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THE EFFECT OF TEMPERATURE ON THE FLUORESCENCE KINETICS OF SPINACH CHLOROPLASTS

S. W. THORNE AND N. K. BOARDMAN

Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra (Australia)

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SUMMARY

1. The fluorescence kinetics of spinach chloroplasts were studied over the temperature range +40° to −196°. At temperatures above −5°, the ratio of the steady-state level of fluorescence (F_∞) to the initial level (F_0) was determined by the intensity of the excitation light. At −5°, F_∞/F_0 was independent of excitation intensity and F_∞ was equal to the fluorescence yield in the presence of dithionite (F_{reduced}). In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, there was a rapid rise in the fluorescence to the steady-state level. The fluorescence rise ($F_\infty - F_0$) in untreated chloroplasts was dependent on pH with maxima at pH 6.1 and pH 7.2; F_0 was independent of pH.

2. The biphasic kinetics, observed at 20° were reproducible provided the chloroplasts remained in the dark for a few min between excitations, but at −5° the initial biphasic kinetic curve was replaced by an exponential curve at subsequent excitations. Illumination of the chloroplasts at −5° by far-red light restored the chloroplasts to their initial state, and a biphasic kinetic curve was then obtained at the second excitation. An action spectrum for the far-red restoration showed a peak at 707 nm. The rate of the far-red response declined gradually at temperatures below −5°, and it was completely inhibited at −30°.

3. Over the temperature range −40° to −196° exponential fluorescence kinetics were obtained, but only at the first excitation. Subsequent excitations gave an immediate rise to the F_∞ level. The rate of the initial exponential rise in fluorescence declined with decreasing temperature.

4. The results are discussed in terms of a sequential series of carriers (E,Q,P) on the reducing side of Photosystem II. Analysis of the kinetic curves obtained at −5° indicated a P/Q ratio of 6, and 1 Q per 140 chlorophylls. E is present to a smaller extent than Q. The quantum efficiency for the reduction of Q by Photosystem II declined with temperature between −5° and −196°. To explain the temperature responses, it is postulated that oxygen interacts at 3 sites with the electron transport chain of chloroplasts.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

INTRODUCTION

On illumination of dark-adapted isolated chloroplasts with red light, the fluorescence rises from an initial level (F_0) to a steady-state level (F_∞). The time course of the fluorescence rise is biphasic at room temperature, but exponential at $-196°$ (see ref. 1) MALKIN AND KOK[2] and MALKIN[3] have analysed the time course of fluorescence in terms of a quencher Q (*cf.* ref. 4) and a secondary oxidant P. MALKIN AND KOK[2] concluded that the ratio of Q to P was unity and that each was present in an amount of one per 70 chlorophylls.

On the other hand, fluorescence kinetic measurements in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) showed a rapid rise in the fluorescence from F_0 to F_∞, and suggested that the primary oxidant pool was much smaller (one per 500 chlorophylls)[5]. Studies of the oxygen gush from algae with single flashes of light[6,7] also pointed to a small primary oxidant pool, (*ca.* 1 per 500 chlorophylls) and a larger secondary oxidant pool (*ca.* 1 per 30 chlorophylls). From a study of the reduction of the secondary pool with single flashes of varying duration, FORBUSH AND KOK[8] concluded that the secondary pool was heterogeneous, one-third reacting more rapidly with the primary pool than the remainder. Analysis of fluorescence-rise curves obtained with a series of brief flashes indicated a value of about 18 for the proportion of secondary pool to primary pool.

MURATA *et al.*[9] observed that the steady state fluorescence yield of isolated chloroplasts decreased at lower light intensities. The effect was explained by a back-reaction of the primary photochemical products, because it was considered that in the absence of a Hill oxidant there was no non-cyclic electron flow. However, as pointed out by MALKIN[10], oxygen may act as an electron acceptor in aerobic suspension of chloroplasts (MEHLER reaction[11]). At low light intensities the steady state level of fluorescence is low because the rate of oxidation of Q^- and P^- by oxygen (as the ultimate electron acceptor) is comparable with the rate of the light-induced reduction of Q.

In the present study, we have examined the influence of temperature on the fluorescence kinetics of isolated chloroplasts over the range of $+40°$ to $-196°$. Under certain conditions the oxidation of P^- by oxygen is a rate limiting step and the light-induced reduction of Q may be separated from the reduction of P. From the effect of temperature both on the fluorescence kinetics and the recovery of the chloroplasts in the dark, we postulate that oxygen interacts at 3 sites with the photosynthetic electron transport chain.

MATERIALS AND METHODS

Spinach chloroplasts were isolated as described previously[12] and resuspended in 0.05 M phosphate buffer, pH 7.2, containing 0.3 M sucrose and 0.01 M KCl.

