

# Changes in P-700 Oxidation during the Early Stages of the Induction of Photosynthesis<sup>1</sup>

Jeremy Harbinson\* and Clifford L. Hedley

ATO-DLO, Postbus 17, 6700 AA Wageningen, The Netherlands (J.H.); and John Innes Institute and Agricultural and Food Research Council Institute of Plant Science Research, Colney Lane, NR4 7UH, United Kingdom (C.L.H.)

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Following dark adaptation, the response to irradiance of chlorophyll (Chl) fluorescence, the light-induced absorbance change around 820 nm (to measure reaction center Chl of photosystem I [PSI] P-700 oxidation), and CO<sub>2</sub> fixation were examined in pea (*Pisum sativum* L.) leaves under a range of conditions. Initially, P-700 oxidation is restricted by a lack of regeneration of PSI electron acceptors, and the increase of oxidized P-700 (P-700<sup>+</sup>) that occurs during approximately the first 60 s of irradiance is largely independent of the resistance to electron flow between the two photosystems. Under these conditions, the quantum efficiency for linear electron flow is directly positively related to P-700<sup>+</sup> accumulation, which is in contrast to the direct negative correlation that is the most frequently reported relationship between P-700<sup>+</sup> accumulation and the quantum efficiency for linear electron flow.

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The activation of photosynthesis in dark-adapted materials, such as leaves or chloroplasts, following the start of irradiance is known as photosynthetic induction. The changes that occur during induction have been so extensively studied that they have become a paradigm for photosynthetic control (e.g. Horton, 1985), even though a sudden, discontinuous start of irradiance is not an obviously natural event, although sunflecks do represent a broadly similar effect.

During induction, large changes occur in the fluxes through the various pathways located in the thylakoids, stroma, and cytosol, and in the redox state of electron carriers involved in these pathways (e.g. Satoh et al., 1977; Satoh and Katoh, 1980; Heber et al., 1982; Andreeva and Tikhonov, 1983; Quick and Horton, 1984; Horton, 1985; Stitt and Schreiber, 1988; Laisk et al., 1989; Foyer et al., 1992). These changes precede and, by various mechanisms, lead to the balanced conditions of steady-state photosynthesis. The processes that cause these changes are continually operating to determine the state of the photosynthetic system in an intact leaf, within which photosynthesis must respond to a continually changing internal and external environment.

Most studies of photosynthetic induction have dealt with changes in Chl fluorescence, O<sub>2</sub> evolution, CO<sub>2</sub> fixation, enzyme activity, and metabolite pool size (see refs. above for

examples). Because of technical reasons, not all measurements are made on leaves in air, and little attention has been paid to changes around PSI.

P-700 is the reaction center Chl of PSI (Mathis and Pailotin, 1981; Rutherford and Heathcote, 1985; Golbeck, 1987), and it has been shown that during induction large changes occur in absorbance changes or electron spin resonance signals attributed to the presence of P-700<sup>+</sup> (Maxwell and Biggins, 1976; Andreeva and Tikhonov, 1983; Harbinson and Woodward, 1987; Schreiber et al., 1988). The forces producing these changes are not well understood.

The amount of P-700<sup>+</sup> within any photosynthetic tissue is determined by the balance between the photochemical oxidation of P-700 and the reduction of any P-700<sup>+</sup> so formed (Foyer et al., 1990). Following excitation of the PSI pigment bed, P-700 oxidation occurs with a quantum efficiency of 1 (Hiyama, 1985), provided there is an available electron acceptor; in the absence of an electron acceptor pool, no stable P-700 oxidation can occur (Rutherford and Heathcote, 1985; Golbeck, 1987). This is important because although it may be obvious that the redox state of P-700 is strongly influenced by irradiance and electron transfer from PSII, it may also be affected by the redox state of the Fd pool and thus directly by the metabolism of the chloroplast. Therefore, information provided by measurements of the P-700 oxidation state may be ambiguous and must be treated with care. However, this also implies that, besides being a valuable tool with which to monitor the relationship between PSI and PSII (Weis et al., 1987; Harbinson et al., 1989), changes in the redox state of P-700 may be a useful means with which to probe the relationship between the thylakoids and the stroma.

Once oxidized, P-700 is reduced by electrons derived from either PSII via linear electron flow or from PSI via cyclic electron flow. Whatever their source, all electrons must pass

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Abbreviations: A<sub>0</sub>, primary electron accepting Chl of PSI; ΔA<sub>820</sub>, light-induced absorbance change around 820 nm; LED, light-emitting diode; PAQF, photosynthetically active quantum flux; PQH<sub>2</sub>, plastoquinol; P-680, reaction center Chl of PSII; P-700, reaction center Chl(s) of PSI in reduced state; P-700<sup>+</sup>, oxidized P-700; Φ<sub>CO<sub>2</sub></sub>, the quantum efficiency of CO<sub>2</sub> fixation, given by the rate of CO<sub>2</sub> fixation divided by the irradiance; Φ<sub>PSI</sub>, the quantum efficiency of PSI for electron transport; Φ<sub>PSII</sub>, the quantum efficiency of PSII for electron transport; Q<sub>A</sub>, primary electron-accepting plastoquinone of PSII; q<sub>N</sub>, nonphotochemical quenching of Chl fluorescence; q<sub>P</sub>, photochemical quenching of Chl fluorescence; t<sub>1/2</sub>, half-time.

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\* Corresponding author; fax 31-8370-12260.

through the PQH<sub>2</sub> pool and the Cyt *b<sub>6</sub>/f* complex en route to P-700<sup>+</sup> (e.g. Whitmarsh and Cramer, 1979; Bendall, 1982; O'Keefe, 1988). Under most conditions, the rate-limiting step of thylakoid electron transport chain is the reaction between PQH<sub>2</sub> and the Cyt *b<sub>6</sub>/f* complex, the rate of which is determined by the concentration of PQH<sub>2</sub> (Rich, 1982) and the intra-thylakoid pH (Rich and Bendall, 1981; Bendall, 1982). Whenever electron transport is limited between the two photosystems, it is the kinetics of this reaction that largely determines the capacity for electron transport. The reduction of P-700<sup>+</sup>, which occurs with a *t*<sub>1/2</sub> of several milliseconds in vitro (Haehnel, 1984) and in vivo (Maxwell and Biggins, 1977; Harbinson and Hedley, 1989; Schreiber et al., 1989; Genty et al., 1990), is therefore sensitive to both intra-thylakoid pH (Tikhonov et al., 1984) and the redox state of the PQH<sub>2</sub> pool (Stiehl and Witt, 1969; Siggel, 1976), although the latter has been poorly documented either in vivo or in vitro (Amesz et al., 1971, 1972).

It is possible to use the reduction kinetics of the P-700<sup>+</sup> pool following a light-to-dark transition to estimate the resistance for electron flow between the two photosystems (e.g. Harbinson and Hedley, 1989; Genty et al., 1990). Such a measurement can be used to determine whether the degree of oxidation of P-700 is consistent with measured *t*<sub>1/2</sub> for P-700<sup>+</sup> reduction (Harbinson and Hedley, 1989). For example, if P-700 oxidation was to be restricted by a shortage of electron acceptors, changes in the pool of P-700<sup>+</sup> could occur in the absence of changes in the *t*<sub>1/2</sub> for P-700<sup>+</sup> reduction as the shortage of acceptors was relieved.

When dark-adapted leaves are subjected to an actinic irradiance, the P-700<sup>+</sup> pool slowly increases to a maximum level over several seconds to several minutes, depending on the origin and history of the leaf (Andreeva and Tikhonov, 1983; Harbinson and Woodward, 1987). Superimposed upon this broad, general increase are generally one or more subsidiary maxima whose location and size is again influenced by the origin and history of the leaf. Based on biochemical and physiological observations, it is generally proposed that the slow increase of the P-700<sup>+</sup> pool is caused by a shortage of electron acceptors on the acceptor side of PSI (Andreeva and Tikhonov, 1983; Harbinson and Woodward, 1987; Laisk et al., 1991; Foyer et al., 1992; Heber et al., 1992). Although this is a plausible proposition, none of these authors attempted to analyze more carefully the changes in the P-700<sup>+</sup> pool during induction to verify that this was indeed the case.

