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PHOTOSYNTHETIC ELECTRON TRANSPORT AND ELECTROCHROMIC EFFECTS AT SUB-ZERO TEMPERATURES

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SUMMARY

Spinach chloroplasts, suspended in a liquid medium containing ethyleneglycol, showed reversible absorbance changes near 700 and 518 nm due to *P*-700 and "*P*-518" in the region from −35 to −50 °C upon illumination. The kinetics were the same at both wavelengths, provided absorbance changes due to Photosystem II were suppressed. At both wavelengths, the decay was slowed down considerably, not only by the System I electron acceptor methyl viologen, but also by silicomolybdate. The effect of the latter compound is probably not due to the oxidation of the reduced acceptor of Photosystem I by silicomolybdate, but to the enhanced accessibility of the acceptor to some other oxidant.

In the presence of both an electron donor and acceptor for System I, a strong stimulation of the extent of the light-induced absorbance increase at 518 nm was observed. The most effective donor tested was reduced *N*-methylphenazonium methosulphate (PMS). The light-induced difference spectrum was similar to spectra obtained earlier at room temperature, and indicated electrochromic band shifts of chlorophylls *a* and *b* and carotenoid, due to a large potential over the thylakoid membrane, caused by sustained electron transport. It was estimated that steady-state potentials of up to nearly 500 mV were obtained in this way; the potentials reversed only slowly in the dark, indicating a low conductance of the membrane. This decay was accelerated by gramicidin D. The absorbance changes were linearly proportional to the membrane potential.

INTRODUCTION

Several studies have shown that most secondary photosynthetic electron

Abbreviations: DAD, 2,3,4,6-tetramethyl-*p*-phenylenediamine (diaminodurene); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; *P*-518, pigment showing a light-induced absorbance increase near 518 nm; *P*-700, reaction center chlorophyll, primary electron donor of Photosystem I; PMS, *N*-methylphenazonium methosulphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-penylenediamine; tricine, *N*-tris(hydroxymethyl)methylglycine.

transport reactions are almost completely inhibited below −35 °C. Among these are the photooxidation of cytochrome *f* by Photosystem I [1, 2] and the dark oxidation of the reduced acceptor of Photosystem II [3–5]. In a previous study [6], we have shown that spinach chloroplasts cooled to −50 °C display a reversible photooxidation of *P*-700 upon illumination. Measurements of the kinetics of the absorbance changes of *P*-518, which are thought to be due to an electrochromic shift, mainly of a carotenoid, caused by the generation of an electrical potential across the thylakoid membrane [7, 8], showed that the absorption shift of *P*-518 at −50 °C was caused by both photosystems. The System I component of *P*-518 showed the same kinetics as *P*-700. This was also true in the presence of methyl viologen or ferricyanide, which substances caused a considerable reduction of the rate of dark decay of both *P*-700 and *P*-518 [6]. These observations indicate that, in the absence of an external electron acceptor, the only reactions of System I that occur at −50 °C are the transfer of an electron from *P*-700 to an acceptor, and a back reaction of the photoproducts. Methyl viologen and ferricyanide react with the reduced acceptor, so that P^+-700 cannot react back in the dark.

In this paper we report experiments at −35 to −45 °C, in the presence of artificial electron donors as well as electron acceptors of Photosystem I. The results indicate that chloroplasts suspended in a mixture of ethyleneglycol and water sustain fairly high rates of electron transport with suitable donor-acceptor systems. The electron transport was accompanied by large absorbance changes of *P*-518, corresponding to estimated membrane potentials of up to nearly 500 mV. The changes reversed only slowly upon darkening, indicating a low permeability of the thylakoid membrane.

MATERIAL AND METHODS

Unless noted otherwise, spinach chloroplasts were prepared as described earlier [9] and stored on ice in the dark before use in a buffer solution containing 0.05 M tricine, 0.01 M KCl, 0.002 M $MgCl_2$ and 0.4 M sucrose (0.4 M sorbitol in some experiments), pH 7.8. The chloroplast suspension was diluted just before each experiment with glycol to a final concentration of 53 % (v/v).

Unless otherwise indicated, the final chlorophyll concentration, determined according to Whatley and Arnon [10], was 0.5 mM. Measurements of absorbance changes were performed in the same way as described earlier [6]. The optical pathlength was 1.3 mm. Unless otherwise indicated, illumination of the samples was provided by a combination of a Schott AL 630 nm interference filter, RG-610, 3 mm filter, and an interference filter with long-wave cut-off at 645 nm, intensity 14 n-einstein $\cdot$ $cm^{-2} \cdot s^{-1}$. Flash-induced absorbance changes were measured with a single-beam spectrophotometer [9]; an RG 680, 3 mm was used for the illumination. In some experiments an averager (Nuclear Chicago model 7100) was used.

