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THE CAROTENOID SHIFT IN *RHODOPSEUDOMONAS SPHAEROIDES* CHANGE INDUCED UNDER CONTINUOUS ILLUMINATION

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SUMMARY

The spectrum of the carotenoid shift generated under continuous illumination in the G1C mutant of *Rhodopseudomonas sphaeroides*, which has a single carotenoid, has been examined under a variety of conditions expected to alter the size of the membrane potential. If the difference spectrum observed was due to a species with the spectrum of the bulk pigment, it would correspond to a change of a variable proportion of the pigment to a form absorbing at a higher wavelength. The maximal change induced by light could be described as a shift of about 10 % of the pigment by 7 nm to the red, assuming that the shifted species was spectrally identical to the bulk carotenoid.

It is concluded that the changes seen are not easily compatible with a progressive red shift in the whole spectrum with increasing applied potential as would be expected from a simple linear electrochromic mechanism; alternative hypotheses are discussed.

INTRODUCTION

Light-induced shifts in chlorophyll and carotenoid spectra in both photosynthetic bacteria and chloroplasts are now well documented [1–11]. The shift in the carotenoid spectrum (the "carotenoid shift") in photosynthetic bacteria has been shown to occur in most species and to have characteristics which suggest a relation to changes in the transmembrane electric field. The action of uncoupling agents and ionophores [1, 2], the rapid rise on flash illumination [2–4], the effect of phosphorylating conditions on the decay of the change after a single flash [5, 6] and the

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid; MOPS, 3-(*N*-morpholino)propane sulphonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

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generation of a carotenoid shift in *Rhodospirillum rubrum* by ATP in the dark [3], all support this conclusion. Perhaps the most compelling evidence for a relation to membrane potential was the demonstration of a linear relationship between the carotenoid shift and changes in transmembrane potential of known value induced by ionic diffusion gradients [7].

From the shape of the spectrum of the carotenoid shift in *Rhodopseudomonas sphaeroides* only some 10 % of the total carotenoid complement would appear to be involved in the light-induced change, that proportion being shifted by between 7 and 10 nm [8–10]. However, by analogy with the "518 change" in chloroplasts, other authors [11] have assumed that the carotenoid shift represents a shift of all the pigment by a small amount.

It has been suggested that the carotenoid shift may involve an electrochromic interaction, implying a progressive shift of the carotenoid spectrum with increasing field strength [11]. A progressive shift of all the pigment would induce a progressive change in shape of the carotenoid spectrum which would be readily observable if the shift was as large as the 7 nm indicated by the difference spectrum. The work of Amesz et al. [8] failed to detect such a progressive shift in *Rps. sphaeroides* wild type. Comparison of the spectra of the shift generated after each of a series of saturating flashes in an essentially single carotenoid strain of *Rps. sphaeroides* (strain G1C) led to a similar conclusion [10]. The absorption change showed a small progressive shift with increasing flash number, but the shift after the first flash was larger, and that on subsequent flashes much smaller, than was compatible with a simple linear band shift of pigment with the spectrum of the bulk carotenoid.

Using this same mutant, *Rps. sphaeroides* G1C, we have now extended these observations to the spectral changes under steady-state illumination and these are reported below.

METHODS

Cultures of the mutant *Rps. sphaeroides* G1C were grown and chromatophores made, essentially as previously described [12]. Spectra were recorded using a spectrophotometer linked to a DEC PDP 11/10 computer as described previously [13], with the addition of a DECtape unit on which spectra could be stored and accessed for processing by BASIC and FORTRAN language programmes. A modification to the software permitted the rapid scanning of a series of spectra and their subtraction from a reference spectrum. Exciting light was provided by a 55 W quartz halogen lamp from which infrared light was selected by a Wratten 88A filter. The photomultiplier was protected from stray actinic light by a Corning blue glass filter (No. 9782).

