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MEMBRANE-POTENTIAL- AND SURFACE-POTENTIAL-INDUCED ABSORBANCE CHANGES OF MEROCYANINE DYES ADDED TO CHROMATOPHORES FROM *RHODOPSEUDOMONAS SPHAEROIDES*

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(1) Three analogs of merocyanine dyes added to suspensions of chromatophore vesicles showed absorbance changes responding to the change in surface potential induced by salt addition and to the change in membrane potential induced by illumination. (2) The extent of the light-induced absorbance changes of the dyes was linearly related, in the presence and absence of uncouplers, to that of carotenoid spectral shift which is an intrinsic probe of the intramembrane electric field. (3) Comparison of the merocyanine absorbance changes induced by salt addition with those induced by illumination indicated that the surface potential change in the outer surface of chromatophore membranes during illumination was very small. (4) Judging from the spectra of these absorbance and from the low permeabilities of the dyes to membrane, the absorbance change are attributed to change in distribution of the dyes between the medium and the outer surface region in chromatophore membranes. The extent of the light-induced absorbance changes of merocyanine dyes depended on the salt concentration of the medium. The types of dependence were different among three merocyanine analogs. This is explained by the mechanism mentioned above assuming appropriate parameters. It is suggested that, under continuous illumination, an equilibrium of the electrochemical potential of H^+ is reached between the bulk aqueous phase and the outer surface region in the membrane where the merocyanine dyes are distributed.

Introduction

In energy transduction in biomembranes, electron transport induces an electrochemical potential difference of H^+, in which electrical potential difference is one of the parameters. The potential difference between the two surfaces of the membrane and that between the two aqueous phases separated by the membrane (membrane potential) are important in energy transduction in biomembranes. The two potential differences do not necessarily coincide. The surface potential, the potential difference between the bulk aqueous phase and the membrane surface, is formed due to the presence of fixed electrical charges on the membrane. If the surface potentials on either side of the membrane are different, the membrane potential and the surface-surface potential difference are not equal.

The transmembrane electrical potential difference can be directly measured by an electrode method in some cases. In vesicular membranes which are too small for the electrode method and have no intrinsic probes, the electrical potential changes can be measured by the responses of added extrinsic probes [1, 2]. Various optical probes have been developed [2,3–6] for the rapid and continuous measurement of the

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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membrane potential and are used in photosynthetic membranes [7–10].

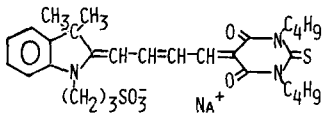
Chromatophores of *Rhodopseudomonas sphaeroides* have an intrinsic electrical potential probe (carotenoid spectral shift). Carotenoid pigments change their absorption spectra in response to changes in the intramembrane electric field induced by changes either in surface potential on salt addition or in membrane potential on illumination or on application of a diffusion potential. It serves as an intrinsic probe to indicate the potential between two surfaces of the membrane (Ref. 11 and Fig. 2). Negatively charged merocyanine dyes, which have been used as probes of membrane potential [3,5,6], were shown to respond to the change in surface potential [13,14], i.e., to act as an extrinsic probe of the potential difference between the dye-distribution site within the membrane near the outer surface and the bulk aqueous phase on the same side. The characteristics of merocyanine response to the surface potential and membrane potential are compared with those of carotenoid spectral shift in chromatophores of *Rps. sphaeroides*.

Materials and Methods

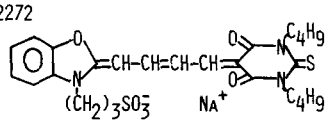
Rps. sphaeroides was grown photosynthetically as described previously [11]. Cells were harvested and washed with a medium containing 2 mM Tricine-NaOH (pH 8.0), 0.01 mM $MgSO_4$ and 1 mM KCl. Chromatophores were prepared by differential centrifugation (between $20\,000 \times g$, 30 min and $100\,000 \times g$, 1.5 h) after the French press disruption of cells at 1000 kg/cm^2 in the medium mentioned above, washed once with the same medium and suspended in the medium.

