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ELECTROCHROMIC ABSORBANCE CHANGES OF PHOTOSYNTHETIC PIGMENTS IN *RHODOPSEUDOMONAS SPHAEROIDES*

I. STIMULATION BY SECONDARY ELECTRON TRANSPORT AT LOW TEMPERATURE

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SUMMARY

Light-induced absorbance changes were measured at temperatures between −30 and −55 °C in chromatophores of *Rhodopseudomonas sphaeroides*. Absorbance changes due to photooxidation of reaction center bacteriochlorophyll (*P*-870) were accompanied by a red shift of the absorption bands of a carotenoid. The red shift was inhibited by gramicidin D. The kinetics of *P*-870 indicated electron transport from the "primary" to a secondary electron acceptor. This electron transport was slowed down by lowering the temperature or increasing the pH of the suspension. Electron transport from soluble cytochrome *c* to $P\text{-}870^+$ occurred in less purified chromatophore preparations. This electron transport was accompanied by a relatively large increase of the carotenoid absorbance change. This agrees with the hypothesis that *P*-870 is located inside the membrane, so that an additional membrane potential is generated upon transfer of an electron from cytochrome to $P\text{-}870^+$.

A strong stimulation of the carotenoid changes (more than 10-fold in some experiments) and pronounced band shifts of bacteriochlorophyll *B*-850 were observed upon illumination in the presence of artificial donor-acceptor systems. Reduced *N*-methylphenazonium methosulphate (PMS) and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) were fairly efficient donors, whereas endogenous ubiquinone and oxidized PMS acted as secondary acceptor. These results indicate the generation of large membrane potentials at low temperature, caused by sustained electron transport across the chromatophore membrane. The artificial probe, merocyanine MC-V did not show electrochromic band shifts at low temperature.

INTRODUCTION

Upon illumination of intact cells and cell-free preparations of various species of

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Abbreviations: PMS, *N*-methylphenazonium methosulphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

higher plants, algae and purple bacteria, absorbance changes have been observed [1–5] that cannot be ascribed to an oxidation-reduction reaction of a specific electron carrier. It is generally accepted that these changes are due to changes in the absorption spectrum of photosynthetic pigments that reflect the generation of a potential across the thylakoid or chromatophore membrane.

On basis of experiments with spinach chloroplasts and model systems, it was concluded [6–9] that the absorbance changes are due to electrochromic effects on the absorption spectra of the pigments involved, such as carotenoid and chlorophylls, caused by the electric field of the membrane potential. These effects result among other things in shifts of the absorption bands, the extent of which is an increasing function of the membrane potential. However, a direct demonstration that this is indeed the case by analysis of the kinetics and extent of the absorbance changes in vivo has not been possible so far. A quantitative analysis of this type appeared to be possible for the purple bacterium *Rhodopseudomonas sphaeroides* [4, 10]. The result, however indicated that, in the blue region of the spectrum, the absorbance changes reflected a shift by about 10 nm of the bands of spheroidene, the major carotenoid in this bacterium [11], towards longer wavelengths. Such a shift would presumably be too large to be explained by an electrochromic effect induced by a membrane potential. Moreover, only the size, but not the location of the spectrum appeared to be dependent on the intensity of illumination. This suggested a fixed shift of variable number of pigment molecules rather than a variable shift of the absorption spectrum of a fixed number of molecules upon variation of the electric field [4, 10]. More recently similar conclusions were obtained from experiments with a mutant of *Rps. sphaeroides* containing neurosporene [12, 13].

As reported in a previous paper [14] chloroplasts illuminated in the presence of an artificial donor and acceptor system at sub-zero temperature are able to generate relatively high membrane potentials. Since the accompanying pigment changes are both large and slow in these conditions, they can be measured easily with high precision. Therefore we decided to apply the same technique to chromatophores of *Rps. sphaeroides* in order to reinvestigate the nature of the pigment shifts in this bacterium.

The results, to be described in this paper, indicate that large membrane potentials, accompanied by band shifts of carotenoid and bacteriochlorophyll can indeed be obtained with chromatophores. An analysis of these changes will be given in a subsequent paper.