RESULTS AND DISCUSSION

Secondary electron acceptors

We reported earlier [6], that the kinetics of the light-induced absorbance changes of *P*-700 at −50 °C are similar to those of the System I component of the

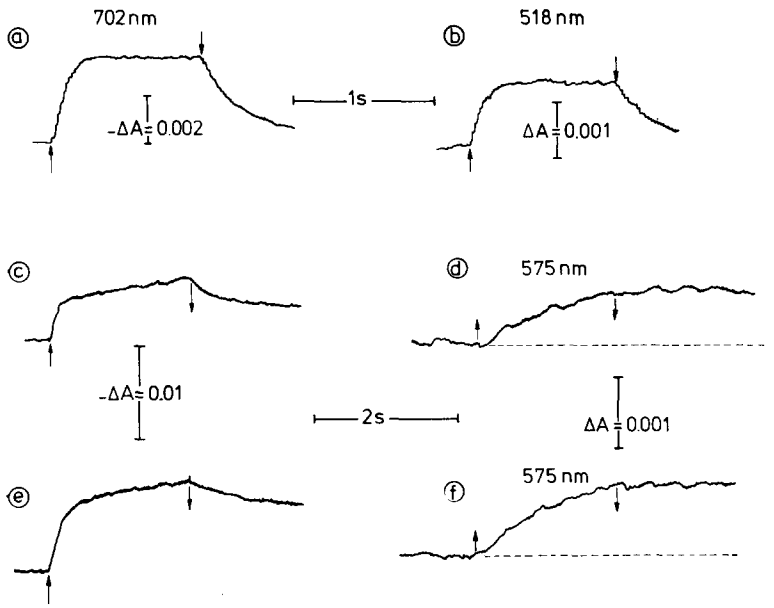

Fig. 1. Absorbance changes of chloroplasts suspended in tricine buffer and glycol and cooled down to the temperature indicated below. Recordings a and b are the average of 10 recordings obtained with a cycle of 1 s light, 7 s dark. Recordings c and d, single recordings in the presence of 10 μM methyl viologen; e and f, 0.1 mM methyl viologen. Temperature: a and b, −50 °C; c–f, −37 °C. Upward pointing arrows mark the onset, downward pointing ones cessation of illumination. To facilitate comparison, the absorbance changes of *P*-700 were plotted upside down in this figure.

absorbance changes at 518 nm (due to *P*-518). More accurate experiments by means of an averager are shown in Fig. 1, recordings a and b. Within the error of measurement, differences in kinetics were not observed. Analysis of the kinetics obtained upon darkening for these and similar recordings showed one rapid and at least one slower component in the decay of both *P*-700 and *P*-518. As stated in the Introduction, the decay probably represents a back reaction between P^+-700 and the acceptor of Photosystem I. The half-time of the rapid component was 0.16 s in the region between −50 and −30 °C, independent of temperature. Above −30 °C the decay of *P*-518 became slower and its amplitude larger, presumably because of secondary electron transport.

Fig. 1, recordings c and e, and Fig. 2 (insert) show the kinetics of *P*-700 in the presence of methyl viologen; recordings d and f show corresponding measurements at 575 nm, due to reduction of this electron acceptor [11]. With a low concentration of methyl viologen, especially at low temperature, the decay of only part of P^+-700 was slowed down upon a brief illumination, apparently because the time was too short for a complete oxidation of the electron acceptor of System I. Fig. 2A shows the proportion of reaction centers which had reacted with methyl viologen (10 μM) as a function of illumination time at −37 °C. Fig. 2B illustrates the temperature dependence of the reaction. Even at −60 °C, the decay of P^+-700 indicated that methyl viologen reduction still occurred, although with a low rate. The reaction did