Time courses of salt- and light-induced absorbance changes of carotenoid and merocyanines were measured with a Hitachi 356 spectrophotometer on a dual-wavelength mode (488 minus 506 nm for carotenoid, 550 minus 590 nm for NK2274, 570 minus 610 nm for NK2272 and 606 minus 582 nm for NK2273) as described previously [11,13]. For measuring the salt-induced absorbance changes, 2–150 μl of 3 M KCl or 1 m $MgSO_4$ were added to chromatophores suspended in 3 ml of a solution containing 2 mM Tricine-NaOH (pH 8.0), 0.01 mM $MgSO_4$ and 1 mM KCl. Absorption spectra of merocyanine dyes

NK2273



NK2272



NK2274

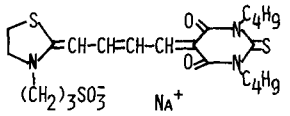


Fig. 1. Structural formulae of merocyanine-thiobarbital dyes.

were measured with a Union Giken SM-401 spectrophotometer equipped with a spectral data processor SM-540.

Merocyanine dyes (three merocyanine-thiobarbital analogs; NK2274, NK2272 (merocyanine-540) and NK2273) have similar formulae and have a negative charge at one end of the molecule due to a sulfonate group (Fig. 1). They were obtained from Nippon Kanko-shikiso Co. Ltd. (Okayama-shi, Okayama 700, Japan).

Results

At pH 8.0, chromatophore membranes have net negative charges on their surfaces and are at negative surface potentials (Fig. 2, see also Ref. 11). Addition of salt induced a positive shift of surface potential of the outer surface and increased the distribution of anionic dye (NK2272) to the membrane [13], reflected in the increased $\Delta A_{570-610}$ (Fig. 3b). The salt addition also changed the intramembrane electric field when the membrane potential was fixed by the presence of CCCP (Fig. 2, dashed line). Then absorbance decreased due to a blue shift of the carotenoid spectrum which corresponded to the outside-positive shift of the intramembrane electric field (Fig. 3a). In contrast, an inside-positive shift of membrane potential was induced by illumination [1] and resulted in a red shift of the carotenoid spectrum (an increase in

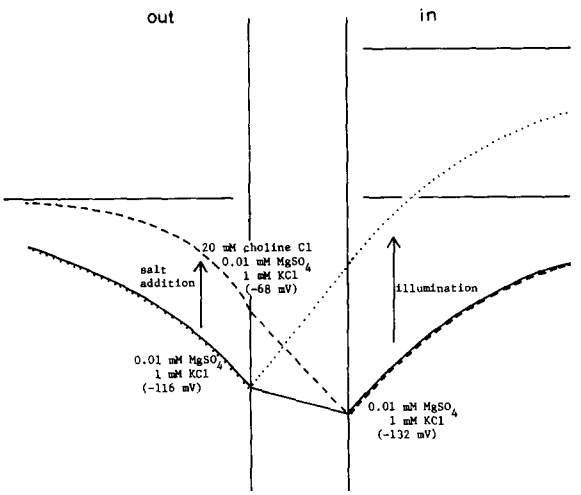


Fig. 2. Schematic diagram of the potential profiles of chromatophore membranes of *Rps. sphaeroides* before and after salt addition (to the outside phase) and before and during illumination. Horizontal lines show the potentials in the bulk phases. (- - - - - -) Potential change induced by salt addition. In this case, the membrane potential between the outer and inner bulk aqueous phases is maintained at zero with CCCP. The arrow designated 'illumination' shows the inside-positive shift of the membrane potential induced by illumination without CCCP (· · · · · ·). The surface potentials were calculated from the Gouy-Chapman equation with the surface charge densities of $-1.9 \cdot 10^{-3}$ and $-2.9 \cdot 10^{-3}$ elementary charge/Å^2 for outer and inner surfaces, respectively [11,12]. The change in the surface charge density induced by the surface pH change was not taken into consideration.

