Biochimica et Biophysica Acta, 292 (1973) 140–151

BBA 46454

THE KINETICS OF LIGHT-INDUCED CHANGES OF C-550, CYTOCHROME b_{559} AND FLUORESCENCE YIELD IN CHLOROPLASTS AT LOW TEMPERATURE

WARREN L. BUTLER*, JAN W. M. VISSER and HUGO L. SIMONS
Biophysical Laboratory, State University, Schelpenkade 14 A, Leiden (The Netherlands)
(Received August 14th, 1972)

SUMMARY

The kinetics of the photoreduction of C-550, the photooxidation of cytochrome b_{559} and the fluorescence yield changes during irradiation of chloroplasts at −196 °C were measured and compared. The photoreduction of C-550 proceeded more rapidly than the photooxidation of cytochrome b_{559} and the fluorescence yield increase followed the cytochrome b_{559} oxidation. These results suggest that fluorescence yield under these conditions indicates the dark reduction of the primary electron donor to Photosystem II, $P680^+$, by cytochrome b_{559} rather than the photoreduction of the primary electron acceptor.

The photoreduction of C-550 showed little if any temperature dependence over the range of −196 to −100 °C. The amount of cytochrome b_{559} photooxidized was sensitive to temperature decreasing from the maximal change at temperatures between −196 to −160 °C to no change at −100 °C. To the extent that the reaction occurred at temperatures between −160 and −100 °C the rate was largely independent of temperature. The rate of the fluorescence increase was dependent on temperature over this range being 3–4 times more rapid at −100 than at −160 °C. At −100 °C the light-induced fluorescence increase and the photoreduction of C-550 show similar kinetics. The temperature dependence of the fluorescence induction curve is attributed to the temperature dependence of the dark reduction of $P680^+$.

The intensity dependence of the photoreduction of C-550 and of the photooxidation of cytochrome b_{559} are linear at low intensities (below 200 $\mu W/cm^2$) but fall off at higher intensities. The failure of reciprocity in the photoreduction of C-550 at the higher intensities is not explained by the simple model proposed for the Photosystem II reaction centers.

INTRODUCTION

Fluorescence yield changes in chloroplasts have been assumed to reflect changes in the redox state of the primary electron acceptor of Photosystem II, usually denoted Q after the work of Duysens and Sweers[1]. The hypothesis that the

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

* On leave from University of California, San Diego, Department of Biology, La Jolla, Calif., U.S.A.

oxidized state of the acceptor, Q, quenches fluorescence while the reduced state, QH, does not has been remarkably successful in relating fluorescence yield changes to the primary photochemical activity of Photosystem II and the subsequent electron transport reactions.

More recently light-induced absorbance changes of cytochrome b_{559} and of a newly discovered component, C-550, have been related to the primary photochemical activity of Photosystem II (refs 2–4). The photoreduction of C-550 and the photooxidation of cytochrome b_{559} which occur at −196 °C were shown to represent the electron accepting and electron donating ends of the same photoreaction[5] which can be written as:

$$D \cdot P \cdot A \xrightarrow{h\nu} D \cdot P^{+} \cdot A^{-} \rightarrow D^{+} \cdot P \cdot A^{-}$$

where D is cytochrome b_{559}, A is C-550 and P is assumed to be the reaction center chlorophyll of Photosystem II, P680.

Fluorescence yield changes have been correlated with absorbance changes of C-550 in a wide variety of experiments[5–7]. (For such comparisons C-550 and Q should be assayed at low temperature to avoid secondary biochemical effects of membrane potential and pH gradient on the measurements of C-550 and fluorescence yield[8]). The one-to-one correspondence between absorbance measurements of C-550 and fluorescence yield measurements of Q established the equivalence of C-550 and Q; both being directly related to the primary electron acceptor of Photosystem II.