In this paper we intend to examine the changes in the P-700<sup>+</sup> pool during induction to show that the changes in pea (*Pisum sativum*) leaves are due to metabolic changes and that other possible causes, such as changes in either the resistance for electron flow between the two photosystems or the capacity for electron transfer by PSI, are either nonexistent or of lesser importance. An implication of these results is that during photosynthetic induction the site of limitation of electron transport changes from the acceptor side of PSI to the donor side. These results are consistent with biochemical measurements made during induction in vivo (Siebke et al., 1991; Foyer et al., 1992) and are significant in explaining control of photosynthesis during induction.

## MATERIALS AND METHODS

### The Use of *A*<sub>820</sub> to Measure the Redox State of P-700 in Leaves

These measurements will be made using a recently introduced technique employing light-induced *A*<sub>820</sub> (Harbinson and Woodward, 1987; Schreiber et al., 1988). The difference spectra of the light-induced absorbance changes in leaves, chloroplasts, and algae in the near IR (Inoue et al., 1973; Schreiber et al., 1988; Klughammer and Schreiber, 1991) are similar to those reported for P-700<sup>+</sup>, although a thorough analysis of the absorbance changes from intact chloroplasts demonstrated the existence of changes due to plastocyanin and Fd around 820 nm in addition to those of P-700 (Klughammer and Schreiber, 1991). The behavior of the  $\Delta A_{820}$  in leaves during irradiation with far-red light and light that excites both photosystems, its response to DCMU, its relationship to Chl fluorescence, its behavior in leaves containing no PSI, and other experiments supporting in various ways the usefulness of the  $\Delta A_{820}$  to monitor oxidation of P-700 have been described elsewhere (Inoue et al., 1973; Harbinson and Woodward, 1987; Weis et al., 1987; Schreiber et al., 1988; Harbinson et al., 1989; Harbinson and Hedley, 1989; Genty et al., 1990; Malkin et al., 1991).

Both by calculation and measurement, it appears that possibly 35% of the *A*<sub>820</sub> attributed to P-700 is actually due to plastocyanin (Harbinson and Hedley, 1989; Klughammer and Schreiber, 1991). Interference from plastocyanin does not, however, pose a serious problem because (a) the apparent equilibrium constant between reduced plastocyanin and P-700<sup>+</sup> is close to 1 when the PSI donor pool is reduced (Joliot and Joliot, 1984; see, also, Delosme, 1991) and (b) the *t*<sub>1/2</sub> for P-700<sup>+</sup> reduction by [plastocyanin]<sup>-</sup> is very rapid (<500  $\mu$ s) (Haehnel, 1984), compared with the rate of P-700 excitation (approximately  $10^3$  s<sup>-1</sup> at 2000  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> PAR). The absorbance changes due to plastocyanin will therefore largely parallel those of P-700.

Absorbance changes due to Fd can also contribute to the  $\Delta A_{820}$  under certain conditions, for example, when electron transport from the Fd pool is blocked (Klughammer and Schreiber, 1991). Other Chl and phaeophytin species also produce absorbance changes in the near IR that are very similar to P-700. These include Chl singlets and triplets, P-680, *A*<sub>0</sub>, and phaeophytin (Doring et al., 1967; Klimov et al., 1986; Nuijs et al., 1986a, 1986b). However, most of these species are very short-lived ( $10^{-11}$  to  $10^{-7}$  s; Kramer and Mathis, 1980; Nuijs et al., 1986a; Shuvalov et al., 1986; Mathis et al., 1988; Schatz et al., 1988; Genty et al., 1992) and will not contribute to the absorbance change produced by P-700<sup>+</sup>, which has a lifetime of  $10^{-3}$  to  $10^{-2}$  s (Haehnel, 1984) at the irradiances of up to 2000 to 3000  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> used in whole-leaf photosynthesis experiments. The case with P-680 is possibly more difficult. When the PSII donor side is functioning normally, P-680<sup>+</sup> is rereduced with *t*<sub>1/2</sub> values of 20 to 250 ns (Brettel et al., 1984); this is too fast to allow any significant accumulation of P-680<sup>+</sup> under the irradiances normally employed in the steady-state irradiation of whole leaves. However, when the donor side of PSII is inactive (at low pH, for example), P-680<sup>+</sup> rereduction via a back-reaction from Q<sub>A</sub><sup>-</sup> occurs with complex multiphasic

kinetics with  $t_{1/2}$  values of up to 200  $\mu\text{s}$ , 500 to 700  $\mu\text{s}$ , or 5 ms, although the slowest phase is only 10% of the total at pH 4 (Schlödter and Meyer, 1987). If these kinetics were to occur *in vivo*, the slowest reduction kinetics could interfere with the use of  $\Delta A_{820}$  measurements to monitor P-700 oxidation or reduction, although given the small size of the 5-ms component, this interference would not be marked. Donor-side restriction of PSII has been proposed as a mechanism for the nonphotochemical quenching of PSII (Krieger et al., 1992; Rees et al., 1992), although a careful analysis of the Chl fluorescence decay kinetics from leaves produced no evidence for the modulation of nonphotochemical quenching of PSII by donor-side inactivation (Genty et al., 1992). Nonetheless, the possibility of a measurable contribution by P-680 oxidation or reduction to  $A_{820}$  remains a possibility.

Critically, however, the good linear correlations between the  $\Phi_{\text{PSI}}$  calculated from  $\Delta A_{820}$  measurements and  $\Phi_{\text{PSII}}$  and  $\Phi_{\text{CO}_2}$  (in the absence of photorespiration) (Weis et al., 1987; Harbinson et al., 1989; Genty et al., 1990; Harbinson et al., 1990a, 1990b; Peterson, 1991), or photosynthetic energy conversion (Malkin et al., 1991) suggest that the  $\Delta A_{820}$  is quantitatively measuring P-700 oxidation. Some nonlinear relationships between  $\Phi_{\text{PSI}}$  and other estimates of photosynthetic efficiency have been reported (Harbinson et al., 1990; Weis et al., 1990; Harbinson and Foyer, 1991), but in these cases there were good reasons why this should be so (e.g. cyclic electron flow around PSI).

The absence of other components that would affect the usefulness of the  $\Delta A_{820}$  to measure P-700 oxidation is also supported by the simple monophasic character of the dark relaxation of the  $\Delta A_{820}$  following the removal of irradiance (Harbinson and Hedley, 1989); any other components changing with a different  $t_{1/2}$  to P-700<sup>+</sup> would be detectable by this means. These results show that the  $\Delta A_{820}$  is a reliable indicator of P-700 oxidation and that other photosynthetic components do not interfere with this use. Changes due to plastocyanin appear to shadow those of P-700, and although this would affect the use of this technique to quantify the absolute amount of P-700, it does not affect its use to determine relative changes in the P-700 oxidation state. The absorbance changes due to Fd are small at 820 nm and appear to be confined to situations where electron transport from the acceptor side of PSI is restricted (Klughammer and Schreiber, 1991).

The  $\Delta A_{820}$  is therefore a useful means to both qualitatively and quantitatively estimate the oxidation state of P-700 *in vivo*. The usefulness of this technique is enhanced because light at these wavelengths is extensively scattered by leaves. This scattering is wavelength dependent and is greatest at wavelengths where the absorption is lowest (Ruhle and Wild, 1979); using leaves with a low Chl content, Ruhle and Wild (1979) found a path length increase of up to six times due to scattering. Since leaves absorb only weakly at approximately 820 nm (Rabideau et al., 1946), the absorbance changes due to P-700 obtained at these wavelengths are therefore many times larger than expected.