Fluorescence measurements were made on a fluorescence spectrometer, some details of which were described previously[13]. The cuvette containing the chloroplasts was positioned so that the whole sample was exposed to the light from the excitation monochromator. Light intensities were measured by a Reeder Type RP 2 vacuum thermopile, and cross checked with a Zeiss type VTh. 8 thermopile. A relative scale of light intensities was obtained by directing a sample of excitation light on to the

excitation photomultiplier, which was equipped with a wide calibrated range of gain sensitivities. The excitation wavelength was 650 nm. Under our standard conditions, the excitation band width was ± 1.5 nm, and the light intensity at the surface of the cuvette was 200 erg·cm^{-2}·sec^{-1} ($I_0 \times 1$). Higher ($I_0 \times 3$ and $I_0 \times 10$) and lower ($I_0 \times 0.1$ and $I_0 \times 0.3$) intensities were obtained by varying the width of the slit on the excitation monochromator. Fluorescence kinetics were measured at 683 ± 1.0 nm and recorded on a Rikadenki B-34 recorder at a chart speed of 200 mm/min.

For temperatures in the range −10°–+40°, the cuvette was placed in a jacketed holder and a glycol–water mixture was circulated from a refrigerated bath maintained at the desired temperature to an accuracy of ± 0.2°. For temperature control between −10° and −196° the cuvette was surrounded by a copper case, which was lagged and fitted with appropriate optical windows and connected by a copper rod to a tank. Liquid nitrogen was added to the tank at a rate sufficient to maintain the required temperature at the cuvette. The temperature of the sample was measured to an accuracy of ± 1° by a thermocouple.

Fluorescence measurements in the pH range 5.8–7.8 were made in 0.05 M phosphate buffer. At pH 5.0, 0.05 M acetate buffer was used, and at pH 9.0 the chloroplasts were suspended in 0.05 M glycine buffer. The chloroplast concentration was such as to give an absorbance of 0.1 or 0.2 at 650 nm, when measured on a Cary Model 14R spectrometer, equipped with a scattered-transmission accessory. For measurements at −5°, the chloroplasts were suspended in buffer containing 20 % glycerol and at temperatures below −5°, 63 % glycerol was used.

RESULTS

The results are discussed in terms of the series formulation of the photochemical systems (Fig. 1) (*cf.* ref. 14). The fluorescence intensity of isolated chloroplasts is considered to be determined by the redox level of E, the primary electron acceptor of Photosystem II (possibly equivalent to Q of DUYSENS AND SWEERS[4]). In dark-adapted chloroplasts, E is oxidized and the initial fluorescence (F_0) is quenched. On illumination of the chloroplasts at room temperature, E, Q and P are sequentially reduced and the fluorescence rises to a steady-state level (F_∞), *via* a biphasic kinetic curve. The levels of reduction of E, Q and P at the steady-state are dependent on the light intensity.

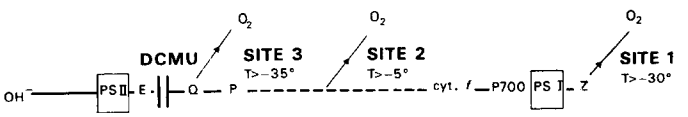

Fig. 1. Scheme of the photosynthetic electron transport chain showing proposed sites of interaction of oxygen. PS, photosystem.

Under certain conditions, the fluorescence-rise curve is monophasic. It is thought that the monophasic curve corresponds to the reduction of E and Q, but not P. E and Q are assumed to be in rapid redox equilibrium. In the presence of DCMU, electron flow from E$^-$ to Q is inhibited, and only E is reduced. Dithionite causes reduction of E, Q and P, since on illumination of chloroplasts in dithionite, the fluorescence rises immediately to a high level (F_{reduced}). In the presence of ferricyanide, E, Q and P remain oxidized, and the fluorescence remains near the F_0 level.

In the present paper, we will refer to a total concentration of quencher $[Q_t]$, equal to the sum of $[E] + [Q] + [P]$ where [E], [Q] and [P] are the concentrations of E, Q and P, respectively. Even though E seems to be the actual quencher of fluorescence of Photosystem II, the concentration of oxidized E is dependent on the concentrations of oxidized Q and oxidized P.

Q^- and P^- are reoxidized by oxygen, either directly or indirectly, and this reoxidation occurs both in the dark and in the presence of light absorbed by Photosystem I.

Effect of light intensity on fluorescence kinetics

Chloroplasts under aerobic conditions and adapted in the dark at 20° for 5 min, gave, on excitation with 650-nm light, fluorescence rise-curves as shown by the solid lines in Fig. 2. The initial levels of fluorescence (F_0) or the levels after addition of sodium dithionite ($F_{reduced}$) are proportional to the excitation light intensity, but the steady-state levels (F_∞) increase to a greater extent than is accounted for by the increase in light intensity. The levels of fluorescence in the presence of ferricyanide ($F_{oxidised}$) approximate to the F_0 values. Similar results were obtained for excitation wavelengths of 436, 470 or 670 nm. Kinetic curves at 20° were repeatable provided the chloroplasts remained in the dark for at least 2 min between excitations.