Bacteriochlorophyll was estimated after extraction into 7 : 2 (v/v) acetone/methanol, from the extinction at 772 nm using the extinction coefficient given by Clayton [14].

Choline chloride was freed of volatile amines as previously described [12].

RESULTS

Previous observations [10] indicated that the steady-state light-induced carotenoid absorption changes in this mutant could be modelled by a shift of from 7 to

11 % of the pigment by 7 nm. In order to observe the spectrum of the carotenoid shift over a range of membrane potentials use was made of the ionophorous antibiotics valinomycin (which modifies the potential by allowing electrophoretic movement of specific alkali metal cations) and nigericin (which modifies the kinetics of the fall of the membrane potential under continuous illumination by catalysing an electroneutral exchange of specific alkali metal cations and protons [2]). Use was also made of the natural decay of the carotenoid shift under continuous illumination; this decay has been ascribed to the concomitant rise of the chemical (ΔpH) component of the proton motive force [15]. Preliminary observations at a relatively slow rate of scanning indicated that a shift of the base line occurred over the time period of such an experiment which made accurate assessment of the shape of the spectrum difficult.

In an attempt to overcome this technical difficulty, short spectra of 32 or 64 nm, scanned at a rapid rate (25 nm/s) were used, allowing a span of 32 nm to be observed in 1.28 s.

Fig. 1 shows light-induced changes in the carotenoid spectrum taken over a period of about 1.5 min of illumination. It should be noted that the series of spectra pass through an isosbestic point which, although away from the base line, is also isosbestic for the spectral change remaining after a dark period following the period of illumination. By the addition of valinomycin and nigericin (Figs. 2 and 3) spectra of differing amplitudes could be measured. These were also seen to pass through an isosbestic point. The range of magnitudes of carotenoid shift covered by these spectra can be seen modelled in Fig. 4, which also shows the absolute spectrum over the carotenoid region, of the chromatophores used in the experiments of Figs. 1–3.

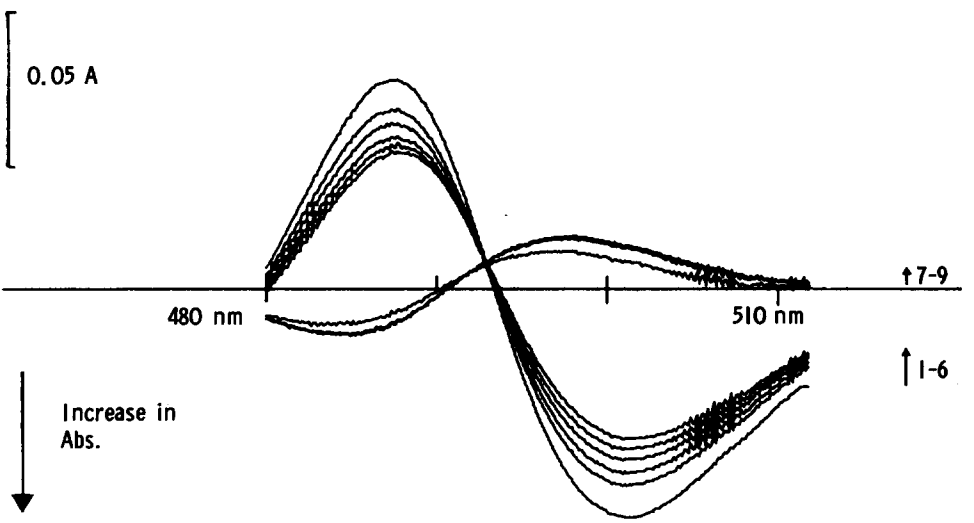

Fig. 1. Spectra of the carotenoid shift during decay under continuous illumination. Chromatophores of *Rps. sphaeroides* G1C were suspended to a concentration of 28 μg/ml bacteriochlorophyll in 50 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 10 % sucrose at pH 7.6. Spectra were taken every 5.75 s after the onset of actinic illumination. The rate of scanning was 25 nm/s. Spectra are: 1–6: difference spectra after the following periods of illumination: 3, 14.5, 26.0, 43.25, 60.5 and 77.75 s. The light was turned off after 85 s. 7–9: difference spectra 10.2, 21.7 and 27.5 s after the end of actinic illumination.