In one set of experiments[9], however, it was found that if cytochrome b_{559} was oxidized prior to freezing to −196 °C the fluorescence yield of the chloroplasts increased only 2-fold due to irradiation at low temperature whereas in the normal case, where cytochrome b_{559} was reduced, the fluorescence yield increased 5 fold, the F_0 level being the same in both cases. The photoreduction of C-550 was independent of the oxidation state of cytochrome b_{559}. In normal chloroplasts the final state of the Photosystem II reaction centers after irradiation at −196 °C is $D^{+} \cdot P \cdot A^{-}$; when cytochrome b_{559} was initially oxidized the final state might be $D^{+} \cdot P^{+} \cdot A^{-}$. It was inferred from these experiments that fluorescence yield was determined by the redox state of the oxidizing side of the Photosystem II reaction centers as well as by the reducing side[9]. Under normal conditions at room temperature appreciable amounts of $P680^{+}$ do not accumulate because of its rapid turnover and fluorescence yield appears to be determined solely by the redox state of the primary acceptor.

In the present work we investigated the kinetics of the light-induced changes of C-550, cytochrome b_{559} and fluorescence yield in the low temperature region between −196 and −80 °C in an effort to further correlate fluorescence yield changes with the electron transport reactions associated directly with the Photosystem II reaction centers. The results provide further evidence that C-550 is the primary electron acceptor of Photosystem II and that the fluorescence yield is determined by the redox state of P680 as well as that of C-550.

METHODS

Spinach chloroplasts were made according to methods described previously[10]. Chloroplast samples suspended in 55% glycerol mixtures in 1 mm × 10 × 30 mm

plastic cuvettes were carefully frozen to −196 °C in complete darkness. The end of the metal cuvette holder was submerged in liquid nitrogen in the vertical cylindrical Dewar with windows at the sample position. A thermocouple adjacent to the cuvette was used to monitor the temperature. Temperatures above −196 °C were obtained by blowing a stream of cold nitrogen gas through the Dewar. Measurements were made only on samples that remained as clear glasses throughout the measurement.

Photometric measurements were made with a simple single-beam spectrophotometer. Steady (unchopped) measuring light for the transmission measurements was provided by a Bausch and Lomb 500 mm monochromator and a tungsten lamp powered with a stabilized DC power supply. The measuring beam was focused on the sample cuvette. The measuring light transmitted by 1-mm samples was measured with a phototube (EMI 9558) protected with a broad band blue (two Corning 4-96 colored glass filters and a 583-nm short pass interference filter) blocking filter. The voltage signal developed across the $10^6\ \Omega$ load resistor of the phototube was taken directly to an oscilloscope which was connected to a fast strip-chart recorder for data recording. Photometric measurements were made by adjusting the phototube power supply to give a 1-V signal. The sensitivity of the oscilloscope amplifier was then increased 100-fold and a d.c. voltage offset was used to position the trace. The time constant of the instrument for the transmission measurements had a half rise time of 0.1 s and a 90% rise time of 0.3 s. Transmission changes are presented as absorbance changes where $\Delta A = 0.43\ \Delta T$.

Actinic light provided by a tungsten lamp and filters was incident on the front surface of the cuvette at a small angle to the measuring beam. The intensity of actinic light at the cuvette was calibrated with a Yellow Springs Radiometer and monitored continuously with a photodiode. The filter for the 630-nm actinic source consisted of two 630-nm interference filters (8 nm passband) and a 644-nm short pass interference filter. The broad band red actinic light used to determine the intensity dependence of the absorbance changes consisted of a Balzers K6 broad band red interference filter and two Schott RG 630/2 cutoff filters. The broad band red source peaked at 645 nm with a 25-nm half width. Some of the fluorescence measurements were made with broad band blue exciting light obtained with Corning 4-96 and 7-59 colored glass filters and a 524-nm short pass interference filter.

Measurements of fluorescence were made with the same optical geometry. The actinic light was used to excite fluorescence and the phototube blocking filter was changed to a Corning 4-77 colored glass filter with a 692-nm interference filter. On turning on the actinic light the fluorescence rises immediately to the F_0 level (for dark adapted samples) and increases with time as the fluorescence yield of the sample increases.

