin
quenches in the pigment-bed. The results, however, do not further clarify the site of constitutive quenching which has been attributed to reaction-center quenching (Adams 1990). Two different sites of quenching for constitutive and zeaxanthin-dependent quenching is consistent with the relationship of $q_0$ and $q_E$ reported earlier by Bilger and Schreiber (1986). Rees et al. (1990) also examined the $q_0$ and $q_E$ relationship and, treating $q_0$ as a single component, postulated that $q_E$ occurs in the pigment bed. Since neither Bilger and Schreiber (1986) nor Rees et al. (1990) reported zeaxanthin changes, their results cannot be directly related to the present results. However, the possibility that zeaxanthin formation was a factor in these other studies cannot be excluded. In intact-leaf systems, high-light intensity alone is sufficient for zeaxanthin formation (Yamamoto 1962). In isolated intact (Type A) chloroplasts, Sokolove and Marsho (1976) showed that zeaxanthin formation can occur without addition of exogenous ascorbate. While available evidence suggests zeaxanthin-dependent quenching and constitutive quenching occur at different sites (Adams et al. 1990, Demmig-Adams et al. 1990), similarities between the zeaxanthin-$SV_E$ and the constitutive-$SV_E$ in this study both in terms of electron-transport mediation and sensitivity to antimycin suggest they are mechanistically similar.

**Antimycin Inhibition of Zeaxanthin-$SV_E$ and Constitutive-$SV_E$.** The mechanism of antimycin action is unclear. Antimycin inhibits both $SV_E$ types under linear and cyclic electron-transport, indicating that the target site is common to both electron-transport systems. In mitochondria, the site appears to be in the cytochrome
b-c_{1} region of the electron-transport chain (Slater 1973). For isolated chloroplasts, Moss and Bendall (1984) speculated that antimycin inhibits a ferredoxin-quinone reductase (FQR) based on partial inhibition of ferredoxin-mediated but not of chemically-mediated cyclic phosphorylation. Oxborough and Horton (1988) observed that increasing MV concentrations increased q_{E} and suggested the effect was mediated by oxidized FQR.

There is as yet no direct evidence that the antimycin target is FQR. Antimycin completely inhibits non-photochemical quenching only when added to dark-adapted or non-energized chloroplasts (Chapter 2). Antimycin does not inhibit electron transport (Oxborough and Horton 1986) or zeaxanthin formation (Chapter 2) but does decrease the q_{E} to ΔpH ratio (Noctor et al. 1990). Available evidence thus appears to suggest three possible alternative mechanisms for conditional inhibition, namely sensitivity to antimycin of a critical component depending on its redox state or protection against antimycin inhibition by the energized state (ΔpH), or both.

Xanthophyll Cycle and SV_{E} in Photoprotection. If SV_{E} has a protective function, the xanthophyll cycle and membrane components that contribute to constitutive SV_{E} together appear to form a system that is able to respond over a wide time scale and to variable extent. Constitutive-SV_{E} responds rapidly but appears limited in size. Zeaxanthin-SV_{E} forms more slowly, parallel with the kinetics of the violaxanthin de-epoxidation of the xanthophyll cycle. Violaxanthin availability
(Siefermann and Yamamoto 1975) determines the fraction of the total violaxanthin pool that can form zeaxanthin. Violaxanthin availability is in turn affected by the redox state of an intersystem electron-transport component, probably PQ. On a longer time scale, growth conditions influence the xanthophyll pool size as well as the violaxanthin availability (Thayer and Björkman 1990). Thus $S_{VE}$ has rapid (constitutive), slow (zeaxanthin formation and availability) and adaptive (pool size) effects. Slowly reversible or irreversible $\Delta p$H-independent zeaxanthin-related non-photochemical fluorescence quenching has been reported in leaves given severe treatments (Demmig et al. 1988). Little is known about the mechanism of this form of zeaxanthin quenching but it may represent a further extension of the overall protective function of zeaxanthin.
CHAPTER 4

RESOLUTION OF LUTEIN AND ZEAXANTHIN USING A NON-ENDCAPPED, LIGHTLY CARBON-LOADED C\textsubscript{18} HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMN.

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ABSTRACT

A new rapid and reproducible high-performance liquid chromatographic method using Spherisorb ODS-1, a non-endcapped, lightly carbon-loaded column material, for the separation of higher-plant chloroplast pigments is described. The method resolves lutein and zeaxanthin, as well as all other major and minor pigments at or near baseline by either of two solvent programs. Program I is faster and more sensitive than program II while the latter resolves pheophytin a and $\beta,\varepsilon$-carotene slightly better than program I. Both programs use an initial buffered aqueous mixture that appears critical for this application of ODS-1. The method is well suited for analysis of xanthophyll-cycle pigment changes.
INTRODUCTION

Baseline separation of plastid pigments from higher plants in a simple, reproducible, one-step method has yet to be reported. Difficulties arise from the wide ranging polarities of the comprising pigments and the limited selectivity of the columns. The carotenes are nonpolar whereas at the other extreme 9'-cis-neoxanthin is polar. Separation of structural isomers such as lutein from zeaxanthin and \( \beta,\epsilon \)-carotene from \( \beta,\beta \)-carotene is usually incomplete in most reversed-phase HPLC procedures. Of several aqueous (Bergweiler and Lütz 1986, Braumann and Grimme 1979, 1981, de las Rivas et al. 1989, Eskins and Harris 1981, Siefermann-Harms 1988, Wright and Shearer 1984, Zapata et al. 1987) and nonaqueous (Khachik et al. 1986, Landis et al. 1983) reversed-phase HPLC methods reported for plant and algal pigments, only one method separates lutein from zeaxanthin at the baseline (Thayer and Björkman 1990). Rapid, quantitative separation of lutein and zeaxanthin has become important for research on photoprotective processes in plants because of the apparent relationship between zeaxanthin and nonphotochemical quenching of excess energy in the antennae chlorophylls of photosystem II (Demmig-Adams et al. 1990). Light induces changes in zeaxanthin levels via interconversions with violaxanthin and antheraxanthin in the xanthophyll cycle (Yamamoto 1979).

Thayer and Björkman (1990) obtained baseline separation of lutein and zeaxanthin with a nonendcapped Zorbax-ODS column. Unfortunately this packing material is not presently being manufactured and therefore is not widely available. We sought an alternative solution and here report a new method using ODS-1, a
nonendcapped and lightly carbon loaded material. The method separates lutein and zeaxanthin and most other chloroplast pigments at or near baseline. Procedures for analyses of pigment extracts from whole leaves and isolated chloroplasts and the applicability of the method for xanthophyll-cycle studies are detailed.
MATERIALS AND METHODS

**Instrumentation.** The chromatographic system was a Beckman/Altex Model 334 Gradient Liquid Chromatograph (Beckman Instruments, Berkeley, CA U.S.A.) equipped with a Waters 990 Photodiode Array Detector (Millipore Corp., Milford, MA U.S.A.). All solvents were HPLC grade and obtained from Fisher Scientific, Fair Lawn, NJ U.S.A.. Pigments standards were quantitated spectrophotometrically with a DW-2000 UV-VIS dual-wavelength dual-beam spectrophotometer (SLM Instruments Inc., Urbana IL U.S.A.).

Spherisorb ODS-1 columns (5 μm particle size, 4.6 mm X 250 mm) were from Alltech Associates, Inc., Deerfield IL U.S.A.. ODS-1 is a nonendcapped, 6 % carbon, spherical silica material manufactured by Phase Separations Ltd., Clwyd U.K.. The guard column was ODS-1 or Adsorbosphere C-18 direct-connect cartridge, also from Alltech Associates.

**Liquid chromatography.** The flow rate for all separations was 2 ml min\(^{-1}\) and all sample injections were 20 μl. Two solvent programs were developed. Program I: Solvent A-1 was ran isocratically from 0 to 4 min followed by a 2.5 min linear gradient to 100 % solvent B. Program II: Solvent A-1 was ran isocratically for 6 min followed by a 10 min linear gradient to 100% solvent C. Solvent A-2 replaced solvent A-1 in some experiments. Solvent mixtures were: A-1, acetonitrile-methanol-Tris-HCl buffer 0.1 M pH 8.0 (72:8:3); A-2, acetonitrile-methanol-Tris-HCl buffer 0.1 M pH 8.0 (75:12:4); B, methanol-hexane (4:1); C, methanol-ethyl acetate (68:32).
The columns were re-equilibrated between samples for a minimum of 10 min with solvent A-1 for both solvent programs. When changing solvent programs the columns were equilibrated with 60 ml of solvent B or C, and then with 30 ml of solvent A-1. This extensive re-equilibration was necessary when changing programs to remove residual effects of the prior solvents B or C. All runs were at room temperature.

**Pigment identification and calibration.** \( \beta,\epsilon \)-Carotene and \( \beta,\beta \)-carotene were obtained from Sigma Chemical Co., St. Louis Mo. U.S.A.. Violaxanthin, lutein, and zeaxanthin were isolated according to Yamamoto et al. (1974). Antheraxanthin, 9'-cis-neoxanthin, lactucaxanthin and pheophytins \( a \) and \( b \) were identified by absorption spectra.

Chlorophylls \( a \) and \( b \) were quantitated according to Vernon (1960). Lutein, violaxanthin, and zeaxanthin standards were in ethanol and \( \beta,\epsilon \)-carotene and \( \beta,\beta \)-carotene were in hexane. Extinction coefficients \( (E^1\%_1cm) \) used for quantitation were: lutein and violaxanthin \( (2550) \), zeaxanthin \( (2540) \), \( \beta,\epsilon \)-carotene \( (2725) \) and \( \beta,\beta \)-carotene \( (2590) \) (Davies 1976). Linearity of the peak area (absorbance units X minutes) calibrations against pigment concentrations was \( r^2 \geq 0.991 \) for all pigment standards. The photodiode-detector wavelength for integration of peak areas was 440 nm. Conversion factors for peak area to nanomol per injection for program I, solvent A-1 were: violaxanthin \( (20.72) \); lutein \( (27.10) \); zeaxanthin \( (26.90) \); chlorophyll \( a \) \( (34.94) \); chlorophyll \( b \) \( (38.53) \); \( \beta,\epsilon \)-carotene \( (18.51) \); \( \beta,\beta \)-carotene \( (18.94) \).
Antheraxanthin was estimated with the conversion factor for lutein. Lactucaxanthin and 9'-cis-neoxanthin concentrations were estimated using the conversion factor for violaxanthin.

**Preparation of isolated chloroplast and leaf-disk samples.** Leaf-disks and isolated chloroplasts with high and low levels of zeaxanthin were prepared to demonstrate the effectiveness of the method for quantitative analysis of the xanthophyll-cycle. Chloroplasts were isolated from market lettuce (*Lactuca sativa* L. cv. Romaine) according to Yamamoto et al. (1972). Prior to isolation, the leaves were dark adapted for 12 h to reduce the background level of zeaxanthin. Addition of 10 mM Na-ascorbate induced zeaxanthin formation in osmotically-shocked chloroplasts suspended in 50 mM sodium citrate buffer at pH 5.0. Zeaxanthin formation was stopped after 10 mins with 1.5 mM DTT. All reactions were 3 ml final volume and chlorophyll concentration was 30 μg total chlorophyll ml⁻¹. Chloroplast suspensions were divided into two microcentrifuge tubes, centrifuged for 5 min and the resulting pellet extracted as described under pigment extractions.

Leaf disks (10 cm², approximately 0.25 g) were punched from fully developed leaves of shade-grown *Anthurium andraeanum* cv. Brown Tulip. Prior to removal of disks, the plant was dark adapted for 12 to 15 h to reduce the background level of zeaxanthin. For light-induced zeaxanthin formation, a leaf disk was floated on water in a water-jacketed beaker and exposed to 2000 μmol photons m⁻² s⁻¹ white light for 20 min from a Model 640-HD lamp (Acme Light, Skokie, IL U.S.A.). The light was
filtered through 2.5 cm of refrigerated circulating water to remove heat. Leaf-disk
temperature remained between 18 and 25° C.