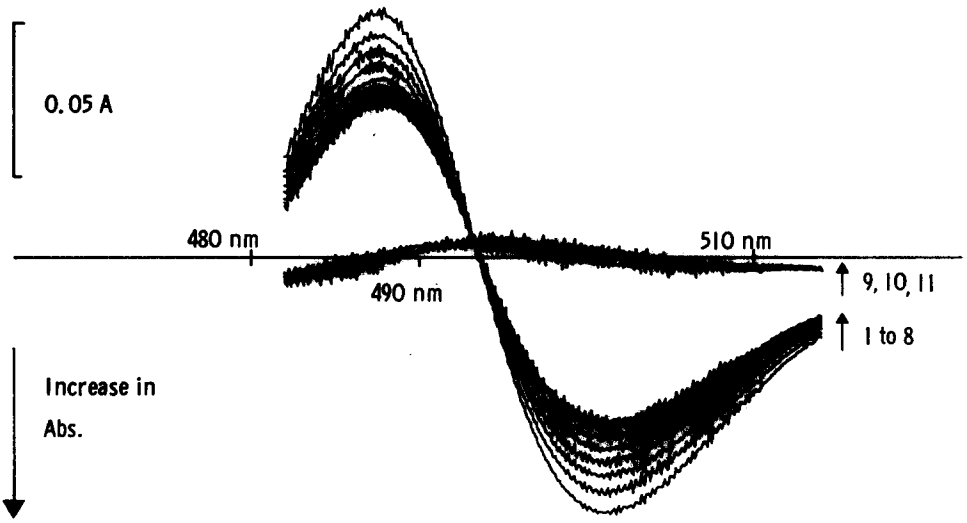

Fig. 2. Spectra of the carotenoid shift during decay under continuous illumination in the presence of nigericin. Conditions as for Fig. 1 but with nigericin (2 μg/ml) present. Spectra are: 1–8: difference spectra every 11.5 s starting 3 s after the onset of actinic illumination. The light was turned off after 85 s. 9–11: difference spectra 10.2, 21.7 and 27.5 s after the end of actinic illumination.

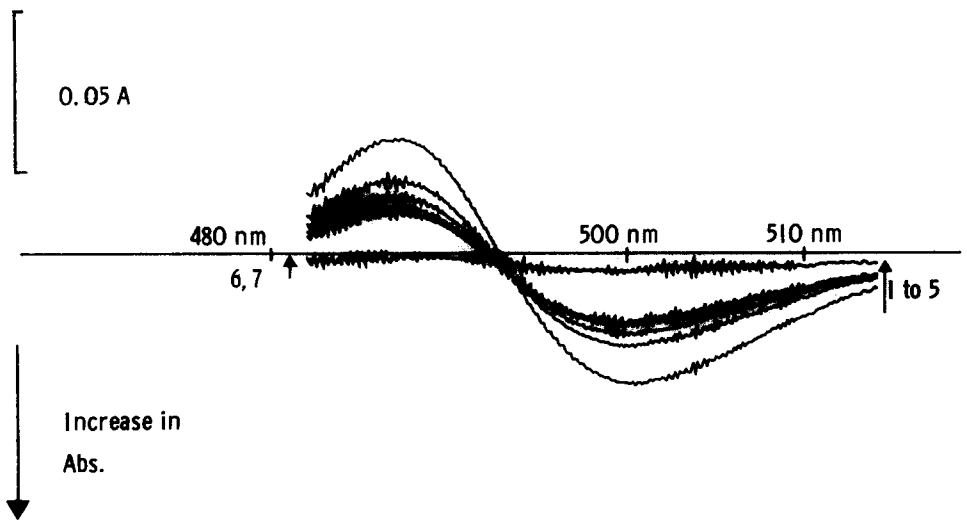

Fig. 3. Spectra of the carotenoid shift during decay under continuous illumination in the presence of valinomycin. Conditions as for Fig. 1 but with valinomycin (5.4 nM) present Spectra are: 1–5: difference spectra every 11.5 s starting 3 s after the onset of actinic illumination. The light was turned off after 56 s. 6, 7: difference spectra 5.5 and 17 s after the end of actinic illumination.