RESULTS AND DISCUSSION

C-550, cytochrome b_{559} and fluorescence yield changes at −196 °C

The difference spectrum of absorbance changes produced by irradiating a sample of chloroplasts at −196 °C is shown in Fig. 1. The bleaching at 556 nm is due to the photooxidation of cytochrome b_{559} while the bleaching at 546 and the absorbance increase at 542 nm are due to the photoreduction of C-550. Time course curves of the absorbance changes at 543 and 556 nm and of the fluorescence yield

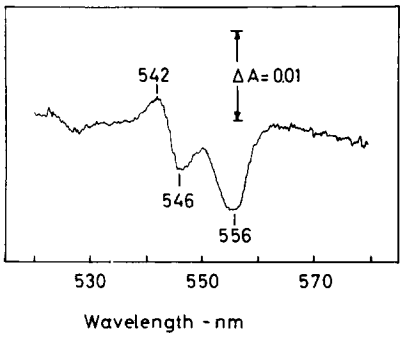


Fig. 1. Light-*minus*-dark difference spectrum of chloroplasts at −196 °C due to irradiation for 30 s with red light at −196 °C (see refs 4 and 5 for methods).

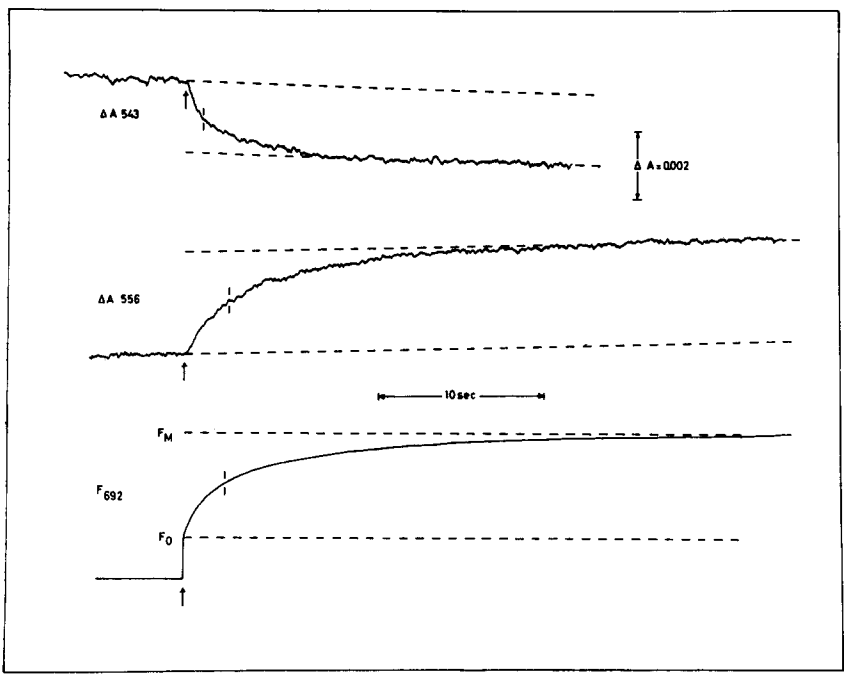


Fig. 2. Time course of transmission at 543 and 556 nm and of fluorescence at 692 nm during irradiation of spinach chloroplasts (1 mg chlorophyll/ml in 1 mm cuvette) with 630 nm actinic light (165 μW/cm^2) at −196 °C. Actinic irradiation on at upward arrows. Vertical lines on curves indicate half times.

change at 692 nm during irradiation of the sample with red (630 nm) actinic light at −196 °C are shown in Fig. 2. Semilogarithmic plots of these time course curves, each being normalized to the 100% change, are presented in Fig. 3. A bleaching at 547 nm (not shown) occurs with precisely the same kinetics as the absorbance increase at 543 nm. The rate of bleaching at 556 nm is appreciably slower than the rate of absorbance increase at 543 nm and the kinetics of the fluorescence yield change are essentially the same as the kinetics of the 556 nm absorbance change. It is apparent that the photoreduction of C-550 is faster than the photooxidation of cytochrome

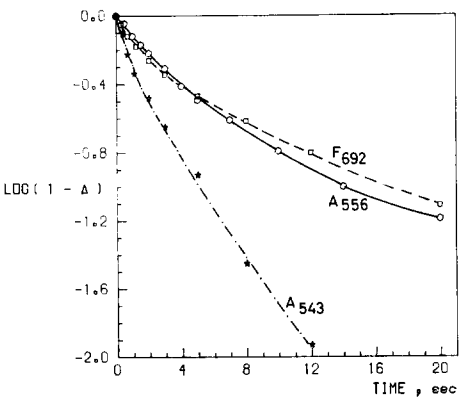


Fig. 3. Semilogarithmic plots of data in Fig. 2. Log $(1-\Delta)$ *vs* time where Δ is the fraction of the maximal change.

b_{559} at -196 °C and that the fluorescence yield increase follows the photooxidation of cytochrome b_{559}, not the photoreduction of C-550.