**Pigment extractions.** Pigments were extracted at room temperature and under
dim laboratory light. Chloroplast pellets (45 µg total chlorophyll) were suspended in
0.25 ml 100 % acetone for 5 min at room temperature with occasional vortex mixing,
centrifuged for 5 min in a microcentrifuge and the resulting supernatant saved. The
acetone-dried pellets were re-extracted as above to ensure complete extraction of
β,β-carotene and β,ε-carotene. The supernatants were pooled and then filtered
through 0.2 µm nylon-66 microcentrifuge filters (Microfilterfuge, Rainin Instrument
Co, Inc., Woburn, MA U.S.A.). Leaf disks were ground in a tissue homogenizer with
25 mg CaCO₃ and 2.5 ml 100% acetone. The extract was divided into two
microcentrifuge tubes and spun for 5 min. The supernatants were removed and the
pellets extracted again with 1.25 ml 100 % acetone each at room temperature for 5
min, with occasional vortex mixing before spinning again for 5 min. The supernatants
were pooled and filtered as described for chloroplast extracts. The pigment extracts
were either analyzed immediately or after 1 to 2 days storage at -20°C under argon.
No pigment degradation was observed during this storage period.
RESULTS AND DISCUSSION

Solvent programs. Fig. 4.1 shows chromatograms of Anthurium extracts using solvent program I. The extracts were prepared from dark-adapted (A) and light-treated leaf disks (B). Fig. 4.1C shows the extract in (B) after acid treatment. The program resolved lutein, zeaxanthin and most of the other plastid pigments at baseline in about 13 min. A low level of zeaxanthin was detectable in the dark-adapted sample. Separation of \( \beta,\beta \)- and \( \beta,\epsilon \)-carotene was incomplete although adequate for detection and estimation. Pheophytins \( a \) and \( b \) were resolved but the former just barely from \( \beta,\epsilon \)-carotene. Fig. 4.2 shows the method also resolves lactucaxanthin in lettuce extracts. Zeaxanthin was undetectable in the dark-adapted lettuce (Fig. 4.2A). Lettuce also apparently lacks \( \beta,\epsilon \)-carotene.

Increasing the hexane content in solvent B from methanol-hexane (4:1) to (3:1) improved the separation of the pheophytins and carotenoids but also introduced a refractive-index change that interfered with the quantitation of the carotenoids (data not shown). The proximity of pheophytin \( a \) to \( \beta,\epsilon \)-carotene with program I is not critical for most applications since pheophytins are not usually detectable at 440 nm in undegraded pigment extracts.
Figure 4.1 Chromatograms of anthurium extracts on column 1 with program I, solvent A-1; (A) Before zeaxanthin formation, (B) after zeaxanthin formation and (C) after acid treatment of the same extract used in B. Abbreviations are: \(N, 9'-\text{cis-neoxanthin}; V, \text{violaxanthin}; A, \text{antheraxanthin}; L, \text{lutein}; Z, \text{zeaxanthin}; Cb, \text{chlorophyll b}; Ca, \text{chlorophyll a}; \beta\epsilon, \beta,\epsilon\text{-carotene}; \beta\beta, \beta,\beta\text{-carotene}; Pb, \text{pheophytin b}; Pa, \text{pheophytin a}.\)
Figure 4.2. Chromatograms of lettuce extracts on column 1 with solvent program I, solvent; (A) Before zeaxanthin formation and (B) after zeaxanthin formation. Abbreviations are the same as Fig. 4.1 and with La for lactucaxanthin.
When maximum separation of the carotenes and pheophytins is important, program II can be used. Fig. 4.3 shows that the separations with program II were comparable to program I except that the pheophytins eluted after the carotenes (Fig. 4.3C), and were more completely resolved from the carotenes. Program II, however, is appreciably longer than Program I.

Separation of lutein from zeaxanthin is reportedly marginal with endcapped materials (Thayer and Björkman 1990, Wright and Shearer 1984). Indeed, endcapped column materials such as Lichrosorb RP-18 or ODS-2, did not separate zeaxanthin and lutein satisfactorily with this method (data not shown). Presumably interaction of these pigments with the exposed silanol sites of the non-endcapped ODS-1 material is important. The aqueous condition in solvent A-I was a key to the successful application of ODS-1 inasmuch as zeaxanthin and chlorophyll b were otherwise unresolved. We speculate that water is required for sufficient interaction between the pigments and the lightly carbon-loaded ODS-1 material.

The Tris buffer in solvent A-1 neutralizes the ODS-1 columns. Without Tris the chlorophylls and carotenoids degraded. Tris also neutralizes acids inherently present in the acetonitrile solvent (Siefermann-Harms 1988). Although chloride ions harm stainless steel we have seen no corrosion in our system. We flush the system with 20 to 30 ml of methanol after each days runs to minimize corrosion and to eliminate residual hexane in the column. This method shows that non-endcapped column materials, previously avoided because of isomerization and degradation of pigments, can be used successfully for pigment separations with proper precautions.
Figure 4.3. Chromatograms of same extracts as Fig. 4.1 on column 1 with program II, solvent A-1. Abbreviations are the same as Fig. 4.2.
Column variability. Column variability is common in HPLC and may be even greater in nonendcapped materials. We tested three ODS-1 columns designated 1, 2, and 3. Whereas columns 1 and 2 gave similar separations, column 3 performed poorly. As shown in Fig. 4.4A, the resolution of zeaxanthin and chlorophyll b was poor and peak sensitivity was low on column 3 (solvent A-1, program II). We found that the poor resolution of column 3 was improved by further increasing the water and methanol content of the mobile phase. Fig. 4.4B shows that using the more aqueous A-2 mixture resolved lactucaxanthin, lutein, zeaxanthin and chlorophyll b adequately but still not as well as on columns 1 and 2. Apparently, increasing the polarity of the mobile phase by increasing the Tris-HCl buffer and methanol higher than in solvent A-1 caused chlorophyll b to interact sufficiently with the stationary phase to separate zeaxanthin. Solvent A-2 also worked satisfactorily with columns 1 and 2, but the resolution and sensitivity for lactucaxanthin, lutein, and zeaxanthin was higher with solvent A-1. Thus we regard solvent A-2 as a second-line solvent to be used only with columns that do not perform satisfactorily with solvent A-1.

The source of the observed column differences is not known. The supplier's test chromatograms for columns 1, 2, and 3 for the separation of ethyl benzene were 75575, 125479, and 101429 plates m⁻¹, respectively. Thus the reported efficiencies do not explain the performance difference between the columns. Column 3, however, separated N,N-diethyl-M-toluamide and toluene by only 0.56 min whereas columns 1 and 2 separated these compounds by 0.91 and 1.22 min, respectively. The separation of these test components appears to correlate with the poorer resolution
of chlorophyll $b$ and zeaxanthin on column 3 (Fig. 4.4A). The increased water-content requirement in the mobile phase suggests column 3 may have slightly less carbon loading than the other columns.
Figure 4.4 Chromatograms of lettuce extracts after zeaxanthin formation on column 3 with program II using (A) solvent A-1 and (B) solvent A-2. Abbreviations are the same as Fig. 4.2.
Retention time and sensitivity of solvent programs I and II. Table 4.1 compares the relative retention times for the major pigments of lettuce on column 1. Retention times varied by less than 0.05 min from the mean in five successive runs for either solvent program. The polar xanthophylls eluted earlier in program II than program I, whereas the chlorophylls and \( \beta,\beta \)-carotene eluted later. The peak heights (sensitivity) of the major nonpolar pigments were significantly higher in program I than program II, whereas the sensitivities for the polar xanthophylls were similar. For example, chlorophylls \( b \) and \( a \) were approximately 2 and 3-fold higher, respectively, for program I than for program II. Also, \( \beta,\beta \)-carotene peaks were over 50% higher in program I than program II. In our studies the rapidity and increased sensitivity of program I outweighed the resolution problem of pheophytin \( a \) and \( \beta,\epsilon \)-carotene. The detectable limit for individual xanthophyll pigments (V, A, or Z) was about 5-7 picomoles per 20 \( \mu l \) injection for program I. This is lower sensitivity than that reported by Thayer and Björkman (1990) but was more than sufficient for studies involving intact leaf tissue and isolated chloroplasts. The sensitivity can be increased by slowing the flow rate in both programs to 1 ml min\(^{-1}\) and proportionally adjusting the changeovers to solvents B or C.

Quantitative Analyses of xanthophyll cycle changes with ODS-1. The following data demonstrates the usefulness of this method for quantitative analysis for the violaxanthin-cycle. Table 4.2 shows the relative pigment content in lettuce chloroplasts before and after dark ascorbate-induced zeaxanthin formation.
Stimulation of violaxanthin de-epoxidation converts violaxanthin to zeaxanthin with virtual mole to mole stoichiometry (Yamamoto 1979). The total relative concentrations of the violaxanthin cycle pigments (V+A+Z) were consistent within standard deviation for before and after zeaxanthin formation. The variance of the individual violaxanthin cycle components (V, A, and Z) in the post-zeaxanthin runs reflected the variance of replicate treatments. All non-violaxanthin cycle pigments remained unchanged. The coefficient of variance for the other major pigments was less than 2.66% for both the before and after runs.

Concluding Remarks. ODS-I, a currently available high-performance liquid chromatographic column material, with the solvent programs described gives rapid and quantitative separation of all major and most minor chloroplast pigments. The method is well suited for studies on the xanthophyll cycle. Although the method has not been thoroughly tested for separation of more complex pigment compositions such as those found in phytoplankton, we have observed good separation of pigments in undegraded extracts of diatoms and several species of brown algae.
TABLE 4.1. RETENTION TIMES (MINUTES) FOR MAJOR LETTUCE CHLOROPLAST PIGMENTS FOR FIVE SUCCESSIVE SEPARATIONS WITH BOTH SOLVENT PROGRAMS I AND II.

<table>
<thead>
<tr>
<th>SEPARATION</th>
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<th>Cb</th>
<th>Ca</th>
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<tr>
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<td>4.52</td>
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TABLE 4.1. (Continued) RETENTION TIMES (MINUTES) FOR MAJOR LETTUCE CHLOROPLAST PIGMENTS FOR FIVE SUCCESSIVE SEPARATIONS WITH BOTH SOLVENT PROGRAMS I AND II.

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**SOLVENT PROGRAM II**

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Abbreviations same as in Fig. 4.2. *Solvent system same as in Fig. 4.1. **Solvent system same as in Fig. 4.3.
TABLE 4.2. RELATIVE PIGMENT CONCENTRATION FOR LETTUCE CHLOROPLASTS BEFORE AND AFTER DARK, pH 5.0, ASCORBATE-INDUCED ZEAXANTHIN FORMATION

ALL VALUES ARE RELATIVE TO CHLOROPHYLL $a$ (mmol mol$^{-1}$ Ca), EXCEPT $Cb/Ca$ WHICH IS (mol/mol).

<table>
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<th>La</th>
<th>L</th>
<th>Ch/Ca</th>
<th>$\beta\beta$</th>
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<td>0.00</td>
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<td>1.53</td>
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TABLE 4.2. (Continued) RELATIVE PIGMENT CONCENTRATION FOR LETTUCE CHLOROPLASTS BEFORE AND AFTER DARK, pH 5.0, ASCORBATE-INDUCED ZEAXANTHIN FORMATION.

ALL VALUES ARE RELATIVE TO CHLOROPHYLL a (mmol mol$^{-1}$ Ca), EXCEPT Ch/Ca WHICH IS (mol/mol).

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<tr>
<td>C.V.(%)</td>
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<td>1.31</td>
<td>0.12</td>
<td>0.62</td>
<td>0.47</td>
<td>1.22</td>
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</table>

†All values are the mean of three individual experiments either before or after zeaxanthin formation. Solvent program I, solvent A-1, and column 1 were used for all runs. Abbreviations same as Fig. 4.2.
CHAPTER 5

DARK INDUCTION OF ZEAXANTHIN-DEPENDENT NON-PHOTO-
CHEMICAL FLUORESCENCE QUENCHING MEDIATED BY ATP.