### Experimental Procedures

All measurements were made on healthy, fully developed leaves of pea (*Pisum sativum*) varieties JI799, XM7405/1, JI73,

or BC1/6RR. Plants were grown in 70% John Innes No. 1 compost and 30% chick-grit in either a controlled environment cabinet (20°C day/15°C night, 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF, or a glasshouse [midspring-summer period only]).

Measurements of the yield of Chl fluorescence were made after the technique of Ogren and Baker (1985) using equipment described previously (Harbinson and Woodward, 1987; Harbinson et al., 1989). The Chl fluorescence was detected by either an S1223 Si PIN or a G1736 GaAsP diffusion photodiode (Hamamatsu, Japan) filtered by an RG9N filter (Schott, Mainz, Germany), situated below the leaf.

The oxidation state of P-700 was measured using the  $\Delta A_{820}$  using equipment basically described by Harbinson and Woodward (1987). This measuring wavelength will also detect changes due to plastocyanin oxidation or reduction, but the plastocyanin changes appear to parallel those of P-700, so for convenience we will only refer to the P-700 component. Irradiance was obtained from either an array of LEDs used without filtration (types H-2K and H-3K, Stanley, Tokyo, Japan, or type TLRA190A, Toshiba, Tokyo, Japan) or from a quartz-halogen bulb filtered with NIR and Red DT filters (Balzers, Lichtenstein) and a special version of a Balzers Cyan DT dichroic mirror obtained from Walz (Effeltrich, Germany). The 820-nm measuring beam was produced by an HLP40RGB emitter (Hitachi, Tokyo, Japan) and was detected by either an S1223 Si PIN photodiode (Hamamatsu) filtered with an RG9N glass filter (Schott) and situated below the leaf, or by an S1223 filtered by an RG780 glass filter (Schott) and situated above the leaf. Except for the results shown in Figure 8, the measuring beam was modulated to allow the rejection of long wavelength Chl fluorescence and actinic light. An error correction circuit was included in the signal recovery circuit to eliminate the small errors introduced by nonlinearities in the photo-diode/filter/amplifier system.

Absorbance changes were also measured at 740 and 700 nm; under aerobic conditions, the former is sensitive to Fd redox changes and an electrochromic shift, and the latter is widely used as a measuring wavelength for P-700 redox changes. To produce a measuring beam at 740 nm, an LED with a broad emission around 730 nm was employed. The output from this LED was modulated and filtered with a 732-nm interference filter (Ealing-Beck, Watford, UK) that, together with the RG9N filter used to screen the photodiode, produced an effective measuring wavelength of around 740 nm. To produce the 700-nm measuring beam, an array of LEDs (HEMT-6000, Hewlett-Packard) was filtered by a 700-nm interference filter (Ealing-Beck) and the radiation was focused onto the surface the leaf. To allow the use of a weak measuring beam, the photodiode detector, which was situated below the leaf, was optically unfiltered and the current-to-voltage converter was fitted with a low-leakage voltage clamp network to prevent amplifier saturation during irradiation by the actinic beam. This measuring beam was not modulated, but the fast turn-off of the LED array used to supply the actinic beam allowed the signal changes due to the removal of the actinic beam to be easily distinguished from those due to the slower reduction of P-700<sup>+</sup> (Harbinson and Hedley, 1989).

Measurements of CO<sub>2</sub> fixation were made concurrently with measurements of leaf absorbance and fluorescence

changes by enclosing leaves of pea varieties JI799 or JI73 in a gas-tight cuvette. The variety JI799 was used in experiments with a low ambient CO<sub>2</sub> concentration because under these conditions it appears better able than most pea varieties to control its electron transport and allow P-700 oxidation. The variety JI73 was used because its slow induction kinetics make it easier to record CO<sub>2</sub> fixation concurrently with the  $\Delta A_{820}$ . The cuvette was fitted with transparent upper and lower windows through which the actinic and measuring beams could be directed at the leaf and through which radiation could reach the photodiode situated below the leaf. The upper window was fitted with a circular mask that allowed 1.25 cm<sup>2</sup> of leaf area to be evenly irradiated by an array of high-intensity LEDs (H-2K and H-3K, peak emission wavelength 660 nm, Stanley, Tokyo, Japan). Different gas mixtures were passed through the assimilation chamber and subsequently analyzed by a CO<sub>2</sub> gas analyzer (Analytical Development Co., Hoddesdon, Hertshire, UK).

Variety XM7405/1 was used in experiments involving treatment of leaves with methyl viologen. This variety carries the argentea allele, whose presence allows the upper epidermis to be easily removed with only minor apparent damage to the underlying mesophyll tissue (Marx, 1982). This allows inhibitors, etc. to be applied directly to the mesophyll cells. Once the upper epidermis had been removed, the leaves were bathed in 10  $\mu\text{mol dm}^{-3}$  methyl viologen (Sigma) for about 10 s before dark adaptation for 5 min and subsequent irradiation. Control leaves were bathed in deionized water and then treated similarly. Treatment with DCMU was achieved by soaking detached leaves of BC1/6 RR in a

saturated aqueous solution of DMCU (Sigma). The leaves were allowed to remain in this solution for 4 h.

## RESULTS AND DISCUSSION

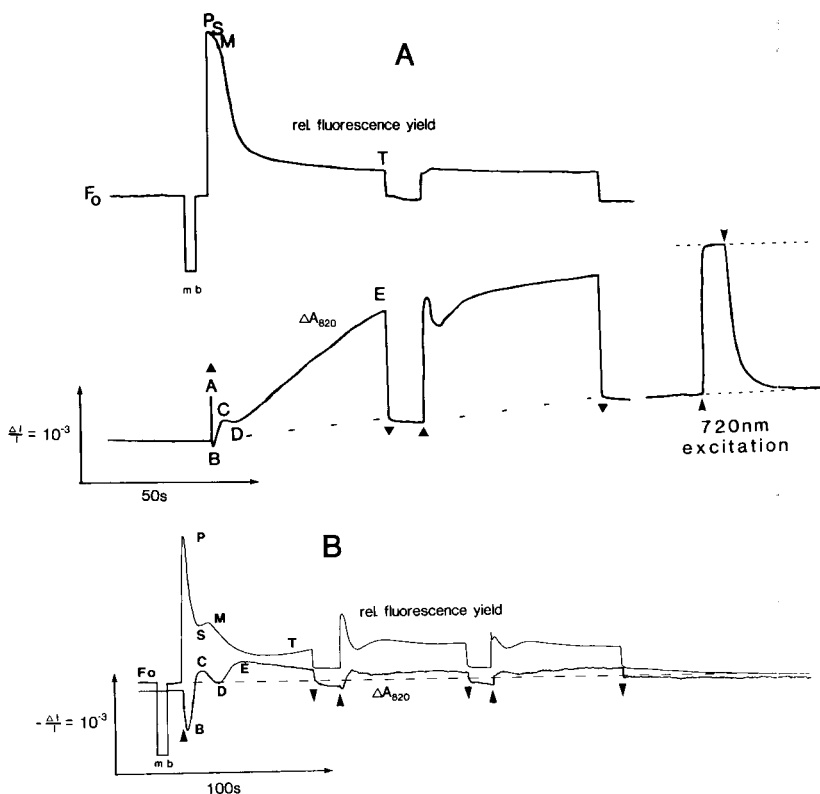
### Changes in the Absorbance and Chl Fluorescence of Leaves Irradiated in Air