The time course of the curves in Fig. 2 must deviate from the inherent kinetic character of the photochemical transformations because the optical density of the sample to the actinic light (approx. 1.2) was great enough to cause a marked exponential decrease of light intensity with sample depth. First-order photochemical transformations in such a sample will appear more second-order in character and the rate of transformation at any time (including $t=0$) will be less than would have been obtained with lower density samples[11]. The reason for using samples of such optical density was to obtain adequate signal-to-noise ratios for the absorbance measurements.

The measurements of absorbance and fluorescence presented in Fig. 2 were all made with identical samples, actinic light and optical geometry so that the rate of change of absorbance and fluorescence could be compared. One other condition should also be met, however, in order to make the kinetic comparison between light-induced absorbance and fluorescence changes in a relatively dense sample. Absorbance measurements give an average of the changes occurring at the different actinic light intensities throughout the sample. Fluorescence measurements are more complex. Strong absorption of the actinic light will cause greater weight to be given to changes occurring in the front part of the cuvette where actinic intensities are higher than average. On the other hand self absorption of the fluorescence will cause greater weight to be given to changes occurring in the rear of the cuvette where intensities are less than average. These two effects cancel each other when the absorbance of the sample to the actinic light is equal to the absorbance to the fluorescence pass band*. Under this condition fluorescence represents a true average of changes

* The increment of fluorescence, dF, emanating from a thin layer of thickness dx a distance x from the front surface of the sample will be $dF = I_x c\alpha_A \phi e^{-c\alpha_F(d-x)} \cdot dx$ where I_x is the intensity of the actinic light at x, c is the concentration of chloroplasts, α_A is the extinction coefficient of the chloroplast for the actinic pass band, ϕ is the quantum yield of fluorescence, α_F is the extinction coefficient for the fluorescence pass band and d is the thickness of the sample, $0 < x < d$. Since $I_x = I_0 e^{-c\alpha_A x}$ where I_0 is the actinic intensity at the front surface of the sample $dF = I_0 c\alpha_A \phi e^{-c\alpha_F} e^{-c(\alpha_A - \alpha_F)x} \cdot dx$. It is apparent that the dependence on sample depth is eliminated when $\alpha_A = \alpha_F$.

occurring through the sample. In our experiments the 630-nm actinic light was absorbed somewhat more strongly than the 692-nm fluorescence band, so that fluorescence measurements might favor regions of the sample that have a slightly higher than average light intensity (and therefore slightly faster kinetics). Thus, if there is a discrepancy in the comparison of the absorbance and fluorescence kinetics, fluorescence changes will appear faster than the corresponding absorbance changes. Experimental tests comparing the kinetics at different fluorescence wavelengths, however, indicated that this discrepancy is negligible with the actinic and fluorescence passbands used.

Temperature dependence of the photoreactions

The influence of temperature on the low temperature photoreactions in the range from −196 to −100 °C is indicated in Figs 4, 5 and 6. The photoreduction of C-550 (Fig. 4) shows little or no temperature dependence over this range. The extent of the absorbance change may be somewhat greater at −196 °C because the absorption bands of the oxidized and reduced forms of C-550 are sharper and better separated at the lower temperature. The rate of photoreduction is essentially independent of temperature: the half times shown in Fig. 4 are equal within the limits of the measurement. The average of a number of such experiments indicate the rate at −100 °C might be approx. 30% faster than the rate at −196 °C which could be due to a more efficient trapping of energy by the Photosystem II reaction centers at the higher temperatures. Sharpening of the chlorophyll absorption bands at −196 °C could result in less resonance interaction between the bulk and reaction center chlorophyll.