This chapter is presented similar to the published account, see ref. Gilmore AM and Yamamoto HY (1992) Dark induction of zeaxanthin-dependent non-photochemical quenching mediated by ATP. Proc Nat Acad Sci USA 89:1899-1903
ABSTRACT

Zeaxanthin-dependent non-photochemical fluorescence quenching (NPQ) is a light-induced activity in plants that apparently protects against photodestructive damage due to excess light. We report a dark-induced NPQ in thylakoids of *Lactuca sativa* L. cv. Romaine. This effect was due to ATP-hydrolysis induced ΔpH and required an active ATPase. The NPQ induction was optimal at a physiological ATP concentration, and occurred under conditions of no forward and little or no reverse electron flow. The ATP-induced NPQ was, aside from a lower overall extent due to the subsaturating ΔpH, in most respects similar to light-induced quenching. Antimycin inhibited the quenching but not the ΔpH. We conclude quenching depends directly on zeaxanthin and lumen pH, indirectly on light, and is completely independent of any light-induced redox changes. We propose that a slow ΔpH-dependent change together with zeaxanthin causes quenching in the pigment bed; apparently antimycin inhibits this change. Furthermore, we suggest from the ability of ATP to sustain quenching in the dark for extended periods that persistent or slowly reversing zeaxanthin quenching often observed under photoinhibitory conditions *in vivo* may be due to ATP-hydrolysis.
INTRODUCTION

High light causes reversible changes in level of zeaxanthin in leaves and isolated chloroplasts via the so-called violaxanthin cycle (Hager 1980, Siefermann-Harms 1977, Yamamoto 1979). Zeaxanthin, formed by de-epoxidation of violaxanthin (Yamamoto et al. 1962) under acidic lumen pH (Hager 1969), enhances thermal or non-radiative dissipation of absorbed energy that is in excess of the photosynthetic capacity. Thus zeaxanthin apparently protects the photosystem against the potentially damaging effects of excess light (Demmig-Adams 1990). 

In vitro, the rate and extent of zeaxanthin formation are functions of lumen pH, ascorbate concentration, and apparently the redox state of plastoquinone (Yamamoto 1979). In vivo, zeaxanthin forms when CO$_2$ becomes limiting (Sapozhnikov 1973, Siefermann 1971, Yamamoto et al. 1962), at high-temperature (Bilger and Björkman 1990, Sapozhnikov 1973) or under other combined stresses (Demmig et al. 1988). Zeaxanthin forming and thus protective capacity, varies with species and growth history. Plants that are adapted to high-light have larger pools of xanthophyll-cycle pigments (violaxanthin + antheraxanthin + zeaxanthin) than the same species grown under reduced light (Thayer and Björkman 1990).

Non-radiative energy dissipation at photosystem II (PSII) can be measured experimentally as non-photochemical quenching (NPQ) of room-temperature fluorescence (Bradbury and Baker 1981, Krause 1988, Schreiber et al. 1986, Weis and Berry 1987). The correlation of NPQ with zeaxanthin formation, reported first by Demmig et al. (1987) in leaves, has since been observed in intact chloroplasts.
Zeaxanthin-dependent NPQ is apparently dependent on the "high-energy" state or $\Delta pH$ inasmuch as the presence of zeaxanthin in the thylakoid does not obligate quenching in the absence of a $\Delta pH$ (Demmig-Adams et al. 1990, Chapter 3). Whether, zeaxanthin and NPQ are correlated under all $\Delta pH$ conditions is controversial (Noctor et al. 1991). Additionally, zeaxanthin-independent or constitutive NPQ that is also present in many systems is $\Delta pH$-dependent (Adams et al. 1990, Demmig-Adams et al. 1990, Noctor et al. 1991, Chapter 3).

Although, lowering of the intrinsic quantum efficiency or "down-regulation" of PSII has been related to $\Delta pH$-dependent quenching (Weis and Berry 1987, Krause et al. 1988), the mechanism of high-energy NPQ is unclear. Zeaxanthin (Bilger and Björkman 1990, Demmig-Adams et al. 1990, Chapter 3) and lumenal-proton concentration (Briantais et al. 1980, Gilmore and Yamamoto 1991, Noctor et al. 1991) can be quantitatively related to NPQ but quenching is not simply due to $\Delta pH$ or zeaxanthin or both. Antimycin inhibits NPQ without inhibiting either $\Delta pH$ (Oxborough and Horton 1987) or zeaxanthin formation (Chapter 3). Antimycin has been proposed to inhibit quenching by inhibiting the oxidation of a ferredoxin-quinone reductase (Oxborough and Horton 1988).

The site of zeaxanthin quenching was determined by Stern-Volmer type analysis of maximal ($F_M$) and dark-level ($F_D$) NPQ (Adams et al. 1990, Bilger and Björkman 1990, Demmig-Adams et al. 1990, Chapter 3) to be in the "pigment bed" according to the Butler-Kitajima (1975) model. In contrast, it appears from a lack
of $F_O$ quenching that constitutive NPQ possibly occurs in the PSII reaction center (Adams et al. 1990, Demmig-Adams et al. 1990).

All zeaxanthin-dependent NPQ reported to date has been light-induced. Hence, it has not been determined whether light has a direct role in NPQ other than generation of the $\Delta pH$ required for NPQ and zeaxanthin formation. Adenosine 5'-triphosphate (ATP) hydrolysis can generate a $\Delta pH$ (Avron and Schreiber 1977, Petrack and Lipman 1961) and with ascorbate present can induce zeaxanthin formation in the dark (Hager 1969). In contrast to zeaxanthin-formation, several previous studies excluded the possibility ATP-hydrolysis can induce NPQ in the dark (Bennet et al. 1980, Horton and Black 1981, Krause et al. 1983a). ATP-hydrolysis in the presence of an electron donor/carrier system induces PSII variable fluorescence $F_V$ and stimulates chlorophyll $a$ (Chl $a$) luminescence by inducing reverse-electron flow to the primary electron-acceptor of PSII or $Q_A$ (Avron and Schreiber 1977, Schreiber 1980). We show herein that ATP-hydrolysis in the presence of a strong electron-acceptor induces zeaxanthin-dependent quenching under conditions of no forward and little or no reverse-electron flow. Other properties of this ATP-induced NPQ are also characterized. Hereafter in this report, NPQ refers to reversible zeaxanthin-dependent non-photochemical quenching unless noted otherwise.
MATERIALS AND METHODS

Chloroplast Isolation. Market lettuce, *Lactuca sativa* L. cv Romaine was dark adapted for at least 12 h at 4°C prior to use. Outer green leaf parts were removed and chilled on ice for 1 h prior to isolation of chloroplasts according to Chapter 1. Reaction mixtures in 3 ml final volume contained 0.1 M sorbitol, 5 mM MgCl₂, 50 mM tricine, 10 mM KCl, and 1 mM KH₂PO₄, adjusted to pH 8.0 with KOH. All reactions were at 25°C and stirred continuously during measurements.

Measurement of Zeaxanthin formation, Room-temperature Chlorophyll Fluorescence and Lumen pH. Room-temperature chlorophyll-fluorescence induction and absorbance readings were measured according to Chapter 1. Relative lumen-acidity was measured in parallel treatments as neutral-red uptake at 520 nm (ΔA₅₂₀) according to Siefermann-Harms (1978).
RESULTS

Preliminary experiments indicated that lettuce thylakoids are similar to pea thylakoids with respect to the effects of artificial electron-transport mediators, uncouplers and antimycin on NPQ (Chapter 3), except that lettuce has low to negligible levels of constitutive NPQ. Importantly, lettuce thylakoids have high violaxanthin-cycle activity and thus high NPQ. The reason for low constitutive NPQ in lettuce is unclear but may be related to lactucaxanthin, a xanthophyll that is unique to *Lactuca sativa* L. and closely related species (Siefermann-Harms et al. 1981). Lactucaxanthin appears to partially substitute for lutein in the light-harvesting chlorophyll \(a/b\) protein complex in lettuce (Siefermann-Harms and Ninnemann 1982).

**ATP-Induced Zeaxanthin-Dependent \(F_M\) and \(F_O\) Quenching.** A three-phased protocol was developed to examine the effects of ATP, in the dark, on NPQ. Zeaxanthin was first light induced with ascorbate and methylviologen (MV) (Chapter 3). Near the end of the light induction, DTT was added to inhibit further zeaxanthin formation (Yamamoto and Kamite 1972) and at the same time to activate the ATPase (Petrack and Lipman 1961). Finally, after a short dark period for \(\Delta pH\) relaxation, ATP was added. Fluorescence quenching and \(\Delta A_{520}\) were monitored following light off. The control treatment was similar except DTT was added before the actinic-light treatment to inhibit all zeaxanthin formation.

Fig. 5.1 shows typical results using the above protocol with zeaxanthin formation induced (ZEAX) or inhibited (DTT). The recorder tracings shown are
after the preliminary actinic treatment. The upper traces show that ATP induced significant \( F_M \) quenching in the ZEAX but not in the DTT treatment. The kinetics and extent of quenching were equal whether \( F_M \) was monitored kinetically with continuous flashes or with a single flash at the end of induction (data not shown). As shown, depleting ATP with hexokinase plus glucose rapidly reversed the quenching. Uncoupling with nigericin while ATP was still present similarly reversed the quenching (data not shown). The induction kinetics varied somewhat between chloroplast samples prepared from different lots of market lettuce but the extents of ATP-induced quenching were similar and all other properties investigated were qualitatively the same.

Only very slight increases in \( F_O \), which are indicative of closing of PSII traps by reverse electron flow to the primary electron acceptor (Schreiber 1984 a,b), were observed, see DTT treatment. Reverse electron flow is stimulated by the presence of an electron donor and carrier system such as DTT and phenazine methosulphate, respectively (Schreiber 1984a). Our system contained both ascorbate and DTT but no artificial electron carrier. The small \( F_O \) changes were examined at high sensitivity (middle traces) under identical conditions used for \( F_M \) measurements but without the saturating flashes which would otherwise interfere with the observations. In the ZEAX sample, \( F_O \) decreased in apparent proportion to \( F_M \) quenching. This apparent relationship between \( F_M \) and \( F_O \) quenching is examined in greater detail later. In the DTT treatment \( F_O \) showed a small rapid rise. Apparently this slight \( F_O \) rise is related to the slow ATP-induced \( F_V \) rise which Schreiber (Schreiber 1984 a,b)
observed in the presence of electron donors and carriers, it was definitely nigericin sensitive and was apparently quenched in the presence of zeaxanthin.