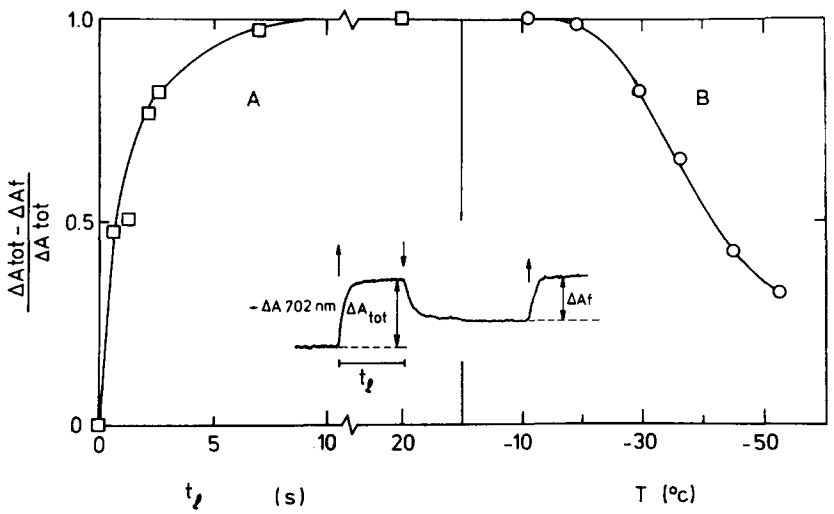

Fig. 2.(A) Fraction of reaction centers of System I that have reacted with methyl viologen (10 μM) as a function of illumination time, t_l. The squares give the fraction of *P*-700 that decayed slowly in the dark (see insert); the solid line was taken from a normalized, hand-smoothed recording of the absorbance increase at 575 nm, due to reduction of methyl viologen. Temperature: −37 °C. (B) Fraction of *P*-700 that decayed slowly as function of temperature, with a fixed illumination period of 2.0 s. Insert, kinetics of *P*-700, at −53 °C recorded at the same sensitivity as for Fig. 1(c). Dark time between two illuminations, 4.0 s. Illumination: 630 nm, 20 neinstein · $cm^{-2} \cdot s^{-1}$.

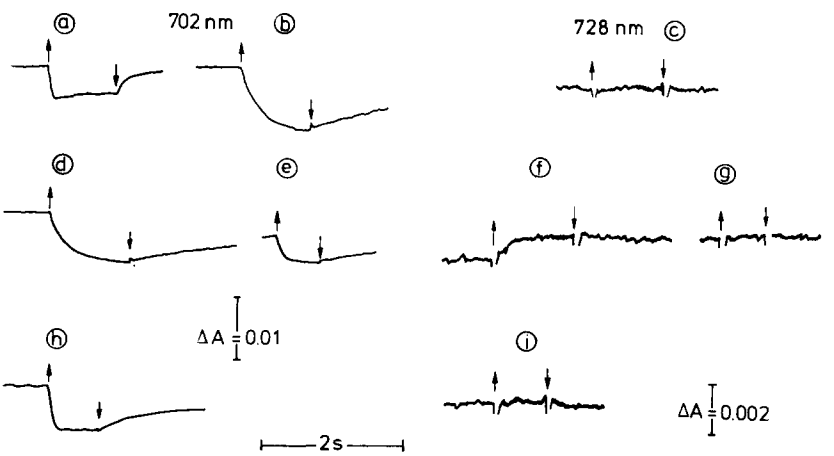

Fig. 3. Effect of silicomolybdate (0.13 g/l, recordings d–i). Recordings d, f, h and i were obtained upon the first illumination after cooling, e and g upon the second illumination, given after 3.2 s darkness. Temperature: a–g, −30 °C, h and i, −50 °C. Recordings a–c show control experiments; a, no additions; b and c, 0.1 mM methyl viologen. At 728 nm, the isosbestic wavelength for *P*-700, the transients upon turning on and off the light are fluorescence artifacts. Corresponding traces at 702 and 728 nm were recorded simultaneously with the same sample. Illumination: 630 nm, 3.5 neinstein · $cm^{-2} \cdot s^{-1}$.

not occur in a frozen sample (without glycol). The amount of *P*-700 oxidized in experiments such as that of recording e of Fig. 1, was about 1 per 400 chlorophyll molecules (calculated on basis of a differential extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ at 702 nm [12]).

Somewhat surprisingly, addition of silicomolybdate, at room temperature an electron acceptor for Photosystem II only [13, 14], produced a similar effect upon the kinetics of *P*-700 and *P*-518 as methyl viologen (Fig. 3). However, measurements of light-induced absorbance changes at 728 nm to observe the formation of the reduction product ("heteropoly blue" [13]), showed that reduction of silicomolybdate only occurred above about −40 °C and only upon the first illumination, whereas the slowing down of the re-reduction of P^+-700 by silicomolybdate occurred at lower temperatures and after the second illumination also. This suggests that silicomolybdate makes the acceptor accessible to some other oxidant for System I, perhaps oxygen. A similar mechanism was proposed for System II at room temperature [15]. The reduction of silicomolybdate upon the first illumination at above −40 °C is probably due to Photosystem II. The amount reduced in this way was 1 per approx. 500 chlorophyll molecules. A differential specific extinction coefficient of 9 $mM^{-1} \cdot cm^{-1}$ at 728 nm was applied for the calculation [13].

Electron donors

The light-induced absorbance increase at 518 nm due to System I was strongly enhanced (up to 20-fold) when both an electron acceptor and donor were present