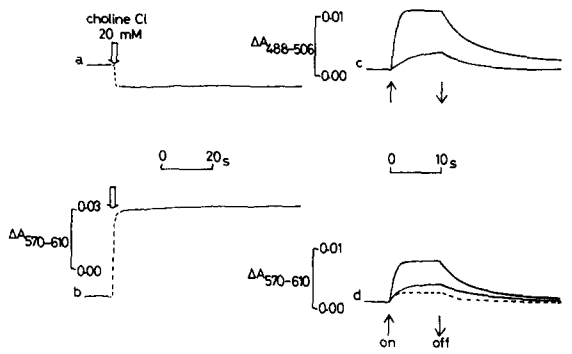


Fig. 3. Time courses of absorbance changes of carotenoid (488 minus 506 nm, upper traces) and merocyanine NK2272 (570 minus 610 nm, lower traces) induced by salt addition and by illumination. Chromatophores (final concentration 10 μM bacteriochlorophyll) were suspended in 3 ml of 2 mM Tricine-NaOH (pH 8.0), 0.01 mM $MgSO_4$, 1 mM KCl and 0.8 μM CCCP (traces a and b) or 2 mM Tricine-NaOH (pH 8.0), 0.01 mM $MgSO_4$ and 10 mM KCl (traces c and d). 3.3 μM NK2272 was present in b and d except in the control (dashed line in d). For traces with smaller deflections in c and d, 0.3 μM valinomycin was present.

$\Delta A_{488-506}$, Fig. 3c; cf. Fig. 2) (In this case CCCP was omitted from the medium.). Upon illumination, merocyanine showed an increase in absorbance which was in the same direction as that seen upon salt addition (Fig. 3d). Prolonged illumination induced a slow increase in NK2272 absorbance which was not completely reversible (not shown). The light-induced responses of merocyanine and carotenoid were suppressed in the presence of valinomycin (Fig. 3c and d). The responses of merocyanine dyes at the concentration used (3.3 μM) did not affect the extent and kinetics of light-induced absorbance changes of carotenoid spectral shift (not shown).

When Triton X-100 (final concentration 0.01%) was added 2–3 min after salt addition, a further increase in merocyanine absorbance was observed to an extent similar to the sum of those induced by the previous dye and salt additions. This fact suggests that the increase in absorbance of NK2272 upon salt addition in the absence of Triton X-100 results from the increase in dye distribution to the outer surface region of chromatophore membranes.

The dependence of the absorbance increase of NK2272 on added salt concentration (Fig. 4) was similar to that of NK2273 in spinach chloroplasts [13]. That divalent ions were more effective than monovalent ions also indicates that the absorbance increase of NK2272 was induced by the change in surface potential. Light-induced membrane potential induced an additional increase in NK2272 absorbance (Fig. 3d; Fig. 4, dashed curve). The extent of the light-induced absorbance change depended on the salt concentration of the medium, but was much smaller than that induced by salt addition.

The spectra of the absorbance changes of NK2272 observed upon salt additions, illumination and application of diffusion potentials showed similar forms with a peak at 570 nm (Fig. 5). The peak wavelength corresponded to that of the peak of monomeric NK2272 adsorbed on liposome membranes [5,6]. Application of the diffusion potential of K^+ by

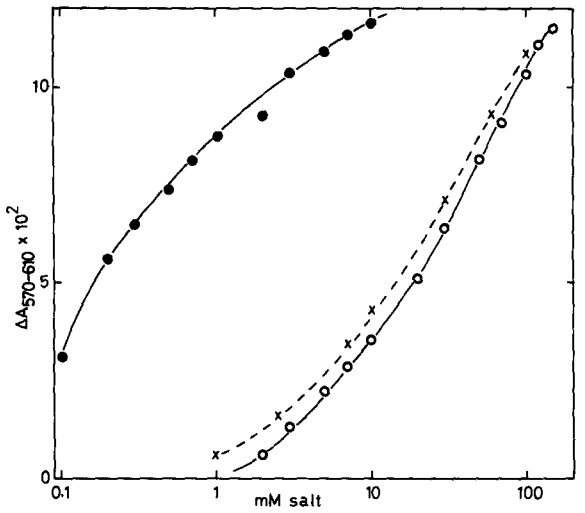


Fig. 4. Dependence of merocyanine absorbance change on concentration of salts added. Chromatophores (final concentration 10 μM bacteriochlorophyll) were suspended in 3 ml of 2 mM Tricine-NaOH (pH 8.0), 0.01 mM $MgSO_4$, 1 mM KCl, 0.8 μM CCCP and 3.3 μM NK2272. Absorbance changes at 30 s after the salt additions were plotted. •———•, $MgSO_4$; o———o, KCl; x- - - - -x, absorbance changes induced by light at various KCl concentrations without CCCP were added to the respective KCl-induced changes (replotted from Fig. 7).

