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Comparison of Permeant Ion Uptake and Carotenoid Band Shift as Methods for Determining the Membrane Potential in Chromatophores from *Rhodopseudomonas sphaeroides* Ga

By STUART J. FERGUSON,*† OWEN T. G. JONES,‡ DOUGLAS B. KELL§‖ and M. CATIA SORGATO*¶

**Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.,*
‡*Department of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, U.K., and*
§*Botany School, University of Oxford, South Parks Road, Oxford OX1 3RA, U.K.,*

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1. A comparison was made of two methods for estimating the membrane potential in chromatophores from *Rhodopseudomonas sphaeroides* Ga. Illuminated chromatophores generated a potential that is apparently much larger when estimated on the basis of the red-band shift of carotenoids rather than from the extent of uptake of the permeant SCN^- ion. 2. In contrast, when the chromatophores were oxidizing NADH or succinate the uptake of SCN^- indicated a larger membrane potential than was estimated from the carotenoid band shift. 3. The extent of SCN^- uptake and the carotenoid-band shift respond differently to changes in the ionic composition of the reaction medium. 4. The effects of antimycin on the carotenoid band shift and SCN^- uptake are reported. 5. It is concluded that the carotenoid band shift and the uptake of SCN^- are responding to different aspects of the energized state.

The quantitative determination of membrane potentials in systems that are too small to be studied by the direct methods of electrophysiology is of crucial importance for the understanding of the coupling of electron transport to ATP synthesis or other energy-linked reactions, as a membrane potential is now widely believed to be an intermediate in energy-coupling reactions. In most systems the only method available for estimating the membrane potential is to measure the uptake of a permeant ion (e.g. SCN^-), which is assumed to come to electrochemical equilibrium with the potential (see, e.g., Rottenberg, 1975). This method further involves the estimation of the internal volume enclosed by the membranes and assumption of equal activity coefficients for the ion inside and outside membrane vesicles or organelles such as mitochondria.

In view of the assumptions necessary with the ion-uptake method it is important to compare the results from this procedure with those from other methods. An alternative method for determining membrane potential has been used for chromatophores from photosynthetic bacteria, in which a light-dependent spectroscopic red-band shift of the carotenoids is measured and compared with the shift induced by K^+ diffusion potentials (Jackson & Crofts, 1969). The results of experiments using this spectroscopic method have indicated that membrane potentials in the range 210–240mV can be maintained in the steady state with illuminated chromatophores from either *Rhodopseudomonas sphaeroides* or *Rhodopseudomonas capsulata* (Jackson & Crofts, 1969; Crofts & Jackson, 1970; Casadio *et al.*, 1974*a*,*b*; Baccarini Melandri *et al.*, 1977).

An early estimate (Schuldiner *et al.*, 1974) of the membrane potential from the extent of SCN^- uptake was made with chromatophores from *Rhodospirillum rubrum* and showed that the light-dependent potential was approx. 100mV. A difficulty in that work was that the chromatophores could not easily be rapidly separated from the suspending reaction medium. However, work using the flow-dialysis technique or an ion-selective electrode, both of which avoid this difficulty, has given a similar estimate of the potential in *R. rubrum* chromatophores, with SCN^- again as the permeant ion (Kell *et al.*, 1978*a*,*b*). By analogy with chromatophores from other bacteria, a potential of 100mV is lower than the value that can be expected to be indicated by the carotenoid shift, but unfortunately the carotenoid band shift is not readily calibrated in *R. rubrum* and so comparison of the two methods is not easy. Recently Michels & Konings

Abbreviation used: Mops, 3-(*N*-morpholino)propanesulphonic acid.

† Present address: Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

‖ Present address: Department of Botany and Microbiology, University College of Wales, Penglais, Aberystwyth, Dyfed SY23 3DA, Wales, U.K.

¶ Present address: Istituto di Chimica Biologica, Via F. Marzolo 3, 35100 Padova, Italy.

(1978*a*) have estimated lower potentials, in the range 50–90mV, for illuminated *R. sphaeroides* chromatophores on the basis of $S^{14}CN^-$ uptake. They have also indicated that the extent of the carotenoid shift corresponded to a potential of up to 220mV.

These discrepancies between the size of the membrane potential calculated on the basis of SCN^- uptake or carotenoid shift prompted us to make a more extensive comparison of the two methods for estimating not only the light-dependent potential, but also the potential generated by both succinate and NADH oxidation. The results of this investigation into which of the two methods is the more reliable are the subject of the present paper. We have used chromatophores from the Ga mutant of *R. sphaeroides* which has spectroscopic properties that make it a favoured organism for much current work on electron transport in photosynthetic bacteria (Dutton *et al.*, 1978; Takamiya & Dutton, 1977).

Materials and Methods

Growth of cells and preparation of chromatophores

The green mutant of *R. sphaeroides*, Ga (Sistrom & Clayton, 1964), was grown photosynthetically in the medium described by Sistrom (1960) illuminated anaerobically by incandescent lamps. The cells were washed in a sodium- and potassium-free medium containing 100mM-choline chloride/20mM-Mops, adjusted to pH7.0 with tetramethylammonium hydroxide. Approximately one-half of the cells were resuspended in the same buffer and disrupted by passing twice through a French pressure cell (1.52×10^5kPa). Chromatophores were prepared from the disrupted cells by differential centrifugation as described by Connelly *et al.* (1973) and were resuspended in the same choline chloride/Mops buffer. The remaining washed cells were resuspended in a medium containing 200mM-sucrose and 20mM-Mops titrated to pH7.0 with tetramethylammonium hydroxide. Chromatophores were prepared by the above procedure and finally resuspended in the sucrose/Mops buffer.

Measurement of the carotenoid absorption shift

Recordings of the light-induced carotenoid shift at 475–490nm were carried out on a dual-beam spectrophotometer with actinic illumination, through a Kodak Wratten filter number 88A which transmits light with wavelengths above 730nm, at 90° to the measuring beam. The photomultiplier was screened from the actinic illumination by 1cm of concentrated $CuSO_4$ solution. The respiration-induced carotenoid shift was measured at the same wavelengths, but without actinic illumination. The sample cell was maintained at room temperature by circulating water.

