

# Chlorophyll Fluorescence Characteristics Associated with Hydration Level in Pea Cotyledons

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## ABSTRACT

In order to study the effects of desiccation on a photosynthetic system, light harvesting and light-induced electron transport processes were examined in pea cotyledons at various moisture levels, using *in vivo* fluorescence excitation spectra and fluorescence induction kinetics. Water sorption isotherms yielded thermodynamic data that suggested very strong water binding between 4 to 11% water, intermediate sorption between water contents of 13 to 22%, and very weak binding at moisture contents between 24 to 32%. The fluorescence properties of the tissue changed with the moisture contents, and these changes correlated generally with the three regions of water binding. Peak fluorescence and fluorescence yield remained at low levels when water content was limited to the tightly bound regions, below 12%. Several new peaks appeared in the chlorophyll *a* excitation spectrum and both peak fluorescence and fluorescence yield increased at intermediate water-binding levels (12–22%). At moisture contents where water is weakly bound (>24%), peak fluorescence and fluorescence yield were maximum and the fluorescence excitation spectrum was unchanging with further increases in water content.

The state of water is an important component in the energy transfer and electron transport system. At hydration levels where water is most tightly bound, energy transfer from pigments is limited and electron transport is blocked. At intermediate water binding levels, energy transfer and electron transport increase and, in the region of weak water binding, energy transfer and electron transport are maximized.

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Organisms that remain viable in the desiccated state are prevented from metabolizing when dry, but regain normal metabolic activity upon hydration. In recent studies (1, 3, 14, 19), it has become increasingly evident that it is not the quantity of water per se which affects enzyme function, organelle integrity and metabolic activity, but the kind of water. At least three different types of water, identified by mobility and thermodynamic characteristics (8, 20), are associated with fully hydrated biological materials and correspond to the strength by which they are sorbed to macromolecular surfaces. Types I, II, and III water refer to water molecules that are bonded to charged moieties (I), water molecules sorbed to uncharged, but hydrophilic surfaces (II), and water condensed over hydrophobic regions (III). Although protein stability is believed to be relatively unaffected over a range of water contents (2, 17), membrane characteristics may be altered by the withdrawal of water (9, 18) and membrane integrity is lost altogether in desiccation intolerant organisms (4–6).

In addition to monitoring the physical state of various tissue components in relation to water content, it is also useful to determine how the status of water affects biologically significant

reactions. The presence of Chl in pea cotyledons provides an opportunity to examine nondestructively electron transport in imbibing tissues by monitoring changes in Chl fluorescence properties. In this study, two fluorescence phenomena of pea cotyledons were investigated: Chl fluorescence excitation spectra and fluorescence induction. Fluorescence excitation spectra provide an indication of the pigments which can transfer absorbed radiant energy to Chl *a* molecules, and fluorescence induction kinetics provide an indication of electron transport associated with PSII (see [12] for review). Fluorescence induction curves of hydrated dark-adapted tissue can be separated into two main parts reflecting the partial reactions of PSII coming to a steady state. The initial rise to a maximal fluorescence level is associated with the reduction of the electron acceptor Q in PSII (10, 15); the subsequent decay in fluorescence has been associated with the membrane energization processes including the generation of transmembrane pH and electrochemical gradients (12).

## MATERIALS AND METHODS

**Plant Material.** Pea cotyledons (var Alaska field pea—Burpee, 1982) with seed coats removed combine the useful features of possessing Chl and withstanding desiccation. For sorption isotherms, the cotyledons were ground into pellets 0.5 to 1 mm in diameter. For fluorescence studies whole cotyledons were used.

Cotyledons or seed particles were incubated over various saturated salt solutions to obtain desired moisture contents up to 25% (19). For higher moisture contents, cotyledons were either incubated over water or partially imbibed on paper towels. Moisture contents were determined gravimetrically. Dry weights were taken after 5 d at 90°C—a time when constant weight was obtained. All moisture contents are expressed on a dry weight basis.