MATERIALS AND METHODS

Rps. sphaeroides was grown anaerobially in the light in the medium given by Cohen-Bazire et al. [15]. After centrifugation the cells were resuspended in 50 mM potassium hydrogen phosphate buffer, pH 7.5 (or, where indicated, in 50 mM Tris buffer, pH 8.0) sonicated for 10 min with a Branson type S 125 sonifier and centrifuged for 30 min at 20 000 $\times g$. The chromatophores were collected by centrifuging the supernatant for 90 min at 110 000 $\times g$ and the sediment was resuspended in buffer. The suspension was stored at −40 °C or at −196 °C.

Changes in absorbance induced by illumination were measured with a split-beam apparatus described elsewhere [16]. The optical pathlength was 1.3 mm. The measuring beam had a halfwidth of 1.6 nm. Most experiments were done at tempera-

tures between −35 and −50 °C. Just before cooling, the chromatophores were mixed with 50–55 % (v/v) ethylene glycol in order to obtain liquid samples.

The pH of the chromatophore suspension was measured at room temperature before mixing with glycol. The effective pH (pH*), after mixing with glycol and cooling to the desired temperature, was taken from data given by Hui Bon Hoa and Douzou [17]. Bacteriochlorophyll concentrations were calculated using the in vivo extinction coefficient of *Rps. sphaeroides* of 95 $cm^{-1} \cdot mM^{-1}$ at 850 nm given by Clayton [18].

RESULTS AND DISCUSSION

Charge separation and secondary electron transfer

Light-induced absorbance changes at sub-zero temperatures in chromatophores of *Rps. sphaeroides* are shown in Fig. 1 at three different wavelengths. The kinetics of the primary donor (*P*-870), measured at 602 nm, were in agreement with a scheme of the type proposed by Slooten [19]:

$$PXA \overset{h\nu}{\rightleftharpoons} P^+X^-A \rightleftharpoons P^+XA^-$$

According to this scheme, electron transport from the reduced acceptor X^- to a secondary acceptor A competes with the back reaction between *P*-870$^+$ and X^-. In agreement with this, the decay of *P*-870$^+$ after the light was turned off was biphasic (recordings a–c). A fraction dependent on illumination time, temperature and pH

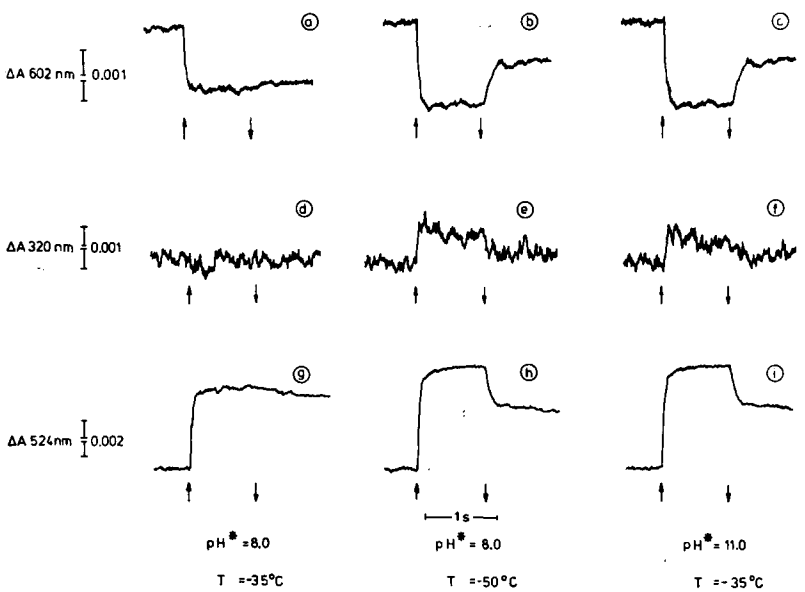

Fig. 1. Kinetics of light-induced absorbance changes of chromatophores of *R. sphaeroides* at the different wavelengths, temperatures and pH*, as indicated. Up- and downward pointing arrows mark the beginning and end of illumination (880 nm; 60 nE/cm^2 per s). Recordings a and g, b and h, and c and i were measured simultaneously. Bacteriochlorophyll concentration: 80 μM. Measurements at pH* 11.0 were done in Tris buffer.