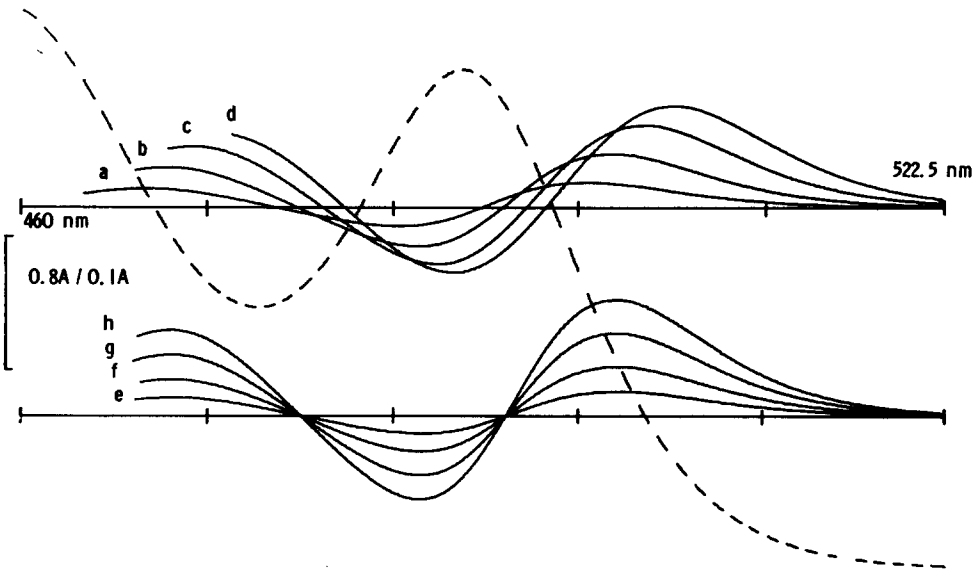

Fig. 4. Model spectra of the carotenoid shift. Conditions as for Fig. 1. The difference spectra were obtained by shifting proportions of the absolute spectrum by the values given below and subtracting the result from the absolute spectrum. The maximum and minimum values of the variable parameters are selected, in each case, to give spectra corresponding to the maximum and minimum changes seen experimentally (from Figs. 2 and 3), assuming that the difference in absorbance at two wavelengths is linearly related to the shift. The values of these parameters are: 7 % of the spectrum by a, 2.5 nm; b, 5.5 nm; c, 9.0 nm and d, 12.0 nm; e, 3 %; f, 6 %; g, 10 % and h, 14 % of the spectrum by 6 nm. The absolute absorbance spectrum is also shown (dashed line).

This figure shows families of synthetic curves for spectra expected from two different models for the carotenoid shift: (a) one involving a progressive shift and (b) one involving two separate pools of pigment, one absorbing at longer wavelength than the other and a shift of pigment between these pools. The change of shape of spectrum with increasing magnitude of change expected from the former model can clearly be seen in the upper set of synthetic curves, in Fig. 4. In particular the point at which the difference spectra cross the base line can be seen to shift to the red. No such change in spectral shape or movement of the zero point can be seen in the spectra of Figs. 1–3. They are, in fact, similar to the bottom set of traces in Fig. 4, corresponding to the change of between 3 and 14 % of the pigment to a form absorbing 6 nm further to the red, and not to the top model representing a progressive shift.