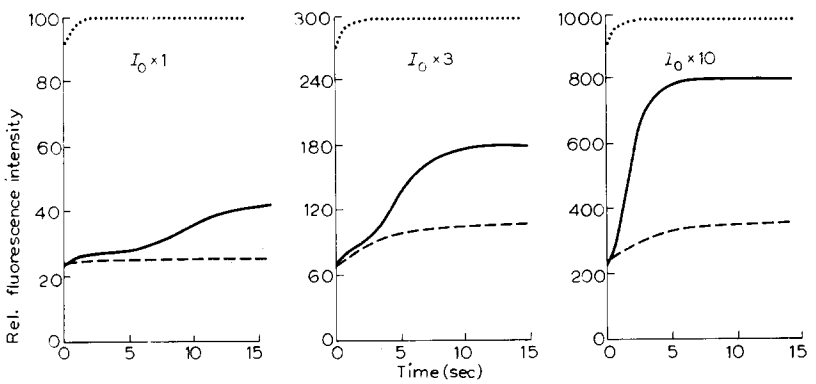

Fig. 2. Fluorescence emission kinetics of dark-adapted spinach chloroplasts, under normal aerobic conditions, in sucrose phosphate buffer at pH 7.2. Fluorescence at 683 nm; excitation at 650 nm with light intensities of $I_0 \times 1$, $I_0 \times 3$, $I_0 \times 10$, where $I_0 = 200$ ergs·cm^{-2}·sec^{-1}. Absorbance 0.2 at 650 nm; ———, no addition; · · · · · ·, in the presence of sodium dithionite; – – – –, in the presence of $1 \cdot 10^{-4}$ M potassium ferricyanide.

The influence of pH on the fluorescence properties of chloroplasts was studied at 2 excitation intensities. F_0 was independent of pH in the range 5.0–9.0, but F_∞ was influenced by pH (Fig. 3). At pH 5.0 or pH 9.0, fluorescence kinetics were absent and the F_∞ levels were equal to the F_0 values. The F_∞–pH curves show maxima at 7.2 and 6.1, with a trough at pH 6.5. Measurements in Tris buffer also gave a maximum for F_∞ at pH 7.2. The ratios $F_{reduced}/F_{oxidised}$ or $F_{reduced}/F_0$ were independent of pH between 5.0 and 7.8, but at pH 9.0 the ratio decreased from its usual value of 4.5 to a value of 2. The loss of fluorescence kinetics at pH 5.0 or pH 9.0 was reversible, if the chloroplasts were returned to pH 7.2. Most of the measurements reported in this paper were carried out at pH 7.2, where the fluorescence rise shows a maximum.

Effect of temperature on fluorescence kinetics

The effect of temperature on the fluorescence kinetics of chloroplasts at pH 7.2 was studied at 3 intensities of excitation at 650 nm (Table I). As the temperature is lowered from 40° to 5°, there is increase in the level of F_∞, while F_0 remains constant. This temperature effect on the fluorescence kinetics is greatest at the lowest light intensity.

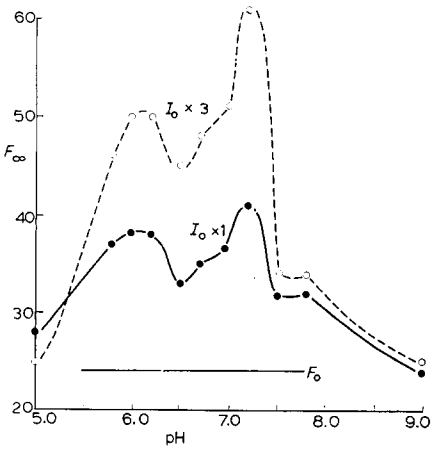

Fig. 3. Influence of pH on the steady-state levels of fluorescence (F_∞) of spinach chloroplasts at 20°. Emission at 683 nm, excitation at 650 nm with $I_0 \times 1$ (●—●) and $I_0 \times 3$ (○—○) where $I_0 = 200\ \text{erg}\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$. Absorbance at 650 nm, 0.2. F_0 and F_∞ values at the higher light intensity ($I_0 \times 3$) should be multiplied by 3. Mean values of F_0 are indicated by the horizontal line.

TABLE I

THE EFFECT OF TEMPERATURE ON THE FLUORESCENCE EMISSION OF SPINACH CHLOROPLAST AT THREE EXCITATION LIGHT INTENSITIES

Spinach chloroplasts in sucrose–phosphate buffer, pH 7.2, at a concentration of 0.2 absorbance units at 650 nm. Excitation at 650 nm with intensities $I_0 \times 1$, $I_0 \times 3$ and $I_0 \times 10$, where $I_0 = 200\ \text{ergs}\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$. Fluorescence amplitudes measured at 683 nm with electrical gain reduced by 1/3 for $I_0 \times 3$ and by 1/10 for $I_0 \times 10$ to give the figures in the table.