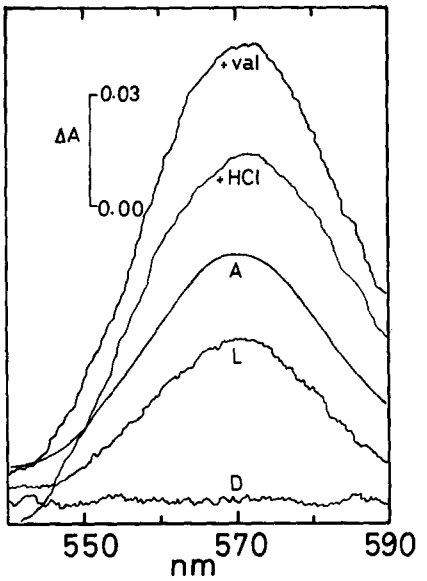


Fig. 5. Spectra of absorbance changes of merocyanine dye in chromatophore suspension. Difference spectra (after minus before treatment) were recorded with a scanning speed of 20 nm/s. Chromatophores (final concentration 10 μM bacteriochlorophyll) were suspended in 2.5 ml of 2 mM Tricine-NaOH (pH 7.85), 0.01 mM $MgSO_4$, 1 mM KCl and 10 μM NK2272. A, absorbance change induced by addition of 30 mM KCl (reduced by a factor of 5); L, light-induced absorbance change (5 s illumination); D, dark control; +HCl, difference spectrum 30 s after addition of 10 μl of 0.1 M HCl (pH shifted from 7.85 to 7.25) in the presence of 0.8 μM CCCP and 10 mM $MgSO_4$ (enlarged by a factor of 5); +val, difference spectrum 30 s after the addition of valinomycin (final concentration 0.8 μM) in the presence of 30 mM KCl.

adding valinomycin induced biphasic responses of NK2272 absorbance. It had a phase of very slow absorbance change. Its extent was almost independent of KCl concentration but was rather dependent on valinomycin concentration [15]. This slow phase may reflect the movement of NK2272 across chromatophore membranes accompanying the valinomycin-K^+ complex. When a diffusion potential was applied by the addition of an acid or base, however, the slow phase of absorbance changes (an absorbance increase at 570 nm by adding acid and a decrease by adding base) was very small.

Although the calibration of the change in membrane potential by the response of NK2272 on application of a diffusion potential may have some ambiguities, the light-induced response of NK2272 was linearly related to the carotenoid absorbance change (Fig. 6), i.e., to the change in membrane potential. Good correlation between the responses of merocyanine and carotenoid was observed in their dependences on light intensity in the presence and absence of CCCP and on the concentration of gramicidin D. These allow at least an indirect calibration of the potential change by the dye absorbance change.

Light-induced membrane potential changes measured by the carotenoid spectral shift had only a slight dependence on the salt concentration of the medium. However, the responses of merocyanine dyes to the change in membrane potential significantly depended on the salt concentration (Fig. 7). The extent of the light-induced absorbance change of NK2272 slightly decreased, while that of NK2273 decreased and that of NK2274 increased with increasing KCl concentration.