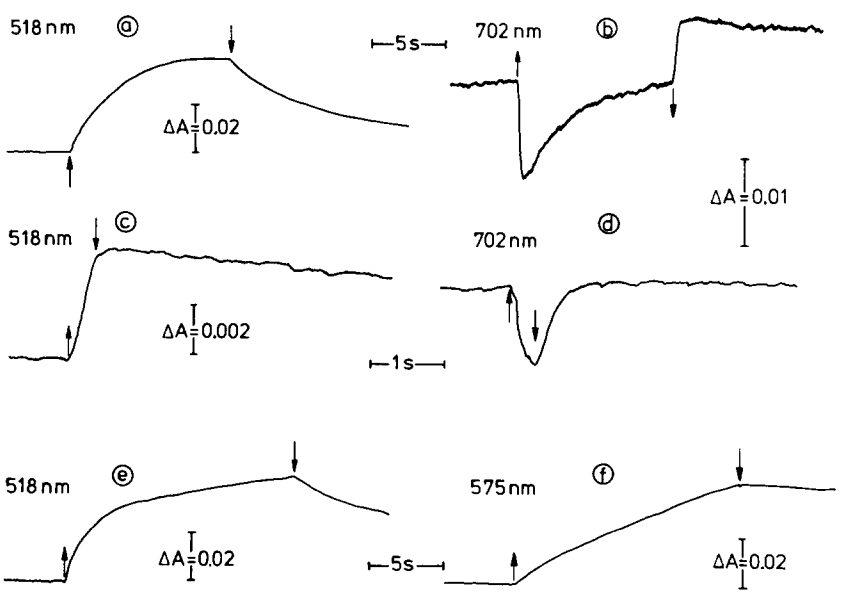

Fig. 4. Absorbance changes in the presence of both an electron donor and acceptor. Additions: a–d, 0.17 mM PMS, 3.3 mM ascorbate, 1.0 mM NH_2OH, 20 μM DCMU; e and f, 0.1 mM TMPD, 0.1 mM methyl viologen. Temperature, −45 °C. For recording e, the slow increase is mainly due to oxidation of TMPD. For recordings a–d, the chloroplasts were preilluminated with 12 short, saturating flashes just before cooling.

(Fig. 4, recording a). This enhancement is apparently due to an accumulation of relatively large amounts of reduced acceptor and oxidized donor on opposite sides of the thylakoid membrane, creating a much larger membrane potential than that caused by the primary photoreaction alone. Re-reduction of P^+-700 in the dark was not accompanied by a reversal of the absorbance change at 518 nm (Fig. 4, recordings c and d), since a reaction with added donor does not bring about a collapse of the membrane potential. The most effective donor tested was reduced PMS; less effective donors were TMPD, DAD and reduced DCIP. Methyl viologen was used as electron acceptor in these experiments; however, with PMS its addition was not necessary to obtain large membrane potentials, even in the presence of a large excess of ascorbate. This indicates that PMS in the oxidized form, even at a low concentration, is also an effective electron acceptor. All measurements with donor and acceptor reported here were made at saturating light intensities. The absorbance changes reversed only slowly upon darkening, indicating a low rate of ion or electron transport through the membrane.

The temperature dependence of the absorbance increase obtained with PMS as donor and acceptor is shown in Fig. 5. Although the rate of electron transport decreased with decreasing temperature (Table I), the steady-state deflection of the change at 518 nm showed a sharp increase down to about −40 °C, due to a decrease of the ionic conductivity and the rate of "cyclic" electron transport through the membrane upon lowering the temperature. With TMPD, electron transport was also measured directly (Fig. 4, recording f) by the absorbance increase at 575 nm, due to oxidation of TMPD [16] and reduction of methyl viologen [11]. The reaction was virtually irreversible. This indicates that in this case at least, the reversal of the *P*-518 change was due to ion transport only, and that oxidized TMPD and reduced methyl viologen did not react with each other, with P^+-700 or with the reduced electron acceptor, presumably because the membrane is impermeable to these substances at low temperature. In the system with PMS and ascorbate direct measurement of this type

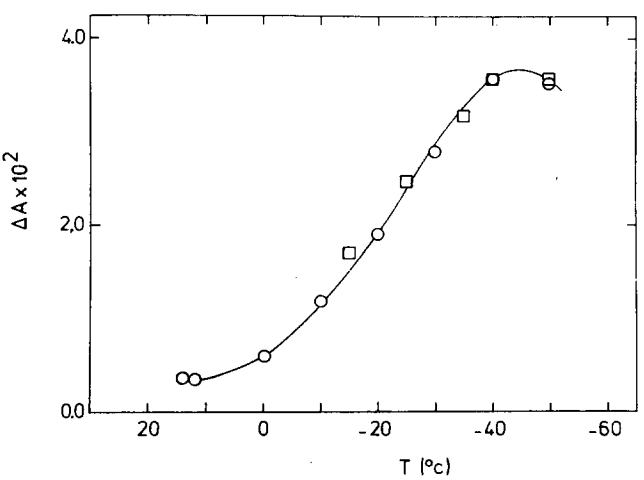

Fig. 5. Temperature dependence of the steady-state absorbance change at 518 nm. Additions: 0.17 mM PMS, 3.3 mM ascorbate. Different symbols refer to different batches of chloroplasts.

TABLE I

Membrane potentials in the steady state and rates of electron transport obtained in the light with various donor and acceptor systems, calculated as for Fig. 7 from the absorbance increase at 518 nm or at 575 nm (using a differential extinction coefficient of 8.6 $mM^{-1} \cdot cm^{-1}$ for TMPD and 2.9 $mM^{-1} \cdot cm^{-1}$ for methyl viologen). Additions: methyl viologen, 0.1 mM; ascorbate, 2 mM; donors, 0.1 mM.

