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Electrical potential changes in the surface and the central region of chromatophore membranes of photosynthetic bacteria detected by the absorbance changes of ethidium and carotenoid

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(1) Changes of local intramembrane electrical field in the surface and central region of the chromatophore membrane during energization were studied both by the measurement of absorbance changes of ethidium, a monovalent cationic dye, and of carotenoid, the intrinsic probe of electrical field. (2) Binding of ethidium to the chromatophore membrane of *Rhodopseudomonas sphaeroides* was found to be dependent on the energization of membrane as well as on the ionic condition of the medium. The dye was released from the membrane when salt was added to the suspension, indicating the electrostatic interaction between the positive dye and the net negative membrane surface. The result was explained by the surface-potential dependent distribution of the dye to the membrane surface, as seen with other charged dyes (Masamoto, K., Matsuura, K., Itoh, S. and Nishimura, M. (1981) Biochim. Biophys. Acta 638, 108–115). (3) Energization of chromatophores by flash-light-induced absorbance change of ethidium showing a similar difference spectrum to that induced by the addition of salts. The release of ethidium by a single turn-over flash of saturating intensity was estimated to be 0.22 ethidium per reaction center. Addition of ethidium (at 200 μM) slightly affected the flash-induced absorbance change of carotenoid which responds to the intramembrane electrical-field change, indicating a low-membrane permeability of the dye. The extent of the absorbance change of ethidium was linear to that of carotenoid, and was abolished in the presence of valinomycin plus K^+. However, the rise and decay kinetics of the absorbance change of ethidium was different from that of carotenoid. (4) These absorbance changes of ethidium and carotenoid can be explained by a model in which ethidium responds to the potential changes in the surface region and carotenoid in the central hydrophobic region of the chromatophore membrane.

Introduction

Membranes of photosynthetic bacteria have an intrinsic carotenoid pool, which responds to the change of intramembrane electrical field. Absorbance change of the carotenoid can be a probe of the intramembrane electrical field change caused by illumination, or by application of diffusion potential or surface potential change [1–6]. This electrochromic spectral shifts of the special carotenoids in the light-harvesting pigment-protein complex [7–10] have been used to monitor the membrane potential changes in photosynthetic membranes [1–6]. On the other hand, in other membrane systems, various extrinsic dyes [11,12]

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Abbreviation: Mes, 4-morpholineethanesulphonic acid.

have been used for the measurement of the electrical-potential changes. However, uncertainty still remains for the response mechanisms of the dyes to membrane potential and the actual potential profile inside the membrane. Study of detailed profile of electrical-potential change inside membrane will be helpful for an understanding of the energy-conversion mechanism in biological membranes [13,14]. Among the dyes used, negatively charged merocyanine dyes added to chromatophore membranes of *Rhodopseudomonas sphaeroides* were shown to probe the potential change of the membrane surface region [15,16]. On the other hand, the absorbance change of carotenoids was suggested to be a response to potential change in the inner part of the membranes [5,6,17].

In this study we have examined the movement of monovalent cationic dye, ethidium, which was shown to be an anisotropic energy-transfer inhibitor (inhibits phosphorylation only when the dye interacts from the outer surface (cytoplasmic side) of the mitochondrial inner membrane [18]). However, the mechanism of its action and its movement at the surface or inside of the membrane does not seem to be fully clarified. This study indicates that the dye shows almost no inhibitory effect on the phosphorylation in chromatophores even after the chromatophores are long incubated with high concentration of the dye. It was concluded that ethidium responded to the intramembrane potential change in the surface region of the membrane in a similar mechanism as the merocyanine dyes [15,17].

Materials and Methods

The cells of *Rps. sphaeroides* Ga (green mutant [20]) were grown in a synthetic medium under incandescent light, and the chromatophores were prepared after disruption of the cells through a French pressure cell in a medium containing 5 mM $MgCl_2$/50 mM Tris-HCl (pH 7.4)/and 100 mM NaCl, as described previously [6]. The chromatophores were washed once with the medium containing 2 mM Mes (pH 6.0)/1 mM NaCl and suspended in the same medium.