**Sorption Isotherms and Sorption Calculations.** The strengths of water binding, derived by the Clausius-Claperyon equation, were calculated using data from water sorption isotherms at 15 and 25°C (19). Pellets were incubated over saturated salt solutions for 5 to 10 d until a constant fresh weight was obtained. Twenty different salt solutions were used. Moisture content determinations were replicated 2 to 3 times at each station. The RH of the salt solutions were obtained from tables in Winston and Bates (21) and Rockland (13).

**Fluorescence Excitation Spectra.** Fluorescence excitation spectra were determined on a Perkin-Elmer fluorescence spectrophotometer model MPF 44B operating in ratio mode. Pea cotyledons at various moisture contents were loaded into a dry sample holder, with the flat side exposed to the light source. Excitation measurements between 300 to 700 nm were recorded, emission wavelength being monitored at 728 nm. The signal gain for each spectrum determination was normalized at the peak excitation wavelength, 437 nm. Each treatment was replicated 4 to 5 times.

**Fluorescence Induction.** Fluorescence induction curves were

measured on a portable Branker fluorometer SF-10 (16). After samples were dark-adapted for 2 to 3 d, red light at an intensity of about  $20 \mu E/m^2 \cdot s$  was shone continuously on the sample and the rise and subsequent decline in fluorescence were followed until there were no further changes in fluorescence intensity. This steady state level was used as a measure of Ft.<sup>1</sup> Each treatment was replicated 5 times.

The increase in fluorescence, termed Fp, was determined by subtracting Ft from maximum fluorescence (Fig. 4A, inset). The area under the decay curve was used as a measure of Fy. Area was calculated by a Zeiss digitizing unit. To avoid variability due to Chl content, both Fp and Fy values are normalized by the corresponding Ft measurement.

To determine the effects of an electron transport inhibitor on the fluorescence decay kinetics, pea cotyledons were soaked in either 1 mM DCMU solution or water for 1 h, dried, and rehydrated to controlled water contents as described above. Fluorescence induction experiments then were performed on these cotyledons.

RESULTS

Thermodynamic data describing the strength of water binding in pea seeds were calculated from moisture isotherms at 15 and 25°C and are shown in Figure 1. Between moisture contents of 1 to 4%, pea pellets were hydrophobic as shown by a positive enthalpy ( $\Delta H$ ) of sorption value. Between 5 and 11% water, water was bound in the pea tissues tightly, with  $\Delta H$  ranging between -4 and -7 kcal/mol of water. At moisture contents between 13 to 22%, an intermediate level of water binding was indicated by enthalpy values ranging between -1.8 and -1.2 kcal/mol. Between 24 and 32% water, a third region with very weak water binding was indicated by  $\Delta H$  values of approximately -0.4 kcal/mol. Above 32% water,  $\Delta H$  was 0 or slightly positive, indicating a region of unbound water.

To compare Chl states in these three regions of water binding, fluorescence excitation spectra for pea cotyledons at seven different water contents were recorded. Representative fluorescence spectra for tissues at 7, 18, and 35% water are shown in Figure 2, A, B, and C, respectively. For each of the hydration levels,

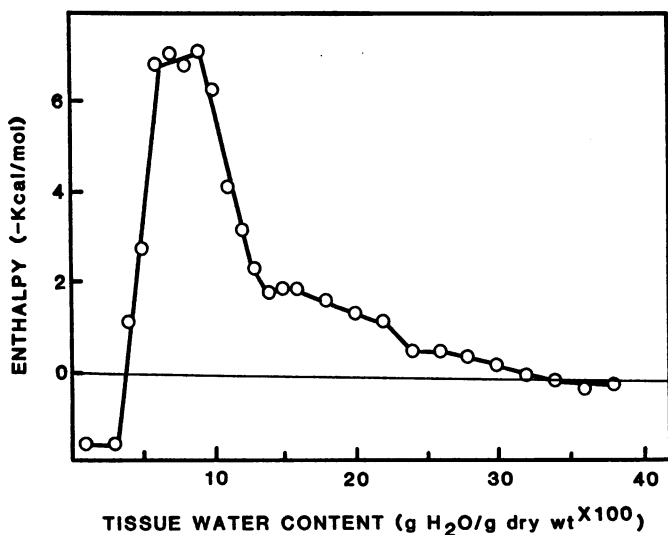


FIG. 1. Differential enthalpy of water sorption at different moisture contents of ground pea cotyledons. Values are calculated from sorption isotherms at 15 and 25°C.