Measurement of $S^{14}CN^-$ and [^{14}C]methylamine uptake

The flow-dialysis method described by Kell *et al.* (1978*a*) and Sorgato *et al.* (1978) was used. Chromatophores in the flow-dialysis cell at room temperature were illuminated by a 300W (incorrectly described as 500W by Kell *et al.*, 1978*a*) tungsten/halogen bulb from which the light was filtered through a Kodak Cinemoid 5A deep-orange filter (transmits above approx. 550nm) and 5cm of water. Where appropriate, daylight was excluded from the cell by covering it with aluminium foil. The extent of $S^{14}CN^-$ or [^{14}C]methylamine uptake was related to the membrane potential ($\Delta\psi$) or pH gradient (ΔpH) with the assumptions discussed elsewhere (Kell *et al.*, 1978*a*; Sorgato *et al.*, 1978), and using a value of 22μl/mg of bacteriochlorophyll for the internal volume of the chromatophores. This value was calculated from the data of Packham *et al.* (1978) assuming a mean chromatophore diameter of 40nm and 1000 molecules of bacteriochlorophyll per chromatophore. The use of alternative estimates of the internal volume is dealt with in the Discussion section. The flow-dialysis experiments were completed approx. 24h after measurement of the carotenoid absorbance changes.

Bacteriochlorophyll and protein determinations

Bacteriochlorophyll was determined after extraction into acetone/methanol (7:2, v/v) and using the millimolar absorption coefficient ($\varepsilon_{772}^{1cm} = 75$) given by Clayton (1963). Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin (Cohn fraction V) as standard.

Respiration rates

A Clark-type oxygen electrode (Hansatech, King's Lynn, U.K.) was used.

Reagents

Chemicals and radiochemicals were obtained from the sources specified previously (Kell *et al.*, 1978*a*; Sorgato *et al.*, 1978). Volatile amines were removed from the choline chloride by rotary evaporation.

Results

Comparison of the light-induced carotenoid absorption shift with $S^{14}CN^-$ uptake

In order to use the carotenoid band shift as a quantitative indicator of $\Delta\psi$ the shift must first be calibrated by generating K^+ diffusion potentials across the chromatophore membrane (Jackson & Crofts, 1969). Fig. 1 shows a calibration plot for chromatophores prepared in, and resuspended in, the choline chloride/Mops medium. These results are similar to those reported originally by Jackson & Crofts (1969) for *R. sphaeroides* and more recently by Takamiya & Dutton (1977) for *R. sphaeroides* Ga. The slope of Fig. 1 is used to give the spectral change per decade of K^+-concentration difference, or per 60mV from the Nernst equation (Jackson & Crofts, 1969).

Continuous illumination of chromatophores from

the same preparation that was used for the experiments in Fig. 1 caused a shift in the carotenoid spectrum (Fig. 2), with a time course resembling that reported by Jackson & Crofts (1969, 1971). After a rapid decay from a spike, the extent of the carotenoid band shift almost came to a steady state, although the residual decay was never completely arrested. At the spike immediately after illumination the shift indicated a $\Delta\psi$ of 220mV, and after 6min corresponded to 130mV, if the shift was measured relative to the baseline before illumination. Alternative estimates of $\Delta\Psi$ of 150 and 190mV can be made by referring to the baseline after addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone as uncoupler, or after darkening subsequent to addition of the uncoupler (Fig. 2). The reasons for failure to return to the original baseline after uncoupling and/or darkening may be connected with the observation that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone does not totally abolish the carotenoid shift (Jackson & Crofts, 1971), and that the same uncoupler can induce proton-diffusion potentials in the dark (Jackson & Crofts, 1969). Therefore in the present paper we shall in general estimate $\Delta\psi$ from the extent of the carotenoid shift relative to the baseline before illumination as seems to have been the practice of other workers (Jackson & Crofts, 1969, 1971; Baccarini Melandri *et al.*, 1977). $\Delta\psi$ estimated at the spike and in the steady state is lower than some values obtained by Crofts & Jackson (1970) under comparable conditions, but is close to $\Delta\psi$ found by them for other preparations of chromatophores.

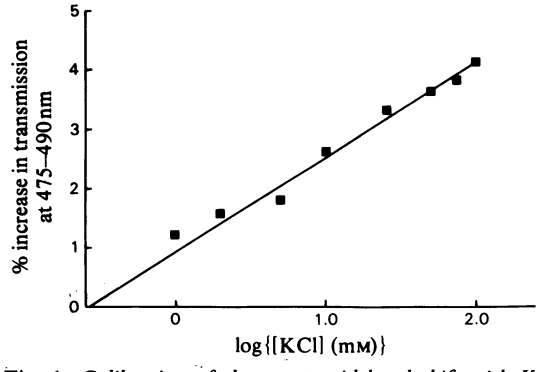

Fig. 1. *Calibration of the carotenoid band shift with K^+ diffusion potentials*
Chromatophores (from the preparation isolated in choline chloride/Mops) containing 26μg of bacteriochlorophyll were suspended in 3ml of the sodium- and potassium-free 20mM-Mops/100mM-choline chloride buffer. KCl pulses were added to give the final K^+ concentration shown on the abscissa. The concentration of valinomycin was 0.66μg/ml.

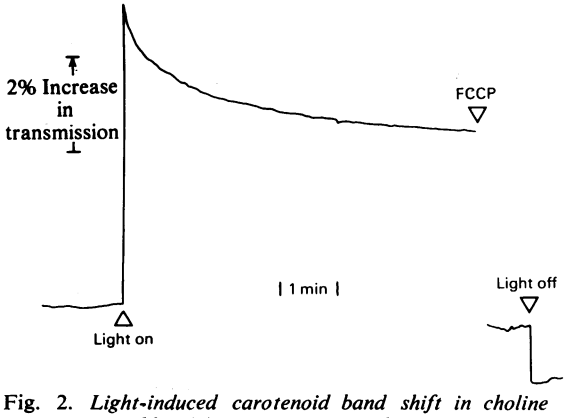

Fig. 2. *Light-induced carotenoid band shift in choline chloride/Mops reaction medium*
Chromatophores (26μg of bacteriochlorophyll) were suspended in 3ml of the choline chloride/Mops buffer; 6.6μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added as indicated. Addition of the same amount of uncoupler to a cuvette containing chromatophores in the dark did not cause any change in transmittance.