decayed with a halftime of about 0.1 s, presumably by the back reaction with X^- [19, 20]. The second component had a halftime of several minutes and was probably due to a reaction between A^- and $P\text{-}870^+$. Recordings d–f of Fig. 1 were measured at 325 nm. At this wavelength, reduction of X is accompanied by an absorption increase whereas oxidation of *P*-870 shows a relatively small decrease in absorption [19]. During saturating continuous illumination at −50 °C and at "normal" pH (pH* 8.0), reduction of X was followed by its reoxidation by A, while *P*-870 remained oxidized in the light (recordings e and b, respectively). At −35 °C this reoxidation was considerably faster and no changes due to accumulation of X^- could be observed at 325 nm (recordings d and a). If the pH was increased to pH* 11.0 at −35 °C, the reaction between X^- and A was slowed down to a halftime of a few seconds (recording f), and the corresponding absorbance change at 602 nm showed a rapid rereduction of part of $P\text{-}870^+$ upon darkening (recording c). About 20 % of *P*-870 remained irreversibly photooxidized even after 15 min of darkness upon the first illumination (not shown) both at pH* 8.0 and pH* 11.0 and at −35 °C as well as at −50 °C; the remaining 80 % was completely reversible in repeated cycles of 2 s light –5 min darkness.

The difference spectrum obtained in such a light-dark cycle at pH* 11.0 is plotted in Fig. 2 (solid line). The ultraviolet and blue region of this spectrum is very similar to difference spectra of reaction center preparations of *Rps. sphaeroides* at room temperature which were ascribed to the formation of P^+X^- [19, 21]. Between 430 and 560 nm additional changes were observed with maxima of 524, 490 and 458 and minima at 509 and 476 nm, which are due to the well-known carotenoid band shift [2, 4, 22]. If gramicidin D was added before cooling, this carotenoid shift was inhibited

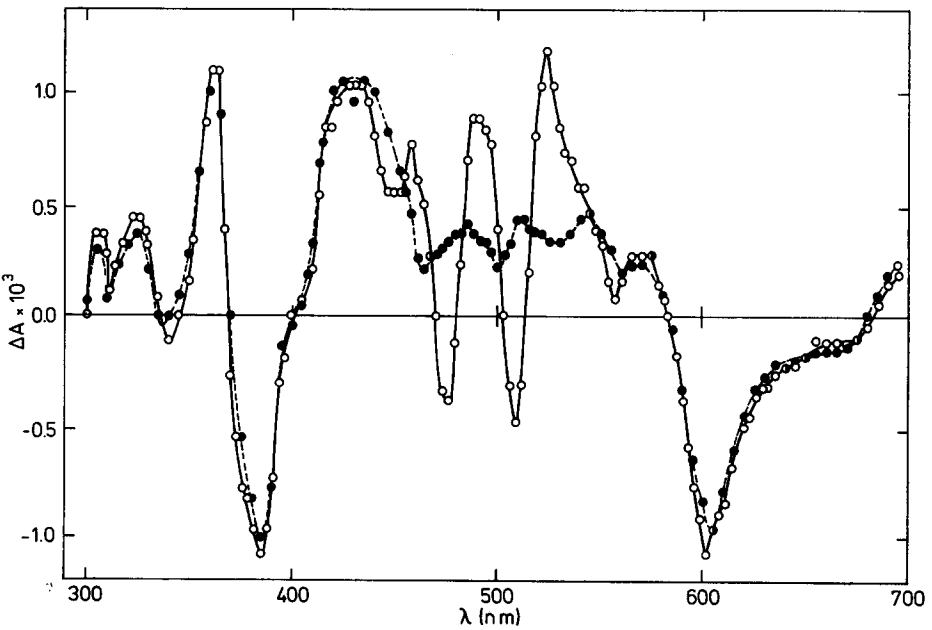

Fig. 2. Light-induced absorbance difference spectrum obtained from the second and subsequent illuminations at −45 °C. Solid line, no additions; broken line, 50 μM gramicidin D present. Bacteriochlorophyll concentration : 80 μM; Tris buffer, pH* 11.0. Illumination: 880 nm; 60 nE/cm² per s.