Donor	−35 °C		−45 °C	
	Rate (μequiv./mg chlorophyll per h)	Membrane potential (mV)	Rate (μequiv./mg chlorophyll per h)	Membrane potential (mV)
PMS (red.)	50	420	16	460
TMPD	20	360	4.0	300
TMPD	27*		4.5*	
DAD	8.6	250	3.2	300
$DCIPH_2$	10	110	2.5	130

* Measured at 575 nm.

was of course impossible, because the total amount of PMS and its reduced form did not change during the reaction.

The absorbance changes at 518 nm were not inhibited by DCMU nor by pre-illumination in the presence of DCMU and hydroxylamine [17] before cooling, apart from a small proportion due to the primary reaction of Photosystem II. This indicates that the stimulated 518 nm increase is caused by Photosystem I only. At 700 nm, the absorbance change in the presence of PMS and ascorbate showed a slow and a rapid phase upon illumination and upon darkening (Fig. 4, recording b). The rapid

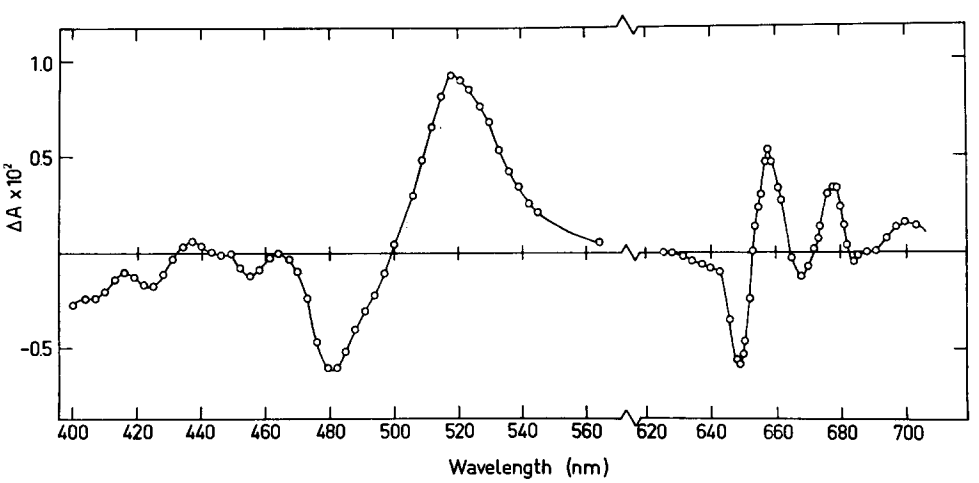

Fig. 6. Spectrum of the slow phase of the light-induced absorbance change (see text) at −35 °C. Chlorophyll concentration: 0.17 mM. Further conditions as for Fig. 5, except that measurements obtained in the region 625–705 nm were obtained by illumination with blue light (480 nm, 8.0 neinstein $\cdot cm^{-2} \cdot s^{-1}$).

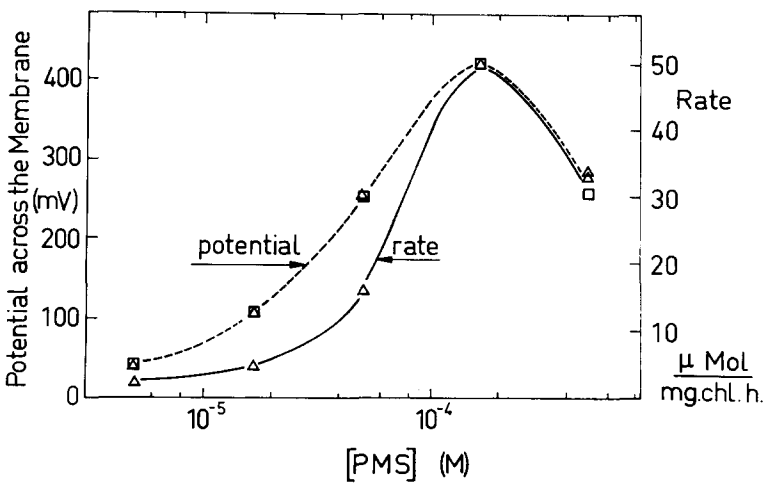

Fig. 7. Rate of electron transport measured from the rate of absorbance change, 0.2 s after onset of illumination and estimated membrane potential obtained in the steady state (see text) as function of PMS concentration. The ascorbate concentration was 20 times that of PMS. Triangles refer to measurements at 518, squares to measurements at 649 nm, recorded simultaneously. Illumination: 690 nm, 10 neinstein · $cm^{-2} \cdot s^{-1}$. Further conditions as for Fig. 6.

changes were due to *P*-700 mainly, as shown by the difference spectrum; the slow changes were of opposite sign and apparently due to an electrochromic shift of a chlorophyll *a* form. Fig. 6 shows the difference spectrum of the "slow" changes, measured between 0.5 and 9 s after onset of illumination. The shape confirms the hypothesis that the absorbance changes are due to a membrane potential across the thylakoid lamellae: it is very similar to spectra obtained earlier with spinach chloroplasts [18] and with the green alga *Scenedesmus obliquus* [19] at room temperature, and indicates electrochromic shifts of chlorophylls *a* and *b* and carotenoid. The shape of the spectrum also indicates that no significant changes in light scattering occurred.