Intensity		*Temperature*			
		40°	*30°*	*20°*	*5°*
$I_0 \times 1$	F_{reduced}	100	100	100	100
	F_∞	26	34	43	70
	F_{oxidised}	25	25	33	37
	F_0	25	24	26	26
$I_0 \times 3$	F_{reduced}	94	100	100	100
	F_∞	40	51	60	81
	F_{oxidised}	27	28	37	40
	F_0	25	24	25	26
$I_0 \times 10$	F_{reduced}	104	104	95	98
	F_∞	71	75	79	89
	F_{oxidised}	34	26	37	53
	F_0	25	24	25	26

The effective concentration of the total quencher may be calculated by assuming first order fluorescence quenching in accordance with the STERN–VOLMER equation[15].

$$F_{\text{reduced}}/F_\infty = 1 + k[Q_t] \tag{1}$$

where F_{reduced} is the fluorescence level in the presence of dithionite, F_∞ is the steady-state level, Q_t is the effective concentration of the total quencher $[E] + [Q] + [P]$ and k is a rate constant. As the light intensity approaches zero, $F \longrightarrow F_0$ and

$$F_{\text{reduced}}/F_0 = 1 + k[Q_t]_m \tag{2}$$

where $[Q_t]_m$ is the maximum concentration of Q_t. At higher light intensities, F_∞ and $[Q_t]$ are a function of the exciting light intensity. From Eqns. 1 and 2:

$$\frac{[Q_t]}{[Q_t]_m} = \left(\frac{F_{\text{reduced}}}{F_\infty} - 1\right) \Big/ \left(\frac{F_{\text{reduced}}}{F_0} - 1\right) \tag{3}$$

Ratios of $[Q_t]/[Q_t]_m$ were calculated from the experimental values of F_0, F_∞ and F_{reduced}. The effect of temperature on the relative quencher concentration in the steady-state is shown in Fig. 4 at 3 excitation intensities. It is clear that as the temperature is lowered, there is a decrease in the relative concentration of Q_t. At any fixed temperature, the relative concentration of Q_t decreases with increasing light intensity.

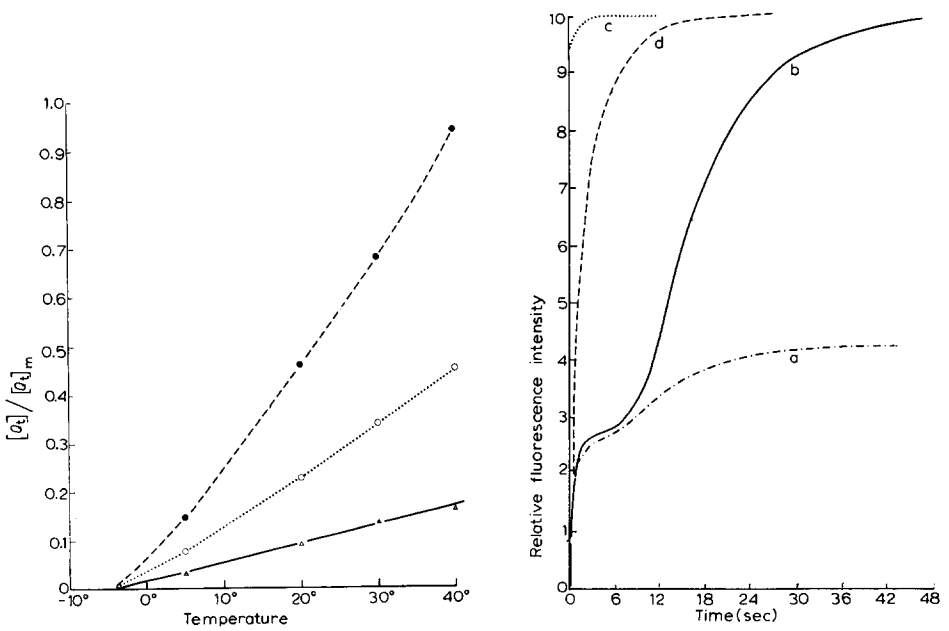

Fig. 4. Fraction of quencher Q_t as a function of temperature and excitation light intensity. $[Q_t]$ is the effective concentration of the total quencher and $[Q_t]_m$ is its maximum concentration. Excitation intensity at 650 nm; ●–●, $I_0 \times 1$; ○···○, $I_0 \times 3$; △—△, $I_0 \times 10$, where $I_0 = 200$ erg·cm^{-2}·sec^{-1}.

Fig. 5. Fluorescence kinetics of spinach chloroplasts in 0.05 M phosphate buffer pH 7.2 and 20% glycerol. Excitation at 650 nm with an intensity of 200 erg·cm^{-2}·sec^{-1}; emission at 683 nm. (a) —·—, 20°; (b) ———, 5°; (d) – – – –, chloroplasts preilluminated at −5°, then returned to darkness for 2 min and illuminated again at −5°; (c) ·····, in the presence of sodium dithionite at −5° or 20°.

It is apparent that the curves converge on the abscissa at a temperature of $-4.5°$. At this temperature, the steady-state level of quencher is reduced to zero, even at low light intensity. We interpret this to mean that the rate of the aerobic reoxidation of $E^- + Q^- + P^-$ at $-5°$ is low compared with the rate of photoreduction.

Fluorescence kinetics at $-5°$

The fluorescence kinetics of chloroplasts at $-5°$ were determined in phosphate buffers containing 20 % (by vol.) of glycerol. The influence of pH on the F_∞ levels at $-5°$ showed a similar pattern to that observed at room temperature. At pH 7.2, the steady-state level of fluorescence corresponded to the F_{reduced} value at 20°, suggesting that Q_t is fully reduced at $-5°$. Initial levels of fluorescence (F_0) were the same at $-5°$ and 20°.