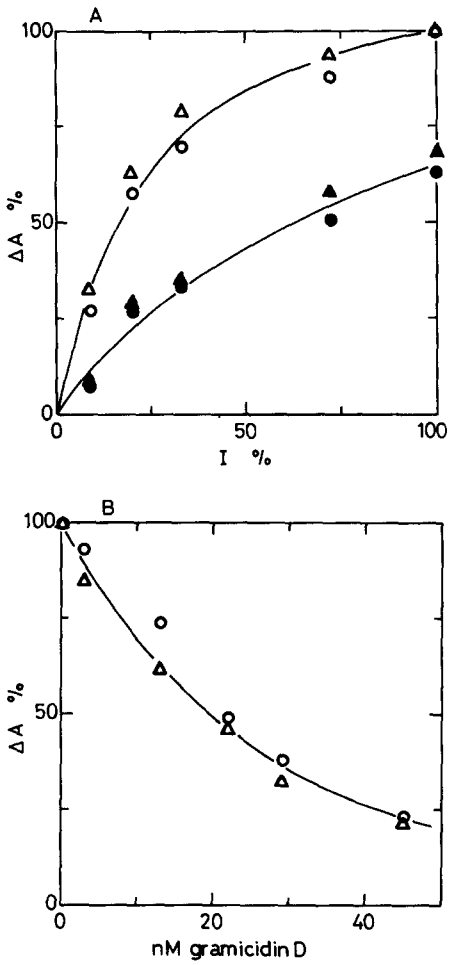


Fig. 6. Dependences of the light-induced absorbance changes of carotenoid and NK2272 on light intensity (A) and on concentration of gramicidin (B). Chromatophores (final concentration 10 μM bacteriochlorophyll) were suspended in 3 ml of 2 mM Tricine-NaOH (pH 8.0), 0.01 mM $MgSO_4$, 10 mM KCl and 3.3 μM NK2272. Absorbance changes: (○, ●) carotenoid, (△, ▲) NK2272. (●, ▲) Absorbance changes in the presence of 0.25 μM CCCP. In A, 100% intensity indicates $5.4 \cdot 10^3$ erg/cm^2 per s. The intensity of illumination in B was at the saturation level.

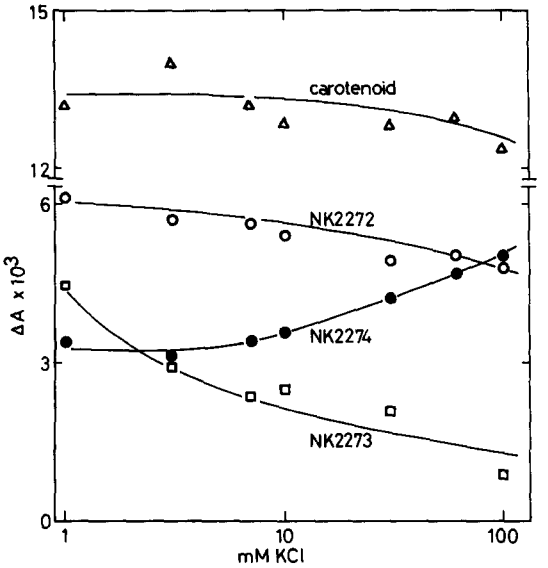


Fig. 7. Dependences of light-induced absorbance changes of carotenoid and merocyanine dyes on the salt concentration. Conditions were the same as those in Fig. 5. Concentration of each dye was 3.3 μM.

Discussion

Salt addition to a suspension of chromatophore membranes induced absorbance changes in merocyanine dyes (Fig. 3). This was due to the increased dye distribution to the membranes [13]. This was supported by the result that the amount of dye in the membrane measured after centrifugation was larger at high salt concentration and the comparison of the spectrum of the absorbance change with that of dye in alcohols of different chain lengths [13]. The spectrum of the light-induced absorbance change of a merocyanine dye (Fig. 5), which reflects the spectrum of monomeric NK2272 [6], was the same as the difference spectrum observed upon salt addition. It indicates that illumination (which induced an inside-positive potential) increased distribution of merocyanine to the membranes (in its monomeric form). That the addition of Triton X-100 after that of salt doubled the absorbance increase suggests the asymmetric distribution of merocyanines (predominantly to the outer surface region of the chromatophore membranes) and the low permeability of the dyes without Triton X-100. It has been reported that the permeability of an amphipathic anion, 8-anilino-1-naphthalenesulfonate, which has a sulfonate group like NK2272, is low but is increased by valinomycin plus K^+ [16]. A similar situation may be responsible for the slow phase of NK2272 absorbance change under prolonged illumination. The low permeability of NK2272 is also indicated by the results of the aniso-

tropic distribution and responses of NK2272 absorbed on axons [5]. This anisotropy of localization and responses was very different from the responses of cyanines and oxonols [5]. If NK2272 is considerably permeable in the time range examined in our experiments, a train of flashes should increase the absorbance changes of NK2272 after saturation of the carotenoid response is reached (i.e., after the extent of the membrane potential becomes almost constant). This situation was observed in the case of oxonols, which have a delocalized charge and are permeable to membranes [10]. The absorbance change of NK2272 by a train of flashes saturated with similar numbers of flashes which were sufficient for the saturation of carotenoid [15]. Therefore, the light-induced absorbance increase of NK2272 can be regarded to be due to the increased distribution of monomeric NK2272 to the outer surface region in the chromatophore membranes.