Fig. 7 shows the dependence on PMS concentration. As Table I and Fig. 7 show, fairly high rates of electron transport were observed, even at −45 °C. The data were calculated from the slope of the absorbance increase at 518 nm after the first second of illumination by comparison with the size of the 518 nm change reflecting the photooxidation of a known amount of *P*-700. The rates of TMPD oxidation were also measured directly at 575 nm. Table I and Fig. 7 also show the estimated membrane potentials obtained upon sufficiently long illumination. These numbers were calculated by comparing the size of the 518 nm change with that obtained in the presence of methyl viologen, but in the absence of donor, upon a sufficiently long illumination which caused complete oxidation of *P*-700, corresponding to a single charge transfer for each System I reaction center. The calculation was based on the estimate given by Schliephake et al. [20] for the membrane potential (50 mV) generrated by a single photoact in the reaction centers of System I and II. More recently, this number was revised by Zickler et al. [21] on the basis of experiments with the voltage-dependent pore-forming antibiotic alamethicin. If the revised number is correct the estimated membrane potentials should be multiplied by a factor of two.

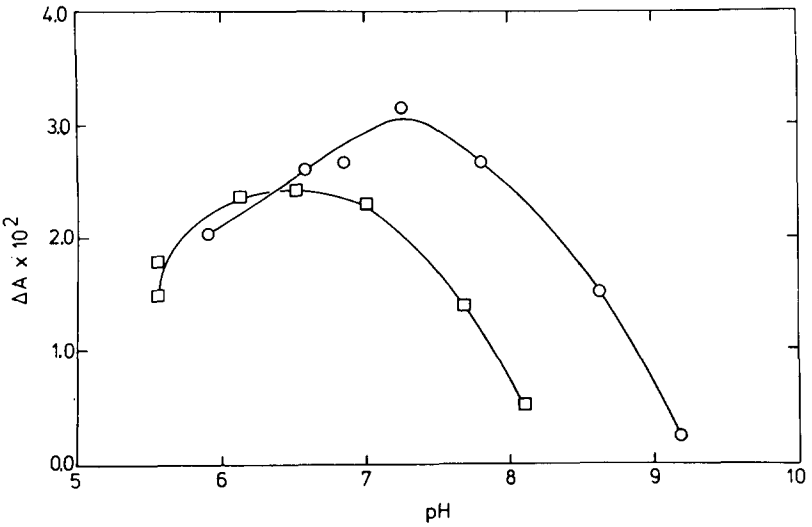

Fig. 8. pH dependence of the steady-state absorbance change at 518 nm. Squares: with phosphate buffer, 60 mM; circles: with tricine buffer (see text). Temperature: −37 °C. Further conditions as for Fig. 5, except that sucrose was replaced by sorbitol in the experiment with tricine.

Since, however, direct measurements with chloroplasts of *Peperomia metallica* are more in agreement with the lower than with the higher estimate (Vredenberg, W. J. and Bulychev, A. A., personal communication), we preferred to use the lower number. A linear relation between the absorbance change and the potential was assumed for the calculation (see below); it was also assumed that the dielectric constant of the membrane was the same as in the experiments of Schliephake et al. [20].

The pH dependence in the presence of PMS and ascorbate is shown in Fig. 8. The squares show data obtained with chloroplasts prepared and stored in phosphate/sucrose buffer, pH 7.0. A few seconds before the addition of glycol, the pH was brought to the desired value by mixing with an excess phosphate buffer of lower or higher pH. The abscissa shows the pH before mixing with glycol and cooling; according to Hui Bon Hoa and Douzou [22] the "effective" pH, pH*, of this system increases by 1.1 unit by these treatments. The pH optimum of the steady-state *P*-518 change, after correction, was about 7.5. Data obtained with tricine (circles) suggest a similar pH optimum, but the correction that should be applied for addition of glycol and cooling is not known in this case.