Fig. 5 shows a comparison between the fluorescence kinetics at 20° and $-5°$, on exposure of dark-adapted chloroplasts to an excitation intensity of 200 erg·cm^{-2}·sec^{-1} at 650 nm. The kinetics are biphasic at both temperatures but the steady-state level of fluorescence is almost 2.5-fold greater at $-5°$, and it corresponds to the F_{reduced} value. Curve c shows the kinetic response on illumination of chloroplasts in the presence of dithionite at either 20° or $-5°$. There is an immediate rise to the F_{reduced} level. If chloroplasts are pre-illuminated at $-5°$ then returned to darkness for 2 min at $-5°$, and illuminated again at $-5°$, then the fluorescence rise-curve follows Curve d of Fig. 5.

The biphasic character of Curve b is absent from Curve d, and the rise in fluorescence is considerably more rapid for Curve d. The time response, however, is slower than for chloroplasts reduced with dithionite. With shorter periods in the dark between the illuminations at $-5°$, time rise curves were obtained which were intermediate between Curves c and d. If the chloroplasts remained in the dark for more than 2 min (*e.g.* 5, 10, 20 or 30 min) after the preillumination, then Curve d was obtained. Curve d was repeatable provided the chloroplasts remained in the dark at $-5°$ for at least 2 min between illuminations.

These results may be interpreted by assuming that E, Q and P are reduced during the first illumination at $-5°$, giving rise to biphasic kinetics. In the subsequent dark period, Q^- and E^- are apparently reoxidised but not P^-, so that in a second illumination at $-5°$, E and Q are reduced and monophasic kinetics are obtained.

Effect of DCMU on fluorescence kinetics at $-5°$

When DCMU is added to chloroplasts, the rise-time of fluorescence at $-5°$ with an excitation intensity of 200 erg·cm^{-2}·sec^{-1} is fast and, in our instrument, not distinguishable from the rise-time in the presence of dithionite (Fig. 6). However, if the excitation intensity is reduced to 20 erg·cm^{-2}·sec^{-1} then the time-rise in the presence of DCMU can be distinguished from the time rise in dithionite. The solid lines in Fig. 6 show the fluorescence kinetics of chloroplasts which were preilluminated at $-5°$, and dark adapted for 5 min at $-5°$. The curves are monophasic at all excitation intensities, but at the lower intensities ($I_0 \times 0.3$ and $I_0 \times 0.1$), the F_∞ levels are considerably below either the F_{reduced} value or the steady-state level of fluorescence in the presence of DCMU. At the lower excitation intensities, the re-oxidation of Q^- is apparently competitive with its light induced reduction. In the presence of DCMU, the rise-time at an excitation intensity of 20 ergs·cm^{-2}·sec^{-1} is faster than the control chloroplasts

excited at 200 erg·cm^{-2}·sec^{-1}. This suggests that there is an electron carrier E between the reaction centre chlorophyll of Photosystem II and Q, in a concentration lower than that of Q. (*cf.* ref. 5).

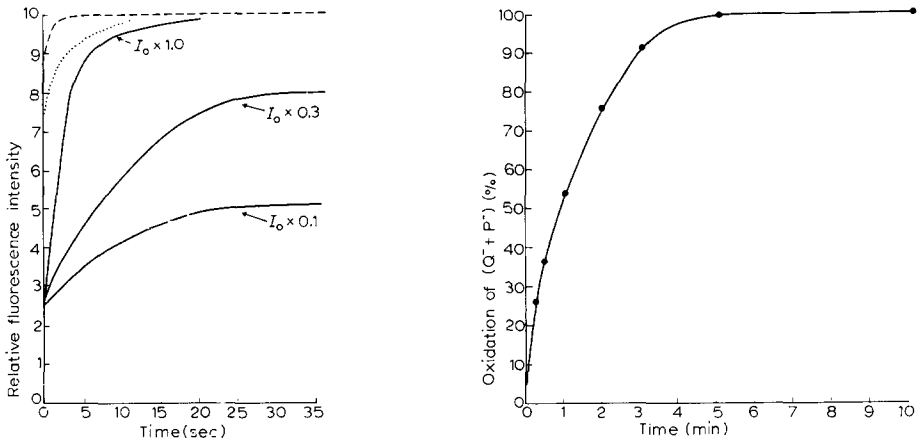

Fig. 6. Fluorescence kinetics of spinach chloroplasts at −5°. The solid lines were obtained without additions and with excitation intensities at 650 nm of $I_0 \times 1$, $I_0 \times 0.3$ and $I_0 \times 0.1$ where I_0 = 200 erg·cm^{-2}·sec^{-1}; – – –, with 1·10^{-4} DCMU or dithionite, excitation intensity $I_0 \times 1$; ·····, with 1·10^{-4} DCMU and an excitation intensity of $I_0 \times 0.1$. Emission at 683 nm.

Fig. 7. Time course for the re-oxidation of Q^- + P^- at −5° by 710-nm light of intensity 200 erg·cm^{-2}·sec^{-1}. Chloroplasts were pre-illuminated with 650-nm light at −5°, exposed to 710-nm light for the desired period, and then excited with 650-nm light and the fluorescence rise curve determined at 683 nm. The percentage oxidation of P^- + Q^- was determined from the area above the rise curve.