Carotenoid spectral shift indicates the intramembrane electric field changes induced by the change either in surface potential or in membrane potential. The light-induced membrane potential of Fig. 3c was 91 mV inside-positive and the salt addition in Fig. 3a induced an increase of 32 mV in the surface potential of the outer surface, according to the calibration under similar conditions [11]. The surface potential increase of 52 mV was calculated from the Gouy-Chapman equation with a net surface charge density of $-1.9 \cdot 10^{-3}$ elementary charge/Å^2 [11] in the case of salt addition in Fig. 3a (see also Fig. 2). However, in the low-salt medium the surface charge density may be smaller than that estimated above due to the lower surface pH [1,17]. Increased distribution of NK2272 to the membranes at higher salt concentrations may result in somewhat higher negative charge density on the surface (it may induce a 25% increase in net negative charge density). Therefore, the calculated value of 52 mV with the constant charge density may be slightly overestimated, which would be partially responsible for the difference from that estimated for the data of Fig. 3a. For the exact calculation of the surface potential changes, information on the dependence of surface charge density on surface pH is needed.

If merocyanine dyes distribute to the outer surface region in chromatophore membranes and change the distribution responding to the change in the electrical potential at their distribution site which lies at a distance d from the outer surface, we can tentatively calculate d assuming that the absorbance change per unit change in electrical potential at the site is the same regardless of the origin of the potential difference. The distance d was assumed to be constant judging from the fact that the absorption peak of the spectrum of a merocyanine dye distributed onto the membranes did not change under various salt concentrations. This implies that the physical parameters of the environment of the distribution site, especially the dielectric constant, are unchanged [13]. The value of d was calculated to be 0.43 Å inside from the outer surface from the comparison of the extent of the light-induced absorbance change of NK2272 (Fig. 3b and d) with that induced by salt additions (i.e., dependence of the merocyanine distribution on the unit change of surface potential). This rather small value of the effective depth of the site of dye distribution was calculated by assuming a uniform low-dielectric layer of 30 Å thickness [18] in the membranes. With the probable existence of a surface region with a higher dielectric constant, the site is more likely to be located deeper in the membrane from the morphological membrane surface. For the tentative calculation of d, we used the values of potential difference between inner and outer surfaces of membranes estimated from the measurements of carotenoid spectral shift induced by illumination and salt addition (Fig. 3a and c). If the light-induced absorbance change of merocyanine is due to the surface potential change in the outer surface, the potential change is calculated to be only +3.8 mV (Fig. 3b and d). This value is fairly small compared with that estimated with an ESR probe on chloroplast membranes [19].

If the situation mentioned above is valid, the salt-induced change in the bulk concentration of dyes should have some relationship with the dependence of the membrane-potential-induced dye response on the bulk salt concentration (Fig. 7), since the extent of the membrane-potential-induced carotenoid absorbance change remained practically constant as the salt concentrations were varied. We previously derived an equation which related the dye absorbance to the surface potential [13]:

$$A = \frac{c\epsilon_b + c\epsilon_m P_o (V_m/V) \exp(-zF\psi_o/RT)}{1 + P_o (V_m/V) \exp(-zF\psi_o/RT)}$$

where c is the total concentration of a dye in the test medium (with a volume of V) ϵ_m and ϵ_b the absorption coefficients of the dye at the distribution site in the membrane (with a volume of V_m) and in the bulk aqueous phase, respectively, z the valence of the dye, ψ_o the surface potential, P_o the partition coefficient of the dye at $\psi_o = 0$ between membrane and aqueous phase, and the other symbols have their usual meanings. The first derivative of the equation with respect to surface potential gives the dependence of absorbance change on surface potential. If the potential change at the distribution site of NK2272 induced by illumination is $\alpha\Delta\psi_m$ where $\Delta\psi_m$ is the light-induced membrane potential and α the relative depth of the site (d/30 Å) in the low-dielectric portion of the membrane from the outer surface, the dependence of the light-induced response of the dye on surface potential is given as follows:

$$\Delta A = [(-zF/RT)\, c(\epsilon_m - \epsilon_b)\, \alpha\Delta\psi_m]$$
$$\times [(1/P_o(V_m/V) \exp(-zF\psi_o/RT)) + 2$$
$$+ P_o(V_m/V) \exp(-zF\psi_o/RT)]^{-1}$$