The temperature dependence of the photooxidation of cytochrome b_{559} is

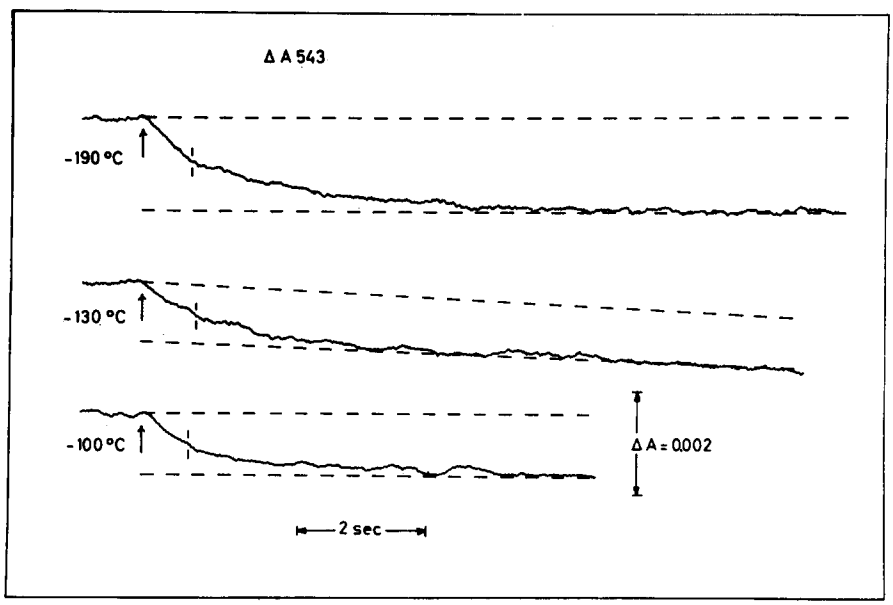


Fig. 4. Time course of transmission at 543 nm during irradiation with 630 nm actinic light at the temperatures indicated. Vertical lines on curves indicate half times.

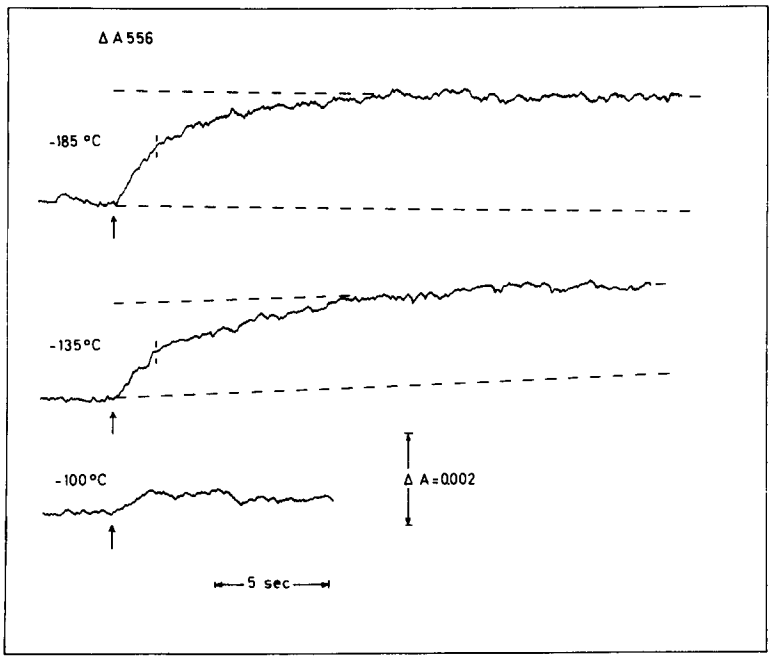


Fig. 5. Time course of transmission at 556 nm during irradiation with 630 nm actinic light at the temperatures indicated. Vertical lines on curves indicate half times.

shown in Fig. 5. The maximal absorbance change at 556 nm is generally found at temperatures between −196 and −160 °C. At −100 °C, however, no absorbance change at 556 nm is found in most experiments; the small change which appears at −100 °C in Fig. 5 is generally not observed. At temperatures between −160 and −100 °C partial absorbance changes are observed. To the extent that the absorbance change