Effect of far-red light

Chloroplasts were preilluminated with 650 nm light for 2 min at −5° and then with far-red light (710 nm) for 5 min at −5°. A second illumination at 650 nm at −5° gave a rise-curve that followed curve b in Fig. 5. Longer periods in the far-red light gave the same result. It appears, therefore, that in far-red light at −5° under aerobic conditions, both P^- and Q^- are re-oxidised, whereas in the dark following illumination with 650-nm light at −5°, Q^- is re-oxidised. The time-course for the re-oxidation of P^- + Q^- in 710 nm light at −5° is shown in Fig. 7. Chloroplasts were preilluminated with 650-nm light at −5°, exposed to 710-nm light for the desired period, and then exposed to 650-nm light and the fluorescence rise-curve determined. The area above the curve was determined as a measure of the amounts of P + Q, which were in the oxidised state after the far-red illumination[2,9]. The half-time for the re-oxidation in 710-nm light of intensity 200 ergs·cm^{-2}·sec^{-1} was about 1 min.

An action spectrum for the far-red restoration was obtained by exposing chloroplasts (preilluminated with 650-nm light) for 2 min at −5° with monochromatic light of constant intensity and of various wavelengths between 690 and 740 nm, (Fig. 8). The action spectrum shows a maximum at 707 nm. This agrees with earlier fluorescence studies at room temperature which showed that the restoration of illuminated chloroplasts was accelerated best by 705-nm light[16]. The action spectrum is considered to represent the difference between Photosystem I and Photosystem II.

During measurements of the response to far-red light it was observed that long exposure to far-red light caused a decrease in the F_∞ level in subsequent exposures to 650-nm light. A comparison was made, therefore, between the following treatments at 20° on the F_∞ levels of chloroplasts: (a) chloroplasts maintained in the dark at 20°, with an occasional half min excitation at 650 nm at an incident intensity of 2000 ergs·cm^{-2}·sec^{-1} to measure F_∞, (b) chloroplasts exposed continuously to 650-nm light of 2000 ergs·cm^{-2}·sec^{-1}, F_∞ being recorded continuously, (c) chloroplasts exposed to 710-nm light of 2000 ergs·cm^{-2}·sec^{-1}, with an occasional excitation at 650-nm for 30-sec periods to measure F_∞, (d) chloroplasts made essentially anaerobic by bubbling with nitrogen gas for 5 min, sealing, and treating as in (c). The time-course of F_∞ levels are plotted in Fig. 9. With chloroplasts maintained in dark, the F_∞ level remained essentially constant over a period of 1 h. Exposure to continuous 650-nm light caused some loss in the F_∞ level, but the deterioration in the red light was small compared to the effect in far-red light. After a period of 1 h in far-red light, the F_∞ level of chloroplasts was only slightly above the F_0 value. Anaerobic conditions largely prevented this loss of fluorescence kinetics in continuous far-red light.

In most of our experiments on the far-red response at −5°, the intensity was 200 ergs·cm^{-2}·sec^{-1} and exposure times of 5 min were sufficient to reoxidize Q^- and P^-. No significant deterioration of the chloroplasts would be expected under these conditions.

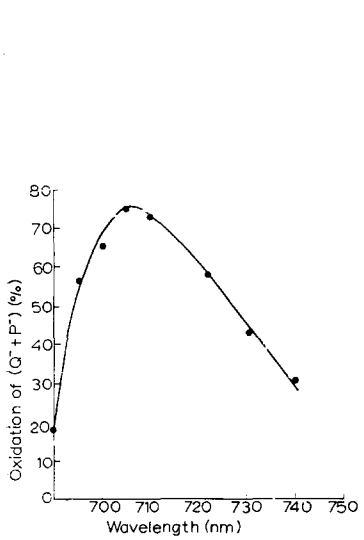

Fig. 8. Action spectrum for the re-oxidation of $Q^- + P^-$ at −5° by far-red light. Bandwidth of far-red light ± 1.5 nm; intensity 200 erg·cm^{-2}·sec^{-1}. Conditions as for Fig. 7.

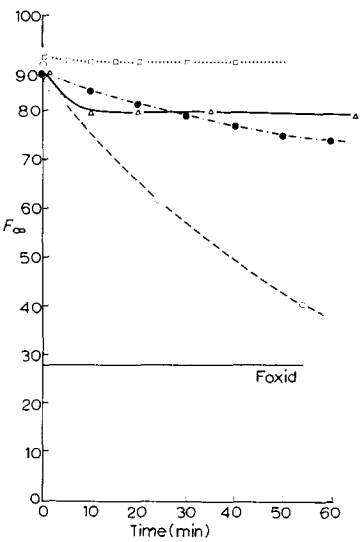

Fig. 9. Time course of steady state levels of fluorescence of spinach chloroplasts at 20°. Excitation at 650 nm of intensity $I_0 \times 10$ where I_0 = 200 erg·cm^{-2}·sec^{-1}. □ · · · □, chloroplasts maintained aerobically in the dark, except for occasional 30 sec exposure at 650 nm to determine F_∞; ●–·–●, exposed continuously to 650-nm light of intensity $I_0 \times 10$; ○---○, exposed continuously to 710-nm light of intensity 2000 erg·cm^{-2}·sec^{-1} except for occasional excitation at 650 nm to determine F_∞; △—△, exposed continuously to 710-nm light under anaerobic conditions with occasional excitation at 650 nm. Emission at 683 nm.