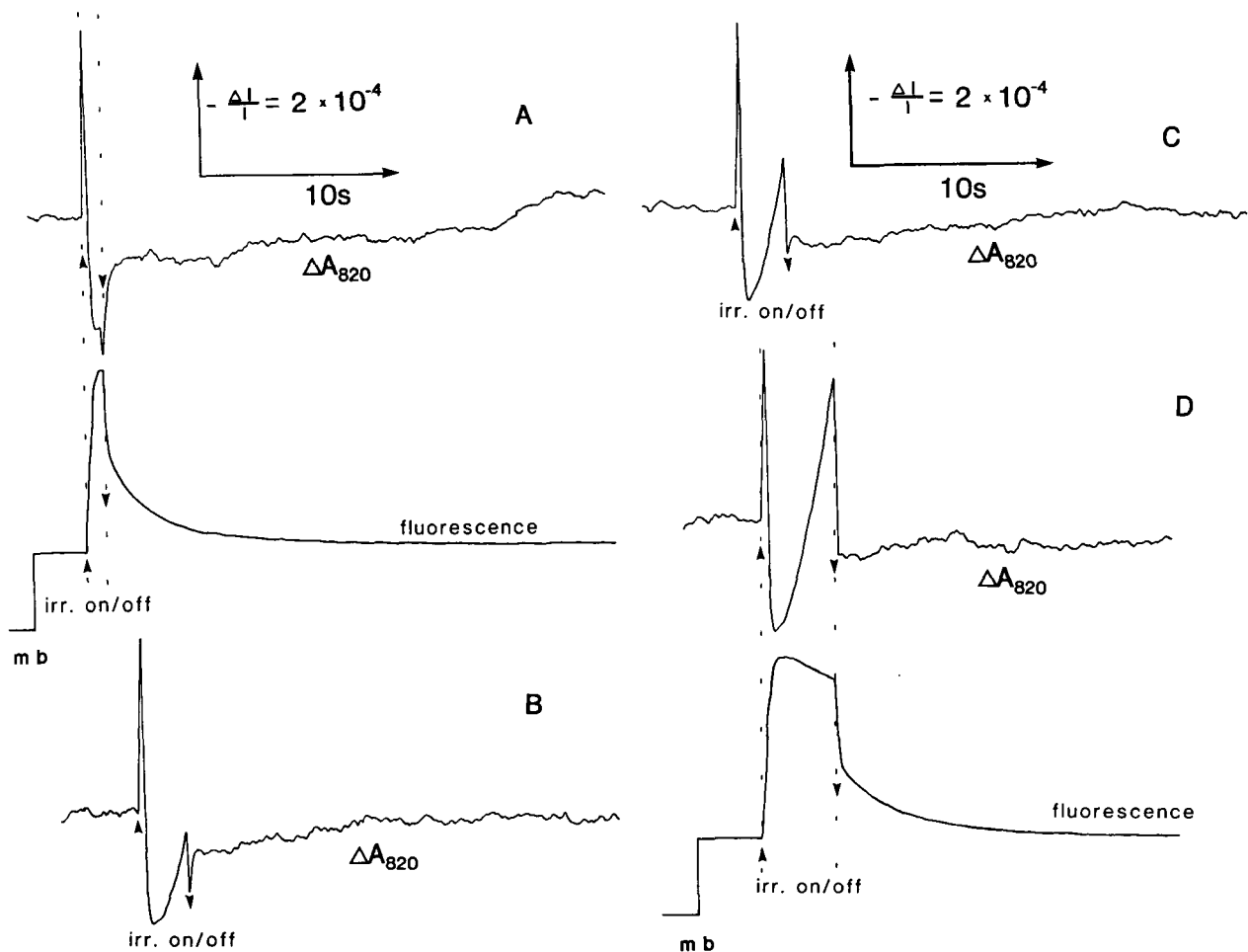
The changes that occur in Chl fluorescence during photosynthetic induction have been well documented, and their relationship to changes in other photosynthetic components and processes has been amply described (e.g. Quick and Horton, 1984; Horton, 1985). Correlating changes in the  $\Delta A_{820}$  with the relative yield of Chl fluorescence from leaves during induction affords a simple means of analyzing how the changes in the  $\Delta A_{820}$  relate to other changes that occur during induction.

Typical  $\Delta A_{820}$  and Chl fluorescence changes recorded from a dark-adapted leaf following the commencement of irradiance are shown in Figures 1A and 2. These results are similar to those reported previously (Harbinson and Woodward, 1987; Schreiber et al., 1988) but are described here in greater detail; the nomenclature used is that of Harbinson and Woodward (1987). Figure 1A shows an example obtained at high irradiance, whereas Figure 1B shows an example obtained at low irradiance in which it is easier to see the pattern of the absorbance changes relative to fluorescence. Figure 2 shows the changes in both Chl fluorescence and the  $\Delta A_{820}$  during short periods of irradiance.

Immediately following the start of irradiance (Fig. 1A) there was a transitory rise in the  $\Delta A_{820}$  (A), which was followed by

**Figure 1.** A, The relationship between the  $\Delta A_{820}$  and the yield of Chl fluorescence in a leaf of pea variety BC1/9RR irradiated with 750  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF centered on 660 nm following a 20-min dark rest. The irradiance was applied for 50 s, then removed for 10 s, and then reapplied for 50 s; the start of irradiance is indicated by  $\blacktriangle$  and the end by  $\blacktriangledown$ . After the last irradiance period, a saturating burst of 720 nm of radiation was applied to completely oxidize the P-700 pool. At the end of each 660-nm irradiance period, the  $\Delta A_{820}$  did not return to the original absorbance change zero. This commonly occurs with leaves, but only the rapid absorbance change, which occurs following the removal of an actinic irradiance, is due to P-700<sup>+</sup> (and plastocyanin) reduction. The fluorescence measuring beam was briefly removed (m b) prior to the beginning of the irradiance to indicate the fluorescence zero. B, As A, but showing the changes produced by a weaker irradiance (120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF) following 120 min of dark rest; the irradiance cycle was 60 s on, 10 s off. Even at this low irradiance, the fluorescence yield does not return to the  $F_0$  level during the dark period, indicating the persistence of some  $Q_A^-$  during this period. To indicate the fluorescence zero, the measuring beam was briefly removed (m b) just before the beginning of the irradiance.





**Figure 2.** The changes of the  $\Delta A_{820}$  (and the yield of Chl fluorescence in A and D only) during brief periods of irradiance applied to a leaf of pea variety BC1/9RR. The irradiance of  $750 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF was terminated during or shortly after the absorbance minimum of the  $\Delta A_{820}$  (B). The leaf was in air and was dark adapted for 20 min before each measurement. The fluorescence measuring beam was switched on at "m b."

a decline (B) to below the absorbance change zero line; the minimum B occurred simultaneously with the Chl fluorescence maximum (P). The magnitude of the absorbance decline to B is dependent on the length of the dark-adaptation time and the particular leaf employed. As fluorescence quenching developed, the  $\Delta A_{820}$  rose to reach a maximum (C) 10 s after the start of irradiation. This was followed by a decline (D) after which the  $\Delta A_{820}$  rose again (E), without interruption, until the irradiation was briefly removed. The changes in the  $\Delta A_{820}$  paralleled those of Chl fluorescence, with the changes B, C, D, and E matching the changes P, S, M, and T of Chl fluorescence. In particular the fluorescence maximum (P) matched the  $\Delta A_{820}$  minimum (B) (Fig. 2), and the secondary fluorescence maximum (M) preceded slightly the inflexion of the  $\Delta A_{820}$  (D) (Fig. 1B).