Linearity of the electrochromic response

Fig. 9 shows the absorbance increase at 518 nm induced by single, saturating 8-μs flashes, measured in the presence of PMS and ascorbate. Photosystem II was made inoperative in these experiments by preillumination in the presence of DCMU and hydroxylamine before cooling [17]. Recording a shows the response to a flash given to a suspension kept in darkness after cooling; recording b was obtained after several seconds of continuous illumination, which generated a membrane potential of 350 mV, as indicated by the size of the absorbance increase at 518 nm produced by

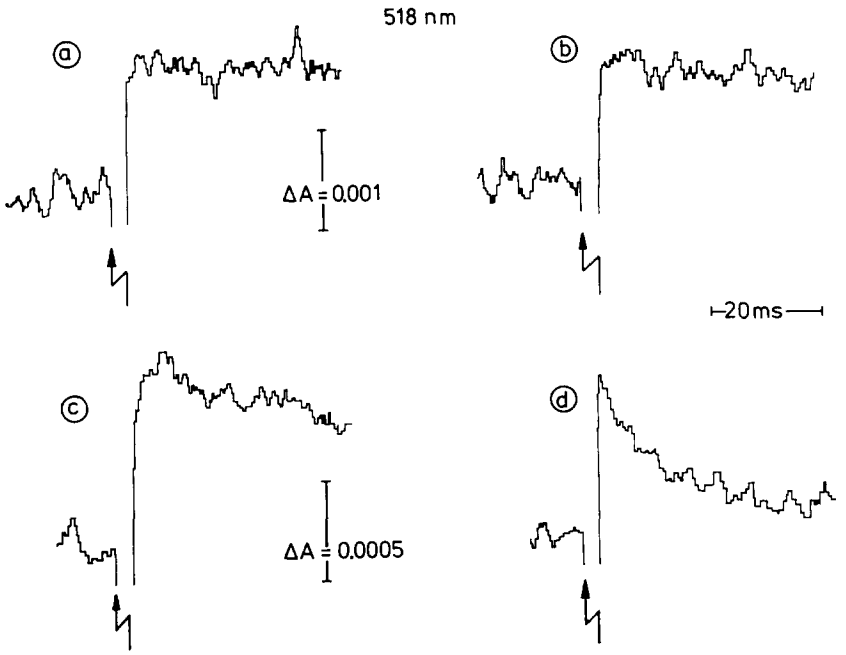

Fig. 9. Absorbance increase at 518 nm brought about by a short saturating flash at −42 °C. Recordings a and b: conditions as for Fig. 4, recording a. Recording b was obtained 0.6 s after continuous illumination. Recording c, no additions; e, 50 μM gramicidin D. Recordings c and d are the average of 10 measurements.

the continuous light (not shown). The flash was given 0.6 s after turning off the continuous light, a time sufficiently long for complete rereduction of P^+-700 and reoxidation of the primary acceptor. Longer dark times did not yield a larger flash-induced response. The absorbance increase at 518 nm induced by the flash, apparently caused by a single turnover of the System I reaction centers, was the same in both experiments, which indicates that the absorbance change is linear with potential up to at least 350 mV. This is consistent with the observed proportionality between the absorbance changes at 518 and 649 nm (Figs. 7 and 11), and agrees with earlier measurements of Reinwald et al. [23] over a smaller potential range at room temperature. At 649 nm, the linearity is explained by the fact that chlorophyll *b*, which is responsible for the absorbance change in this spectral region, has a permanent dipole moment; for carotenoid, the linearity has been explained by the assumption of a relatively large induced dipole moment caused by a local electrical field [8]. If this assumption is correct, the observation that even at 350 mV the deviation from linearity appears to be less than 10 % indicates that the local field is at least five times stronger than that caused by the membrane potential, i.e. $> 7 \cdot 10^6$ V/cm (cf. ref. 8).

Effects of gramicidin and ionic strength of the medium

Gramicidin D enhanced the rate of decay of *P*-518 after a flash (Fig. 9, recording d). In continuous light, it diminished and at sufficiently high concentration abolished the steady-state absorbance changes at 518 and 649 nm as illustrated by

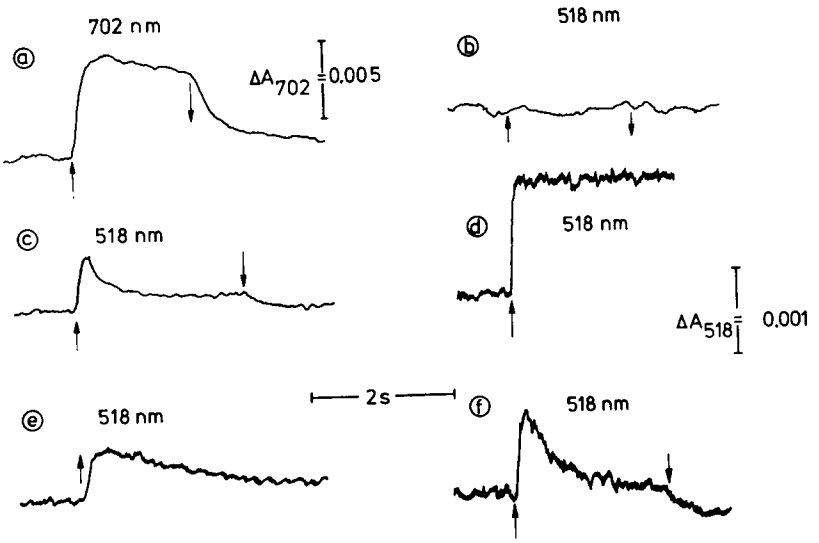

Fig. 10. Light-induced absorbance changes in the presence of gramicidin D (recordings a–c) and carbonylcyanide *m*-chlorophenylhydrazone (recordings d–f). No other additions. Conditions: a and b, gramicidin concentration, 50 μM; c, 5 μM; d–f, carbonylcyanide *m*-chlorophenylhydrazone, 0.1 mM. Temperature: a–d, −45 °C; e, −30 °C; f, −20 °C, d–f, chlorophyll concentration, 0.3 mM.