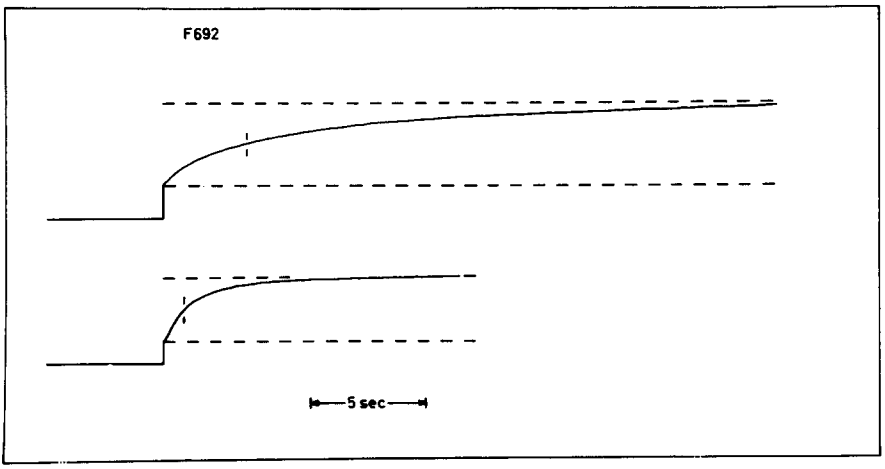
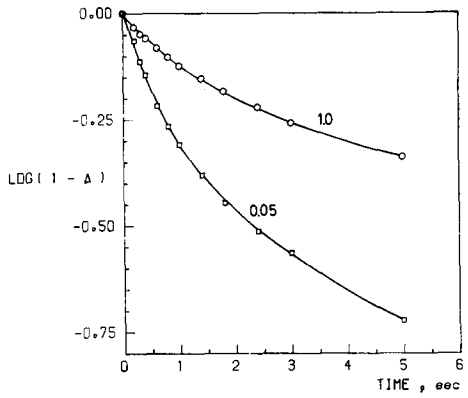


Fig. 6. Time course of fluorescence of chloroplasts (1.0 mg chlorophyll/ml) at 692 nm during irradiation with 630 nm actinic light. Upper curve: −196 °C. Lower curve: −100 °C.

at 556 nm does occur at these intermediate temperatures, the rate of change is essentially independent of temperature. The photooxidation of cytochrome b_{559} shows a marked dependence on temperature in terms of the extent of the reaction but not in terms of the rate. We interpret these data to indicate two possible electron donors to $P680^+$, cytochrome b_{559} and an unknown donor. At temperatures below -160 °C only cytochrome b_{559} functions. At temperatures above -160 °C the unknown donor starts to function and to the extent that it is oxidized, cytochrome b_{559} is spared. At -100 °C only the unknown donor is oxidized.

The effect of temperature on the light-induced fluorescence yield change is shown in Fig. 6. The rate of the fluorescence increase shows an appreciable temperature dependence being about 3–4 times faster at -100 °C (based on half times) than at -196 °C. The approach to the final F_M level is also much slower at the lower temperatures. At -100 °C the time course of the fluorescence yield increase is very similar to the time course of the photoreduction of C-550. These fluorescence measurements were made at a sample concentration of 1 mg chlorophyll/ml in order to be comparable to the absorbance measurements in Figs 4 and 5. Fluorescence measurements, however, can be made at much lower chlorophyll concentrations where the optical density of the sample does not affect the kinetics of the light-induced changes. Fig. 7 compares the kinetics of the fluorescence yield increase measured with a concentrated (1 mg chlorophyll/ml) and a dilute (0.05 mg chlorophyll/ml) sample at -196 °C. The half time for the fluorescence increase is 4 times faster with the dilute sample because of the greater average intensity of the actinic light in the dilute sample.



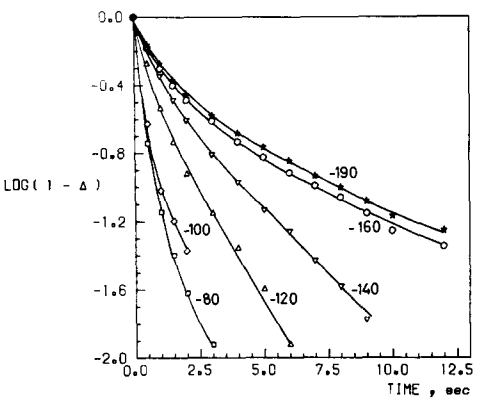


Fig. 7. Semilogarithmic plots of light-induced fluorescence (692 nm) increase of chloroplasts at -196 °C due to irradiation with broad band blue actinic light for a concentrated (1.0 mg chlorophyll/ml) and a dilute (0.05 mg chlorophyll/ml) chloroplast sample (1 mm cuvette).