Illuminated chromatophores, from the same preparation as used in the carotenoid shift experiments and suspended in the choline chloride/Mops medium, took up only a very small amount of $S^{14}CN^-$, as shown by the slight efflux of $S^{14}CN^-$, monitored by flow dialysis, upon adding uncoupler and darkening (Fig. 3). The amount of $S^{14}CN^-$ up-

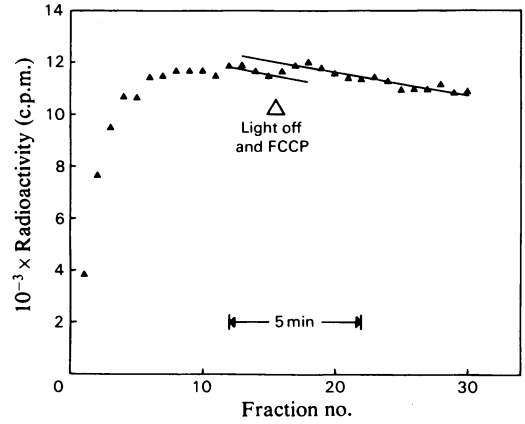

Fig. 3. *Light-induced uptake of $S^{14}CN^-$ by chromatophores suspended in choline chloride/Mops reaction medium*
The upper chamber of the flow-dialysis cell contained in a final volume of 1ml: 20μM-$KS^{14}CN$ (60μCi/μmol), 100mM-choline chloride, 20mM-Mops, pH 7.0, and chromatophores containing 0.83mg of bacteriochlorophyll. The chromatophores were illuminated from the start of the experiment; the light was turned off, daylight excluded, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5μM) was added as shown.

take corresponded to the $\Delta\psi$ of approx. 20mV. This result is clearly at variance with that obtained from the carotenoid band-shift measurements which were made after a similar period of illumination to that used in the flow-dialysis experiments.

The low $S^{14}CN^-$ uptake observed in the presence of 100mM-choline chloride suggested that at such a high Cl^- concentration this anion can move into the lumen of chromatophores at a sufficient rate to neutralize the charge separation achieved by H^+ movement, as also proposed by Jackson & Crofts (1971) and Michels & Konings (1978*a*) for chromatophores. If this is the case then illumination of the chromatophores should generate a substantial ΔpH, and this expectation was confirmed by an experiment to detect [^{14}C]methylamine uptake by flow dialysis. Fig. 4 shows that upon darkening and adding uncoupler to previously illuminated chromatophores there was a marked efflux of [^{14}C]methylamine which corresponded to a ΔpH of 1.9 unit (≡ 115mV).

A high concentration of bacteriochlorophyll (0.83mg/ml) was used for the experiment shown in Fig. 3, as at lower concentrations of bacteriochlorophyll (e.g. 0.2mg/ml) no $S^{14}CN^-$ uptake could be detected in the choline chloride medium, although in the P_i/Tris medium $S^{14}CN^-$ uptake was readily detected with this concentration of bacteriochlorophyll (e.g. Fig. 5). In the absence of detectable $S^{14}CN^-$ uptake at 0.2mg of bacteriochlorophyll/ml of the choline chloride medium it is possible only to place an upper limit on the value of $\Delta\psi$, which in this instance was estimated at approx. 50mV. The measurements made with 0.83mg of bacteriochlorophyll/ml (Fig. 3) showed that $S^{14}CN^-$ uptake corresponded to 20mV, but because the extent of uptake of $S^{14}CN^-$ was so small we were not able to establish that the same value of $\Delta\psi$ was obtained over a range of bacteriochlorophyll concentrations. Therefore we cannot be sure that the light intensity was sufficient at 0.83mg of bacteriochlorophyll/ml to generate the maximum value of the membrane potential. However, as substantial methylamine uptake could be observed upon illuminating chromatophores at 0.83mg/ml (Fig. 4), and as (with a different preparation of chromatophores) an only slightly greater extent of methylamine uptake was observed with 0.2mg of bacteriochlorophyll/ml, it appears that inadequate light-intensity was, at most, only a minor factor in contributing to the low value observed for $\Delta\psi$ (Fig. 3).

Kell *et al.* (1978*a*) observed a substantial light-dependent uptake of $S^{14}CN^-$ into chromatophores of *Rhodospirillum rubrum* that were suspended in a P_i/Tris reaction medium. Fig. 5 shows that when *R. sphaeroides* chromatophores, which had been isolated in Mops/sucrose buffer, were suspended in a similar

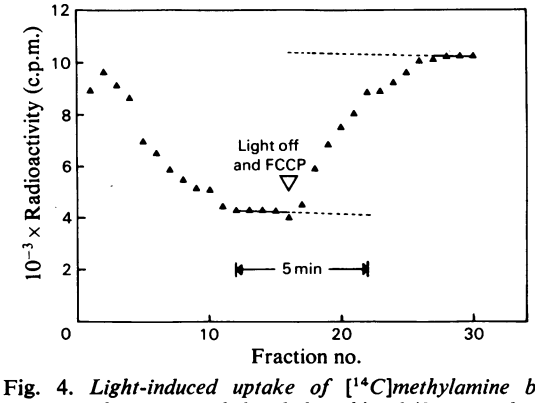

Fig. 4. *Light-induced uptake of* [^{14}C]*methylamine by chromatophores suspended in choline chloride/Mops medium*
The upper chamber of the flow-dialysis cell contained in a final volume of 1ml: 20μM-[^{14}C]methylamine hydrochloride (55.5μCi/μmol), 100mM-choline chloride, 20mM-Mops, pH7.0, and chromatophores containing 0.83mg of bacteriochlorophyll. The chromatophores were illuminated from the start of the experiment; the light was turned off, daylight excluded, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5μm) was added as shown.

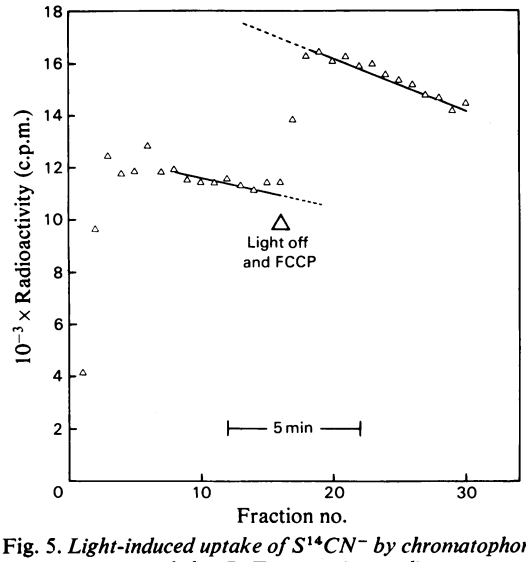

Fig. 5. *Light-induced uptake of* $S^{14}CN^-$ *by chromatophores suspended in* P_i/*Tris reaction medium*
The upper chamber of the flow-dialysis cell contained in a final volume of 1ml: 20μM-K$S^{14}CN$ (60μCi/μmol), 10mM P_i/Tris, 5mM-magnesium acetate and chromatophores (from the preparation isolated in sucrose/Mops) containing 0.125mg of bacteriochlorophyll. The pH was 7.3. The chromatophores were illuminated from the start of the experiment; the light was turned off, daylight excluded, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5μM) was added as shown.