(Fig. 2, broken line). This inhibition is most easily explained if we assume that the shift is due to a delocalized field over the photosynthetic membrane caused by the primary charge separation. With gramicidin, the electric field and consequently the carotenoid shift apparently decays too rapidly to be observed, due to an enhanced permeability of the membrane for ions. The same explanation was applied to similar results obtained with spinach chloroplasts [14].

In the absence of gramicidin, the kinetics of the carotenoid change were the same as those of *P*-870 (Fig. 1, recordings g–i). This also applied for the slow phase of *P*-870$^+$ reduction, which indicates that the permeability of the membrane at low temperature is very small. The reaction between X^- and A was not reflected in the carotenoid shift. This agrees with the assumption that the shift is due to a delocalized membrane potential, and moreover suggests that X and A are both located at the surface, presumably the outer surface [23] of the chromatophore membrane.

The spectrum of the gramicidin-insensitive changes showed maxima at about 485 and 512 nm, which were also observed in reaction center preparation of wild type *Rps. sphaeroides* [20, 24], but not in those from the carotenoidless mutant R26 [25]. This indicates that part of the gramicidin-insensitive absorbance changes in the blue-green region may be due to a red shift of a carotenoid with long-wave absorption maxima at about 440, 473 and 505 nm. The same spectrum was obtained at −35 °C and pH* 8.0, when no accumulation of X^- occurred, indicating that this gramicidin-insensitive carotenoid shift probably reflects a local field of *P*-870$^+$.

Stimulation of pigment shifts

By omitting the final centrifugation, a less purified chromatophore preparation was obtained which contained soluble cytochrome *c*. With this preparation, at −35 °C a biphasic absorbance increase at 524 nm was observed. An initial fast phase was followed by a slower increase, the amplitude of which was about the same as that of

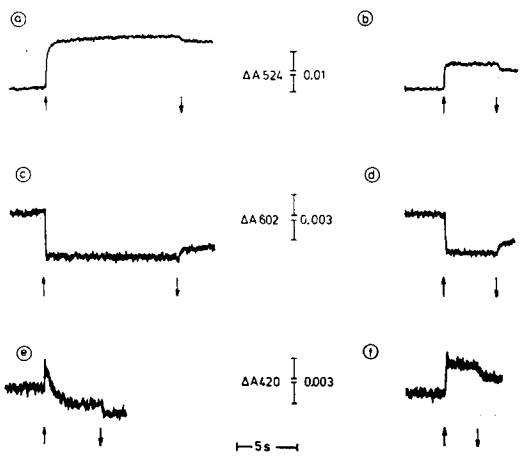

Fig. 3. Kinetics of light-induced absorbance changes at −35 °C in chromatophores which contain soluble cytochrome *c* (see text). Recordings a, c and e: first illumination. Recordings, b, d and f were obtained upon a second illumination after a dark time of 1.7 min. Bacteriochlorophyll concentration: 160 μM. Illumination: 880 nm; 60 nE/cm^2 per s.

the first phase (Fig. 3). Measurements at 602 nm showed that *P*-870 was rapidly oxidized and remained in the oxidized state during illumination. Absorbance changes with the same kinetics as those of the slow phase at 524 nm were detected at around 420 nm and indicated the oxidation of a *c*-type cytochrome, probably cytochrome c_2 (van Grondelle, R., personal communication). The relative amplitudes of the changes at 420 and 602 nm indicated that approximately one cytochrome per three reaction centers was oxidized. Upon a second illumination given after 1.7 min of darkness no cytochrome oxidation was observed and the absorbance changes at 524 nm showed only a fast increase (Fig. 3, recordings f and b, respectively). The kinetics at 602 nm were the same as those obtained upon the first illumination (recording d). Apparently in one-third of the reaction centers a cytochrome is irreversibly oxidized by $P\text{-}870^+$. These reaction centers then undergo a second photochemical charge separation, because X^- is rapidly reoxidized by A under these conditions. The relative amplitudes of the fast and the slow phase at 524 nm indicate that the membrane potential caused by photooxidation of cytochrome is considerably larger than that accompanying photooxidation of the same amount of *P*-870 (see Discussion).