The lower $\Delta A_{520}$ traces in Fig. 5.1 show that ATP induced lumen acidification developed rapidly and faster than $F_M$ or $F_O$ quenching. Apparently, lumen acidification precedes quenching which suggests, consistent with numerous other reports (Adams et al. 1990, Briantais et al. 1980, Demmig-Adams et al. 1990, Gilmore and Yamamoto 1991c, Krause 1988, Oxborough and Horton 1987, 1988, Chapter 3), that quenching involves other slow changes following $\Delta p\text{H}$. The onset of the 520 nm change in the ZEAX treatment was slower than in the DTT sample, although the final extent was the same in both cases. The significance of these differing $\Delta A_{520}$ kinetics is unclear. A shorter actinic illumination was used for the DTT treatment to avoid photoinhibitory damage during ATPase activation. In the absence of zeaxanthin, long light treatments increased $F_O$ indicating photoinhibitory damage (Adams et al. 1990, Bilger and Björkman 1990, Demmig-Adams et al. 1990, Winter and Königer 1989).
Figure 5.1. ATP dark induction of F_M and F_O in thylakoids with high and low levels of preformed zeaxanthin. In the ZEAX treatment, zeaxanthin was light induced for 15 min in the presence of 30 mM ascorbate and 0.1 mM MV, then DTT was added and the illumination was continued for 3 min. In the DTT treatment, the sample was treated first with DTT then illuminated for 3 min. The actinic light intensity was 750 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \). Fluorescence was monitored at 1.6kHz and saturating flashes were 2 s at 3000 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), the first after 4 min dark relaxation. Final concentrations for the additions indicated were 3 mM DTT, 0.3 mM ATP, and 45 units hexokinase plus 10 mM glucose (HEX) was added as indicated. The reaction mixture was the same for the \( \Delta A_{520} \) measurements except for 4 \( \mu \text{M} \) neutral red which was added 2 min after the actinic light period. Final zeaxanthin concentrations were 109 \( \pm 1 \) and 3.5 \( \pm 0.5 \) mmol zeaxanthin mol\(^{-1} \) Chl \( a \) for the ZEAX and DTT experiments, respectively.
ZEAX

DTT

\[
\begin{align*}
F_w & \text{ Relative Units} \\
0.0 & \quad 0.2 & \quad 0.4 & \quad 0.6 & \quad 0.8 & \quad 1.0 \\
\end{align*}
\]

\[
\begin{align*}
F_o & \quad 0.10 F_o \quad \text{ATP} & \quad \text{HEX} & \quad \text{ATP} & \quad \text{HEX} \\
\end{align*}
\]

\[
\Delta A_{520} \quad 0.01 \\
\text{ATP} & \quad \text{HEX} & \quad \text{ATP} & \quad \text{HEX} \\
\]

10 min
Figure 5.2 shows that ATP induced zeaxanthin-dependent $F_M$ quenching showed a sharp optimum at around 0.3 mM ATP. At the optimum, the ATP-induced $\Delta A_{520}$ was approximately 80% of the level under saturating light. At higher ATP concentrations, NPQ was lower and several complex side effects were noted, including acidification of the medium itself. Importantly, the ATP optimum for dark induction of NPQ was within the physiological concentration range present in chloroplast stroma of various species (Hampp et al. 1982, Santarius and Heber 1965, Usuda 1988).

Figure 5.3 shows $S_{VN}$ was highly correlated with zeaxanthin concentration. The equation of the regression line was: $S_{VN} = (6.8 \times 10^{-3})(\text{zeaxanthin, mmol mol}^{-1} \text{Chl } a) + (2.8 \times 10^{-3}); n = 9; r^2 = 0.95$. The line intercepts close to the origin which shows that constitutive NPQ was undetectable in this thylakoid preparation. The linear correlation has a shallower slope than our previously reported results with peas by light induction (Chapter 3). The difference is probably due the subsaturating lumen acidification mentioned above.

**ATP-Sustained Quenching.** Leaves exposed to photoinhibitory conditions (Bilger and Björkman 1990, Demmig-Adams et al. 1989, Demmig et al. 1988) often show a zeaxanthin NPQ that relaxes slowly. We tested the possibility that this slowly relaxing or sustained quenching *in vivo* could be due to dark-sustained ATP-hydrolysis. Thylakoids were treated with actinic light to form zeaxanthin and induce NPQ, DTT was added to activate the ATPase, and ATP was then either added or omitted before turning the light off. In the absence of added ATP (Fig. 5.4A), $F_M$
Figure 5.2. Effects of ATP-concentration on zeaxanthin quenching. Zeaxanthin was preformed in a preliminary light period under the conditions described in Fig. 5.1. ATP at varying concentrations was added after five minutes dark ΔpH-relaxation. $SV_N$ was calculated using the $F_M$ 1 min prior to ATP addition and the $F'_M$ 16 min after ATP addition. The final zeaxanthin concentration for all reactions was $85 \pm 3$ mmol mol$^{-1}$ Chl $a$. 
Figure 5.3. ATP-induced quenching is linearly related to zeaxanthin concentration. All conditions were the same as in Fig. 5.1 (ZEAX) except that subsaturating concentrations of DTT (0 > X < 1.5 mM DTT) were added prior to the actinic light period. $F_M$ was measured 4 min after the end of the actinic light period, $F'_M$ was determined 6 min after addition of ATP.
Figure 5.4. ATP sustains, in the dark, NPQ formed initially in light. In the control (A) no ATP was added prior to turning off the actinic light. In (B) ATP was added at 13 min illumination. MV, DTT, hexokinase/glucose (H), DBMIB (D), and nigericin (N) were added as indicated. Actinic illumination was 16 min at 700 μmol photons m\(^{-2}\) s\(^{-1}\). Final reagent concentrations were as in Fig. 5.1. The initial actinic illumination formed 98 ± 1 mmol zeaxanthin mol\(^{-1}\) Chl \(\alpha\) in both (A) and (B).
relaxed rapidly and \( F_O \) recovered in about one minute after actinic light off. \( F_M \) also recovered completely and was not further increased by hexokinase. As expected, DBMIB increased \( F_M \) and nigericin had no further effect. These results indicate \( \Delta \text{pH} \) relaxed completely following light off, consistent with previous results (Demmig-Adams et al. 1990, Chapter 3). Fig. 5.4B shows that when ATP was added before light off, \( F_O \) and \( F_M \) quenching were sustained in the dark until hexokinase was added. Evidently, the presence of ATP sustained the quenching by maintaining the \( \Delta \text{pH} \).

**Correlation of ATP-Induced \( S_V_N \) and \( S_V_O \).** Light-induced \( F_M \) and \( F_O \) quenching at steady-state are linearly correlated with zeaxanthin (Chapter 3). Whether the quenching is correlated under light-induced kinetic conditions is not easily examined because the slow oxidation of \( Q_A \) and the rapid dissipation of \( \Delta \text{pH} \) make accurate \( F'_O \) measurements difficult. Under ATP-induced conditions these constraints are not present. Fig. 5.5 shows that \( S_V_N \) and \( S_V_O \) observed kinetically are highly correlated. The inset shows the kinetics of the ATP-induced \( S_V_N \) and \( S_V_O \) used in the correlation. The extrapolated threshold for \( S_V_O \) is approximately 0.1 \( S_V_N \). This threshold is probably an artifact of the underlying \( F_O \) rise at low zeaxanthin (Fig. 5.1). Sufficient \( S_V_N \) with its associated \( F_O \) quenching must develop to overcome the ATP-induced \( F_O \) rise. If the threshold is not an artifact, the results suggest that some NPQ occurs in the reaction center. Proportional \( S_V_N \) and \( S_V_O \)
is consistent with earlier studies that conclude most and probably all NPQ occurs in the pigment bed (Noctor et al. 1991, Rees et al. 1990, Chapter 3).

**Effect of Antimycin on ATP-Induced Quenching.** Antimycin inhibits NPQ without affecting ΔpH development (Oxborough and Horton 1987) or zeaxanthin formation (Chapter 3). Antimycin does not reverse NPQ that is pre-developed unless the ΔpH is dissipated (Oxborough and Horton 1987, 1988). It is not clear whether antimycin's inhibitory effect on NPQ is due to a light-induced redox change in an electron-transport carrier or a ΔpH-dependent change or both. Dark induction of NPQ allowed examination of whether antimycin inhibition involved the redox state of an electron transport carrier. Fig. 5.6 shows antimycin inhibited all SV_N development when it was added before ATP was added. Antimycin added after SV_N had developed inhibited further development of SV_N but did not substantially reverse pre-developed SV_N. Fig. 5.6 is in fact similar to Fig. 2.3 in (Chapter 2) which was light-induced. Consistent with earlier studies with light-induced ΔpH (Oxborough and Horton 1987, Chapter 3), antimycin did not inhibit ATP-induced ΔpH (data not shown). Interestingly, ATP induced a rapid F_O increase, up to 9 % in some cases, when NPQ development was inhibited with antimycin, even with zeaxanthin present. This F_O rise was similar to when zeaxanthin formation itself was inhibited with DTT (Fig. 5.1). Thus it appears that this small and rapid ATP-induced F_O increase is detectable when zeaxanthin-dependent NPQ cannot develop due either to the absence of zeaxanthin or presence of antimycin. Interestingly, F_O also increases in
*vivo* when zeaxanthin formation is inhibited with DTT (Adams et al. 1990, Demmig-Adams et al. 1990, Winter and König 1989). However, the *in vivo* rise has in all these cases been attributed to photoinhibitory damage and in no case has there been an implied role of sustained reverse-electron flow.
Figure 5.5. Correlation between ATP-induced $SV_N$ and $SV_O$ in thylakoids with high preformed zeaxanthin. The linear regression equation is $SV_O = 0.234(SV_N) - 0.022$. The inset shows the kinetics of $SV_N$ and $SV_O$ used for the correlation. $SV_N$ and $SV_O$ were determined in separate reactions with and without saturating flashes, respectively. Experimental conditions and reagent concentrations were as in Fig. 5.1 except that zeaxanthin was light induced for 10 min and following the DTT addition, actinic illumination was continued for an additional 3 min. $F_M$ and $F_O$ were measured after 4 min dark $\Delta p\text{H}$ relaxation and ATP was added 1 min later. Final zeaxanthin concentrations were $85 \pm 1$ mmol zeaxanthin mol$^{-1}$ Chl $a$. 

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Figure 5.6. Effects of antimycin (AA) on dark ATP-induced zeaxanthin-dependent NPQ. The experimental conditions were as described in Fig. 5.1. \(SV_N\) was computed using \(F_M\) measured after 5 min of dark relaxation up to the addition of DBMIB (D) and thereafter, the \(F_M\) measured 2 min after the addition of nigericin (N) was used. This procedure accounted for the effects of PQ quenching on \(F_M\). The final zeaxanthin concentration was 100 ± 3 mmol zeaxanthin mol\(^3\) Chl \(a\).
DISCUSSION

In leaves, zeaxanthin formation (Hager 1980, Siefermann-Harms 1977, Yamamoto et al. 1962, Yamamoto 1979) and energy-dependent NPQ (Adams et al. 1990, Bilger and Björkman 1990, Demmig-Adams et al. 1990, Weis and Berry 1987) are both light inducible. These responses, initially characterized independently, are now known to be related in that zeaxanthin formation increases energy-dependent NPQ (Adams et al. 1990, Demmig-Adams et al. 1990, Chapter 3). In thylakoids, with ascorbate present, ΔpH from ATP-hydrolysis or buffering thylakoids at pH 5 can substitute for light in zeaxanthin formation (Hager 1969). The present results establish that ATP can also substitute for light in NPQ. The dark induction is due to ΔpH from hydrolysis-dependent proton translocation and thus requires an activated ATPase.

Under light induction, distinguishing between direct and indirect effects of light is difficult. In a study that preceded the discovery of zeaxanthin NPQ, acid-induced fluorescence quenching was observed in uncoupled thylakoids subjected to pH changes of the suspending medium (Briantais et al. 1980). Irreversible and rapidly reversible quenching components were induced and the latter was concluded to be analogous to the slow P to S light-induced fluorescence decline (Briantais et al. 1979). The dark induction of zeaxanthin NPQ reported here is new. Only a very weak modulated monitoring beam (< 0.15 μmol photons m⁻² s⁻¹), which itself has no actinic effect, was present during F₀ quenching. In the case of Fₘ quenching, which required use of intense saturating flashes, the rate and extent of quenching was
independent of the flash rate. The ATP-induced quenching of $F_O$ in Figs. 5.1 and 5.5 is also a new observation of the kinetics of "energized state" quenching, $F'_{O}$. The proportionality of $F_O$ quenching and $F_M$ quenching under kinetic conditions (Fig. 5.5) strongly support the similar relationship seen under steady-state light-induction (Adams et al. 1990, Bilger and Björkman 1990, Demmig-Adams et al. 1990, Chapter 3). Proportional quenching implies that zeaxanthin-dependent NPQ occurs in the pigment bed. Overall the properties of ATP-induced and light-induced NPQ are similar.