P_i/Tris reaction medium a large amount of light-dependent $S^{14}CN^-$ uptake could be detected, from which $\Delta\psi$ was estimated to be approx. 140mV. The larger extent of $S^{14}CN^-$ uptake in the P_i/Tris medium relative to media containing high concentrations of Cl^- has been attributed to a very limited conversion of $\Delta\psi$ into ΔpH in the P_i/Tris medium (Sorgato *et al.*, 1978; Sorgato & Ferguson, 1978). However, unlike *R. rubrum* chromatophores (Kell *et al.*, 1978*a*), submitochondrial particles (Sorgato *et al.*, 1978) and vesicles from *Paracoccus denitrificans* (Kell *et al.*, 1978*c*), the *R. sphaeroides* chromatophores also generated a substantial ΔpH of approx. 1.4 unit ($\equiv$ 85mV) when energized in the P_i/Tris-based reaction medium, as shown by the light-dependent uptake of [^{14}C]-methylamine under these conditions (Fig. 6).

When a sample of the same preparation of chromatophores that was used for the experiments of Figs. 5 and 6 was illuminated in the spectrophotometer, using the P_i/Tris reaction medium, the carotenoid-band shift shown in Fig. 7 was observed. Direct calibration of the carotenoid shift in P_i/Tris medium with K^+-diffusion potentials was not attempted as this would involve unacceptably large variations in the ionic strength. However, as the chromatophores used for the experiment shown in Fig. 7 were prepared from the same batch culture of cells as the chromatophores used in the experiments of Figs. 1 and 2 (see the Materials and Methods section), an approximate relationship between the carotenoid shift in P_i/Tris and $\Delta\Psi$ can be made by reference to Fig. 1 with proportional correction for the amount of bacteriochlorophyll present. On this basis $\Delta\psi$ values of 450 and 300mV can be estimated at the spike and in the steady state of the carotenoid shift respectively. Again there is a very poor quantitative agreement between the two methods for estimating $\Delta\psi$, although both methods do indicate a higher $\Delta\psi$ in P_i/Tris relative to the choline chloride/Mops reaction medium.

In order to observe substantial amounts of $S^{14}CN^-$ (or methylamine) uptake the concentrations of bacteriochlorophyll used in the flow-dialysis experiments were higher than those used in the carotenoid band-shift experiments. It is important therefore to consider in some detail whether the discrepancy between the values of the membrane potential obtained from the two types of experiment might, at least in part, be ascribed to use of a light intensity that was insufficient to generate the maximum membrane potential in the flow-dialysis experiments.

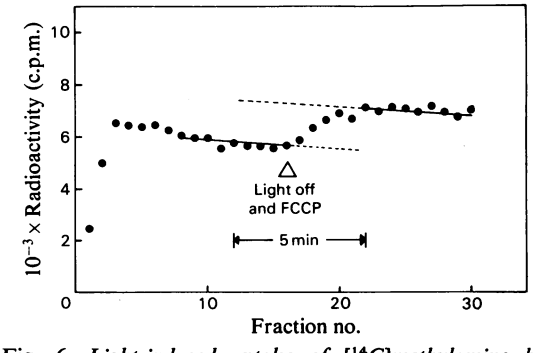

Fig. 6. *Light-induced uptake of [^{14}C]methylamine by chromatophores suspended in P_i/Tris reaction medium*
The upper chamber of the flow-dialysis cell contained in a final volume of 1ml: 20μM-[^{14}C]methylamine hydrochloride (55.5μCi/μmol), 10mM-P_i/Tris, 5mM-magnesium acetate and chromatophores (from the preparation isolated in sucrose/Mops) containing 0.5mg of bacteriochlorophyll. The pH was 7.3. The chromatophores were illuminated from the start of the experiment; the light was turned off, daylight excluded, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5μM) was added as shown.

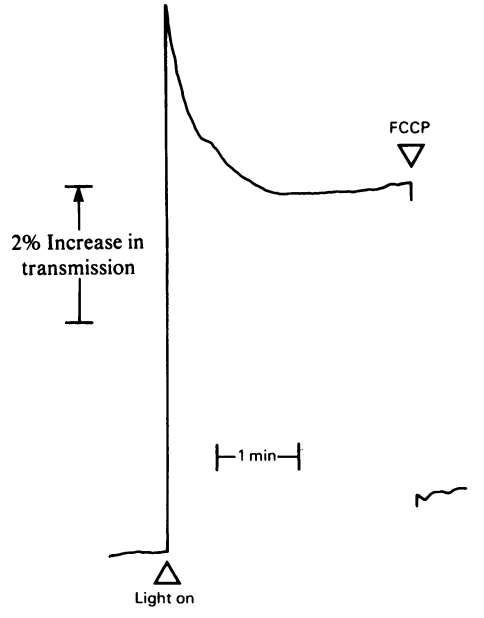

Fig. 7. *Light-induced carotenoid band shift in P_i/Tris reaction medium*
Chromatophores (from the preparation isolated in sucrose/Mops) containing 17μg of bacteriochlorophyll were suspended in 3ml of the 10mM-P_i/Tris, 5mM-magnesium acetate buffer, pH7.0; 6.6μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added as indicated. No change in the spectrum was observed upon addition of additional Mops/sucrose buffer to give the same final concentration as was introduced into the flow-dialysis cell (cf. Fig. 5) from the stock suspension of chromatophores.

The light-induced uptake of SCN^- into the chromatophores suspended in the P_i/Tris medium was measured with a bacteriochlorophyll concentration of 0.125mg/ml (Fig. 5) and also (not shown) at 0.25mg/ml. In these two experiments 47% and 64% of the total $S^{14}CN^-$ was taken up upon illumination, corresponding to a potential of 140mV in each case. The $S^{14}CN^-$ uptake is very sensitive to small changes of $\Delta\psi$ in this range (see Fig. 1 of Kell *et al.*, 1978*a*), and thus if the light intensity had been insufficient to support the full extent of $S^{14}CN^-$ uptake, the membrane potential measured with 0.25mg of bacteriochlorophyll would have been lower than that measured with 0.125mg. It should also be pointed out that under the same experimental conditions the light-induced uptake of $S^{14}CN^-$ into *R. rubrum* chromatophores was found to indicate a constant $\Delta\psi$ over a range of bacteriochlorophyll concentrations from 0.05 to 0.25mg of bacteriochlorophyll/ml (Kell *et al.*, 1978*a*). It is also noteworthy that the flow-dialysis cell is cylindrical (1.2cm in diameter) and the contents rapidly stirred to an extent that a small vortex was formed. Hence in a flow-dialysis experiment the chromatophores were only very occasionally exposed to light that had already passed through 1cm of the chromatophore suspension.