When the artificial electron donor TMPD was added to "purified" chromatophores, the carotenoid changes were stimulated by a factor of 7 at −35 °C. In Fig. 4 the kinetics of this stimulation are shown, measured at 460 nm. Oxidation of TMPD could be followed optically at 559 nm [26]. These wavelengths were chosen such that both reactions could be observed independently of each other. In addition, the changes measured at 285 nm showed the reduction of ubiquinone to ubihydroquinone. It was calculated from these observations that about 15 electrons could be transferred from

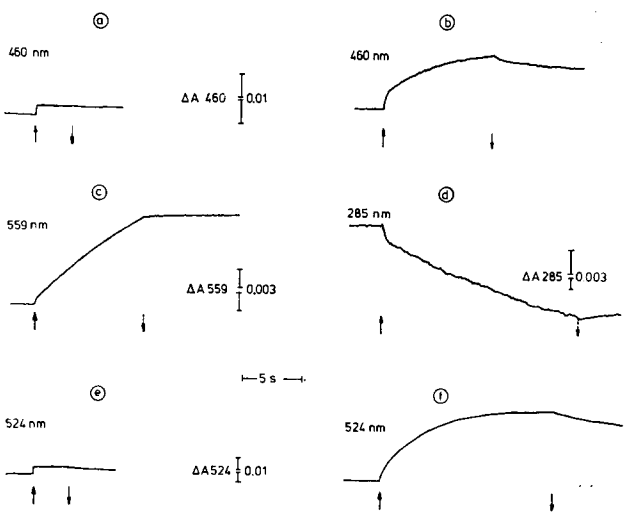

Fig. 4. Light-induced absorbance changes at −35 °C. Recordings a and e were obtained by recording the absorbance changes at 460 and 524 nm, respectively, and subtracting from those the signals observed upon illumination in the presence of 50 μM gramicidin D. The other recordings were obtained in the absence of gramicidin. Illumination: 880 nm; 60 nE/cm^2 per s. Recordings b–d, 0.1 mM TMPD; recording f, 160 μM PMS and 3.3 mM ascorbate. Illumination: 880 nm; 6 nE/cm^2 per s. Bacteriochlorophyll concentration: a–d, 160 μM; e and f, 80 μM.

TMPD to ubiquinone per reaction center. The carotenoid shift was inhibited by gramicidin, but the electron transport was not affected.

A still larger stimulation was obtained when prior to freezing partly reduced PMS was added (Fig. 4, recording f). The spectrum of the maximum light-induced absorbance changes obtained in this way is shown in Fig. 5. In addition to a shift of the carotenoid bands, a red shift of bacteriochlorophyll absorbing near 850 nm (*B*-850), a broad absorbance increase around 900 nm and a blue shift at 790 nm were observed. In agreement with earlier observations [4, 10] a close relationship between the *B*-850 and the carotenoid shift was observed in all our experiments.

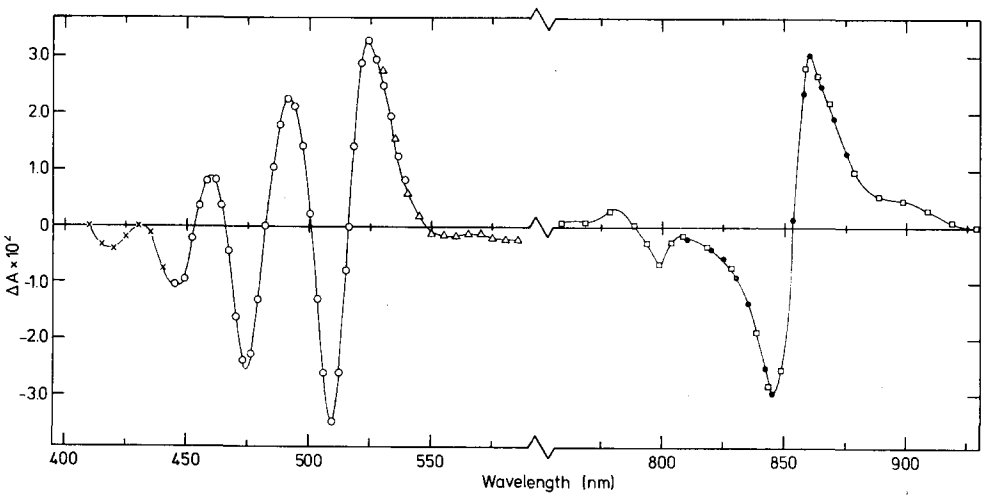

Fig. 5. Absorbance difference spectrum at −35 °C induced by 5 s of illumination in the presence of 160 μM PMS and 3.3 mM ascorbate. Illumination: 880 nm, 6 nE/cm^2 per s (420–580 nm); 470 nm, 8 nE/cm^2 per s (720–960 nm). Bacteriochlorophyll concentration: 80 μM. Different symbols refer to different samples.