Similar behaviour was observed under a wide variety of conditions (Table I) affecting the membrane potential. These include the presence of the uncoupling agent FCCP, or of the inhibitor of electron flow antimycin. The carotenoid shift spectra always showed an isosbestic point and the dark residual spectrum was also isosbestic or nearly isosbestic, at the same point. No difference was observed when spectra were taken over either 32 or 64 nm, and the middle carotenoid peak showed behaviour similar to that of the long wavelength peak.

When chromatophores made in a K^+-containing medium are suspended in a K^+-free medium addition of valinomycin induces a membrane potential of opposite

TABLE I

BEHAVIOUR OF THE CAROTENOID SHIFT SPECTRUM UNDER VARIOUS CONDITIONS

Chromatophores of *Rps. sphaeroides* G1C were suspended to a concentration of 26 μg/ml bacteriochlorophyll in 50 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 10 % sucrose at pH 7.6. Spectra of 64 nm were taken at a rate of 25 nm/s, one spectrum every 6.3 s. Column 4 refers to the absorbance change at the start of illumination and column 5 to the change after 58 s of illumination. Peak of bulk carotenoid spectrum was at 490 nm in the sample used for these measurements.

Condition	Absorbance (nm)			Increase in absorbance (A) at 502–488 nm	
	Maximum	Minimum	Isosbestic	Start of illumination	After 58 s of illumination
First illumination	502	488	493.5	0.091	0.073
Fourth illumination	502	488	493.5	0.038	0.029
7 % actinic light	501.5	488	493.5	0.083	0.072
3.6 μM antimycin	501.5	488	492.5	0.046	0.026
0.2 μM FCCP	501	488	492.5	0.044	0.025
5.4 nM valinomycin	502	488	493.5	0.049	0.016
2 μg/ml nigericin	502	488.5	494.5	0.102	0.045

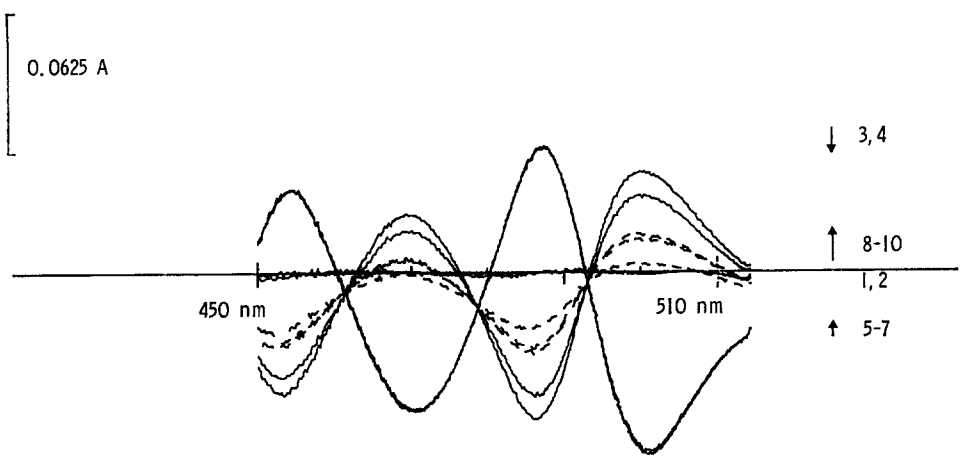

Fig. 5. Spectra of the carotenoid shift induced by valinomycin and by light. Chromatophores of *Rps. sphaeroides* G1C were suspended to a concentration of 40 $\mu g \cdot ml^{-1}$ BChl in 50 mM MOPS, 50 mM choline chloride at pH 7.0. Spectra shown are: 1,2, difference spectra 5 and 25.6 s after the reference spectrum respectively. No additions, no illumination. After 2, 5 μl of a $mg \cdot ml^{-1}$ valinomycin (final concentration 1.8 μM) was added. 3,4, difference spectra 35.9 s and 56.5 s after the reference spectrum. After 4, continuous illumination started. 5–7, difference spectra 66.8 s, 77.1 s and 87.4 s after reference (after about 8, 18 and 28 s of illumination). After 7, the light was turned off. 8–10, difference spectra 97.7 s, 108 s and 118.3 s after reference (about 8, 18 and 28 s after end of illumination).