The finding that the light intensity was sufficient to develop the full membrane potential does not necessarily imply that electron transport was occurring at its maximum rate. There is increasing evidence that the non-ohmic nature of the protic resistance of energy-conserving membranes results in the upper value of the membrane potential being maintained over a range of electron-transport rates (e.g. Baccarini Melandri *et al.*, 1977; Kell *et al.*, 1978*d*). It is of particular interest that Baccarini Melandri *et al.* (1977) found that the extent of the carotenoid band shift was decreased by only approx. 15% upon diminishing the light intensity by 100-fold. This means that even if the effective light intensity were lower in the flow-dialysis experiments, an appropriate and compensating decrease in the light intensity used in the carotenoid band-shift experiments would not remove the discrepancy in the values of $\Delta\psi$ obtained from the two types of experiment.

Effect of antimycin on the light-induced carotenoid shift and on $S^{14}CN^-$ uptake

Baltscheffsky (1976) has suggested that, at least in *R. rubrum*, only the antimycin-sensitive component of the carotenoid shift is an indicator of membrane potential, but Jackson *et al.* (1975) have presented evidence which they take to be incompatible with this view. In view of this disagreement, and the finding from earlier work that antimycin abolished the light-dependent uptake of $S^{14}CN^-$ into *R. rubrum* chromatophores (Kell *et al.*, 1978*a*), the effect of antimycin on both the carotenoid shift and $S^{14}CN^-$ uptake in *R. sphaeroides* chromatophores was examined.

Fig. 8 shows the time course of the carotenoid shift that was observed in P_i/Tris buffer in the presence of antimycin, which inhibits electron flow between the *b* and *c* cytochromes. This result is consistent with the data of Jackson & Crofts (1971) who showed that antimycin: (i) had no effect on the fastest phase of the development of the carotenoid shift; (ii) partially inhibited the slowest phase, and partially decreased the extent of an intermediate phase; (iii) prevented the decay phase of the carotenoid shift. Thus as shown in Fig. 8 the final extent of the light-induced carotenoid change is reached almost immediately after illumination of the chromatophores. The extent of this change, measured relative to the baseline before illumination, corresponded to a $\Delta\psi$ of 260mV compared with 300mV in the same preparation of chromatophores without antimycin (Fig. 7). In agreement with Jackson & Crofts (1971) no carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-insensitive carotenoid shift was observed during

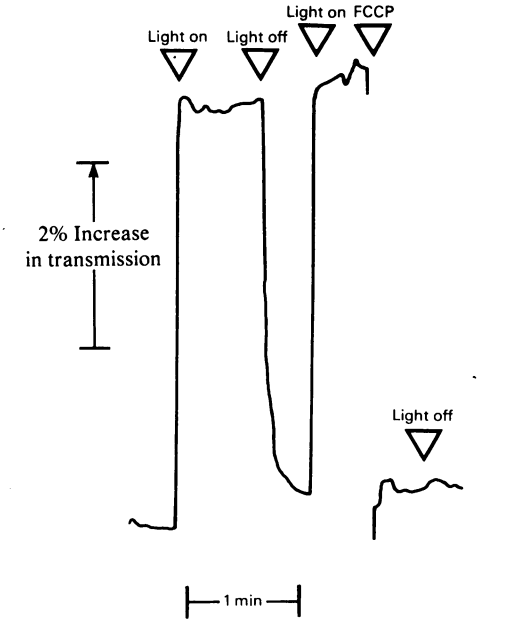

Fig. 8. *Effect of antimycin on the light-induced carotenoid-band shift*

Chromatophores (from the preparation isolated in sucrose/Mops) containing 17μg of bacteriochlorophyll were suspended in 3ml of the 10mM-P_i/Tris, 5mM-magnesium acetate buffer, pH7.0; 2μg of antimycin was present. The light was turned off and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (6.6μM) was added as indicated.

continuous illumination when antimycin was present (Fig. 8).

The effect of antimycin on the uptake of $S^{14}CN^-$ into the same preparation of chromatophores is shown in Fig. 9. The amount of $S^{14}CN^-$ in the outflow from the flow-dialysis cell reached a minimum, corresponding to the maximum uptake of $S^{14}CN^-$ by the chromatophores, but this was followed by a slow increase in the radioactivity in the outflow before addition of uncoupler and darkening. The maximum value of $\Delta\psi$ reached can be estimated from Fig. 9 at approx. 115mV compared with 140mV in the absence of antimycin. The results indicate that unlike in *R. rubrum* antimycin does not abolish the light-dependent uptake of $S^{14}CN^-$ into *R. sphaeroides* chromatophores, and thus there would be no justification for taking only an antimycin-sensitive component of the carotenoid shift as the indicator of $\Delta\psi$ in *R. sphaeroides*.

It remains to be explained why the extent of $S^{14}CN^-$ uptake decreased during continuous illumination (Fig. 9). In mitochondrial membranes antimycin is known to have a secondary uncoupling effect when used in molar excess over its binding site in the cytochrome bc_1 complex (see, e.g., Wikström, 1978). Therefore it is apposite to consider if the data in Fig. 9 might be explained in terms of a slowly developing uncoupling effect of antimycin. The titre of antimycin used, 32μg/mg of bacteriochlorophyll is not unusually high and is less than the 50μg/mg of bacteriochlorophyll used, for instance, by Dutton & Prince (1975). Some clue that our titre of antimycin was not unduly excessive was that the maximum inhibition (65%) of succinate oxidation catalysed by the chromatophores was obtained with 8μg of antimycin/mg of bacteriochlorophyll, which is indicative of the amount of antimycin required to block fully the antimycin-sensitive light-driven cyclic electron flow. A further indication that antimycin was not acting as an uncoupler came from an experiment which was identical with that shown in Fig. 9 except that 100μM-phenazine methosulphate was also present to provide a by-pass around the site of antimycin inhibition. Under these conditions the light-dependent uptake of $S^{14}CN^-$ was fully restored, corresponding to $\Delta\psi$ of 140mV. The amount of antimycin used in the experiment to measure its effect on the carotenoid shift was greater, 120μg/mg of bacteriochlorophyll, than that used in the $S^{14}CN^-$ uptake experiment and yet a stable carotenoid shift was obtained for at least 2min. Any uncoupling effects of antimycin would have been expected to have had a more pronounced effect on the carotenoid shift owing to the larger molar ratio of antimycin used.