Fig. 8. Semilogarithmic plots of fluorescence increase of chloroplasts (0.05 mg chlorophyll/ml) due to irradiation with broad band blue actinic light at the temperatures indicated.

The kinetics of the fluorescence induction measured with dilute samples (0.05 mg chlorophyll/ml) at a series of temperatures between -196 and -80 °C are shown in Fig. 8, and a plot of half time *vs* temperature is presented in Fig. 9. The main region of temperature sensitivity is between -160 and -100 °C. The kinetics appear more first order at the higher temperatures. At the lower temperatures the

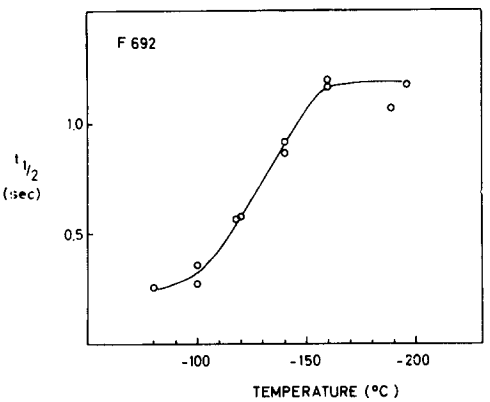


Fig. 9. Half times of light-induced fluorescence increase as a function of temperature.

slow approach to the final F_M level causes the semilogarithmic plot to bend away from a straight line.

The temperature dependence of the fluorescence induction curve in the low-temperature range has been reported previously[12–15]. Kok[12] and Murata[13] noted that the rate of the fluorescence rise in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was approx. 10 times slower at −196 °C than at room temperature. Kok suggested that the number of trapping centers might increase by a factor of 10 on cooling to −196 °C. Murata suggested that DCMU might not block electron transport between Q and the pool of secondary electron acceptors at −196 °C so that the fluorescence induction curve represented a larger pool of reducible acceptors at low temperatures. Thorne and Boardman[14] measured the fluorescence rise at a series of low temperatures and found that the rate at −60 °C was 3.5 times faster than the rate at −196 °C. They assumed that the fluorescence increase at −196 °C followed the reduction of Q *plus* the first pool of secondary electron acceptors; thus a temperature dependence for the reduction of the secondary electron acceptors could be postulated. Malkin and Michaeli[15] also measured fluorescence induction as a function of temperature and found a sharp increase in the induction time in a narrow temperature range below −100 °C (similar to Fig. 9 of this paper). They proposed that there was such a temperature dependence for the quantum yield of the primary photoreduction of Q. These various explanations of the temperature dependence of the fluorescence induction all assumed that fluorescence yield was determined solely by the redox state of the primary electron acceptor. Our results show that the photoreduction of the primary acceptor has no such temperature dependence but that the fluorescence increase follows the oxidation of a secondary electron donor to Photosystem II and presumably reflects the dark reduction of the oxidized primary electron donor, $P680^+$. At temperatures below −160 °C cytochrome b_{559} is the electron donor to $P680^+$, electron transport occurring presumably *via* a tunnelling mechanism with little temperature dependence over this range[16]. As the temperature is increased in the range from −160 to −100 °C another electron donor which reduces $P680^+$ more rapidly than cytochrome b_{559} does, takes over from cytochrome b_{559}. Above −100 °C $P680^+$ is reduced entirely

by this other donor at a rate 3–4 times faster than the rate of reduction by cytochrome b_{559}.