Removal of the irradiance at point B of the  $\Delta A_{820}$  resulted first in an absorbance decrease (Fig. 2A), consistent with the disappearance of a P-700<sup>+</sup> pool, which was followed by a slower absorbance increase. As the duration of the irradiance was lengthened, the absorbance decrease following the re-

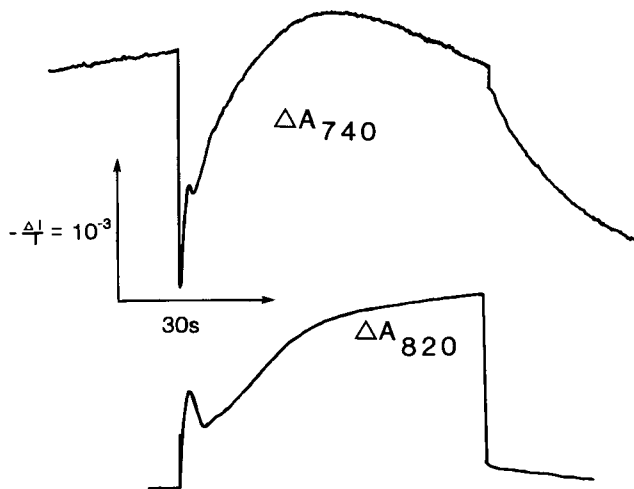
moval of irradiance (P-700<sup>+</sup> reduction) got larger, whereas the slower phase of absorbance increase (not P-700<sup>+</sup> related) diminished (Fig. 2, A–D). In all the examples shown there is a degree of apparent irreversibility, or slow reversibility, in the  $\Delta A_{820}$  in the brief time scale following the cessation of irradiance. Because such changes can also be produced with inert materials, such as polytetrafluoroethylene sheets instead of a leaf, they may be of no biological significance. The results shown in Figure 2 were obtained from a leaf in air; in pure N<sub>2</sub> there was no absorbance change at B that could be attributed to the reduction of P-700<sup>+</sup>; only the slower absorbance increase remained (results not shown).

A contribution to the absorbance rise from B to E from plastocyanin seems certain, but given the low apparent equilibrium constant between plastocyanin and P-700, this absorbance change will shadow that of P-700. Of the other absorbance changes due to Chls and phaeophytin, only a contribution from P-680 oxidation seems possible under the conditions of these experiments. This could only occur if the donor side of PSII were to be inhibited by a low intra-

thylakoid pH, and would be associated with nonphotochemical quenching of PSII Chl fluorescence (Kreiger et al., 1992; Rees et al., 1992). Nonphotochemical quenching does develop during induction (e.g. Quick and Horton, 1984), but once the  $\Delta A_{820}$  reaches E it can be used to estimate  $\Phi_{PSI}$  (Foyer et al., 1992), implying that from this point any interference from P-680 is minimal. Interference from P-680 therefore could only be transitory, appearing when fluorescence quenching developed, but disappearing before the  $\Delta A_{820}$  maximum at E was reached; this seems an unlikely situation. Additionally, the slowest phase of P-680<sup>+</sup> rereduction in donor side-inhibited PSII, the one that would contribute most significantly to the  $\Delta A_{820}$ , is only 10% of the total pool (Schloder and Meyer, 1987), so its contribution to the  $\Delta A_{820}$  would not be large.

A comparison of the  $\Delta A_{820}$  and the  $\Delta A_{740}$  (Fig. 3) shows strong minima in the  $\Delta A_{740}$  that correspond to the minima at B and D in the  $\Delta A_{820}$ . When the irradiance was stopped, a smaller absorbance decrease was produced at 740 nm than was produced at 820 nm; this is consistent with 740 nm being close to the isosbestic wavelength for P-700.

The distinctive relationships between the  $\Delta A_{820}$  and Chl fluorescence, and the  $\Delta A_{820}$  and the  $\Delta A_{740}$  are important because of what is understood about the significance of changes in the yield of Chl fluorescence during photosynthetic induction on the one hand and the possible significance of the  $\Delta A_{740}$  change on the other. The fluorescence maximum (P) has been associated with a period of minimum O<sub>2</sub> evolution and even with a transitory net O<sub>2</sub> uptake (Quick and Horton, 1984; Malkin, 1987). The subsequent decline in the yield of Chl fluorescence, due to the development of both photochemical and nonphotochemical quenching during the transition from P to T, is associated with a rise in the O<sub>2</sub> evolution rate, although this increase briefly arrests if a secondary fluorescence maximum (M) develops (Quick and Horton, 1984; Malkin, 1987). The fluorescence maximum (P)



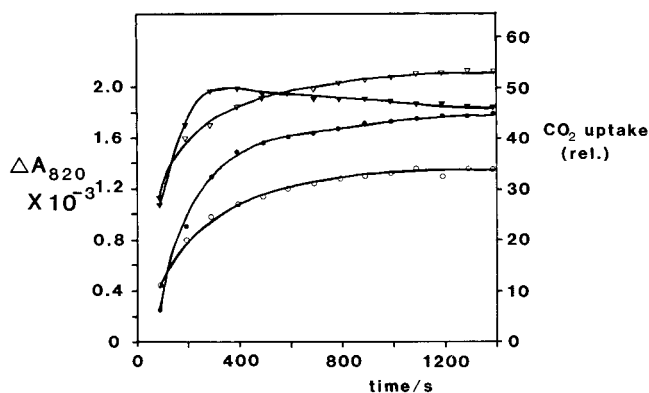
**Figure 3.** The  $\Delta A_{820}$  and the  $\Delta A_{740}$  obtained from pea leaf variety BC1/9RR that had been dark adapted for 10 min prior to irradiation with  $750 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF. The time and absorbance scales apply to both absorbance changes.

has also been associated with a transient rereduction of the Cyt *f* pool following its oxidation after the beginning of irradiance (Sato et al., 1977; Sato and Katoh, 1980; Ruhle et al., 1987). The correlations between the changes in Chl fluorescence and the  $\Delta A_{820}$  indicates that the  $\Delta A_{820}$  minimum (B) is associated with the period of minimum linear electron flow, which appears to be due to a restriction of electron flow lying on the acceptor side of Cyt *f*. The inflexion in the  $\Delta A_{820}$  at D (which occurs concurrently with the fluorescence peak, M) is associated with a subsequent brief decline in the rate of linear electron flow during induction. The broad rise in the  $\Delta A_{820}$  to E is associated with the increase of linear electron transport during induction (Foyer et al., 1992).

Of the two non-P-700 components causing the changes at 740 nm, only that due to Fd persists above 800 nm (Klughammer and Schreiber, 1991). Given the similarity of the  $\Delta A_{740}$  and the  $\Delta A_{820}$  during the first seconds following the beginning of irradiance, it may be that the decline of the  $\Delta A_{820}$  to B is due to Fd reduction in the absence of any significant oxidation of P-700<sup>+</sup>. The increase of the  $\Delta A_{820}$  was also correlated with an increase in the  $\Delta A_{740}$  from the minimum achieved shortly after the start of irradiance (Fig. 3). This increase in the  $\Delta A_{740}$  may be due to a decrease in the pool of reduced Fd, and the parallel rise of the  $\Delta A_{820}$  may therefore be due to a relief of an inhibition of electron flow on the acceptor side of Fd. The absorbance decline to B is small compared with the changes associated with P-700 oxidation. So, although it is difficult to determine when the Fd-linked change disappears during the B-to-E transition, its small size would not seriously distort the interpretation of the absorbance changes.

#### The Relationship between the $\Delta A_{820}$ and the Rate of CO<sub>2</sub> Fixation

The association between the  $\Delta A_{820}$  increase and electron flow can be further demonstrated by comparing the  $\Delta A_{820}$  and the rate of CO<sub>2</sub> fixation during photosynthetic induction (Fig. 4). An O<sub>2</sub> concentration of 2% was used in this case, so photorespiration will therefore be largely abolished (Edwards and Walker, 1983) and the CO<sub>2</sub> fixation rate will be an approximate measure of NADPH demand (or supply). These measurements were made on a leaf of pea variety JI799 and at low CO<sub>2</sub> partial pressure to slow down the rate of rise of both CO<sub>2</sub> fixation and the  $\Delta A_{820}$ . The results show that the rise in the  $\Delta A_{820}$  closely matched the rise in CO<sub>2</sub> fixation rate at both 90 and 180 ppm CO<sub>2</sub>. At a CO<sub>2</sub> concentration of 180 ppm, both the rate of CO<sub>2</sub> fixation and the  $\Delta A_{820}$  rose more quickly than at 90 ppm. At 180 ppm, the  $\Delta A_{820}$  reached a maximum at 390 s, after which it began to decline; during this decline, the rate of CO<sub>2</sub> fixation continued to increase. At 90 ppm, the  $\Delta A_{820}$  rose to a maximum but did not exhibit any subsequent decline. This rise to the maximum  $\Delta A_{820}$  is equivalent to the B-to-E transition (see Fig. 1A) that typically occurs within about 100 s in most C<sub>3</sub> species at atmospheric CO<sub>2</sub> and O<sub>2</sub> concentrations following a dark rest of 20 min. Clearly there is a relationship between increasing P-700 oxidation and the rise of CO<sub>2</sub> fixation and, therefore, the regeneration of NADP<sup>+</sup>. This supports the proposition that the increase in the  $\Delta A_{820}$  from B to E is associated with an