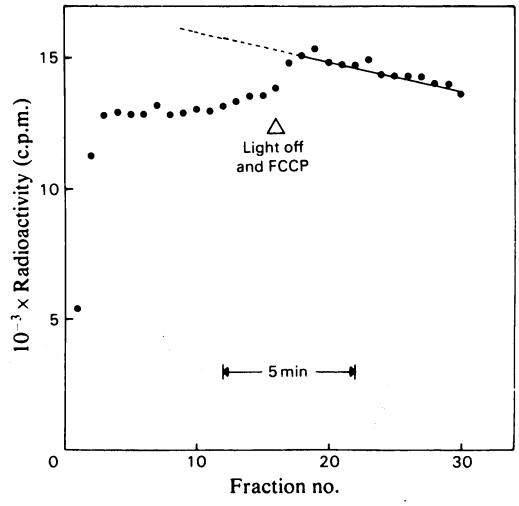

Fig. 9. *Effect of antimycin on the light-induced uptake of $S^{14}CN^-$*

The upper chamber of the flow-dialysis cell contained in a final volume of 1ml: 20μM-$KS^{14}CN$ (60μCi/μmol), 10mM-P_i/Tris, 5mM-magnesium acetate and chromatophores (from the preparation isolated in sucrose/Mops) containing 0.125mg of bacteriochlorophyll. The pH was 7.3 and 4μg of antimycin was added 1min before the chromatophores were illuminated at the start of the experiment. The light was turned off, daylight excluded, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5μM) was added as shown.

Comparison of the carotenoid shift and $S^{14}CN^-$ uptake as indicators of membrane potential generated by oxidation of either NADH or succinate

The data presented so far could be taken to indicate either that $S^{14}CN^-$ uptake underestimates $\Delta\psi$ or that the carotenoid shift responds to factors other than bulk-phase membrane potential. In this last respect it has been suggested that at least a part of the carotenoid-band shift may reflect local charge separation within the reaction centres rather than a bulk-phase transmembrane potential (e.g. Chance & Baltscheffsky, 1975). If this were the case it might be predicted that energization of the chromatophore membrane other than by illumination would cause a less extensive carotenoid shift. Fig. 10(*a*) shows that addition of succinate to a suspension of chromatophores produced only a very small carotenoid shift compared with the shift observed on illuminating an identical sample of the same preparation of chromatophores (Fig. 7). Similarly oxidation of NADH also induced a very small carotenoid shift (Fig. 10*b*), which is emphasized by the magnitude of the carotenoid shift that was observed on illuminating the chromatophores after addition of NADH (Fig. 10*b*). In other experiments it was shown that the carotenoid changes after addition of the respiratory-chain sub-

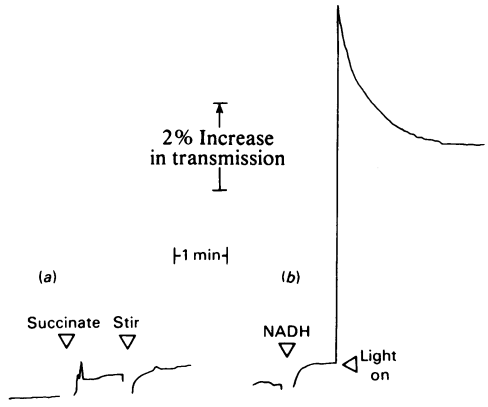

Fig. 10. *Respiration-induced carotenoid band shift*
Chromatophores (from the preparation isolated in sucrose/Mops) containing 17μg of bacteriochlorophyll were suspended in 3ml of the 10mM-P_i/Tris, 5mM-magnesium acetate buffer, pH7.0; (*a*) 10mM-sodium succinate was added as shown; (*b*) 1% (v/v) ethanol, 0.15mg of alcohol dehydrogenase were also present and 0.6mM-NAD^+ was added to start the reaction. The chromatophores were subsequently illuminated as indicated.

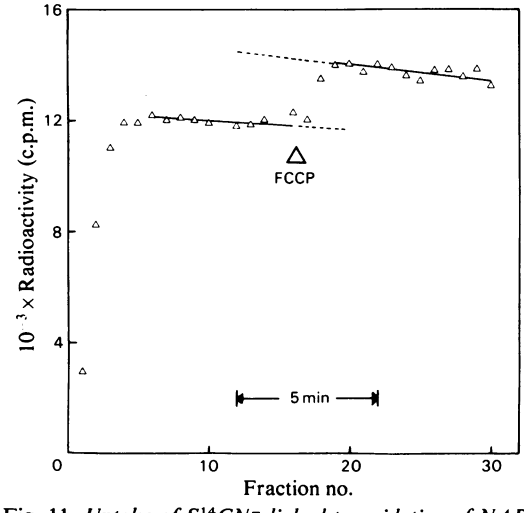

Fig. 11. *Uptake of $S^{14}CN^-$ linked to oxidation of NADH*
The upper chamber of the flow-dialysis cell contained in a final volume of 1ml: 20μM-$KS^{14}CN$ (60μCi/μmol), 0.6mM-NAD^+, 1% (v/v) ethanol, 0.1mg of alcohol dehydrogenase, chromatophores (from the preparation isolated in Mops/sucrose) containing 0.25mg of bacteriochlorophyll, 10mM-P_i/Tris and 5mM-magnesium acetate. The pH was 7.3 and daylight was excluded from the cell throughout the experiment. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5μM) was added as shown. The respiration rate under these conditions was 0.32μg-atom of O/min per mg of bacteriochlorophyll.