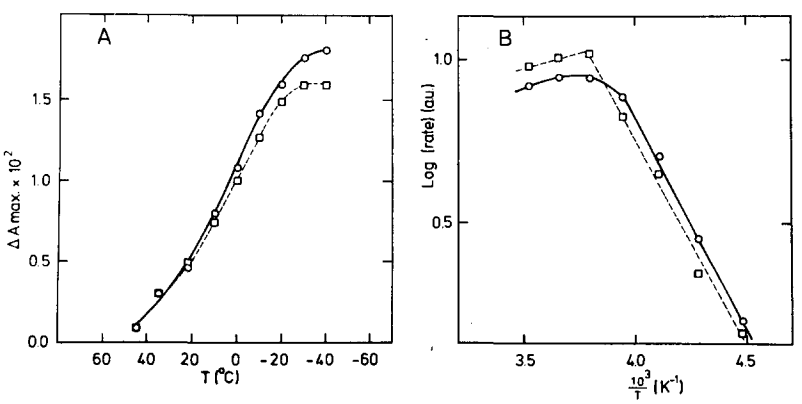

Fig. 6. (A) Temperature dependence of the maximal absorbance change measured simultaneously at 524 nm (○) and 860 nm (□) in the presence of PMS and ascorbate. (B) Arrhenius plot of the initial rate of these changes. Illumination: 600 nm; 10 nE/cm^2 per s.

Fig. 6 shows the temperature dependence of the maximal extent and the rate of the stimulated absorbance increase measured simultaneously at 524 and 860 nm in the presence of PMS. It should be noted that in these experiments the intensity of the actinic light was non-saturating for the rate of electron transport. However, if at low temperatures, higher light intensities were applied, the maximum absorbance changes were lower and the signal decreased during illumination. Measurements at other wavelengths indicated that the effect was due to a decay of the membrane potential. The cause of this phenomenon was not further investigated. It was not observed when TMPD was added instead of PMS.

In order to obtain additional evidence that the changes in pigment absorption discussed above reflect the generation of a membrane potential, we studied the optical changes of the artificial probes merocyanine MC-V [27] and 3,3'-dipropylthiodicarbocyanine (diS-C_3-(5), ref. 28), respectively. Recordings a–d of Fig. 7 compare the absorbance changes of merocyanine with those of carotenoid. Recordings a and b, measured at room temperature, show a clear discrepancy in the kinetics of carotenoid and merocyanine, the absorbance changes of the latter compound being much slower than of the natural probe, both upon illumination and upon darkening. At −35 °C, absorbance changes that could be attributed to merocyanine were not observed at all (recording d).

Results obtained with the fluorescent probe 3,3'-dipropylthiodicarbocyanine are illustrated by recording f. This dye showed a clear fluorescence decrease upon illumination in the presence of chromatophores at −30 °C, but its response was much slower than that of the carotenoid absorbance change. At −45 °C fluorescence changes were not observed.