polarity to that induced in the light [7]. In Fig. 5, spectra resulting from such a reversed membrane potential are compared with the light-induced change in the same sample. It can be seen that the shape of the spectrum of the change induced by the diffusion potential was a mirror image of that induced by illumination, and that the two sets of spectra have a common isosbestic point. The fact that the isosbestic is away from the base line may reflect a dilution artifact or a shift of the base line during the course of the experiment.

DISCUSSION

From these results we may recognize two statements about the carotenoid change which are mutually incompatible: (1) The shift of 7 nm observed is a true shift of the spectrum. (2) The shift observed is a progressive shift. Assuming for the moment that the shift is as large as 7 nm, then the above observations favour the alternative hypothesis put forward by Amesz and co-workers [8], that there are two pools of carotenoid; the empirical evidence may then be interpreted as showing that the longer wavelength form is favoured by a transmembrane potential. Not enough is known as yet of the state of the carotenoid molecules in the membrane to suggest a mechanism for this effect, but a number of restrictions can be imposed on any possible mechanism: (a) over the physiological range, the amount of long wavelength form must be proportional to the membrane potential. This effectively precludes mechanisms in which the membrane potential appears as a linear term modifying the free energy of a simple reaction converting the carotenoid between the two forms observed; (b) from the experiments on reversed potentials one must assume that some long wavelength form exists in the dark; (c) the response to the electric field must occur in less than 1 μs [2]; (d) the shape of the spectrum of the long and short wavelength forms must be similar, though displaced on the wavelength scale; (e) the nature of the change should not depend critically on the species of pigment present, since similar shifts with respect to the bulk spectrum are seen in chromatophores with widely varying carotenoid types.

It seems likely from the above considerations that the change would be related to the environment of the carotenoid molecule rather than to the chemistry of the molecule itself. We have made preliminary studies of the effect of molecular environment on the spectrum of purified neurosporene (Buckberry, P. and Crofts, A. R., unpublished results). As would be expected for an apolar molecule like neurosporene [16] the shift in spectrum in a variety of solvents is related to the difference in refractive index rather than the dielectric constant of the solvent. On incorporation in phospholipid liposomes the spectrum was displaced to the red by 9 nm (compared to the spectrum in light petroleum), but no additional displacement was observed on (a) varying the net charge on the liposomes by incorporating up to 10 % dicetyl phosphate or cetyl pyridinium chloride, (b) varying the ζ-potential by varying the ionic composition of the aqueous phases on either side of the liposome membrane or (c) by applying diffusion potentials across the membranes of the liposomes.

Schmidt and co-workers [11] have attempted to explain the linearity of the carotenoid shift with the membrane potential, by suggesting that a permanent field of $2 \cdot 10^6$ V/cm exists across the chromatophore membrane. We have used the relationship between the size of the shift and the membrane potential, derived by these

authors, to calculate the shift expected from a given membrane potential. In making these calculations we have assumed that the appropriate physicochemical characteristics of neurosporene (the predominant carotenoid in *Rps. sphaeroides* G1C) are similar to those of lutein for which data are available [11].

The relationship derived by Schmidt et al. [11] is

$$h\Delta\nu = -|\vec{\mu}_e - \vec{\mu}_g| F \cos\theta - \tfrac{1}{2}(\alpha_e - \alpha_g)F^2$$

where $\Delta\nu$ is the shift in spectrum of a molecule with orientation θ with respect to the field, F is the field strength, μ is the dipole moment, α the polarizability and subscripts e and g refer to excited and ground states of the molecule respectively; h is the Planck constant.