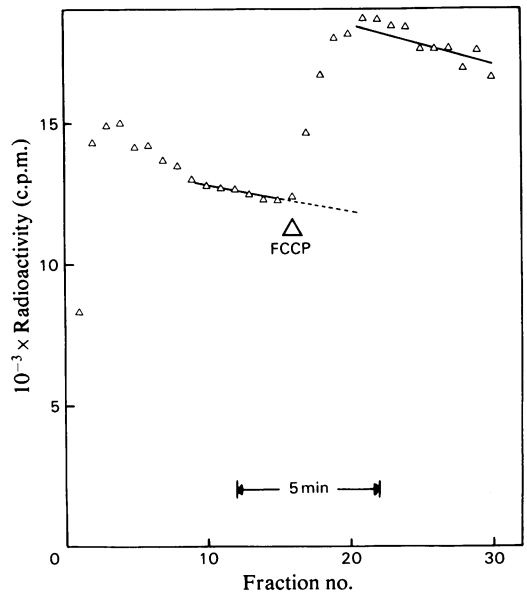

Fig. 12. *Uptake of $S^{14}CN^-$ linked to oxidation of succinate*
The upper chamber of the flow-dialysis cell contained in a final volume of 1ml: 20μM-$KS^{14}CN$ (60μCi/μmol), 10mM-sodium succinate, chromatophores (from the preparation isolated in Mops/sucrose) containing 0.25mg of bacteriochlorophyll, 10mM-P_i/Tris and 5mM-magnesium acetate. The pH was 7.3 and daylight was excluded from the cell throughout the experiment. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (5μM) was added as shown. The respiration rate under these conditions was 1.3μg-atom of O/min per mg of bacteriochlorophyll.

strates were reversed by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Flow-dialysis experiments revealed that the chromatophores accumulated considerable amounts of $S^{14}CN^-$ upon addition of NADH or succinate in the dark (Figs. 11 and 12). Table 1 summarizes the values for $\Delta\psi$ obtained with a single preparation of chromatophores from both the carotenoid shift and $S^{14}CN^-$ uptake using light, NADH or succinate as the source of energy. The $S^{14}CN^-$-uptake method gives a higher value for the substrate-dependent membrane potential, whereas carotenoid change gives the larger value for the light-dependent membrane potential. The finding that SCN^- uptake indicates a larger $\Delta\psi$ than the carotenoid shift when NADH or succinate is substrate strongly militates against an argument that SCN^- uptake systematically underestimates $\Delta\psi$.

As judged by the extent of $S^{14}CN^-$ uptake the respiration-dependent membrane potential is not

Table 1. *Comparison of the values of* $\Delta\Psi$ *determined from the carotenoid shift with the values found from* $S^{14}CN^-$ *uptake*

The same preparation of chromatophores, isolated in Mops/sucrose, was used for all the experiments, which were carried out in 10mM-P_i/Tris/5mM-magnesium acetate, pH7.3. Experimental conditions are as described in Figs. 5, 7, 10, 11 and 12 and the values of $\Delta\Psi$ were calculated as described in the text.

	$\Delta\Psi$ (mV)	
Mode of energization	Carotenoid change	$S^{14}CN^-$ uptake
Light	300	140
NADH	22	95
Succinate	28	120

very much less than the light-induced potential. At first sight this observation may seem surprising as the rate of photophosphorylation catalysed by chromatophores is severalfold higher than the rate of oxidative phosphorylation. For example, K. M. Plewis & O. T. G. Jones (unpublished work) found that chromatophores from *R. sphaeroides* GVP catalysed a photophosphorylation rate of 52nmol/min per mg of protein (equivalent to 856nmol/min per mg of bacteriochlorophyll), whereas the rates of NADH and succinate driven oxidative phosphorylation were 18.4nmol/min per mg of protein and 10.6nmol/min per mg of protein with respective P/O values of 0.32 and 0.2. However, the relationship between the rate of ATP synthesis and the magnitude of the membrane potential (or protonmotive force) is complex (Baccarini Melandri *et al.*, 1977; Kell *et al.*, 1978*d*), and has not yet been fully characterized. There is at present no evidence that a decrease in phosphorylation rate should necessarily be matched by a proportional decrease in membrane potential or protonmotive force, and thus the comparable sizes of the light-induced and succinate-oxidation-dependent $S^{14}CN^-$ uptake (Table 1) do not mean that the rates of ATP synthesis under these two sets of conditions will be comparable.

Discussion

The conclusion from the experiments in the present paper is that the carotenoid band shift and $S^{14}CN^-$ uptake respond to different events. Ideally $S^{14}CN^-$ uptake should reflect only the difference in membrane potential between the two bulk aqueous phases on either side of the chromatophore membrane provided that (i) SCN^- does not bind to the membrane in significant amounts, (ii) SCN^- does not perturb the potential and (iii) SCN^- movement is sufficiently rapid to permit full extent of uptake to be observed during the period of the experiment. It has been discussed at length elsewhere (Kell *et al.*, 1978*a*; Sorgato *et al.*, 1978) that these criteria are met by the SCN^- ion-uptake procedure; here we would just point out that studies with an ion-specific electrode (cf. Kell *et al.*, 1978*b*) have shown that SCN^- is taken up into illuminated *R. sphaeroides* chromatophores in Tris/acetate buffer with a half-time of 45s (D. B. Kell, unpublished observations). We defer consideration of whether our quantitative estimates of $\Delta\psi$ from $S^{14}CN^-$ uptake are likely to be valid, but will analyse our results with the carotenoid band shift on the basis that SCN^- uptake is at least a reliable guide to the relative magnitude of $\Delta\psi$.

If SCN^- uptake is taken as the indicator of $\Delta\psi$, an explanation must be sought for the light-induced carotenoid shift which involves factors other than $\Delta\psi$. The same problem of whether the carotenoid change might respond to other factors than membrane potential has also arisen in studies with thylakoids in which ion-uptake studies indicate a very small $\Delta\psi$ during steady-state illumination (Rottenberg *et al.*, 1972; Schröder *et al.*, 1972; Vredenberg & Tong, 1975). Rumberg & Muhle (1976) have pointed out that in thylakoids a carotenoid shift could be sensitive to a potential difference between the two surfaces of the membrane in circumstances where no bulk-phase membrane potential existed. Specifically they suggested that the large influx of protons into thylakoids results in an increase in positive charge on the interior surface of the membrane, while the external surface retained a more negative charge. Thus a gradient of potential between the two surfaces is detected by the carotenoids although $\Delta\psi$ is zero. A similar explanation could explain the observation in the present work of a carotenoid shift in illuminated chromatophores suspended in choline chloride (Fig. 2), since a large ΔpH, and thus substantial proton accumulation inside the chromatophores, is developed under these conditions. A contribution to carotenoid shifts from changes in surface potential has also been suggested by Young (1974).