Fluorescence properties at −5° to −60°

As the temperature was lowered below −10°, the area above the fluorescence rise curve diminished and at −30°, the rise curve was monophasic. These observations suggest that electron flow from Q^- to P is inhibited at −30°.

Experiments described earlier indicated that Q^- is reoxidized in the dark at −5° within a period of 5 min. As the temperature was lowered further the reoxidation of Q^- was found to occur down to −25°, but below that temperature the re-oxidation was gradually inhibited, and at −35° it did not occur. A second excitation of chloroplasts at −35° gave an immediate rise in the fluorescence to the F_{reduced} level.

The far-red response in which P^- is re-oxidised at −5° by exposing chloroplasts to 710-nm light for 5 min declined gradually as the temperature was lowered. Chloroplasts were cooled at −5°, and excited with 650-nm light to reduce Q and P. The temperature was then lowered to the desired level (*e.g.* −40°, −30°, −20°, −10°) and the chloroplasts illuminated with 710-nm light for 5 min. The chloroplasts were then warmed to −5° in the dark, and a fluorescence rise curve determined by excitation with 650-nm light. If the chloroplasts had been illuminated at −10° with 710-nm light, the subsequent rise curve was biphasic, but if the far-red treatment had been given at −30° or lower temperature the rise curve was monophasic. We interpret these results as indicating that the re-oxidation of P^- by far-red light is inhibited at −30°.

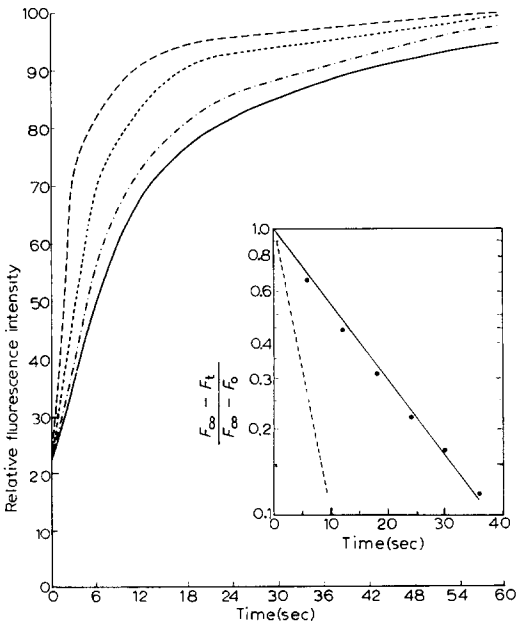

Fig. 10. Fluorescence kinetics of spinach chloroplasts at low temperature. Chloroplasts were dark-adapted for 5 min at 20° before cooling. Suspension medium 0.05 M phosphate buffer pH 7.2 and 63% glycerol. Fluorescence emission at 683 nm; excitation at 650 nm with intensity 200 erg·cm^{-2}·sec^{-1}. – – –, −60°; -------, −120°; —·—, −155°; ———, −196°. Insert shows log plots of $(F_\infty - F_t)/(F_\infty - F_0)$ against time, where F_t is the fluorescence level at time t; – – – –, −60°; ———, −196°.

Fluorescence kinetics at −60° to −196°

The fluorescence kinetics of spinach chloroplasts determined at −60°, −120°, −155° and −196° are shown in Fig. 10. Dark re-oxidation of Q^- was completely inhibited at these temperatures, and subsequent excitations gave an immediate rise in the fluorescence to the F_∞ level. As the temperature is lowered, there is a decline in the initial slope of the fluorescence curve and an increase in the area bounded by the curve, the ordinate and the F_∞ level (Fig. 10 and Table II). In the insert in Fig. 10, the curves have been replotted on a log scale. A linear relationship is obtained indicating that the curves in Fig. 10 are exponential. This suggests that the slower rise in fluorescence as the temperature is lowered is due to a falling probability for the transfer of an electron from the excited chlorophyll molecule of the reaction centre of Photosystem II (chlorophyll a_2*) to Q. Relative quantum efficiencies for the reduction of Q are shown in Table II. The quantum efficiency for the reduction of Q at room temperature or −5° is taken as 0.97, the loss, 0.03 being due to the probability of fluorescence emission or internal loss within chlorophyll a_2*, with Q in the oxidized state. The quantum efficiency for the reduction of Q at −196° is 4.4 times less than at −5°.