<sup>1</sup> Abbreviations: Ft, terminal fluorescence; Fp, peak fluorescence; Fy, fluorescence yield.

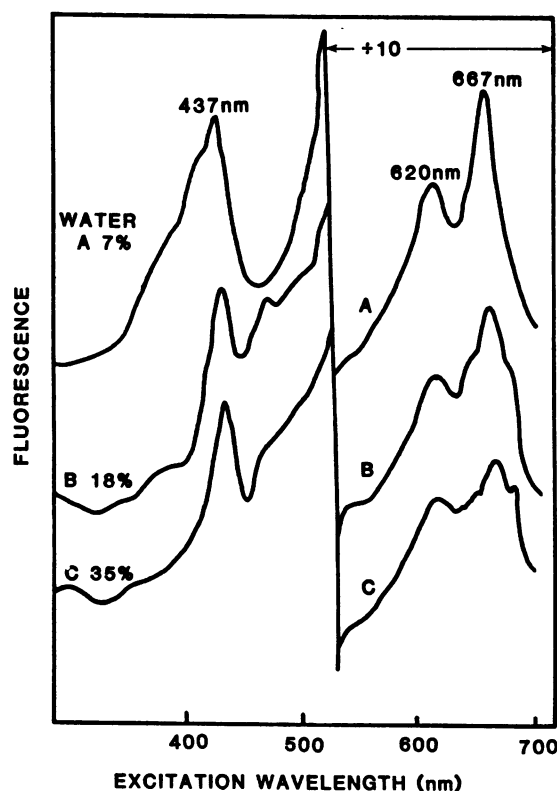


FIG. 2. Fluorescence excitation spectra of Chl in pea cotyledons at A, 7%; B, 18%; and C, 35% water. The emission wavelength is set at 728 nm. Signal gain is reduced by 10 at wavelengths above 540 nm.

major peaks were observed at 437, 620, and 667 nm. However, an increase in hydration from 7 to 18% produced several noticeable changes in the excitation spectrum including a large increase in the 450 to 490 nm range with a peak near 480 nm, the appearance of new peaks near 653 and 684 nm, and a decline in the 400 to 420 nm region. The excitation spectrum at the 35% hydration level was approximately similar to the excitation spectrum of the 18% water sample.

Differences in the excitation spectrum of Chl a with moisture content are plotted in Figure 3. Changes in spectra are plotted as changes in ratios between paired wavelengths. Relatively small differences in spectra were observed from peas at 1 and 7% water. However, several of the ratios changed steadily as moisture content was raised above 7%. Between 18 and 23% water, these trends changed abruptly. For example, the ratio between fluorescence at 482 nm, a carotenoid and/or Chl b absorbing region, and 437 nm rose steadily with increasing moisture content up to 18% water and then leveled off (Fig. 3B). The ratio between peak fluorescence in the red and in the blue region (Fig. 3C) remained unchanged for moisture contents below 18%, but abruptly changed to a new lower level when water content exceeded 23%. The differences between peak height at 620 and 667 (Fig. 3D) remained relatively unchanged with water content. When moisture content exceeded 12%, auxiliary peaks at 653 and 684 appeared (Fig. 3, E and F).