The absorbance changes of ethidium induced by salt-additions were measured with Hitachi 557 dual-wavelength spectrophotometer. The reaction mixture contained chromatophores of 10 μM bacteriochlorophyll/200 μM ethidium bromide (Sigma)/10 mM Tris-HCl (pH 7.8)/2 mM NaCl in 3.0 ml. For the difference spectra, the spectrum before salt addition was subtracted from one after the salt addition.

The amount of dyes bound to the chromatophore membranes was estimated from the concentration of dyes in the supernatants after centrifugation for 2 h at $144000 \times g$ (the absorption coefficient of the dye in the medium was determined to be 0.0059 μM^{-1} at 478 nm from a calibration experiment). The pH of the medium in the binding experiment was 4.4 which was adjusted by adding 1 M HCl after preparation of the reaction mixtures. The flash-induced absorbance changes of ethidium and of carotenoid were measured with a local designed single beam spectrophotometer, equipped with a signal averager (Kawasaki M-100E and TMC-200) and a flash lamp (half-intensity duration of 2 μs, Sugawara PS-271). The excitation light from the flash lamp passed through Toshiba R-68 and Wratten 88A filters was strong enough to excited all the reaction centers in the medium. Flash intensity was changed by the use of neutral density filters. The photomultiplier was protected from the excitation light by a Corning C.S.4-96 glass filter. The flash-induced absorbance change of ethidium was obtained by subtracting the absorbance change of the suspension without ethidium from that with ethidium. The signals were averaged for four times. The extent of the absorbance change at 20 ms after the flash excitation was used to minimize the interference due to absorption changes of other components.

Bacteriochlorophyll was determined in acetone/methanol (7 : 2, v/v) extracts by the method according to Clayton [19].

The amount of reaction center in the chromatophore was estimated from the measurement of absorbance change under multi-flashes in the presence of antimycin A with the redox difference absorption coefficient of the reaction center ($\Delta\epsilon_{605-542} = 29.8\ mM^{-1} \cdot cm^{-1}$) [20]. The reaction mixture contained chromatophores corresponding to 20 μM bacteriochlorophyll/100 mM KCl/20 mM 4-morpholinepropanesulphonic acid-NaOH (pH 7.0)/1 μM valinomycin/2 μM antimycin A

and redox reagents (10 μM diaminodurene/1 mM sodium ascorbate/0.1 mM potassium ferrocyanide; under argon gas phase; the redox potential of 350 mV) in 5.0 ml.

Phosphorylation was measured by monitoring pH change of the medium with a pH meter (Hitachi-Horiba F7-ssII) according to the method of Nishimura [21]. The reaction mixture contained 15 μM bacteriochlorophyll/100 mM KCl/10 mM $MgCl_2$/0.33 mM ADP/3.3 mM pottasium phosphate/0.33 mM sodium succinate in 5.0 ml at pH 7.40 (25°C). The rates of photophosphorylation under continuous illumination were compared before and after the addition of ethidium (final concentration 200 μM).

Results

Surface-potential dependence of ethidium binding

Ionic dyes electrostatically interact with net negatively charged membrane surfaces in a physiological pH region and change their distribution to photosynthetic membranes depending on surface potential, which can be changed by changing pH or ionic concentrations of the medium [16,22,23]. Ethidium, monovalent cationic dye, showed a similar spectral shift to longer wavelengths when bound to chromatophore membranes as seen in the experiment with mitochondrial membranes [24]. Addition of $MgCl_2$ induced the absorbance change which indicated the increase of the bulk aqueous concentration of the dye (absorbance increase, near 465 nm) and a decrease of the membrane-bound form (absorbance decrease, near 545 nm) (the difference spectra before and after $MgCl_2$ addition, Fig. 1B). The extent of release of ethidium from the membrane mainly depended on the concentration and valence of the added cations (data not shown; $MgCl_2$, when added at concentrations less than 1 mM, showed the same effectiveness as KCl at 20–50-fold higher concentrations). This fact indicates that the binding of ethidium depends on the surface potential of chromatophore membranes, as seen with other ionic dyes [15,16].