Effect of ferricyanide on fluorescence at −100 °C

The above results on the temperature dependence of cytochrome b_{559} photooxidation could be taken to suggest that the donor which function above −100 °C is the normal physiological donor to Photosystem II and that electron donation by cytochrome b_{559} at lower temperature is an artificial reaction. If so, the normal donor might be a stronger oxidizing agent than ferricyanide in which case we would expect ferricyanide to have no effect on fluorescence induction at −100 °C. Fig. 10

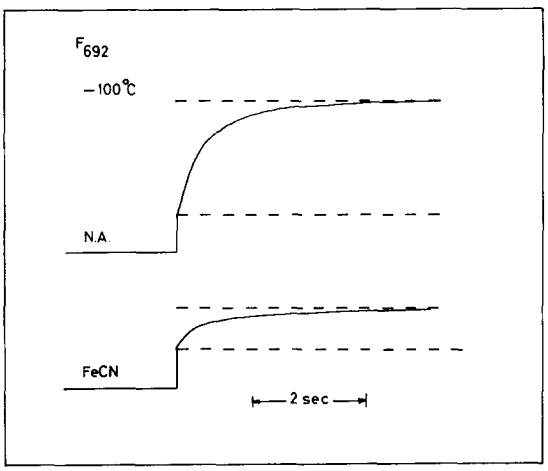


Fig. 10. Time course of fluorescence increase of chloroplasts (0.05 mg chlorophyll/ml) at −100 °C frozen without (N.A.) and with (FeCN) 2 mM potassium ferricyanide.

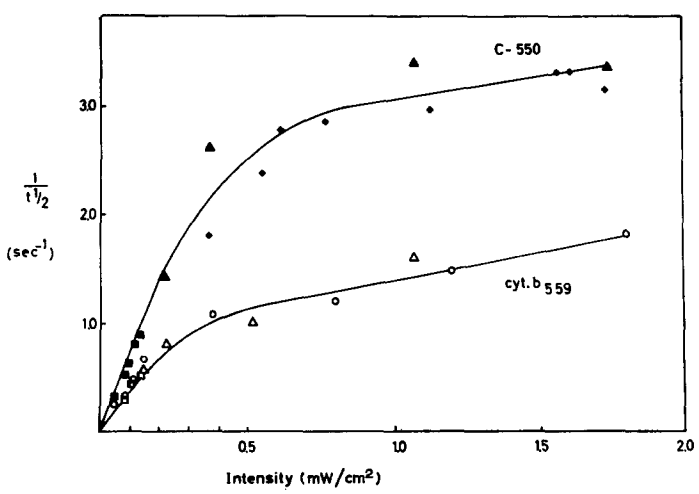


Fig. 11. Intensity dependence of the rates (reciprocal half times) of the transmission changes at 543 and 556 nm. Irradiation with the broad band red actinic source.

shows that the ferricyanide effect on fluorescence is essentially the same at −100 as it was at −196 °C (ref. 9); in the presence of ferricyanide added before freezing the ratio F_M/F_0 is decreased from about 4.0 to 2.0. The basis of the ferricyanide effect on fluorescence is thus subject to the same speculations that were given previously[9]. A redox titration curve at −100 °C, however, might indicate the redox potential of the electron donor functioning at that temperature.

Intensity dependence of the photoreactions at −196 °C

The intensity dependence for the photoreduction of C-550 and the photooxidation of cytochrome b_{559} are shown in Fig. 11. At the lower intensities, below 200 μW/cm^2, the quasi rate constants (reciprocal half times) of both photoreactions are proportional to light intensity and extrapolate through zero. At higher intensities, however, the photoreactions appear to be less efficient, with the rate constants showing less dependence on intensity. It is difficult to reconcile this intensity dependence with the simple D·P·A model proposed for the Photosystem II reaction centers. On the basis of that model

$$D.P.A \underset{k_{-1}}{\overset{h\nu}{\rightleftarrows}} D.P^+.A^- \xrightarrow{k_2} D^+.P.A^-$$

we would expect the rate of photoreduction of C-550 to be proportional to light intensity and that the rate of photooxidation of cytochrome b_{559} should level off at a maximal rate determined by k_2. The falling off of the intensity dependence of C-550 reduction at higher intensities would be explained if the back reaction, k_{-1}, was stimulated at higher intensities. Such a stimulation might occur if P^+ still absorbed and acted as an energy trap, *i.e.* excitation of P^+ might facilitate a back reaction with A^-, but we have no direct evidence for such a photochemical process.

ACKNOWLEDGEMENTS

We are indebted to Mr R. Lozier for the measurement of Fig. 1. This work was carried out under a National Science Foundation Senior Postdoctoral Fellowship (to W.L.B.). The work was supported in part by the Netherlands Foundation for Biophysics, financed by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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