In fluorescence induction experiments, the degree of hydration effected both fluorescence rise and the rate of fluorescence decay in dark-adapted tissue (Fig. 4). When moist ( $\geq 33\%$  water) cotyledons were exposed to light (Fig. 4A), the initial rate and amplitude of fluorescence rise was very high and decay was rapid. Fluorescence decay continued for about 35 to 40 min. Initial fluorescence rise, decay rate, and length of time for decay decreased with lower moisture content down to about 10% water,

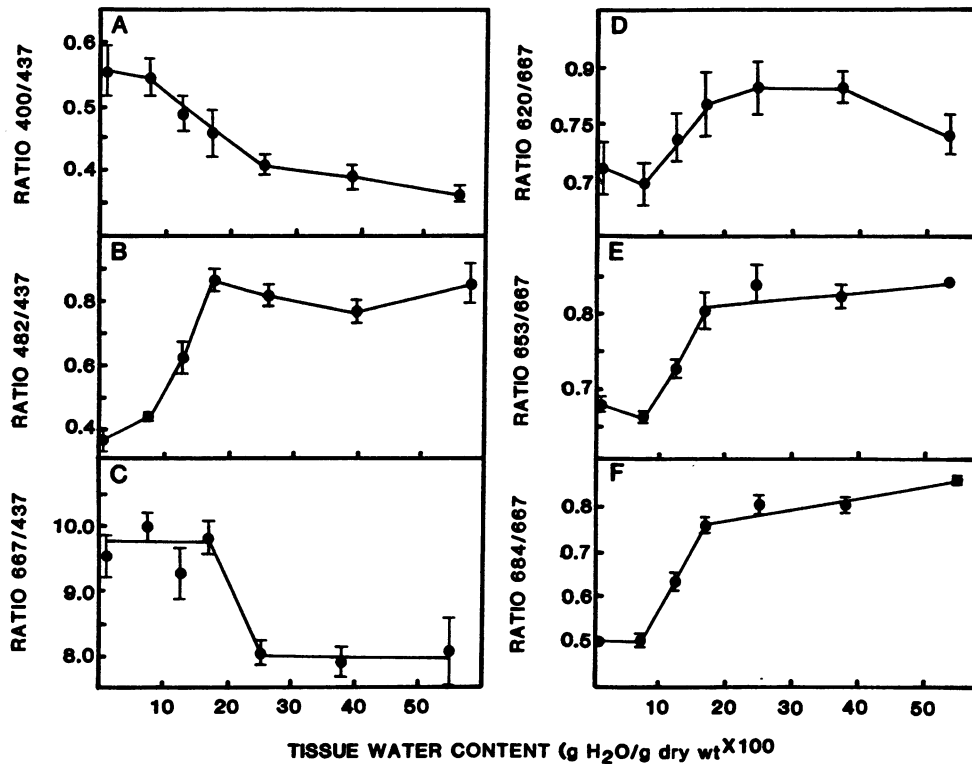


FIG. 3. Changes in the pea cotyledon fluorescence excitation spectrum with moisture content. Fluorescence values were determined from excitation spectra similar to those in Figure 2. Fluorescence at wavelengths as indicated on the ordinate were normalized to peak fluorescence values at either 437 or 667 nm. The error bars represent the SE of 4 to 5 replicates.