Whereas the magnitude of the light-induced carotenoid shift may be suggested to respond to both membrane potential and surface potential, the small carotenoid shift, but relatively large $\Delta\psi$, with NADH or succinate oxidation is not readily accommodated within such a mechanism. The very small substrate-induced carotenoid shift raises the possibility that the carotenoid changes are, at least in part, responding to changes in the reaction centres. The same interpretation might also be applied to the results of Baccarini Melandri *et al.* (1977) and of Casadio *et al.* (1978). These workers found that with chromatophores from *R. capsulata* the light-induced carotenoid shift corresponded to a potential of 240mV, whereas under the same conditions the extent of quenching of 9-aminoacridine fluorescence corresponded to 199mV (Baccarini Melandri *et al.*, 1977). Under the same reaction conditions ATP hydrolysis caused a shift

which corresponded to a potential of 30mV, but the 9-aminoacridine fluorescence indicated a pH gradient of 160mV. Thus, although a similar pH gradient was seen with either light or ATP as the energy source, the carotenoid change was markedly less when the membrane was energized by ATP rather than light. It is also noteworthy that Case & Parson (1973) found it difficult to interpret their experiments on carotenoid band shifts in *Chromatium vinosum* in terms of a uniform transmembrane electric field.

The notion that the carotenoid changes are influenced by molecular events within the reaction centres gains some support from work with chromatophores from a mutant of *R. sphaeroides*, PM-8, which lacks reaction centres, but has a normal complement of light-harvesting bacteriochlorophyll and carotenoids. These PM-8 chromatophores were found to show no carotenoid shift in response to K^+ diffusion potentials (Sherman & Clayton, 1972). It has also been argued that the linear response of carotenoids to a membrane potential (e.g. Jackson & Crofts, 1969, Fig. 1) can be explained by the existence of a local field arising, for example, from an association between the carotenoids and chlorophyll (Sewe & Reich, 1977). If the linear response in the dark to K^+ diffusion potentials does depend on the interaction of carotenoids with a permanent local field, it could be that the nature of this interaction changes upon illumination, thus making the dark calibration of the carotenoid shift inappropriate for the light-induced shifts. However, although an explanation on these lines could help to explain why the carotenoid shifts indicate a larger light-induced $\Delta\psi$ than $S^{14}CN^-$ uptake, the K^+ diffusion calibration procedure should still be appropriate for the dark NADH- or succinate-dependent shifts, in which case Table 1 would indicate that $S^{14}CN^-$ uptake overestimates the potential. Interpretation of the carotenoid shift would be faciliated if the physical basis of the shift were not still uncertain (Crofts & Wood, 1978; Symons *et al.*, 1977). On the basis of the data in the present paper it seems appropriate to agree with Chance (1977) that 'what, where and how the carotenoids measure is undetermined'. It is also appropriate to point out that the arguments discussed in the present paper relate to the steady state. As pointed out by Rumberg & Muhle (1976), the carotenoid change could reflect changes in membrane potential during short flashes of light when changes of surface potential might not occur.

The values of $\Delta\psi$ and ΔpH estimated from the extent of SCN^- and methylamine uptake require further comment. The magnitude of the total protonmotive force ($\Delta\psi+\Delta$pH), 225mV, estimated in the P_i/Tris reaction medium is considerably higher than the values (100–138mV) found by Michels & Konings (1978*a*) for illuminated *R. sphaeroides* chromatophores in a variety of reaction media. Several factors may be suggested to account for this discrepancy. As we were not primarily concerned with the absolute values of $\Delta\psi$ and ΔpH in the present work we used an internal volume of 22μl/mg of bacteriochlorophyll, which was calculated from the data of Packham *et al.* (1978), who prepared chromatophores by sonication. This is a rather low value for the internal volume and so might lead to an overestimate of $\Delta\psi$ and ΔpH when, as in the present work, chromatophores are prepared with a French pressure cell. Parenthetically we note that Casadio *et al.* (1974*b*) estimated the internal volume of *R. capsulata* as 150μl/mg of bacteriochlorophyll, but the data of Packham *et al.* (1978) for *R. capsulata* give a value of approx. 25μl. Michels & Konings (1978*a*) calculated their data using a value of 1.2μl/mg of protein, and this would correspond to 34μl/mg of bacteriochlorophyll in our chromatophores, which were found to have approx. 35μg of bacteriochlorophyll/mg of protein. As substitution of the internal volume used by Michels & Konings (1978*a*) for our value would decrease the total protonmotive force by only approx. 20mV the difference in the magnitude of the protonmotive force cannot be ascribed to differences in the estimated internal volume alone. Michels & Konings (1978*a*) observed rather considerable variation (40%) in the size of the protonmotive force depending on the nature of the reaction mixture. Thus as the reaction mixture (P_i/Tris/magnesium acetate) used in the present work was different from the reaction mixtures used by Michels & Konings (1978*a*), this may be a reason for the discrepancy. Alternatively, compared with the present work the very different buffers used by Michels & Konings (1978*a*,*b*) for isolating and storing chromatophores might also contribute to the differences in the protonmotive force. This last factor might also be relevant in considering why the *R. sphaeroides* chromatophores used in the present work generated a much higher ΔpH than the *R. rubrum* chromatophores (Kell *et al.*, 1978*a*) or submitochondrial particles (Sorgato *et al.*, 1978) in a very similar reaction medium.

Michels & Konings (1978*a*) offered no evidence that the light intensity was sufficient at the high concentrations of chromatophores used in their flow-dialysis experiments. Hence another reason for the lower values of $\Delta\Psi$ reported by Michels & Konings (1978*a*) could be a consequence of a failure to use a saturating light intensity.

We discussed earlier possible interpretations of the effect of antimycin on light-induced $S^{14}CN^-$ uptake. A further point of interest is to consider how a membrane potential can be maintained for several minutes in the presence of antimycin, as after a very short time cytochrome *c* should be fully oxidized and electron flow terminated (Jackson & Dutton, 1973), so that just as when the light is turned off the potential should decay rather rapidly. Two possibilities are

that either the potential is maintained by some electrons leaking past the site of antimycin inhibition, or that a distinct antimycin-insensitive pathway for the electrons is available (Jackson, 1974). The second explanation might also provide an alternative explanation for the decay of $S^{14}CN^-$ uptake. If electrons passing through the antimycin-insensitive pathway are able to flow to oxygen (cf. Connelly *et al.*, 1973) at a comparable rate with the rate of cyclic electron flow, then, under the conditions used for the experiment of Fig. 9, electrons may be gradually lost to oxygen from the cyclic electron-transport system, thus causing a decay in $\Delta\psi$ as the rate of cyclic electron flow decreases. The effects of antimycin merit further investigation.

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