While induction of a $\Delta p$H by ATP-hydrolysis is not surprising, dark induction of zeaxanthin NPQ was not predictable from the literature. Earlier studies reported that ATP-hydrolysis induced reverse-coupling reactions which caused a biphasic increase in the dark-level fluorescence (Avron and Schreiber 1977, Schreiber 1984 a, b) and stimulated delayed light-emission from PSII (Schreiber 1980). These effects required the presence of an electron donor such as DTT or ascorbate plus an electron carrier such as phenazine methosulphate (Avron and Schreiber 1977, Schreiber 1980, Schreiber 1984a,b). The absence of artificial electron carriers in the present severely inhibited reverse electron flow, although ascorbate and DTT were present. The $F_O$ signal after each flash indicated that $Q_A$ remained almost completely oxidized during ATP induction (Fig. 5.1). This condition was apparently favored not only by the absence of added electron carriers but more certainly by the presence of the strong electron acceptor, MV. The highly oxidized conditions probably explain the lack of uncoupler-insensitive ATP-induced quenching of the type
previously reported to be due to state transitions (Horton and Black 1981, Krause and Behrend 1983). Importantly, NPQ induction under both dark (this study) and light conditions (Chapter 3) strongly suggests that the redox state of an electron transport carrier is not critical.

While neither a direct light effect nor the redox state of an electron transport carrier appear critical for development of NPQ, it remains that zeaxanthin and ΔpH are insufficient for quenching. This is clearly seen in the effect of antimycin which inhibits NPQ without inhibiting either ΔpH (Oxborough and Horton 1987) or zeaxanthin (Chapter 3) formation. When NPQ can develop, zeaxanthin (Demmig-Adams et al. 1990, Chapter 3) and lumen protons (Briantais et al. 1980, Noctor et al. 1991) behave as Stern-Volmer type quenchers. The events that ultimately translate these parameters into quenching is unknown. The faster kinetics of lumen acidification over NPQ development, with zeaxanthin preformed (Fig. 5.1, Noctor et al. 1991), suggests that a critical change follows ΔpH. We propose, that a slow pH-sensitive conformational change is involved. This conformational change is hypothesised to result in zeaxanthin-dependent quenching in the pigment bed. Antimycin presumably inhibits this conformational change. The relationships of ΔpH to zeaxanthin formation and NPQ, including the possible site of antimycin inhibition are shown schematically in Fig. 5.6.

Zeaxanthin has been correlated with both rapidly relaxing (Adams et al. 1990, Demmig-Adams et al. 1990) and sustained types of NPQ (Demmig-Adams et al. 1989, Demmig et al. 1988). Whereas rapidly-relaxing NPQ is ‘high-energy’ quenching,
the nature of sustained quenching is undefined. It was suggested that the same conformational changes in the light-harvesting chlorophyll-protein complexes that cause NPQ are somehow sustained under photoinhibitory conditions even after ΔpH-relaxation (Demmig-Adams 1990). Interestingly, this slow reversing quenching was reported to reverse in synchrony with the reversion of zeaxanthin back to violaxanthin, which would be consistent with a slowly reversing ΔpH. In leaves, sustained NPQ was observed under conditions of limited CO₂ assimilation, such as light exposure under 2% O₂, zero CO₂ (Demmig-Adams et al. 1989), high-light (Bilger and Björkman 1990, Demmig et al. 1988), and water stress (Demmig et al. 1988). Here, exogenous ATP at concentrations naturally occurring in chloroplast stroma simulated sustained quenching (Fig. 5.4). Hence, we suggest that slowly relaxing quenching may be due in part to ΔpH maintained by ATP-hydrolysis under conditions of limited ATP consumption by dark photosynthetic reactions. An integrated feedback mechanism which dissipates excess light energy from PSII under conditions of "excess" ATP is implied.
Figure 5.7. Schematic of light- and ATP-induced zeaxanthin-dependent NPQ. A low lumen pH and ascorbate (Asc) are required for zeaxanthin (Zeax) formation from violaxanthin (Viol) via the violaxanthin de-epoxidase (VDE) and for NPQ induction; uncouplers therefore inhibit both Zeax and NPQ formation. In contrast with NPQ, Zeax after its formation in thylakoids is not reversed by uncoupling. DTT inhibits Zeax formation but not ΔpH or NPQ directly. Antimycin (Anti-A) is proposed to inhibit after ΔpH and Zeax formation because it does not inhibit either but does inhibit NPQ.
CHAPTER 6

A LINEAR MODEL, WHICH INCLUDES ANTERAXANTHIN,
CLOSELY PREDICTS XANTHOPHYLL-DEPENDENT
NON-PHOTOCHEMICAL QUENCHING.

This chapter is presented as prepared for publication in Photosynth Res.
ABSTRACT

Whether quenching is linear with both zeaxanthin and lumen proton concentration is controversial. We hypothesized that if quenching is indeed linear with zeaxanthin and lumen proton concentration, then nonphotochemical quenching can be described by a linear model. To test this hypothesis a data matrix of nonphotochemical quenching, zeaxanthin, and lumen proton concentration, measured as neutral red uptake, was obtained with chloroplasts of *Pisum sativum* L. cv. Oregon and *Lactuca sativa* L. cv. Romaine. In order to account for zeaxanthin-independent or constitutive quenching, both the product of (neutral red uptake x zeaxanthin concentration) and neutral red uptake were treated as independent variables. Multiple-regression analyses yielded predictive equations with high correlations for both peas and lettuce. However, a linear model which includes antheraxanthin with a quenching efficiency equal to zeaxanthin, excluded neutral red uptake as an independent variable. It is possible to describe all quenching as simple linear equations with high correlations by assuming that quenching is the product of (neutral red uptake x zeaxanthin plus antheraxanthin concentration). These results support the view that zeaxanthin plus antheraxanthin and lumen proton concentration both linearly increase quenching. The results also suggest that quenching by antheraxanthin accounts for what was previously termed constitutive quenching.
INTRODUCTION

In higher plants, excess light can lower the photon or quantum yield of photosynthesis. This lowering of the quantum efficiency, generally termed photoinhibition of photosynthesis, has in some cases been attributed to an increase in nonradiative dissipation of excitation energy in the pigment antennae complexes (Björkman 1987, Krause and Weis 1991). Nonradiative dissipation in the antennae is believed to protect against overexcitation of the reaction centers. Alternatively or additionally, photoinhibition results from overexcitation of the photosystem II (PSII) reaction centers and leads to reaction center inactivation (Cleland et al. 1986, Kyle 1987, Guenther and Melis 1990). The mechanism behind the induction and reversal of reaction center inactivation is believed to involve damage and repair of the Q_B-binding or D1 protein (Guenther and Melis 1990, Krause and Weis 1991, Kyle 1987). The excess light conditions that induce these two type of responses can differ. Whereas, damage to the D1 protein generally occurs under extreme conditions of excess-light and is observed primarily in vitro (Baker 1991, Kyle 1987), nonradiative dissipation is readily observed in vivo as a diurnal response (Baker 1991, Björkman 1987, Demmig-Adams 1990).

Nonradiative dissipation processes can be monitored by measuring non-photochemical chlorophyll fluorescence quenching, NPQ (Bradbury and Baker 1981, Schreiber et al. 1986). Although several components comprise NPQ (for a review see Krause and Weis 1991), the major component is ΔpH-dependent or ‘high-energy’ NPQ (Briantais et al. 1979, 1980). High-energy NPQ also comprises of both
zeaxanthin (Z)-dependent and independent (constitutive) components (Adams et al.
1990, Bilger and Björkman 1990, Demmig-Adams et al. 1990, Chapter 3). Both
components have similar sensitivity to uncouplers (Demmig-Adams et al. 1990,
Chapter 3) and antimycin and are inducible under linear and cyclic electron transport
(Chapter 3). In this communication, unless stated otherwise, NPQ means ΔpH-
dependent NPQ.

Lumen proton concentration, measured as quenching of 9-aminoacridine
fluorescence (Briantais et al. 1979, 1980), has been shown to linearly increase NPQ.
More recently, (Z)-concentration or [Z] (Bilger and Björkman 1991, Demmig-Adams
et al. 1990, Chapters 3 and 5) has also been shown to linearly increase NPQ, the
latter under a saturating light-induced ΔpH (Chapter 3) or a subsaturating ATP-
induced ΔpH (Chapter 5). However, other reports (Noctor et al. 1991, Rees et al.
1989, Ruban et al. 1991) suggest a nonlinear relationship where [Z] increases
quenching only at subsaturating and not at saturating ΔpH. If NPQ is linear with
both [Z] (Bilger and Björkman 1991, Demmig-Adams et al. 1990, Chapters 3 and 5)
and lumen proton concentration or [H+] (Briantais et al. 1979, 1980) it should be
possible to describe NPQ as a simple linear model.

Gilmore and Yamamoto (1991c) reported that NPQ was linear with [Z] at any
given lumen [H+] , measured as neutral red uptake, and conversely that NPQ was
linear with lumen [H+] for any given [Z]. Herein, we present a linear model, based
on the relative lumen [H+] measured as neutral red uptake (Siefermann-Harms
1978), and the product of (lumen [H+] x zeaxanthin concentration [Z]) as
independent parameters. Inclusion of the lumen $[H^+]$ as an independent variable accounts for any zeaxanthin-independent or constitutive NPQ. We also show that including antheraxanthin (A) as a quencher with equal efficiency to zeaxanthin allows the prediction of NPQ with a simple linear equation, where all measured NPQ is accounted for by calculating the product of (lumen $[H^+] \times [Z + A]$). Importantly, (A) appears to quench with the same efficiency as (Z) and accounts for the so-called constitutive NPQ component in both peas and lettuce.
MATERIALS AND METHODS

**Chloroplast Isolation.** Chloroplasts were isolated according to Chapter 1. The reactions were stirred continuously at 18°C.

**Measurement of Room-temperature Chlorophyll Fluorescence, Lumen [H⁺], and De-epoxidation.** Induction of room-temperature chlorophyll-fluorescence (Schreiber et al. 1986) was measured according to Chapter 1. Actinic PFD's were controlled with neutral density filters (Wratten 96, Kodak).

A data matrix of SVN-values with corresponding xanthophyll compositions and relative lumen [H⁺]'s was generated. Five separate reactions were used for both the pea and lettuce data, each reaction comprised of five light periods at five different PFD's; this yielded a matrix of 25 SVN values for both peas and lettuce. The series of five light periods were each separated by 5 min dark periods; the first light period was 20 min and the other four were for 5 min. The first light period was at a PFD saturating for ΔpH and the following four light periods were at progressively lower subsaturating PFD's. Zeaxanthin formation was controlled with various subsaturating DTT concentrations added prior to illumination. After 19 min illumination in the first light period the DTT concentration of all reactions was brought up to 1.5 mM DTT. The weak 1.6kHz PAM measuring beam (<0.15 μmol photons m⁻² s⁻¹) excluded additional actinic effects by the measuring beam at subsaturating actinic PFD's. Saturating pulses (2 s, 3000 μmol photons m⁻² s⁻¹) were limited to 60 s intervals during the light periods to minimize lumen pH perturbation at low actinic
PFD's. All $S_{SV}$ values were calculated at steady-state using the $F_M$ after 5 minutes of dark $\Delta p\text{H}$ relaxation after a given light period. $F'_M$ was determined after 20 min and 5 min illumination for the first and all subsequent light periods, respectively.

Steady-state relative lumen $[H^+]$ was measured with a DW 2000 UV-VIS spectrophotometer (SLM-Aminco, Urbana, IL, USA) in parallel experiments by neutral red uptake as the steady-state $\Delta A_{520}$ according to Siefermann-Harms (1978). Neutral red concentration was 4 $\mu$M. All $\Delta A_{520}$ measurements were with 1.5 mM DTT added prior to illumination because absorbance changes due to de-epoxidation (Yamamoto et al. 1972) would interfere. All reported light-induced $\Delta A_{520}$ readings were obtained by subtracting both the $\Delta A_{520}$ due to neutral red absorbance in the dark (Siefermann-Harms 1978) and the $\Delta A_{520}$ without neutral red for each given PFD. The final $\Delta A_{520}$ readings were normalized relative to Chl $a$. Final pigment concentrations were determined at the end of each reaction by HPLC according to Chapter 4. All quantitative pigment values are expressed in mmol's respective pigment mol$^{-1}$ chlorophyll $a$ (Chl $a$).