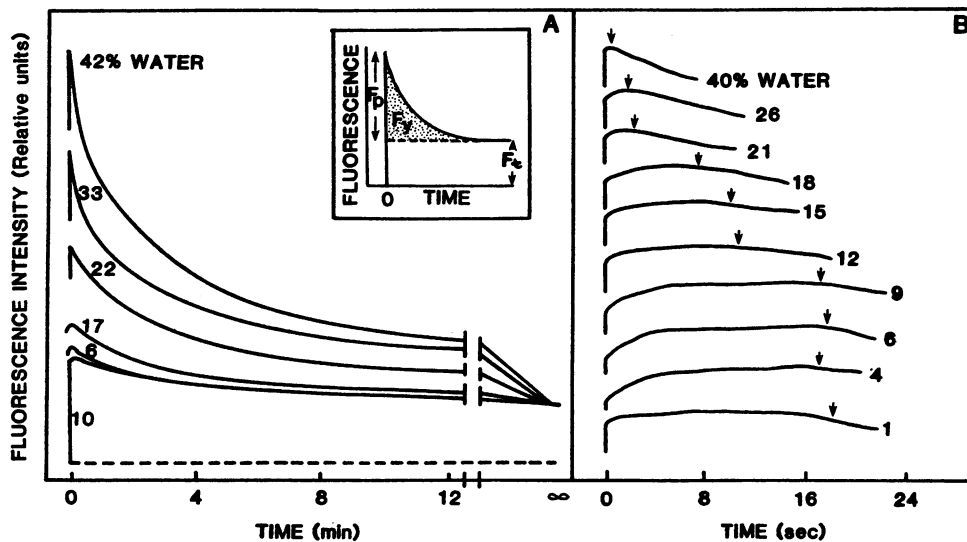


FIG. 4. Sample fluorescence induction tracings of dark-adapted pea cotyledons. A, Cotyledons are exposed to light until fluorescence intensity no longer changes. The inset represents a typical curve showing how Ft, Fp, and Fy are determined. B, Fluorescence rise was recorded using a fast chart speed. Arrows represent time required to reach peak fluorescence.

after which the trend was reversed. Tracings of the rapid fluorescence rise (Fig. 4B) demonstrated that as moisture content decreased, the rate of fluorescence rise (or the onset of fluorescence decay) was depressed. Moist tissues (40–21.4% water) required only 1 to 3 s for the onset of fluorescence decay, while tissues at moisture contents between 18.4 and 12.3% required 8 to 10 s. In dry tissues, below 9.4% water, decay was delayed for 16 to 19 s.

Electron transport, quantified by the parameters Fp and Fy, changed with water content (Fig. 5, B and C). No evident relationship between Ft and water content was observed (Fig. 5A). The variability that was observed was probably due to differences in Chl content among the samples. The relationship between Fy and water content is described in Figure 5B. Fy decreased when moisture content increased from 1 to 5%, and remained low until moisture contents reached 11%. At moisture

contents above 11%, Fy increased abruptly and then maintained a high level at moisture contents above 17%. Similar trends were observed for Fp (Fig. 5C). However, Fp increased abruptly at 13% and leveled off at 22% water as opposed to the 11% and 17% observations described for Fy.

The recovery of peak fluorescence excitation values was measured after a 1-min pulse of light (data not shown). Recovery rate was rapid for seeds between 4 to 30% water and was complete within 7 to 8 min. Recovery of variable fluorescence from seeds with <4% water was slower and incomplete (about 70% Fp after 10 min).

DCMU, which inhibits the transfer of electrons from acceptor Q in PSII to the electron transport chain, had no apparent effect on Fp at any moisture content, but decreased Fy at moisture contents greater than 18% (Table I) Fy was reduced by 67% in DCMU-treated cotyledons with 50% moisture.