The relationship between the amount of dye released and the absorbance change at 540 nm was studied by measuring the effect of KCl concentration in the medium (Fig. 2). After addition of the salt, the absorbance change of ethidium in the

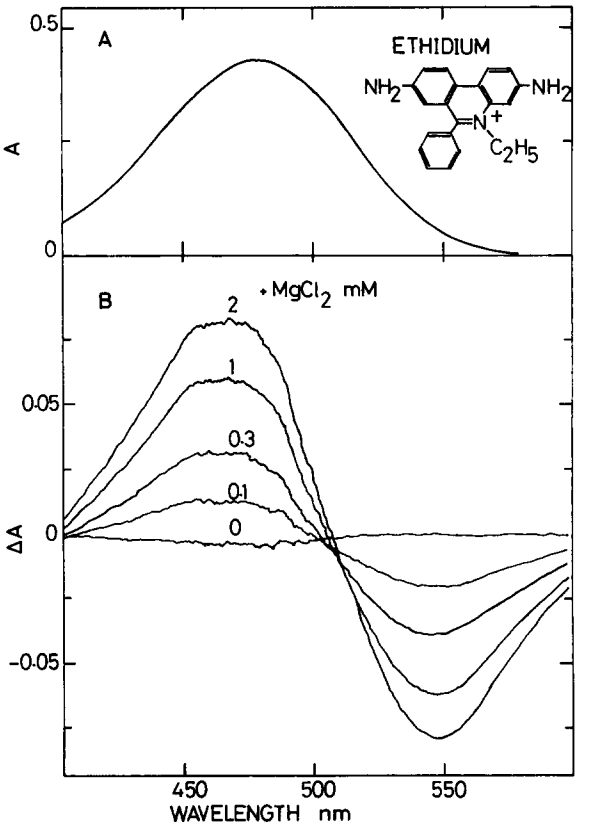

Fig. 1. Release of ethidium from chromatophore membranes by $MgCl_2$ addition. The reaction mixture contained 10 μM bacteriochlorophyll, 200 μM ethidium, 10 mM Tris-HCl (pH 7.8), 2 mM NaCl and 0.02% Triton X-100 in 3.0 ml. (A) Absorption spectrum of ethidium in a solution; (B) difference spectra. The difference spectra were obtained by subtracting the spectrum before the salt-addition from the spectra after the salt-addition.

chromatophore suspension was measured, and then the suspension was centrifuged to measure the amount of dye released into the supernatant. This experiment was done in the presence of 0.02% Triton X-100 which was expected to make membranes more permeable to ions. Thus, the amount of released dye will indicate the changes of binding to both the inner and outer surfaces of the chromatophore membrane [5].

The difference absorption coefficient at 540 nm was determined to be −0.008 per μM ethidium released ($\Delta\epsilon_{540} = -0.008\ \mu M^{-1} \cdot cm^{-1}$). Scatchard plots of the binding of ethidium were analyzed at pH 4.4, at which pH, almost equal to the isoelec-

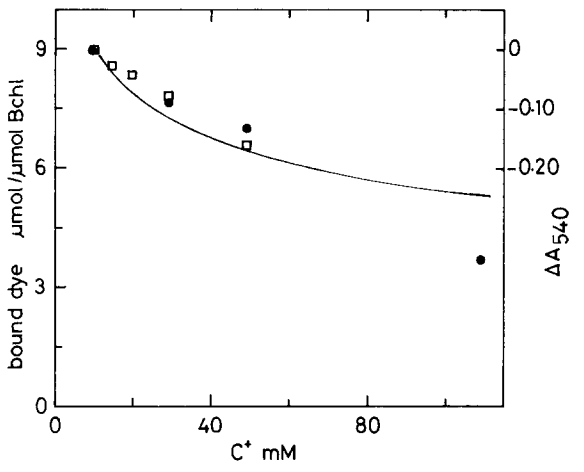

Fig. 2. Salt-dependent ethidium release and absorbance decrease at 540 nm. Conditions were similar to those of Fig. 1, except the addition of KCl. The absorbance decrease (□) were measured from spectra similar to that in Fig. 1B. After the absorbance measurements, the amounts of the dye bound (●) were estimated from the amounts of the dye in the supernatants after centrifugation. For the calculation of the curve, see Discussion.