**Statistical Analysis.** Data was analyzed with an SAS/STAT software package (SAS Institute Inc., Cary, NC, USA).
RESULTS

Data Set for Multiple-Linear Regression Analyses. As stated in the materials and methods a data matrix of five de-epoxidation levels by five PFD's with corresponding ΔA₅₂₀ levels was obtained. Table 6.1 shows the pigment data for pea and lettuce chloroplasts. Low but detectable levels of (A) and (Z) were present in the dark in both species. The dark-levels can vary between plant samples and chloroplast isolations, generally ranging from 0 to 15 mmol of either (Z) or (A) mol⁻¹ Chl a (unpublished data). The levels of (Z) formation, varied with the DTT-treatment conditions, ranging from 7.79 to 37.91 and from 8.79 to 94.58 mmol mol⁻¹ Chl a, for peas and lettuce, respectively.

The total level of (A) in light-treated peas varied from 9.34 to 18.30 mmol mol⁻¹ Chl a. Compared to peas, the changes in lettuce for (A) were greater, varying between 11.48 and 31.79 mmol mol⁻¹ Chl a. In both peas and lettuce, (A) substantially outweighed (Z) when de-epoxidation was inhibited with >0.40 mM DTT. Thus, (A) was a significant component under these conditions where it represented up to 18 and 20% of the total [V + A + Z] in peas and lettuce, respectively. The accumulation of (A) at subsaturating DTT concentrations was counter to what was expected from in vitro studies with the partially purified de-epoxidase enzyme. Yamamoto and Higashi (1978) reported that the kinetics of de-epoxidation of (A) were five times faster than (V). Thus, it was not expected that (A) would accumulate to a substantial degree under any circumstance. The accumulation of (A) suggests
the relative de-epoxidation rates of (A) and (V) were changed by subsaturating DTT concentrations.

The (V + A + Z) pool was constant regardless of DTT treatment in both species. The total maximal [Z + A] formed in lettuce was twice the amount formed in peas. The other major carotenoid pigments, namely, lutein, 9'-cis-neoxanthin, β,β-carotene, in both peas and lettuce remained constant relative to Chl a with C.V. (%) values of less than 2.

Table 6.2 shows the corresponding ΔA_{520} and SV_N data at varying steady-state PFD's in the respective DTT treatment conditions from Table 6.1. The ΔA_{520} levels were light-saturated at about 300 μmol photons m^{-2} s^{-1} and reached steady-state within five minutes at each PFD for both peas and lettuce. The saturating ΔA_{520} levels used in these experiments was twice the saturating PFD for both peas and lettuce. The maximal level of SV_N was about 1.4 times higher in lettuce than in peas.

**Regression Analysis of the Effects of De-epoxidation and ΔA_{520} on SV_N.**

Gilmore and Yamamoto (1991c) reported that SV_N is linear with both [Z] and ΔA_{520}. In accordance with this observation, linear regression equation (1) represents SV_N as the simple product of (Z) concentration in mmol mol^{-1} Chl a and ΔA_{520}:

\[
SV_N = x_1 (ΔA_{520})[Z] + a
\]  

(1)
where $x_1$ is the coefficient for the independent variable $(\Delta A_{520})[Z]$ and $a$ is the SV$_N$ intercept. Table 6.3 shows that the linear correlations were relatively low (Peas, $r^2 = 0.717$ and Lettuce, $r^2 = 0.906$). The standard error values and the corresponding $p$-values for $a$ were high in both peas and lettuce and implied that the predicted SV$_N$-intercept was not statistically valid. The predicted SV$_N$ with this equation is not consistent with the experimental observations of significant levels of SV$_N$ at low $[Z]$, especially in peas. It appears that SV$_N$ is not simply a product of the two parameters, in either peas or lettuce.

Based on the observation that there were significant levels of SV$_N$ even when $[Z]$ was low it was proposed that inclusion $\Delta A_{520}$ as an independent variable may account for the constitutive SV$_N$ and accurately predict SV$_N$ at any given combination of $[Z]$ or $\Delta A_{520}$. Thus in the following equation,

$$SV_N = x_1 (\Delta A_{520})[Z] + x_2 (\Delta A_{520}) + a$$  \hspace{1cm} (2)$$

$x_1$ is the coefficient for $(\Delta A_{520})[Z], x_2$ is the coefficient for $(\Delta A_{520})$, and $a$ is the SV$_N$ intercept. The resulting multiple-regression equations in Table 6.4 have significant correlations, $r^2 = 0.967$ and $r^2 = 0.975$, for peas and lettuce, respectively. The standard error and $p$-values for $a$ in Table 6.4 were more significant in both peas in lettuce than the comparable values for $a$ in Table 6.3. Equation (2) more accurately predicted SV$_N$ at any given $[Z]$ or $\Delta A_{520}$ than equation (1). Inclusion of $\Delta A_{520}$ as an independent variable accounted for any predicted SV$_N$ at $[Z] = 0$. 

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Figure 6.1. The effects of varying zeaxanthin concentration on actual and predicted $S_{V_N}$ in peas and lettuce for three different steady-state $\Delta A_{520}$ levels. (A and B) show the actual (symbols) and predicted (lines) $S_{V_N}$ for peas and lettuce, respectively, from the data sets in Tables 6.1 and 6.2 and the multiple-regression equations in Table 6.4, respectively.
Figure 6.1 compares the actual data at three different $\Delta A_{520}$ levels from Tables 6.1 and 6.2 versus the predicted $SV_N$ with equation (2) from Table 6.4, for peas and lettuce, respectively. Figures 6.1A and 6.1B show that with the multiple-regression equation (2), we see a close fit between the actual and predicted data. Apparently, peas (Fig. 6.1A) have significantly higher levels of predicted quenching at $[Z] = 0$ than lettuce (Fig. 6.1B).

**Antheraxanthin and $SV_N$.** As shown in Table 6.1, when de-epoxidation is titrated with DTT, (A) accumulates to relatively high levels. Demmig-Adams (1990) and Bilger and Björkman (1991) suggested that (A) may quench fluorescence similar to (Z). However, there is presently no experimental or statistical evidence to support this proposal. Interestingly, Demmig-Adams et al. (1990) suggested that (A) may be equal to (Z), whereas, Bilger and Björkman (1991) have shown that NPQ correlates linearly with the so-called epoxidation state or EPS; EPS is a measure of the relative number of epoxide end-groups to the non-epoxide endgroups of the sum of the total violaxanthin cycle pigments including ($V + A + Z$). Thus, a correlation between NPQ and EPS in itself assumes that (A) quenches with one-half the efficiency of (Z), because (A) has one half the number of epoxide end-groups as (V). It was of interest to determine whether inclusion of (A) as a quencher would account for the constitutive $SV_N$ and allow $SV_N$ to be described as a simple linear equation, i.e., rule out the necessity of $\Delta A_{520}$ as an independent variable. The effects of
assuming (A) quenches with one-half the efficiency of (Z) were calculated using equation (3). Assuming,

\[ SV_N = x_I (\Delta A_{520})[Z + 0.5A] + a \]  \hspace{1cm} (3)

did increase the correlation significantly over equation (1); the correlations were \( r^2 = 0.860 \) and \( r^2 = 0.970 \), in peas and lettuce, respectively. The effects of assuming (A) quenches with equal efficiency to (Z) were tested in equation (4). Assuming,

\[ SV_N = x_I (\Delta A_{520})[Z + A] + a \]  \hspace{1cm} (4)

increased the correlations even further to \( r^2 = 0.943 \) and \( r^2 = 0.982 \), for peas and lettuce, respectively; the actual equations based on the general form equation (4) are shown in Table 6.5. Based on the observation that the correlation coefficients were improved statistically and that no \( SV_N \) is predicted at \([Z + A] = 0\) with equation (4), it appears that (A) quantitatively accounts for what was previously termed constitutive \( SV_N \) in both peas and lettuce. The fact that inclusion of \( \Delta A_{520} \) as an independent variable is no longer necessary to explain the constitutive quenching, serves as further evidence supporting this possibility. From results presented in Table 6.5, it appears that all \( SV_N \) can be accounted for by \([Z + A]\) and that both lumen \([H^+]\) and \([Z + A]\) linearly determine \( SV_N \) in peas and lettuce.
In order to more conclusively determine whether the linear-regression equations in Table 6.5 ruled out the significance of $\Delta A_{520}$ as an independent variable the effects of including $[Z + A]$ in a multiple-regression equation were calculated using the equation of the general form:

$$SV_N = x_1 (\Delta A_{520})[Z + A] + x_2 (\Delta A_{520}) + a$$  \hspace{1cm} (5)

The multiple regression equations with $[Z + A]$ in Table 6.6 were more highly correlated than the multiple-regression equations with $[Z]$-only in Table 6.4, for both peas and lettuce. Notably, (A) is the only pigment that increased the multiple-regression correlation because it was the only other pigment besides (Z) or (V), that changed during the light treatments. Considering $(\Delta A_{520})$ as an independent variable in Table 6.6 increased the multiple-regression correlation coefficient in peas from 0.943 to 0.980, when compared to the linear-regression correlation with peas in Table 6.5. This contrasts the improvement from $r^2 = 0.718$ to $r^2 = 0.967$ with peas when comparing the linear- and multiple-regression correlations with $[Z]$ alone in Tables 6.3 and 6.4, respectively. Thus, the data suggests that (A) accounts for a very significant component of the variation when compared to (Z) alone. Also, if it is assumed that $[Z + A] = 0$ in either equation in Table 6.6, no $SV_N$ is predicted even for the saturating $\Delta A_{520}$ levels in peas or lettuce. Since this was also the case with the equations in Table 6.5, addition of $\Delta A_{520}$ as an independent variable is not necessary to account for the constitutive $SV_N$ in either peas or lettuce. However,
equation (5) does allow for a better closeness of fit in peas than equation (4). Also, in Table 6.6 with lettuce it is obvious from the fact that the correlation was not improved by the (\Delta A_{520})-component and from the high \(p\)-values for the (\Delta A_{520})-component that it was not significant as an independent variable. Therefore, the simple linear equations used in Table 6.5 are favored because they are simpler and conceptually account for the combined linear effects \(\Delta A_{520}\) and \([Z + A]\) on \(S V_N\).

In order to demonstrate the linear-model and the effects of (A) on \(S V_N\) Fig. 6.2 was constructed to shows the actual data (from Tables 6.1 and 6.2) and the predicted \(S V_N\) from Table 6.5 for both peas and lettuce at three different \(\Delta A_{520}\) levels. Examination of Figs. 6.2A and 6.2B indicates that both peas and lettuce appear to have significant \([Z + A]\) thresholds at the saturating \(\Delta A_{520}\) as well as the two subsaturating \(\Delta A_{520}\) levels. Thus it is clear that when the sum of \([Z + A]\) is plotted against \(S V_N\) that no \(S V_N\) is predicted below \([Z + A] = 0\). Because no quenching is seen until the threshold \([Z + A]\) is reached for any given \(\Delta A_{520}\), it appears that \((Z)\) and \((A)\) are actually quenching; this contrasts the converse view that quenching may be due to the stoichiometric decrease in \([V]\). The significance of the \([Z + A]\) threshold is unclear; however, it may prevent unnecessary dissipation of excess light energy at limiting PFD's with low levels of \(\Delta pH\) and de-epoxidation, where the accumulation of excess energy would be unlikely. Further, it appears that although lettuce does have higher maximal \([Z + A]\) and \(S V_N\) capacities than peas, the predicted \(S V_N\)-type ratio at a saturating \(\Delta A_{520}\) is significantly lower than in peas, i.e. \(1.52 \times 10^{-2}\) and \(2.47 \times 10^{-2}\) (\(S V_N\)-units / mmol \([Z + A]\) mol\(^{-1}\) Chl \(a\)), for lettuce.
and peas, respectively. The significance of these \( S V_N \)-type ratios is unclear but it may indicate that the quenching efficiency of \([Z + A]\) is species specific and or related to the growth light regime. These possibilities, however, will require more intensive comparative studies with plants from similar and different species adapted to varying growth-light regimes.