**Figure 4.** The relationship between the duration of irradiance and the  $\Delta A_{820}$  ( $\nabla$ ,  $\nabla$ ) and the relative rate of  $\text{CO}_2$  fixation ( $\bullet$ ,  $\circ$ ) for a leaf of pea variety J1799 subjected to an irradiance of  $1420 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF following a 20-min rest. The leaf was exposed to a gaseous phase comprising either 90 ppm  $\text{CO}_2$ , 2%  $\text{O}_2$ , remainder  $\text{N}_2$  ( $\nabla$ ,  $\circ$ ), or 190 ppm  $\text{CO}_2$ , 2%  $\text{O}_2$ , remainder  $\text{N}_2$  ( $\blacktriangledown$ ,  $\bullet$ ). Complete oxidation of the P-700 pool in this leaf produced an absorbance change of  $2.3 \times 10^{-3}$ ; an increasing absorbance represents an increase in the pool of P-700<sup>+</sup>.

increase in electron transport activity, and the sensitivity to  $\text{CO}_2$  concentration implies that the transition from B to E is associated with the metabolic activity of the Calvin cycle.

Under steady-state conditions and during the later stages of induction, it has been shown that the ratio P-700:(P-700<sup>+</sup> + P-700) correlates with  $\Phi_{\text{PSII}}$  in air or similar conditions, and with  $\Phi_{\text{CO}_2}$  under nonphotorespiratory conditions (Weis et al., 1987; Harbinson et al., 1989; Genty et al., 1990; Harbinson et al., 1990a, 1990b; Peterson, 1991). This ratio can in these and similar circumstances be used to estimate  $\Phi_{\text{PSII}}$ , with an increased pool of P-700<sup>+</sup> causing a proportional fall in the quantum efficiency for PSI electron transport. The results shown in Figure 4 are contrary to this; Figure 5 shows this explicitly. In this figure the relationship between the  $\Delta A_{820}$  and the rate of  $\text{CO}_2$  fixation is shown for the results obtained from a leaf of pea variety J1799 (in 2%  $\text{O}_2$ , 360 ppm  $\text{CO}_2$ , balance  $\text{N}_2$ ) as shown in Figure 4, and also during the B-to-

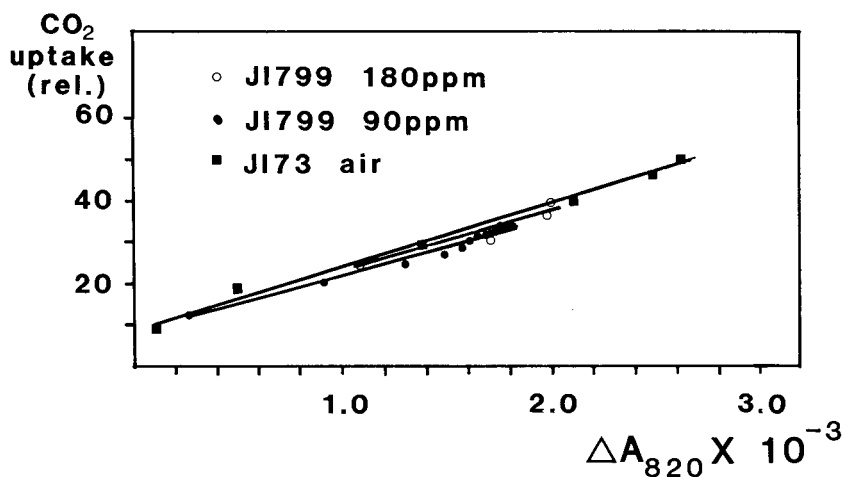
E transition from a leaf of pea variety J173 measured in 360 ppm  $\text{CO}_2$ , 20%  $\text{O}_2$ , balance  $\text{N}_2$  following a dark rest of 1 h. During the B-to-E phase of the  $\Delta A_{820}$ , the increase of P-700<sup>+</sup> accompanies a rise in the quantum yield for  $\text{CO}_2$  fixation and, therefore, in the yield of linear electron transport.

In using the pool of P-700<sup>+</sup> to calculate  $\Phi_{\text{PSII}}$  under steady-state (or similar) conditions, it is assumed that the P-700 pool is quantum efficient and once it is photochemically excited it will become oxidized (Hiyama, 1985) and, therefore, quantum inefficient (e.g. Harbinson et al., 1989). If, however, PSI were to be photochemically incompetent or was unable to be oxidized due to a lack of an acceptor pool (e.g. Fd), then no P-700<sup>+</sup> would be formed during irradiation, there would be no electron transport, and the quantum efficiency of PSI would be zero. A release of these restrictions would allow both more electron transport and more P-700 oxidation. Under these circumstances, increasing P-700<sup>+</sup> would be associated with increased electron transport or quantum efficiency (Fig. 5).

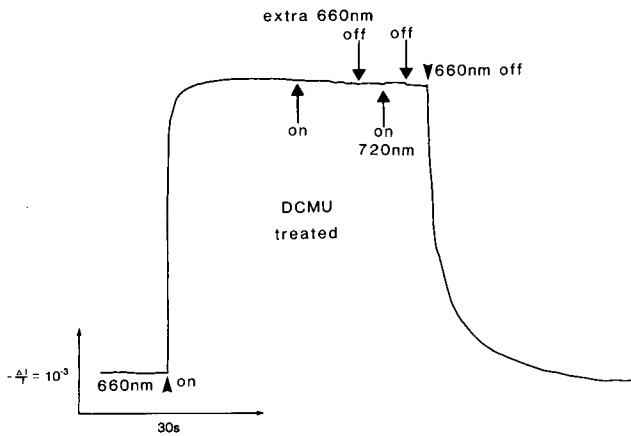
The  $A_{740}$  during induction may be associated with a transient increase in Fd reduction (Klughammer and Schreiber, 1991). Reduction of Fd suggests that PSI is photochemically competent and that it is a shortage of electron acceptors after PSI, caused by reduction of the Fd pool, that is responsible for the lack of P-700 oxidation and the concurrent lack of linear electron transport. The relationship between the rise of the  $\Delta A_{820}$  from B to E and the rise of the rate of  $\text{CO}_2$  fixation is consistent with this proposition, implying an involvement of the activation of the Calvin cycle in determining the increase of the P-700<sup>+</sup> pool.

#### The Effects of Methyl Viologen and DCMU on the $\Delta A_{820}$

The responses of the  $\Delta A_{820}$  in leaves to DCMU and methyl viologen treatment are relevant to the problem of the location of the restriction upon electron transport during induction. DCMU blocks electron transport from  $Q_A$  to the plastoquinone pool, and therefore blocks linear electron transport. In its presence, the  $\Delta A_{820}$  rose to a steady value without any of the detail found in control leaves (Fig. 6); adding either 720 or 660 nm of light to the leaf once the steady-state  $\Delta A_{820}$  had been achieved produced no further absorbance changes.



**Figure 5.** The relationship between the relative rate of  $\text{CO}_2$  fixation and the  $\Delta A_{820}$  during photosynthetic induction as the  $\Delta A_{820}$  rose to the maximum (E); no data from after this maximum are included. Data from two leaves are shown; one leaf, from a plant of pea variety J1799, was exposed to two gaseous phases of 190 or 90 ppm in 2%  $\text{O}_2$ , remainder  $\text{N}_2$ ; the second leaf, of J173, was exposed to air. The leaves were dark adapted for 20 min prior to being irradiated with  $1420 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF.