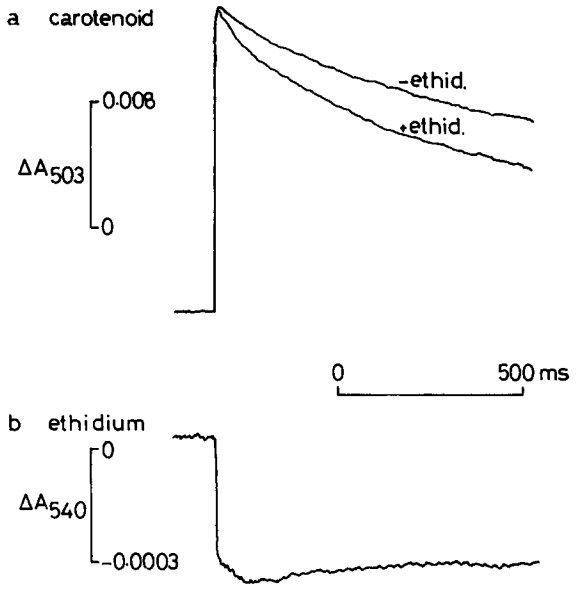

Fig. 3. Flash-induced absorbance changes of ethidium and carotenoid. The reaction mixture contained 20 μM bacteriochlorophyll, 200 μM ethidium, 10 mM Tris-HCl (pH 7.8), 2 mM NaCl, and redox reagents (10 μM diaminodurene, 1 mM sodium ascorbate, and 0.1 mM pottasium ferrocyanide) in 5.0 ml under an argon gas phase. (A) Absorbance change of carotenoid at 503 nm; (B) absorbance decrease of ethidium at 540 nm (release of it from the membrane). The absorbance change of ethidium was obtained by subtracting the absorbance change without ethidium from that with ethidium.

tric pH of the chromatophore membrane [5,6], only negligible surface potential can be expected. The result gave the amount of binding site (N) as 12.5 μmol ethidium/μmol bacteriochlorophyll and the binding constant (K_b) of 0.006 μM^{-1}.

Flash-induced absorbance changes of ethidium and carotenoid

Flash excitation of the chromatophores in the presence of ethidium induced the absorption change with a maximum at about 540 nm (Figs. 3 and 4). This spectrum was almost the same as the one (at longer-wavelengths side) of Fig. 1B indicating a release of ethidium from the chromatophore membranes by the flash excitation (Fig. 2). The absorbance changes below 520 nm were not measured due to a large interference by carotenoid absorbance changes. Half-response time of the absorbance change of ethidium was 0.4 ms. This is faster than that of a merocyanine dye [14]. On the other hand, the recovery rate of ethidium absorbance change was low (only a 10% recovery was seen at 2 s after a flash, in contrast to a considerable recovery of the carotenoid absorbance change at this time, also see Fig. 6). At the high concentration (200 μM), ethidium slightly increased the decay of the carotenoid absorbance change (Fig. 3).

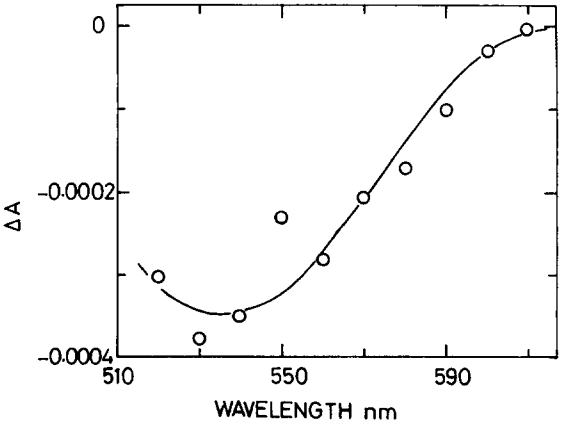

Fig. 4. Spectrum of the flash-induced absorbance change of ethidium. Conditions were the same as those in Fig. 3.