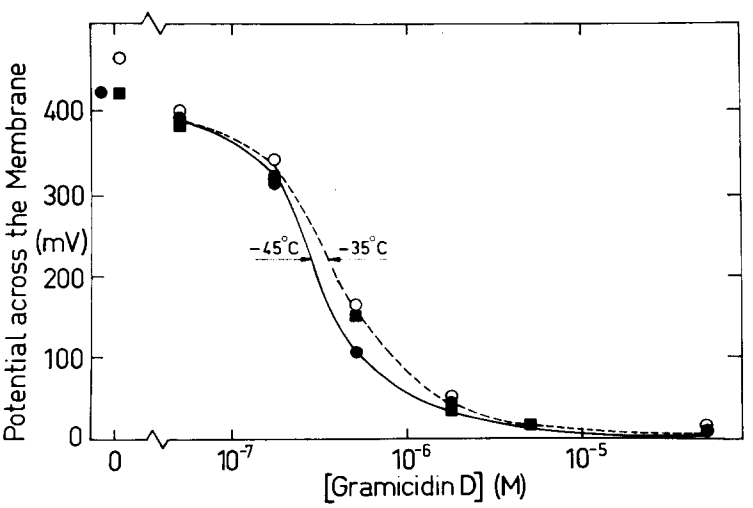

Fig. 11. Effect of gramicidin concentration on the steady-state membrane potential in the light at two different temperatures. Circles: measured at 518 nm; squares: 649 nm. Additions: 0.17 mM PMS, 3.3 mM ascorbate. Further conditions as for Fig. 7.

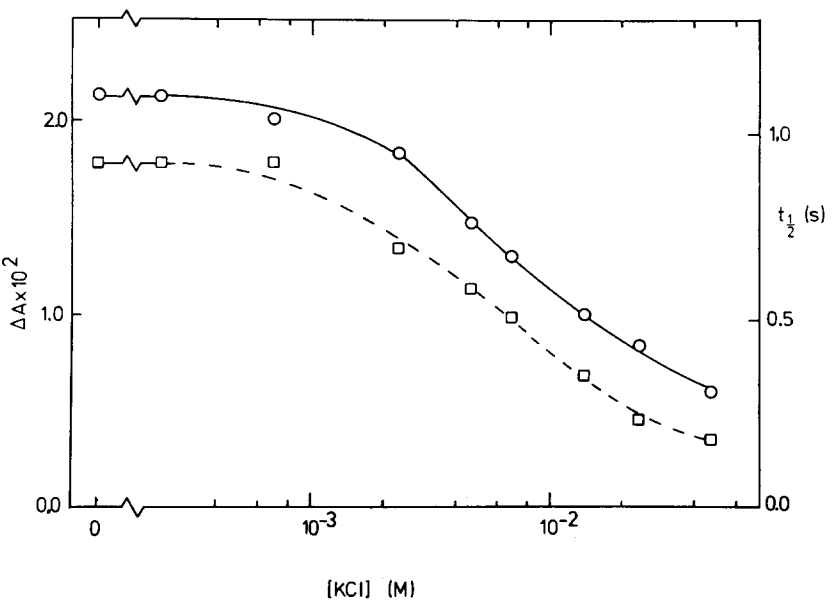

Fig. 12. Effect of KCl concentration on the steady-state absorbance change at 518 nm (circles) and on the first half-time of the reversal upon darkening (squares). Additions: 0.17 mM PMS, 3.3 mM ascorbate, pH 7.8. The chloroplasts were prepared and suspended in 0.4 M sucrose instead of the complete buffer solution; KCl was added immediately before the experiment. Temperature: −35 °C.

Figs. 10 and 11. These effects are presumably due to an increased ionic conductivity of the membrane. The kinetics of *P*-700 were not markedly affected. The concentration of gramicidin needed was higher than that known to produce comparable effects at room temperature [7]. A 50 % decrease of the absorbance changes was brought about by a concentration of gramicidin of approximately one molecule per reaction center of System I (Fig. 11). Carbonylcyanide *m*-chlorophenylhydrazone induced an effect comparable to gramicidin (Fig. 10), but only at relatively high temperatures (above about −35 °C).

The effect of ionic concentration is shown in Fig. 12. As could be expected, the steady-state signal decreased, and the rate of the dark decay increased with increasing KCl concentration.

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