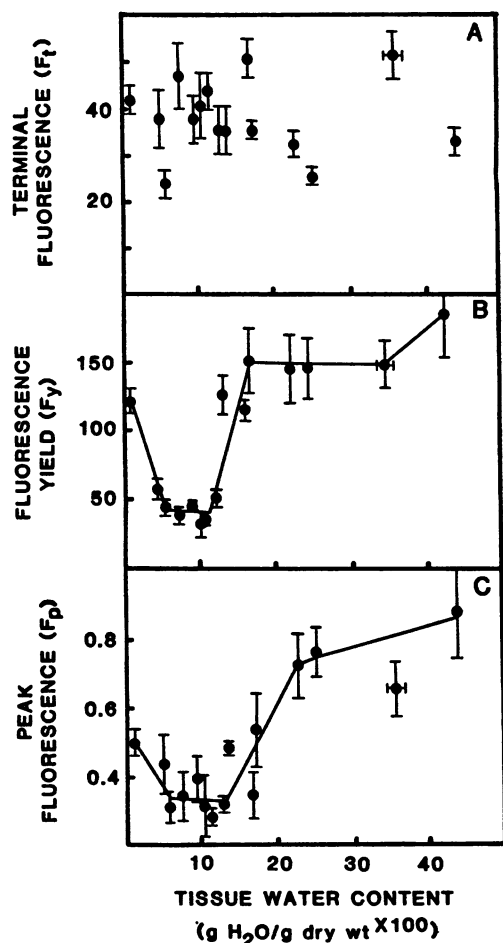


FIG. 5. Changes in fluorescence induction parameters with water content in pea cotyledons: A, Ft; B, Fy; C, Fp. Values are determined from curves similar to those in Figure 4A. Error bars represent SE calculated from 5 replicates.

Table 1. Effects of DCMU on Chl Fluorescence Induction Parameters in Pea Cotyledons of Different Water Contents

Water Content	Preimbibition Treatment	Fp	Fy
%			
1.8	H <sub>2</sub> O	0.66 ± 0.03 <sup>a</sup>	19.3 ± 3.9
	DCMU	0.66 ± 0.03	17.1 ± 2.9
9.7	H <sub>2</sub> O	0.53 ± 0.03	7.7 ± 1.4
	DCMU	0.57 ± 0.07	7.5 ± 2.4
18.1	H <sub>2</sub> O	0.47 ± 0.01	33.1 ± 2.7
	DCMU	0.50 ± 0.03	20.2 ± 1.0
28.2	H <sub>2</sub> O	0.67 ± 0.03	52.9 ± 3.9
	DCMU	0.70 ± 0.07	38.1 ± 3.3
50.5	H <sub>2</sub> O	1.03 ± 0.10	159.7 ± 32.8
	DCMU	1.00 ± 0.10	52.1 ± 3.0

<sup>a</sup> SE calculated from three samples.

DISCUSSION

Hydration-dependent changes in light-induced energy transfer and electron transport seem to reflect the three levels of water binding.

Three main regions of water binding in pea cotyledons, detected by thermodynamic methods, occur between moisture contents of 5 to 11%, 13 to 22%, and 24 to 32%. These different regions are considered to be associated with different levels of

hydrophilic and hydrophobic interactions at different levels of water abundance (1, 14). Other studies using biological materials have reported three levels of water binding with water contents that roughly coincide with those mentioned here (3, 14, 17, 19).

In pea seeds, we do not detect water binding at water contents of 1 to 4% and >32% (Fig. 1). The former region is probably a reflection of the hydrophobic nature of starch when very dry, while the latter region reflects the presence of unbound or free water.

The fluorescence excitation characteristics of Chl show some striking changes with differences in water content. A comparison of the spectral ratio curves shown in Figure 3 with the enthalpy curve in Figure 1 suggests that the major spectral shifts coincided with the transition in water binding from tightly bound to moderately and/or weakly bound water. The spectral changes characterized in Figure 3 could be due to several hydration-dependent phenomena including hydration-dependent shifts in the Chl absorption spectra or hydration-dependent changes in the efficiency with which accessory pigments can transfer their excitation to Chl. Our data cannot distinguish between these possibilities. However, the disappearance of the shoulder at 400 nm (Fig. 3A) and the appearance of the shoulder at 684 nm (Fig. 3E) with increasing water content suggest shifts in the Chl absorption spectrum. These shifts imply hydration-induced conformational changes in the Chl which ultimately affect the wavelengths at which it absorbs light and fluoresces (12). The wavelength of the red fluorescence excitation maximum at 667 nm is shifted to the blue compared to the *in vivo* Chl fluorescence maximum (685 nm). This suggests that in the dry state, Chl in pea seeds is more like free pigments in organic solvents (maxima at 663–666 nm) than those bound in pigment-protein complexes. The increase in fluorescence excitation at 684 nm suggests a shift towards the red as the tissue became hydrated. A similar phenomenon is observed when water is added to Chl in organic solvents (11; unpublished data) and suggests a recovery of Chl native state under aqueous conditions. The increase in the 450 to 500 and 653 nm region of the excitation spectrum when hydration level increased from 8 to 18% water suggests that either a carotenoid or a Chl *b* contribution to the fluorescence excitation depends on hydration level. Wiltens *et al.* (20) suggested that energy transfer efficiency between the red algal phycoobilins and Chl *a* decreased with desiccation, but only at very low water contents.