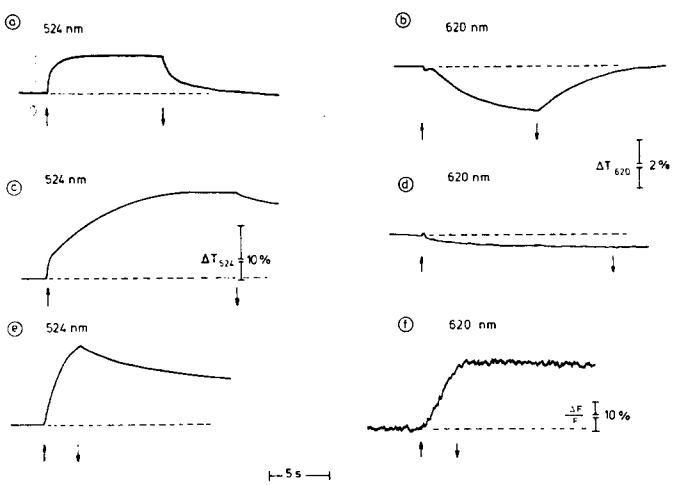

Fig. 7. Light-induced absorbance and fluorescence changes in the presence of artificial probes. Recordings a–d: 20 μM merocyanine MC-V. Recordings a and b were measured at −20 °C, c and d at −35 °C in the presence of PMS and ascorbate. The changes shown in recordings c and d were also observed in the absence of MC-V. Bacteriochlorophyll concentration: 190 μM. Recordings e and f were measured at −30 °C in the presence of 10 μM 3,3'-diprophylthiodicarbocyanine. Bacteriochlorophyll concentration: 120 μM.

DISCUSSION

The stimulation of the pigments shifts described here is very similar to that reported earlier with spinach chloroplasts, suggesting the same explanation [14]: Sustained photochemical electron transport between the donor system (cytochrome *c*, TMPD or reduced PMS) and the electron acceptors (ubiquinone and PMS) creates a potential difference across the chromatophore membrane. This potential difference reaches a high value during illumination and decays only slowly upon darkening due to the low permeability of the membrane at low temperatures. Gramicidin D, which is known to increase the membrane permeability, abolished the pigment shifts, but electron transport between TMPD and ubiquinone was not inhibited.

It has been estimated [29] that a single charge separation at each reaction center creates a membrane potential of about 100 mV, although it is not completely clear how this number should be interpreted, in view of more recent evidence [23]. Our data, which showed more than 10-fold stimulation (see also the subsequent paper) of the carotenoid shift in some cases, then might suggest a membrane potential of more than 1 V. Such a high potential, however, appears to be unrealistic not only because it may exceed the dielectric breakdown potential of the membrane [30], but also because one might expect that the energetics of the primary charge separation would become unfavorable for electron transfer. However, it is possible that the potential for a single photoact is lower than 100 mV, at least for our chromatophores, and moreover, for high potentials the relation between the absorbance changes and the membrane potential may deviate significantly from linearity.

It should be noted that closer inspection of the difference spectra shown in Figs. 2 and 5 shows that the absorbance changes of the carotenoid are not only stimulated in the presence of a donor-acceptor system, but that the bands in the latter spectrum are also slightly displaced towards longer wavelengths. This observation, together with other data, will be discussed in the subsequent paper. It will be shown that it can be explained by the assumption [6–9] that the absorbance changes are caused by a gradual shift of the absorption bands, which increases with increasing membrane potential.

A stimulation of the carotenoid change, by a factor of 1.8, was also observed when cytochrome *c* acted as electron donor to $P\text{-}870^+$. This stimulation was relatively large, if it is considered that cytochrome oxidation, accompanied by a second charge separation, occurred only in about one-third of the reaction centers. Apparently, electron transfer from cytochrome to $P\text{-}870^+$ by itself produces an increase in membrane potential also, which suggests that *P*-870 is located inside the chromatophore membrane, whereas cytochrome *c* appears to be situated at the inner surface of the chromatophore. The same hypothesis was used by Jackson and Dutton [23] to explain the kinetics of the carotenoid change induced by a flash at room temperature. The observation that the cytochrome is released during high-speed centrifugation seems to indicate that the vesicles are damaged by this treatment.

The fluorescence decrease observed with the artificial probe 3,3'-dipropylthiodicarbocyanine is in agreement with the notion that illumination in the presence of a donor-acceptor system at −35 °C induces a potential across the chromatophore membrane. According to Sims et al. [28] the amount of dye that is bound to the membrane varies with the electric field, and because the fluorescence of the membrane-bound

fraction is quenched, the dye acts as an indicator for the membrane potential. The delay at −30 °C and the absence of response at −45 °C can be explained by temperature and viscosity effects on the diffusion of the dye. The results obtained with merocyanine MC-V seem to rule out the possibility (see also ref. 27) that the absorbance changes of this probe reflect an electrochromic response to a membrane potential. Instead, they may suggest a similar mechanism as for 3,3′-dipropylthiodicarbocyanine.

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