Response of the absorbance change of ethidium to the membrane potential was checked by changing the flash light intensity. The absorbance change of ethidium was compared with that of carotenoid (Fig. 5), since the latter is known to be proportional to the applied membrane potential under usual conditions (1–5). The extent of the absorbance changes of ethidium (at 540 nm) and carotenoids (at 503 nm) in Fig. 5 was read from those at 20 ms after the flashes. The decay of membrane potential (judged from the decay of carotenoid absorbance change; Fig. 3) was very small within this time range. The extent of absorbance change of ethidium was proportional to that of carotenoid. This suggests that the absorbance change of ethidium depends on membrane potential. The change of the absorbance induced by a 1 mV change of membrane potential was much smaller than that induced by a 1 mV change of surface potential (see Figs. 1 and 5). A similar phenomenon was observed with merocyanine dyes [15]. The result suggests the difference response mechanism of the dye from that of carotenoid. At saturating flash-light intensity, the absorbance change of ethidium (−0.00041) corresponded to a release of 0.26 nmol ethidium/100 nmol bacteriochlorophyll. It corresponds to 0.22 ethidium released per reaction center, because the chromatophore preparation used contained 1 reaction center/117 bacteriochlorophyll. The amount of ethidium released by the single flash was 0.03% of the total dyes bound to chromatophores.

The absorbance change of ethidium was abolished in the presence of valinomycin (Fig. 6b). This also suggests that the flash-induced ethidium release is driven by the change of membrane potential. However, suggesting different response mechanisms of ethidium and carotenoid to membrane potential, the rise and decay kinetics of the absorbance change of ethidium did not follow those of carotenoid absorbance change, and the extents of accumulated absorbance changes of ethidium and carotenoid did not show a linear relationship each other (Fig. 6a).

Effect of the dye on photophosphorylation

Photophosphorylation was a little inhibited by addition of high concentration of ethidium (at a final concentration of 200 μM) to the reaction mixture. The activity decreased from 0.35 nmol ATP per min per nmol bacteriochlorophyll to 0.30 when ethidium was added (15% inhibition). Long preincubation (5 h at 0°C) of chromatophores

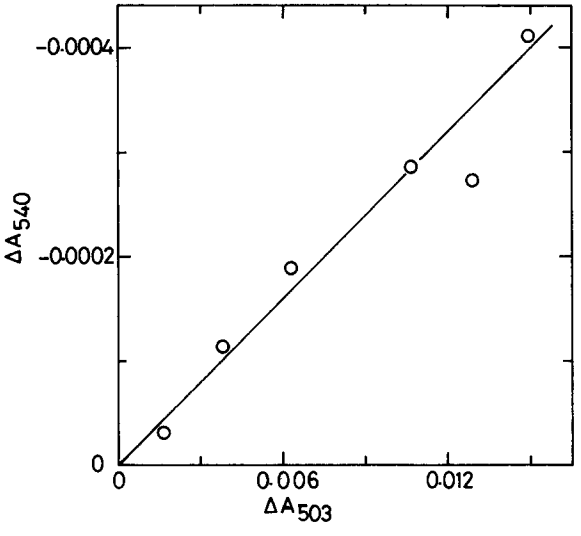

Fig. 5. Linear relationship between the flash-induced absorbance change of ethidium and that of carotenoid. Experimental conditions were the same as those in Fig. 3. Absorbance changes of ethidium were measured at 540 nm and those of carotenoid at 503 nm with varying the flash-light intensities.