TABLE II

EFFECT OF TEMPERATURE ON $dF/dt \int (F_\infty - F)\, dt$

Conditions as for Fig. 10. ϕ is the relative quantum efficiency for the reduction of Q

	Temperature				
	−5°	*−60°*	*−120°*	*−155°*	*−196°*
dF/dt	1.0	0.77	0.40	0.28	0.22
$\int (F_\infty - F)dt$	1.0	1.3	2.2	3.4	4.4
$\frac{dF}{dt} \times \int (F_\infty - F)dt$	1.0	1.0	0.88	0.95	0.97
ϕ	0.97	0.75	0.44	0.29	0.22

DISCUSSION

At room temperature in the dark, E, Q and P are oxidised and cytochrome *f* and P-700 are mainly reduced (Fig. 1). Light absorbed by Photosystem II (650 nm) causes a reduction of E, Q and P by electron flow from water, and the fluorescence yield of the chloroplasts increases. Light absorbed by Photosystem I (710 nm) causes a reduction of Z and an oxidation of the electron carriers between the photoacts. This results in a decline in the fluorescence yield. In a dark period following illumination with Photosystem II light, E^-, Q^- and P^- are re-oxidised presumably by molecular oxygen (Mehler reaction) since the decline in fluorescence yield is inhibited under anaerobic conditions. The effect of far-red light in depressing the fluorescence yield is also dependent on the presence of oxygen.

The difference in the temperature coefficients of the dark oxidation of P^- and its oxidation in far-red light suggests that oxygen interacts with the electron transport chain at more than one site (Fig. 1). To explain the dark oxidation of P^-, we

propose that oxygen interacts with a component of the electron transport chain at Site 2 situated between P^- and Photosystem I.

Light absorbed by Photosystem I will drive the reduction of Z and the oxidation of P-700. Turnover of oxidised P-700 and the re-oxidation of the electron carriers between the photosystems will depend on the re-oxidation of Z^-. It seems likely that oxygen can act as the ultimate electron acceptor for Photosystem I (at Site 1), and interaction of oxygen at this site would then explain the re-oxidation of P^- in far-red light.

It appears that we must postulate a further site of interaction with oxygen (Site 3) to account for the re-oxidation of Q^- in the dark, which is not inhibited until the temperature falls to $-35°$.

The oxidation of Q^- in the dark, and P^- in far-red light have relatively low temperature coefficients, which suggest that the rate of these oxidations may be limited by a diffusion-controlled reaction. Diffusion of oxygen itself may be the rate-limiting step in the re-oxidation of Q^- in the dark and P^- in far-red light. On the other hand, in the dark re-oxidation of P^-, which has a higher temperature coefficient, the rate limiting step may be enzyme-catalysed.

As shown previously by MURATA *et al.*[9] and by MALKIN AND KOK[2], the area bounded by the fluorescence curve, the ordinate and the F_∞ level may be used to calculate the size of the pools of Q and P. MALKIN AND KOK[2] measured the quantum requirement for the reduction of Q and P at room temperature by green light as 2 quanta/equiv. Since green light is absorbed equally well by Photosystems I and II, their measurements indicate a quantum efficiency of unity for the reduction of Q and P by light absorbed by Photosystem II.

The fluorescence curves obtained at $-5°$ may be used to calculate the pool sizes of both P and Q, after making a small correction for the E pool. We calculated the size of the Q pool, relative to the chlorophyll content of chloroplasts, from the area above the monoexponential fluorescence curve measured at $-5°$ at the second excitation (Curve d, Fig. 5) and the number of quanta absorbed by the sample. Assuming a quantum efficiency of 0.5 for the reduction of Q by 650-nm light absorbed by chloroplasts at $-5°$, we obtained a chlorophyll/Q ratio of 140 or one equivalent of Q for every 70 moles of Photosystem II. The size of the pool of P + Q was calculated from the area above the biphasic curve, obtained at the first excitation of dark-adapted chloroplasts at $-5°$ (Curve b, Fig. 5). A value of 20 was found for the chlorophyll/P + Q ratio; this gives a P + Q/Q ratio of 7, a P/Q ratio of 6. As mentioned earlier, the pool of E which is needed to account for the rapid rise of fluorescence in the presence of DCMU is small, compared with the pool of Q.

From fluorescence measurements at room temperature, MALKIN AND KOK[2] and MALKIN[3] obtained a P + Q pool of 1 equiv./35 moles chlorophyll. Because of the monoexponential nature of the fluorescence induction curve at $-196°$ and the fact that the area above the curve was about half that obtained at room temperature, MALKIN AND KOK[2] assumed that the pool of Q was about one-half that of the P + Q pool. However, from the present studies it is concluded that the quantum efficiency for the reduction of Q decreases as the temperature is lowered, and at $-196°$ it is 4.4 times lower than at $-5°$. This would mean that MALKIN AND KOK[2] overestimated the size of the Q pool by a factor of 4.4.

KOK *et al.*[17] studied the reduction of 2,6-dichlorophenolindophenol in both

spinach chloroplasts and *Scenedesmus* particles after brief and long flashes. They interpreted their results in terms of a small pool of one per 1250 chlorophylls and a larger pool of one per 70 chlorophylls. FORBUSH AND KOK[8] concluded that the larger pool was heterogeneous, one-third of it reacting more rapidly with the reduced small pool.

KOK *et al.*[17] and MALKIN[18] have discussed the possibility that E is on the oxidising side of Photosystem II, with DCMU inhibiting electron flow between water and E. Although we have placed E on the reducing side of Photosystem II, the fluorescence results reported in this paper do not exclude E being on the oxidising side.

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