We have made two assumptions with regard to the physical structure of the membrane. The width of the insulating layer of the membrane was taken to be 40 Å (see ref. 17) and the carotenoid molecules are assumed to lie at an angle of 45° to the plane of the membrane as suggested by the work of Breton [18]. These calculations lead to the conclusion that, assuming a permanent field of $2 \cdot 10^6$ V/cm, the shifts in the absorbance spectrum corresponding to membrane potentials of 400 and 200 mV would be 2.2 and 1.0 nm, respectively. These values are clearly inadequate to account for a shift of 7 nm.

It is perhaps interesting to consider the magnitude of the permanent field that would be required to cause such a strong shift. Calculation of the size of the permanent field as the size of field necessary to shift the absolute neurosporene spectrum by the difference between the absorbance spectrum in light petroleum (b.p. 40–60 °C) and in G1C chromatophores (23 nm) indicates a value of about $6.3 \cdot 10^6$ V/cm. Assuming that this is the permanent field experienced by the neurosporene molecules in vivo a shift of 6 nm corresponding to a membrane potential of 400 mV is obtained. Without more detailed knowledge of the structure of the membrane such calculations are necessarily speculative but would suggest at least that for the model of Schmidt et al. [11] a permanent field of at least three times the size that they proposed must be assumed.

For large shifts of absorbance bands the linear relationship between the change in wavelength and the change in absorbance will be lost [19]. Model studies have indicated that the absorbance change at 503–488 nm remains linear with the shift in wavelength up to a shift of 9 nm.

One alternative possibility that must be considered is that the estimates for the parameters of the carotenoid shift used above are erroneous. This could be the case if either the absorbance maximum of the carotenoid or the point at which the difference spectra cross the base line are displaced from their true position; it might then be that the change in shape of the carotenoid shift difference spectrum was too small for these experiments to detect. However, consideration of the absorbance spectrum of the mutant *Rps. sphaeroides* R26 [14] which lacks coloured carotenoids, suggests that the position of the longest wavelength neurosporene peak should not be significantly displaced and we have previously reported spectra of the flash-induced carotenoid shift which were compensated for changes seen in the R26 mutant [10].

It is also possible that a spectrally distinct part of the carotenoid complement, with absorption maxima slightly ($\approx$ 3 nm) to the long wavelength side of the measured maximum of the bulk carotenoid, could be the species undergoing a change.

This could account for the difference spectra observed within an electrochromic mechanism of the sort suggested by Schmidt et al. [11], and would also be compatible with the anomalous progressive shift observed in our earlier work on the change induced by successive flashes [10]. If this were the case, measurement of the apparent shift with respect to the observed spectrum would give an overestimation of the actual shift, and an underestimation of the amount of pigment involved. De Grooth and Amesz [20] using wild type *Rps. sphaeroides* have recently reported experiments at −30 °C which lend support to this hypothesis, and have proposed that the observed spectrum is contributed by more than one spectrally distinct pool of carotenoid, of which the long wavelength component is that undergoing a change. However, if such a mechanism is to be accepted, it must be shown that the spectral characteristics are compatible with the rather small shift indicated by our present results ($\pm$0.3 nm from the apparent isosbestic), and that the suggested multiplicity of components can be accommodated in the observed spectrum. A proper analysis is difficult with the wild type *Rps. sphaeroides* because of the varied carotenoid complement. Elsewhere we report an analysis of the in vivo spectrum of neurosporene in the G1C mutant used here, which argues against a multiplicity of components [21]. However, while our analysis showed that the in vivo spectrum was no more complex than that of neurosporene in solution, we recognize that it is always possible to fit spectra by increasing the number of the contributing bands, so that we cannot exclude the possibility of spectrally distinct components. A mechanism based on the electrochromic response of a spectrally distinct component as suggested by de Grooth and Amesz [20], is in many respects more appealing than the alternative discussed above, since it provides a viable explanation of the observed phenomena at a molecular level.

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