The putative quenching by \((A)\) in this report apparently explains the lack of constitutive \( S V_N \) reported in Chapter 5 with lettuce and apparently also excludes any significance of lactucaxanthin with regards to the lack of constitutive NPQ as previously suggested. The subsaturating ATP-induced \( \Delta pH \) was apparently below the threshold for expression of the normally low to negligible levels of constitutive quenching. Thus relatively high levels of de-epoxidation must occur in lettuce to detect ATP-induced \( S V_N \). These reported \((A)\) and constitutive NPQ levels are consistent with those reported in DTT treated leaves (Bilger et al. 1989, Demmig-Adams et al. 1990) and chloroplasts (Demmig-Adams et al. 1990). In other studies (Adams et al. 1990, Bilger and Björkman 1990) quantitation of \((A)\) was not considered and thus it is difficult to compare \((A)\) and constitutive NPQ data. In addition, this study indicates that the \([Z + A]\) concentration, not the relative epoxidation state (Bilger and Björkman 1991) correlates with NPQ. This study apparently confirms the suggestion of Demmig-Adams (1990) that the equivalent of ‘one-half a zeaxanthin molecule’ may suffice for quenching; although it is obvious that antheraxanthin is much more than half a zeaxanthin molecule.
Figure 6.2. The effects of varying zeaxanthin plus antheraxanthin concentration on actual and predicted SVₙ in peas and lettuce for three different steady-state ΔA₅₂₀ levels. (A and B) show the actual (symbols) and predicted (lines) SVₙ for peas and lettuce, respectively, from the data sets in Tables 6.1 and 6.2 and the linear-regression equations in Table 6.5, respectively.
DISCUSSION

This study shows that xanthophyll-dependent high-energy quenching can be described with linear equations in pea and lettuce chloroplasts. Apparently, quenching can be modelled most simply as the product of lumen \([H^+]\) and \([Z + A]\). This direct implication of \((A)\) as a component of NPQ is new. The possible quenching role of \((A)\) is significant because it implies that all NPQ is xanthophyll-dependent. This study also suggests that NPQ reported in many earlier studies with intact and broken chloroplasts may have been mediated by constitutive levels of \((A)\) and or \((Z)\), assuming no de-epoxidation had occurred unknowingly during the light treatments. The latter case is a possibility in several studies of NPQ which used intact chloroplasts (Barber et al. 1974a,b, Krause et al. 1983, Krause and Laasch 1987, Oxborough and Horton, 1988, Schreiber and Neubauer 1987, 1990). Sokolove and Marsho (1976) showed that de-epoxidation could be stimulated without ascorbate in intact chloroplasts, presumably by endogenous stromal ascorbate. It is well known that ascorbate is a necessary addition for induction of de-epoxidation in broken chloroplasts (Hager 1969, Yamamoto et al. 1972). Interestingly, Briantais et al. (1979) found it necessary to add exogenous ascorbate to their broken chloroplasts in order to invoke any significant NPQ.

Although, this Chapter and Chapters 3 and 5 suggest all NPQ is related to a common mechanism, the current literature includes several hypothetical models to explain the relationship between constitutive NPQ and \((Z)\) NPQ. Demmig-Adams et al. (1990) suggested that constitutive NPQ was mechanistically distinct from \((Z)\)
NPQ, occurred in the reaction center of PSII, and was possibly related to the dissipative electron-cycle around PSII proposed by others (Horton and Lee 1985, Schreiber and Neubauer 1987). Schreiber and Neubauer (1987) recently suggested that the constitutive NPQ may be due to chlorophyll fluorescence quenching by carotenoid triplets and that (Z) added to the capacity for fluorescence quenching by increasing the carotenoid triplet capacity.

Another, model proposed by Noctor et al. (1991) and Rees et al. (1989) and later re-emphasized by Ruban et al. (1991) implies that (Z) acts as a quenching amplifier only at subsaturating ΔpH and that all NPQ is due to conformational changes in the light-harvesting chlorophyll-protein complexes. Thus their studies reported that the relationship between NPQ and (Z) was nonlinear at a saturating ΔpH. Also, in the cases of Rees et al. (1989) and Noctor et al. (1991) there was no apparent correlation between (A) and the level of NPQ in the absence of (Z). Although there is no clear explanation for the differences between Rees et al.'s (1989) and Noctor et al.'s (1991) data and this study, they clearly have a different experimental approach wherein they isolate chloroplasts from light-treated, DTT-treated, or dark-adapted leaves, whereas, in this study the de-epoxidation levels were controlled entirely in vitro with chloroplasts isolated from dark-adapted leaves.

This proposal that (A) is equal to (Z) may indicate why (V) is the main component of the cycle when the ΔpH relaxes. Assuming (A) and (Z) are equivalent, then reversion back to (V) allows the plant to avoid unnecessary energy dissipation at sub-saturating light intensities and low levels of ΔpH where accumulation of excess
energy is unlikely. It is well known that \((A)\) rarely accumulates to significant levels and is normally a minor component of the \((V + A + Z)\) pool in higher plants and green and brown algae. There is a rare exception to this trend in the Rhodophyta where \((A)\) is the major carotenoid pigment in several species of the genus \textit{Gracilaria} and in at least one species of \textit{Acanthophora} (Aihara and Yamamoto 1968, Brown and McLachlan 1982). The possible quenching function of \((A)\) in the Rhodophyta remains undetermined. The normally low levels of \((A)\) accumulation in higher plants are probably mostly owing to the biochemistry of the de-epoxidase enzyme as mentioned in the results section.

This study extends the early observations (Briantais et al. 1979, 1980), which employed 9-aminoacridine to show a linear relationship between lumen \([\text{H}^+]\) and Stern-Volmer type quenching, to xanthophyll-dependent NPQ. The main overriding assumption in this study is that \(\Delta A_{520}\) represents the relative steady-state lumen \([\text{H}^+]\). Siefermann-Harms (1978) showed that \(\Delta A_{520}\) due to low concentrations of neutral red (3 to 5 \(\mu\)M) with 30 \(\mu\)g Chl mL\(^{-1}\) could be used as an indicator of steady-state lumen \([\text{H}^+]\) by describing neutral red uptake with a dimer model. She concluded that a dimer model for neutral red uptake reflected the lumen \([\text{H}^+]\) similar to 9-aminoacridine. Here it is noted that 9-aminoacridine fluorescence is quenched in the presence of ascorbate and thus excluded from use in this study. Therefore, neutral red uptake was used as the probe for lumen \([\text{H}^+]\). This application of neutral red differs from the transient change at 548 nm described by Junge et al. (1986) which does not reflect the steady-state lumen pH. Regardless of the actual quantitative
relationship between $\Delta A_{520}$ and lumen [$H^+$], it is obvious that $SV_N$ is highly correlated to $\Delta A_{520}$ in a linear manner for any given level of $[Z + A]$. Since Briantais et al. (1980) used an SV-type calculation equivalent to $SV_N$ as a probe for lumen [$H^+$], it might be considered that the linearity between $SV_N$ and $\Delta A_{520}$ lends credence to Siefermann-Harms (1978) report.

The fact that $SV_N$ is linear with $\Delta A_{520}$ for any given $[Z + A]$ suggests that the lumen [$H^+$] may be causing a stoichiometric $Mg^{2+}$-efflux as suggested by Briantais et al. (1979). Perhaps the lumen [$H^+$] dependent change leads to a static quenching complex in the pigment bed. Such a conformational change would be consistent with Chapter 5 where $(Z)$-dependent quenching did not require reverse electron-flow or light-induced redox changes.
<table>
<thead>
<tr>
<th>Condition</th>
<th>[V]</th>
<th>[A]</th>
<th>[Z]</th>
<th>[Z + A]</th>
<th>[V + A + Z]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0) Dark Sample</td>
<td>76.08</td>
<td>8.58</td>
<td>6.07</td>
<td>14.65</td>
<td>90.73</td>
</tr>
<tr>
<td>1) No DTT</td>
<td>41.26</td>
<td>13.89</td>
<td>37.91</td>
<td>51.80</td>
<td>93.06</td>
</tr>
<tr>
<td>2) No DTT</td>
<td>42.06</td>
<td>10.66</td>
<td>34.35</td>
<td>45.01</td>
<td>87.07</td>
</tr>
<tr>
<td>3) +0.25 mM DTT</td>
<td>45.30</td>
<td>9.34</td>
<td>32.81</td>
<td>42.15</td>
<td>87.45</td>
</tr>
<tr>
<td>4) +0.55 mM DTT</td>
<td>57.88</td>
<td>18.30</td>
<td>15.15</td>
<td>33.45</td>
<td>91.33</td>
</tr>
<tr>
<td>5) +0.68 mM DTT</td>
<td>66.82</td>
<td>16.67</td>
<td>7.79</td>
<td>24.46</td>
<td>91.28</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>90.15</td>
</tr>
<tr>
<td><strong>S.D.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.17</td>
</tr>
<tr>
<td><strong>C.V.(%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.40</td>
</tr>
</tbody>
</table>
TABLE 6.1. (Continued) [V], [A], and [Z] in Peas and Lettuce
When De-epoxidation is Titrated with Various DTT Levels.

<table>
<thead>
<tr>
<th>Condition</th>
<th>[V]</th>
<th>[A]</th>
<th>[Z]</th>
<th>[Z + A]</th>
<th>[V + A + Z]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0) Dark Sample</td>
<td>159.84</td>
<td>4.58</td>
<td>3.94</td>
<td>8.52</td>
<td>168.36</td>
</tr>
<tr>
<td>1) No DTT</td>
<td>66.07</td>
<td>11.48</td>
<td>94.58</td>
<td>106.06</td>
<td>172.13</td>
</tr>
<tr>
<td>2) +0.10 mM DTT</td>
<td>89.86</td>
<td>27.62</td>
<td>54.76</td>
<td>82.38</td>
<td>172.24</td>
</tr>
<tr>
<td>3) +0.25 mM DTT</td>
<td>115.14</td>
<td>31.79</td>
<td>25.23</td>
<td>57.02</td>
<td>172.16</td>
</tr>
<tr>
<td>4) +0.40 mM DTT</td>
<td>127.38</td>
<td>30.37</td>
<td>16.16</td>
<td>46.53</td>
<td>173.91</td>
</tr>
<tr>
<td>5) +0.66 mM DTT</td>
<td>142.73</td>
<td>20.39</td>
<td>8.79</td>
<td>29.18</td>
<td>171.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MEAN 171.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S.D. 1.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C.V.(%) 0.97</td>
</tr>
</tbody>
</table>

Experimental conditions as described in methods, with DTT added prior to illumination when indicated.
TABLE 6.2. Data Matrix Used in Multiple and Linear-Regression Analyses.

<table>
<thead>
<tr>
<th>PFD, μmol m⁻² s⁻¹</th>
<th>(ΔA₅₂₀ X 10⁴)/μg Chl a</th>
<th>SVₙ=</th>
<th>Con. 1</th>
<th>Con. 2</th>
<th>Con. 3</th>
<th>Con. 4</th>
<th>Con. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>700</td>
<td>7.83</td>
<td>0.979</td>
<td>0.962</td>
<td>0.805</td>
<td>0.652</td>
<td>0.461</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>7.28</td>
<td>0.846</td>
<td>0.705</td>
<td>0.685</td>
<td>0.570</td>
<td>0.382</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>5.12</td>
<td>0.508</td>
<td>0.355</td>
<td>0.344</td>
<td>0.277</td>
<td>0.173</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>3.81</td>
<td>0.288</td>
<td>0.138</td>
<td>0.135</td>
<td>0.124</td>
<td>0.055</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>2.69</td>
<td>0.105</td>
<td>0.040</td>
<td>0.024</td>
<td>0.021</td>
<td>0.010</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 6.2. (Continued) Data Matrix Used in Multiple and Linear-Regression Analyses.