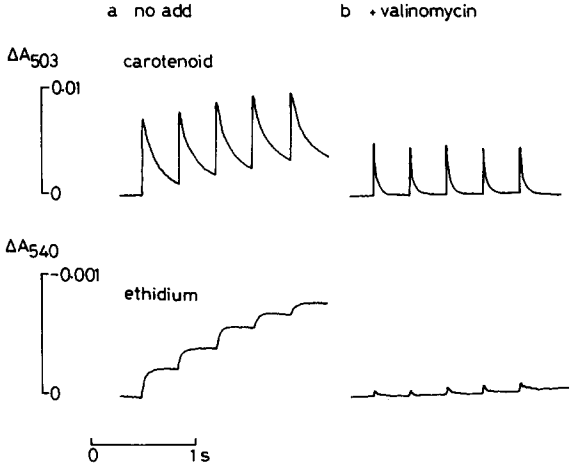

Fig. 6. Absorbance changes of carotenoid and ethidium by multi-flashes. Conditions were the same as those in Fig. 3, except the presence of 5 mM KCl (a and b) and 0.05 μM valinomycin (b).

with 4 mM ethidium did not increase the inhibition (measured with 200 μM of ethidium; data not shown). In mitochondrial membranes (right-side-out, in contrast to the inside-out orientation of chromatophore membrane), 200 μM ethidium is reported to inhibit fully the succinate-supported phosphorylation. On the other hand, in sub-mitochondrial particles (inside out) no inhibition of phosphorylation is reported [18]. From these facats, Higuchi [18] calls ethidium an 'anisotropic' phosphorylation inhibitor. The low sensitivity of phosphorylation in chromatophores may reflect slow permeation of ethidium into the intravesicular space of chromatophores or may indicate different nature of the components (which ethidium binds to) working in the energy coupling in chromatophores from that of mitochondria.

Discussion

Surface-potential-dependent binding of ethidium to chromatophore membranes

The monovalent cation, ethidium, was released from the chromatophore membranes by the salt additions (Figs. 1 and 2), suggesting an electrostatic interaction between ethidium and membrane surface, which is determined by the surface potential value [15,22,23]. The binding of ethidium to the surface is assumed to be expressed quantitatively by an equation combining Gouy-Chapman surface potential equation with Langmuir's absorption isotherm.

Langmuir's absorption isotherm equation gives the mount of dye bound to chromatophores (b; expressed in μmol dye per μmol bacteriochlorophyll):

$$b = K_b C_s (N - b) \tag{1}$$

where K_b is binding constant (μM^{-1}; C_s the dye concentration at the surface of chromatophore membranes (μM); and N the number of binding sites on the membrane (μmol dye/μmol bacteriochlorophyll). C_s is related to its bulk aqueous concentration (C_b; μM) by the Boltzman equation with the value of surface potential (ψ_0 in mV) [15]. C_b can be calculated from the difference between C_t (μM) (the total concentration of the dye) and bB (B(μM) (the bacteriochlorophyll concentration).

The binding of the cationic dye, ethidium, to the surface induces the reduction of the number of negative surface charges on the membrane. This effect is given by the Gouy-Chapman equation which relates surface potential value to the net surface charge density and the bulk concentration of salt. In the case of a symmetrical monovalent ion salt, such as KCl, at 25°C,

$$\sigma + \frac{b}{2S} = \frac{1}{136} C^{1/2} \sinh \frac{\psi_0}{51.4} \tag{2}$$

where σ is the surface charge density of the chromatophore membranes (electronic charge per 0.01 nm^2); S a unit area of chromatophore membranes expressed in nm^2 per 100 bacteriochlorophyll (surface area on both sides of the membrane is taken into account ($2S$)); and C the concentration of monovalent salt in the bulk medium, measured in M. From these equations, a relationship as follows is obtained,

$$b = K_b (N - b)(C_t - bB) \times \exp\left(-2 \sinh^{-1}\left(C^{-1/2}(136)\left(\sigma + \frac{b}{2S}\right)\right)\right) \tag{3}$$

We used -0.24 (e/nm^2) as σ, a mean value of those of the inner and outer surfaces of the chromatophore membrane [5,6]. The salt-dependent release of ethidium suggests that the ethidium binding sites are net negative and negative even after the maximal binding of ethidium under the present experimental conditions. The data of the ethidium release in Fig. 2 were explained by Eqn. 3 with a combination of values of $K_b = 0.001$, $N = 15$ and $S = 4000$ (Fig. 2). The deviation observed at high salt concentrations may reflect the assumption of fixed K_b and N values, since the surface charge densities will vary with the change of surface pH, which is also a function of surface potential and changes by the salt addition [25].