**Figure 6.** The  $\Delta A_{820}$  produced following the irradiation of a pea leaf that had been soaked in a saturated solution of DCMU for 4 h prior to the experiment. The leaf was irradiated with  $750 \mu\text{mol m}^{-2} \text{s}^{-1}$  of radiation centered around 660 nm (obtained by filtering a quartz-halogen bulb with a Balzers red DT mirror and a special version of a Balzers cyan dichroic mirror obtained from Walz). During this irradiance, the leaf was also briefly subjected to additional irradiances centered on 720 and 660 nm.

Upon removal of all actinic light, the  $\Delta A_{820}$  decayed slowly, in contrast to control leaves, where the decay of the  $\Delta A_{820}$  following 660-nm irradiation was very rapid. These results suggest the following: (a) the transition from A to E found in the  $\Delta A_{820}$  records from control leaves require electron transport; (b) the rapid reduction of P-700<sup>+</sup> found in control leaves requires linear electron flow to have been occurring before the cessation of irradiance; (c) the  $\Delta A_{820}$  produced by 660 nm of light is saturable; no P-700 remains to be oxidized by additional far-red or other light.

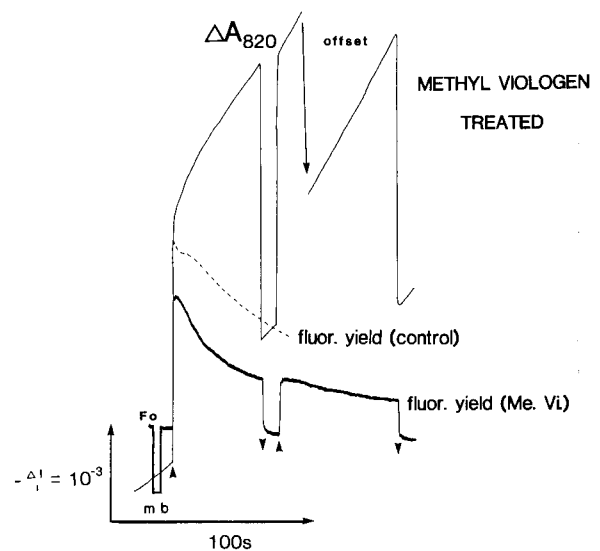
Methyl viologen is an electron acceptor that removes electrons from P-430 (also known as Fe-S<sub>A</sub> and Fe-S<sub>B</sub>) (Hiyama and Ke, 1971; Golbeck and Cornelius, 1986), an electron acceptor that lies on the reducing side of PSI but precedes Fd. In leaves treated with methyl viologen, the A-to-E transition of the  $\Delta A_{820}$  was eliminated and replaced by a simple, rapid rise to a steady-state value (Fig. 7), which in the example shown is super-imposed upon a base-line drift. In contrast to DCMU-treated material, the  $\Delta A_{820}$  relaxed rapidly upon the cessation of irradiance. This implies that PSI is usually competent for electron transport within the time resolution of these measurements and that a deficiency of electron acceptors after P-430 is important in determining the development of the characteristic  $\Delta A_{820}$  changes found in control leaves. The methyl viologen treatment also resulted in a loss of the Chl fluorescence S-M-T transition and a diminution of the  $F_m:F_o$  ratio (Fig. 7). Both observations are consistent with the proposition that during the B-to-E transition of the  $\Delta A_{820}$ , P-700 oxidation is limited by a shortage of acceptors.

The S-M-T transition is thought to be caused by the complex interaction between the two components of Chl fluorescence quenching,  $q_P$  and  $q_N$ , and the Calvin cycle (e.g. Horton, 1985, and refs. therein; Stitt and Schreiber, 1988); methyl viologen would eliminate this interaction. Similarly, the immediate and sustained capacity for electron transport upon

irradiation could be expected to reduce  $F_m:F_o$  under conditions of nonsaturating irradiance.

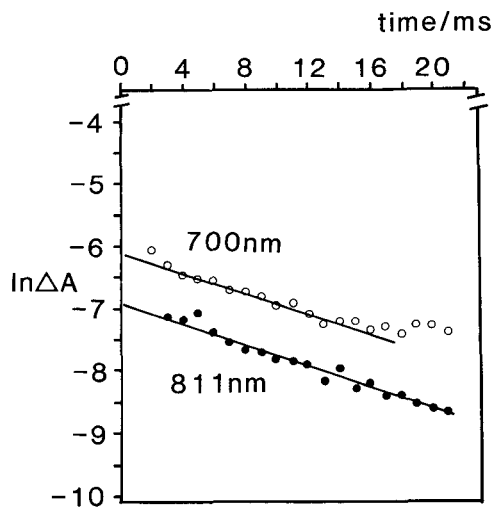
### Significance for Regulation of Electron Transport during Induction: The Acceptor Side of PSI

The results described above suggest that during photosynthetic induction, P-700 oxidation is limited by a shortage of electron acceptors. The relief of this restriction in parallel with the rise of CO<sub>2</sub> fixation implies an involvement of the Calvin cycle in this process. However, Satoh (1981) has produced evidence for the fluorescence DPS transient being due, at least in part, to the activation of the Fd-NADP<sup>+</sup> reductase. Foyer et al. (1992) have shown that in dark-adapted pea leaves there is a delay in the reduction of the NADP pool for about 20 s following the start of irradiance. Therefore, it is possible that the absolute minimum of the  $\Delta A_{820}$  (B) and the earliest stages of the rise to E are associated with a reduction of electron transport to NADP<sup>+</sup> due to the relative inactivity of Fd-NADP<sup>+</sup> reductase. This would be consistent with a possible site of restriction upon electron transport on the acceptor side of PSI. In intact, CO<sub>2</sub>-fixing chloroplasts and leaves, however, there is a rapid rise in the NADPH:NADP<sup>+</sup> ratio 20 s after a dark-to-light transition (Takahama et al., 1981; Heber et al., 1982; Foyer et al., 1992). This is followed by a decline in the NADPH:NADP<sup>+</sup> that parallels the rise in O<sub>2</sub> evolution and the rise of the quantum yield for electron transport by PSII (Foyer et al., 1992). So, although there may be a restriction of the reoxidation of reduced Fd due to the inactivity of NADP<sup>+</sup>-Fd oxidoreductase initially, the rapid reduction of the NADP pool implies that



**Figure 7.** The effect of  $10 \mu\text{mol dm}^{-3}$  (aqueous) methyl viologen on the  $\Delta A_{820}$  and Chl fluorescence from a leaf of pea variety XM7405/1 (with upper epidermis removed) irradiated with  $1420 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF. Because of the background drift of the  $\Delta A_{820}$  during the measurement, the irradiance was interrupted for 10 s to allow reduction of P-700<sup>+</sup> pool. A fluorescence trace obtained from a leaf of XM7405/1 that had been treated with distilled deionized water is also shown.





**Figure 8.** A semi-logarithmic plot of the  $A_{700}$  and  $A_{811}$  from a pea leaf of variety BC 1/6RR following a light-to-dark transition. The irradiance was  $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ . To allow easier comparison with  $A_{811}$ , the changes measured at 700 nm are shown inverted. Following a light-to-dark transition, the steady-state pool of oxidized P700 will be reduced by reducing equivalents derived from the PQH<sub>2</sub> pool and the  $A_{811}$  will decrease and the  $A_{700}$  will increase as the P-700<sup>+</sup> is reduced.