<table>
<thead>
<tr>
<th>PFD, μmol m⁻² s⁻¹</th>
<th>(ΔA₃₂₀ X 10⁴) μg Chl⁻¹</th>
<th>SVₙ</th>
<th>Con. 1</th>
<th>Con. 2</th>
<th>Con. 3</th>
<th>Con. 4</th>
<th>Con. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce 750</td>
<td>9.27</td>
<td></td>
<td>1.396</td>
<td>1.106</td>
<td>0.563</td>
<td>0.500</td>
<td>0.211</td>
</tr>
<tr>
<td>94</td>
<td>8.37</td>
<td></td>
<td>1.225</td>
<td>0.851</td>
<td>0.489</td>
<td>0.399</td>
<td>0.177</td>
</tr>
<tr>
<td>23</td>
<td>5.91</td>
<td></td>
<td>0.865</td>
<td>0.467</td>
<td>0.310</td>
<td>0.160</td>
<td>0.076</td>
</tr>
<tr>
<td>12</td>
<td>4.17</td>
<td></td>
<td>0.565</td>
<td>0.238</td>
<td>0.180</td>
<td>0.059</td>
<td>0.000</td>
</tr>
<tr>
<td>6</td>
<td>3.11</td>
<td></td>
<td>0.321</td>
<td>0.107</td>
<td>0.066</td>
<td>0.011</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Con. (1-5) refers to DTT inhibition conditions in Table 6.1 for peas and lettuce.
TABLE 6.3. Linear-Regression Model Equations with $(\Delta A_{520})[Z]^*$.

<table>
<thead>
<tr>
<th>PEAS</th>
<th>LETTUCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$SV_N = x_I (\Delta A_{520})[Z] + a$</td>
<td>$SV_N = x_I (\Delta A_{520})[Z] + a$</td>
</tr>
<tr>
<td>$r^2 = 0.718$</td>
<td>$r^2 = 0.906$</td>
</tr>
</tbody>
</table>

Parameter Estimates $\pm$ Standard Error; $t$ for $H_0$; $p$

<table>
<thead>
<tr>
<th>Parameter Estimates $\pm$ Standard Error; $t$ for $H_0$; $p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$x_I = 3.09 \times 10^3 \pm 4.04 \times 10^4; t = 7.646; p \leq 0.0001$</td>
</tr>
<tr>
<td>$a = -3.66 \times 10^2 \pm 6.49 \times 10^2; t = -0.564; p \leq 0.5785$</td>
</tr>
</tbody>
</table>

*n = 25 for all equations.
### Table 6.4. Multiple-Regression Model Equations with $(\Delta A_{520})[Z]$ and $\Delta A_{520}^*$.  

<table>
<thead>
<tr>
<th>PEAS</th>
<th>LETTUCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$SV_N = x_1 (\Delta A_{520})[Z] + x_2 (\Delta A_{520}) + a$</td>
<td>$SV_N = x_1 (\Delta A_{520})[Z] + x_2 (\Delta A_{520}) + a$</td>
</tr>
<tr>
<td>$r^2 = 0.967$</td>
<td>$r^2 = 0.975$</td>
</tr>
</tbody>
</table>

Parameter Estimates ± Standard Error; $t$ for $H_0$; $p$  

<table>
<thead>
<tr>
<th>Parameter Estimates ± Standard Error; $t$ for $H_0$; $p$</th>
<th>Parameter Estimates ± Standard Error; $t$ for $H_0$; $p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$x_1 = 1.73 \times 10^3 \pm 1.77 \times 10^4; t = 9.791; p \leq 0.0001$</td>
<td>$x_1 = 1.42 \times 10^3 \pm 6.26 \times 10^5; t = 22.667; p \leq 0.0001$</td>
</tr>
<tr>
<td>$x_2 = 9.71 \times 10^2 \pm 7.59 \times 10^3; t = 12.795; p \leq 0.0001$</td>
<td>$x_2 = 4.77 \times 10^2 \pm 6.05 \times 10^3; t = 7.883; p \leq 0.0001$</td>
</tr>
<tr>
<td>$a = -3.71 \times 10^1 \pm 3.47 \times 10^2; t = -10.686; p \leq 0.0001$</td>
<td>$a = -2.29 \times 10^1 \pm 3.64 \times 10^2; t = -6.306; p \leq 0.0001$</td>
</tr>
</tbody>
</table>

$^*n = 25$ for all equations.
TABLE 6.5. Linear-Regression Model Equations with \((\Delta A_{520})[Z + A]^*\).

<table>
<thead>
<tr>
<th>PEAS</th>
<th>LETTUCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(SV_N = x_1 (\Delta A_{520})[Z + A] + a)</td>
<td>(SV_N = x_1 (\Delta A_{520})[Z + A] + a)</td>
</tr>
<tr>
<td>(r^2 = 0.943)</td>
<td>(r^2 = 0.982)</td>
</tr>
</tbody>
</table>

Parameter Estimates ± Standard Error; \(t\) for \(H_0; p\)

<table>
<thead>
<tr>
<th>PEAS</th>
<th>LETTUCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(x_1 = 3.16 \times 10^3 \pm 1.62 \times 10^4; t = 19.488; p \leq 0.0001)</td>
<td>(x_1 = 1.64 \times 10^3 \pm 4.63 \times 10^5; t = 35.471; p \leq 0.0001)</td>
</tr>
<tr>
<td>(a = -2.79 \times 10^1 \pm 3.74 \times 10^2; t = -7.461; p \leq 0.0001)</td>
<td>(a = -2.37 \times 10^1 \pm 2.13 \times 10^2; t = -11.124; p \leq 0.0001)</td>
</tr>
</tbody>
</table>

\(*n = 25\) for all equations.
TABLE 6.6. Multiple-Regression Model Equations with (ΔA_{520})[Z + A] and (ΔA_{520})*.

<table>
<thead>
<tr>
<th>PEAS</th>
<th>LETTUCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$SV_N = x_1 (ΔA_{520})[Z + A] + x_2 (ΔA_{520}) + a$</td>
<td>$SV_N = x_1 (ΔA_{520})[Z + A] + x_2 (ΔA_{520}) + a$</td>
</tr>
<tr>
<td>$r^2 = 0.980$</td>
<td>$r^2 = 0.982$</td>
</tr>
</tbody>
</table>

Parameter Estimates ± Standard Error; $t$ for $H_0$; $p$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PEAS Estimate</th>
<th>LETTUCE Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$x_1$</td>
<td>$2.26 \times 10^{-3} \pm 1.72 \times 10^{-4}$; $t = 13.132; p \leq 0.0001$</td>
<td>$1.66 \times 10^{-3} \pm 6.19 \times 10^{-5}$; $t = 26.770; p \leq 0.0001$</td>
</tr>
<tr>
<td>$x_2$</td>
<td>$5.24 \times 10^{-2} \pm 8.28 \times 10^{-3}$; $t = 6.321; p \leq 0.0001$</td>
<td>$-2.19 \times 10^{-3} \pm 6.15 \times 10^{-3}$; $t = -0.356; p \leq 0.7255$</td>
</tr>
<tr>
<td>$a$</td>
<td>$-3.71 \times 10^{-1} \pm 2.70 \times 10^{-2}$; $t = -13.725; p \leq 0.0001$</td>
<td>$-2.29 \times 10^{-1} \pm 3.10 \times 10^{-2}$; $t = -7.404; p \leq 0.0001$</td>
</tr>
</tbody>
</table>

*n = 25 for all equations.
CHAPTER 7

CONCLUSIONS AND FUTURE RESEARCH
CONCLUSIONS

The role of photosynthetic electron-transport in higher plants is two-fold, generating reducing potential as well as the $\Delta pH$ required for ATP synthesis. Both reducing potential and ATP are required for the primary photosynthetic objective, namely, carbon fixation. However, the light which a plant absorbs often exceeds the plants photosynthetic capacity. Unless excess absorbed light energy is dissipated harmlessly, the possibility of damage to the photosynthetic system arises. One response to a limitation in CO$_2$-fixing capacity is an increase in the $\Delta pH$. Increases in the $\Delta pH$ trigger a protective 'down-regulation' of PSII photochemistry. This down-regulation is associated with a decreased quantum yield of photosynthesis as well as an increase in the thermal dissipation of excess energy.

Thermal dissipation of excess energy or so-called high-energy quenching (HEQ) has been correlated with the $\Delta pH$ and the xanthophyll zeaxanthin. Although this xanthophyll-associated HEQ phenomenon was studied extensively in intact leaf systems and isolated chloroplasts, much of the biochemistry remained unclear. The findings of this dissertation support the original premises that it is possible to study and characterize the mechanism of HEQ in isolated chloroplasts and that the biochemistry revealed by these in vitro studies further explains the mechanism in intact systems.

Figure 7.1 shows a schematic model of the HEQ phenomenon as it was developed from these in vitro studies. Both coupled light-driven electron-transport (PSI and PSII) and ATP-dependent proton-pumping can generate the $\Delta pH$ required
for HEQ. Thus, HEQ is primarily controlled by the $\Delta p$H and only indirectly dependent on light. The $\Delta p$H, in the presence of ascorbate (Asc), activates the violaxanthin de-epoxidase (VDE) to form zeaxanthin (Zeax) and antheraxanthin (Anth). DTT inhibits the VDE without affecting the $\Delta p$H or the extent of xanthophyll-dependent HEQ after de-epoxidation has been induced. HEQ is highly correlated with a simple linear model where HEQ is the product of ($\text{lumen} [H^+] \times [\text{Zeax} + \text{Anth}]$). Although, the precise mechanism of HEQ remains unclear, work antimycin shows that other changes are required in addition to $\Delta p$H and de-epoxidation. The dark ATP-induction of HEQ shows that the antimycin-sensitive change is pH-sensitive and not associated with any light-dependent redox changes. These data are consistent with all HEQ being related to a common mechanism that dissipates excess light-energy in the light-harvesting pigment bed.

The evolutionary significance of the violaxanthin-cycle is implicated by its high degree of conservation in all higher plants. The importance of the violaxanthin-cycle and HEQ in higher-plant photosynthesis is signified by their direct link to the high-energy state required for photophosphorylation. By this link, violaxanthin de-epoxidation and HEQ are tuned to limitations in photosynthetic carbon-fixation capacity. The findings in this dissertation support the view that the violaxanthin-cycle and HEQ are part of an important functional adaptation that evolved among higher plants for dissipation of excess light energy via "down-regulation" of PSII photochemistry and an increase in non-photochemical quenching.
Figure 7.1. Schematic summary of HEQ biochemical mechanism.
FUTURE RESEARCH

Principal among the most obvious areas requiring further work at the whole plant level is to determine if dark-sustained ATP-hydrolysis occurs in vivo under photoinhibitory conditions. It should also be determined if inhibition of zeaxanthin-NRD in vivo with DTT (Bilger and Björkman 1990, Winter and König 1990) affects the ATPase activation-state since. DTT does affect ATPase activity in vitro (Petrack and Lipman 1961). Also of importance is the determination if any damage to the Q_B-binding protein is the in vivo cause of destructive photoinhibition and whether zeaxanthin-NRD protects against this damage.

On a biochemical level it will be of interest to determine the exact mechanism of the pH-sensitive antimycin change. Preliminary evidence with the divalent ionophore A23187 are consistent with several earlier studies which indicated that the ΔpH induced Mg^{2+}-efflux is directly linked to HEQ (Briantais et al. 1979, Krause et al. 1983). It is important to investigate whether antimycin affects the ΔpH-induced Mg^{2+}-efflux. Although Oxborough and Horton (1987) showed that antimycin, as does A23187 plus Mg^{2+} (Barber et al. 1974b), uncouples at relatively high concentrations, the counter-ion or actual mechanism behind antimycin’s ΔpH reversal needs to be determined.

As was speculated in Chapter 6, the quenching efficiency of (Z + A) concentration at a saturating ΔpH may be species specific and or affected by a plant’s acclimation to varying growth-light regimes. Perhaps this avenue can be explored with chloroplasts isolated from plants of similar and different species adapted and
acclimated to varying growth-light regimes. Also it may be worthwhile investigating if high levels of antheraxanthin reported in some Rhodophyta (Aihara and Yamamoto 1968, Brown and McLachlan 1982) have a protective role. With this in mind it is also of interest to determine if the diadinoxanthin/diatoxanthin cycle in several divisions of algae (Hager 1975) has a photoprotective function.
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