This equation indicates that at fixed $\alpha\Delta\psi_m$ (condition nearly attained in the experiment in Fig. 7), the difference in $P_o(V_m/V)$ values leads to the different types of responses of dyes to salt concentration. Changes in other parameters such as $\epsilon_m - \epsilon_b$ and α affect ΔA but do not give different types of salt-concentration dependences (as far as these values do not depend on salt concentration). Surface potentials were calculated at each salt concentration by the Gouy-Chapman equation with the constant surface charge density of $-1.9 \cdot 10^{-3}$ elementary charge/Å^2 [11] (Fig. 8). c was 3.3 μM and $\Delta\psi_m$, estimated from the carotenoid absorbance change under the conditions of Fig. 7, was about 120 mV. Although different values of α are expected for different dye species from the suggestion of different dye-distribution sites in membranes [13], a value of 0.014, estimated from the result in Fig. 3, was used for all dyes for simplicity. Then we can calculate the salt-concentration dependence of light-induced absorbance changes giving values of $\epsilon_m - \epsilon_b$ ($\mu M^{-1} \cdot cm^{-1}$) and $P_o(V_m/V)$ for each dye. The values for these parameters were chosen to give a salt dependence similar to the results in Fig. 7. The sets of values of 0.098 and 3 for NK2274, 0.152 and 25 for NK2272, 0.094 and 150 for NK2273, for $\epsilon_m - \epsilon_b$ and $P_o(V_m/V)$, respectively, gave the curves in Fig. 8. The values for NK2273 were similar to those previously obtained [13]. The increase in P_o (ratio of 1 : 8 : 50 for NK2274 : NK2272 : NK2273) is as expected from the dye formulae which indicated increased lipophilicity for the latter species (Fig. 1). Fig. 8 explains the data of Fig. 7 semiquantitatively. However, there remains some uncertainty in choosing the values used, which would enlarge or reduce the scales of the abscissa or ordinate of Fig. 8.

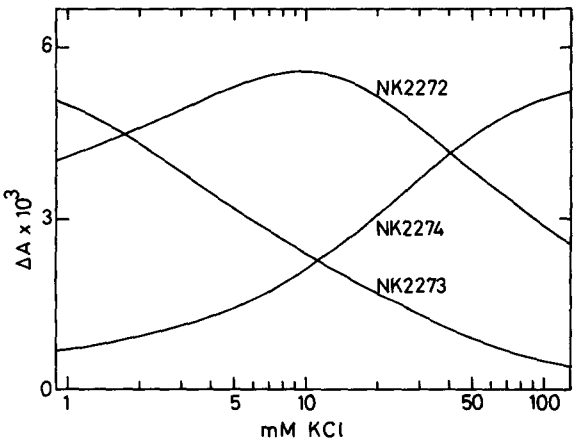


Fig. 8. Dependences of the extent of light-induced absorbance changes of various merocyanine dyes on the salt concentration. ΔA was calculated according to the equation in the text.

The time courses of absorbance changes of merocyanine dyes were similar to those of carotenoid during illumination under various conditions in spite of the difference in response mechanisms, i.e., electrochromism due to intramembrane electric field changes for carotenoid and solvatochromic effect [8] probably due to the potential-dependent change of partition of merocyanine. On the outer side of vesicles there was no large surface potential change during illumination. The possibility of nonequilibration in the H^+ electrochemical potential between the bulk aqueous phase and the region near the membrane surface during rapid energy conversion has been pointed out [20]. If that is the case, uncouplers should give different effects on the patterns of carotenoid and merocyanine responses during continuous illumination, since the presence of uncouplers accel-

erates the equilibration of H^+ between the bulk aqueous phase and the intramembrane regions. The response of merocyanine should be affected more significantly than that of the carotenoid. However, this was not the case. This suggests that the above-stated nonequilibration does not exist in chromatophore membranes. It can be concluded that the outer surface, i.e., the region which can be accessible to the merocyanine molecules, is in an equilibrium with the outer bulk aqueous phase during energy conversion.

Acknowledgements

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