The characteristic of peak fluorescence and fluorescence yield were altered between water binding regions. The low levels of Fy and Fp, observed when water was most tightly bound, steadily increased as water became moderately bound. Little or no change in Fp and Fy were observed when water was added within the third region of water binding. Changes in Fp and Fy have been attributed to changes in the chloroplast membrane system. The decreasing amounts of Fp and Fy in stressed tissues (7, 20) were attributed to increasing disorganization of chloroplast membranes. Fluorescence decay was not observable in chloroplasts with disrupted membranes (12). Desiccation-induced losses of Fy in algae resulted from changes in the ordering of thylakoid structure (4). The low level of Fy and Fp at low moisture contents (<13% water) in pea suggests that pea chloroplast membranes are not totally dysfunctional in the first region of water binding, but become increasingly functional in the second region of water binding, and are fully capable of electron transport when the tissue is hydrated to region III. The reduction in Fy (Table I) and decrease in the kinetics of fluorescence decline (not shown) observed with DCMU-treated cotyledons having water contents >18% substantiates the relation between electron transport and variable fluorescence observed for pea cotyledons. The lack of effect of DCMU on the decay kinetics of cotyledon tissue having less than 18% water may reflect an inability of DCMU to interact

normally with its usual binding site. The reduction in rate of rise of fluorescence (Fig. 4B) at lower hydration contents is consistent with a depressed rate of PSII-related electron transport (10). X-ray diffraction studies of soybean (18) and trefoil (9) seed phospholipids have shown no drastic changes in membrane structure upon dehydration, but did show a change in the bilayer spacing when moisture content exceeded about 20%. The low level of electron transport is consistent with the interpretation that pea seed membranes remain relatively intact at low water contents.

In green plants the rise to maximal fluorescence is associated with the reduction of the PSII electron acceptor Q (10, 12, 15). In our experiments, this parameter was not affected by the presence of DCMU (Table I), but the rate of fluorescence rise was markedly reduced at low water contents (Fig. 4B) and Fp was more sensitive to low water contents than Fy. These phenomena may be due to the unavailability of water as a substrate in the water splitting reaction. In *Porphyra sanjuanensis*, and *Borya nitida*, both desiccation tolerant plants, low water contents also caused slow rates of fluorescence rise, interpreted as electron donation to PSII being particularly sensitive to desiccation (7, 20). The lower rates and amplitudes of Fp with lower water content may also be due to limited energy transfer from light harvesting pigments to PSII reaction centers.

The reason for the high level of fluorescence decay and peak fluorescence at very low water contents (<5% water) is unknown. The moisture contents where this phenomenon is seen coincide with the moisture contents at which pea tissue is hydrophobic (Fig. 1). This may be a factor in the anomalous results. It is also possible that the light caused some photo-destruction of the Chl in the very dry environment, thus mimicking rapid fluorescence decay. Recovery of peak fluorescence in the dark (not shown) is retarded at these very low water contents.

The Chl fluorescence data presented indicate that in pea cotyledons the steps of light absorption, transfer of excitation energy by light-harvesting pigments, and electron transport are affected by water content. At hydration levels where water is most tightly bound, limited electron transport occurs upon illumination. At intermediate water binding levels, there is a progressive increase in the number of absorbing species that can transfer excitation energy to Chl *a*, and a concomitant increase in electron transport indicated by variable fluorescence measurements. Finally, when water is abundant enough to be bound weakly, these reactions appear to be maximal. The abruptness at which these reactions resume activity suggests that the membrane system in pea cotyledons remains organized upon desiccation and that water bound with intermediate strength permits effective

energy transfer from light-harvesting pigments to reaction centers or electron transport.

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