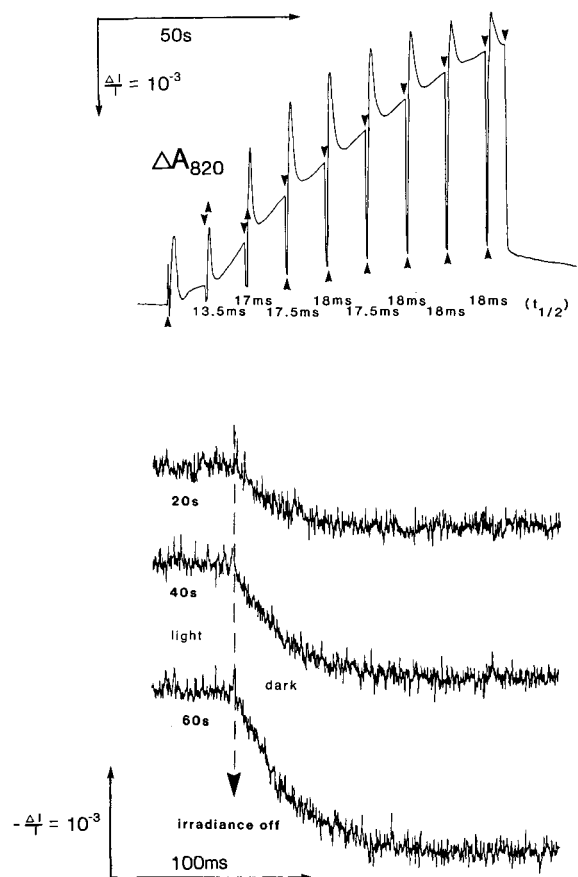
this limitation is short-lived. There must be a restriction of electron transport beyond the NADPH pool that results in its reduction, and as this limitation is relieved, the efficiency of PSII increases, CO<sub>2</sub> fixation increases, and P-700 oxidation proceeds.

#### Significance for Control of Electron Transport during Induction: The Donor Side of PSI

Although the increase in the  $\Delta A_{820}$ , and therefore P-700<sup>+</sup>, is qualitatively due to an increase in electron acceptors for PSI, changes in the resistance for electron flow between PSII and PSI may also contribute to the rise of the  $\Delta A_{820}$ . Changes in the resistance for electron flow between the photosystems will influence the quantum efficiencies of both (Harbinson and Hedley, 1989; Genty et al., 1990). This resistance can be quantified by measuring the  $t_{1/2}$  of P-700<sup>+</sup> reduction using the  $\Delta A_{820}$  relaxation kinetics following a light-to-dark transition (Harbinson and Hedley, 1989; Schreiber et al., 1989). Measurements of the decay of the  $\Delta A_{820}$  and the  $\Delta A_{700}$  obtained under steady-state irradiance (Fig. 8) show them to be pseudo first-order (Harbinson and Hedley, 1989) and with the same  $t_{1/2}$ . In Figure 8, the  $\Delta A_{700}$  changes are inverted to ease comparison with the  $\Delta A_{820}$ ; the  $A_{820}$  was  $1.01 \times 10^{-3}$  and the  $A_{700}$  was  $-2.13 \times 10^{-3}$  (an absorbance increase at 820 nm and a decrease at 700 nm is consistent with P-700<sup>+</sup> formation in the light). The ratio of the absorbance changes at 820 and 700 nm was only about 2, whereas the ratio of the extinction coefficients for P-700 oxidation at 700 and 820 nm is closer to 10 (Mathis and Paillotin, 1981). Using chloroplasts Klughammer and Schreiber (1991) obtained a ratio of 2.5 for the  $A_{700}$  and  $A_{820}$ . They argued that the discrepancy between their ratio and that obtained from P-700-containing particles was

due partly to the presence of plastocyanin in their samples and partly to the greater light scattering within their chloroplast suspension. Because of the lower overall absorbance at 820 nm compared with 700 nm, light scattering would act to increase the apparent extinction coefficient at 820 nm more than at 700 nm. Similar arguments would apply when comparing results obtained from leaves with those obtained from PSI particles.

Measurements of the  $t_{1/2}$  of the  $\Delta A_{820}$  decay following brief interruptions to the irradiance during induction showed only small changes in this  $t_{1/2}$  (Fig. 9) with the increasing  $\Delta A_{820}$ . The decay kinetics of these  $\Delta A_{820}$  changes during brief interruptions to the irradiance show them to be simple and



**Figure 9.** The  $t_{1/2}$  of the decay of the  $\Delta A_{820}$  occurring during brief interruptions in the irradiance of a pea leaf that had been dark adapted for 20 min prior to being irradiated with  $1420 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAQF centered on 660 nm. Following the start of the irradiance, a cycle of 9 s on and 1 s off was employed. Each time the irradiance was interrupted, the decay of the  $\Delta A_{820}$  was recorded using a Thurlby DSA524 digital storage adaptor. The  $t_{1/2}$  values of the decays were measured from a filtered form of this recording. These  $t_{1/2}$  values are indicated beneath the appropriate light off/on event. Three specimen traces of these  $\Delta A_{820}$  decays (measured at 20, 40, and 60 s) show the relaxation of the  $\Delta A_{820}$  from the quasi steady-state level during irradiance to the absorbance change zero following a light-to-dark transition. Superimposed on the changes due to the formation or reduction of P-700<sup>+</sup> is a shift of the absorbance base line; this slowly relaxing change nearly always occurs in leaves.

monotonic (Fig. 9). This implies that there were no significant absorbance changes relaxing with different kinetics to those attributed to P-700 (e.g. a slowly recombining P-680<sup>+</sup>:Q<sub>A</sub><sup>-</sup> component [Schlodder and Meyer, 1987]). The relative constancy of the  $t_{1/2}$  during the B-to-E transition implies that changes in the resistance for electron flow between the photosystems are relatively unimportant in determining the increase in P-700<sup>+</sup>, especially after 20 s (in the example shown).

### The Limitation of Electron Flow during Induction

The absence of any marked change in the  $t_{1/2}$  for P-700<sup>+</sup> reduction in the dark intervals (Fig. 9) implies that no substantial changes occur in the "resistance" for electron flow between the PQH<sub>2</sub> pool and P-700<sup>+</sup> during induction. So changes in the pool of P-700<sup>+</sup> in these data cannot be attributed to changes in the resistance for the supply of reductant to P-700<sup>+</sup> from the PQH<sub>2</sub> pool, although there may be substantial unresolved changes in  $t_{1/2}$  before the earliest useful record made here. Therefore, changes in pool P-700<sup>+</sup> during the early stages of photosynthetic induction *in vivo* are due principally to changes in the restriction of electron flow away from PSI. Since the plastoquinone pool would be extensively reduced during induction (Amesz et al., 1971, 1972), the constancy of  $t_{1/2}$  implies that the intra-thylakoid pH, as sensed by the reaction between PQH<sub>2</sub> and the Cyt *b*<sub>6</sub>/*f* complex (Bendall, 1982), remains largely constant during induction even though large changes in  $q_N$  will be occurring. Although the  $t_{1/2}$  for P-700<sup>+</sup> reduction changes only slightly during the B-to-E transition of the  $\Delta A_{820}$ , it subsequently decreases considerably as photosynthetic induction proceeds (Harbinson and Hedley, 1989). The decline of the  $\Delta A_{820}$  that occurs following E is shown in Figure 4 (180 ppm CO<sub>2</sub> example).

### CONCLUSIONS

The increase of the P-700-related  $\Delta A_{820}$  during the early stages of photosynthetic induction are due largely to the inactivity of those processes in stroma that regenerate oxidized PSI electron acceptors. In the absence of regeneration of the acceptor pool, P-700 oxidation becomes limited on the acceptor side. Changes in the resistance for electron flow into PSI, as measured by the  $t_{1/2}$  for P-700<sup>+</sup> reduction, are small.

During this phase of increasing P-700<sup>+</sup> where P-700 oxidation is blocked on the acceptor side, the  $\Phi_{PSI}$ , as estimated by the fixation of CO<sub>2</sub>, does not linearly relate to the pool of reduced P-700 (see, for example, Genty et al., 1990). Instead, it relates linearly to the pool of oxidized P-700, which then indicates the degree to which PSI is active in electron transport on the acceptor side.

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