Light-induced release of ethidium due to inside positive membrane potential

The analysis above indicates that binding of ethidium to the membrane surface can be quantitatively treated. If the light-induced absorbance decrease of ethidium ($\Delta A = -0.00041$, by a single flash in Fig. 5) can be regarded as a ψ_0-change-induced release of the dye from the outer surface

of the chromatophore membrane to the outer medium, then the combination of Eqn. 1 and the Boltzmann equation will give an estimate of the change of ψ_0. The calculation gave 0.046 mV positive shift of ψ_0 under the condition that $b = 8.3$ μmol ethidium/μmol bacteriolochlorophyll, $C_b = 34$ μM, $B = 20$ μM, and the flash-induced increase in $C_b = 0.051$ μM.

When the ethidium was added to the membrane suspension in the absence of Triton X-100, bound form of ethidium increased very slowly after the addition, which continued at least for a few tens of minutes, probably because the inner surface is difficult to be accessed by the dye. This fact and the small effect of ethidium on the decay rate of flash-induced carotenoid absorbance change (Fig. 3) suggest the slow permeation of ethidium across the chromatophore membrane. This is consistent with the hydrophilic nature of the dye (very slightly soluble in organic solvents). The slow recovery of the bound form of ethidium and the dependence of the absorbance increase on flash number (Figs. 3 and 6a) may reflect the slow permeation of the dye and slow rebinding of the dye under no electrical field.

The carotenoid absorbance change has been considered to reflect the membrane potential in responding to the change of electric field in the hydrophobic central part of the chromatophore membranes [7–10,17]. This suggests that the decay of the light-induced absorbance change of carotenoid is affected by the movements of charges across the hydrophobic central part of the membrane. Ions with high membrane permeability such as tetraphenylboron [17] or valinomycin-K^+ complex are expected to affect the carotenoid decay strongly. These situations have been interpreted by a tripartite capacitor model of membranes [17] (Fig. 7), which assumes the membrane as a series of three capacitors, the first capacitor being composed between the diffuse layer in the outer bulk phase and the layer of hydrophobic ions bound to the membrane just beneath the outer surface (C_1); the second between the layers of hydrophobic charges beneath outer and inner surfaces inside the membrane (C_2); and the third between the charge layer beneath the inner surface and the diffuse layer in the inner bulk medium (C_3).

Ethidium may be estimated to move across the

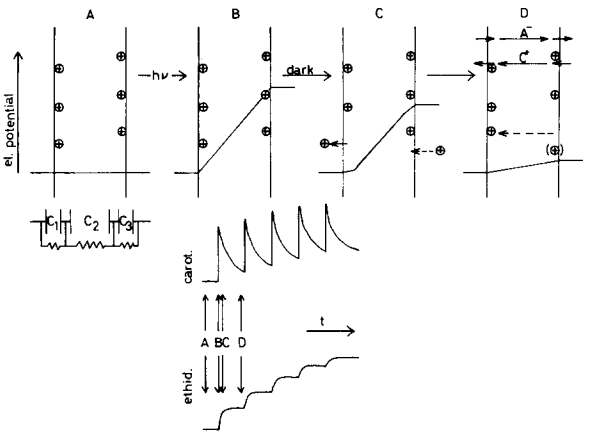

Fig. 7. Tripartite condenser model of chromatophore membranes; this model is based on that in Ref. 17 and 27. A situation before illumination was shown in A. Flash light induces an inside positive membrane potential (B). Release (and binding) of ethidium partially dissipates the potential difference (C). Potential difference in the inner part is diminished by fluxes of other ions (D). The arrows in the membrane model (C and D) indicate fluxes of ions. The arrows with broken line indicate the postulated flux of ethidium. For details, see Discussion.

central part of the membrane very slowly (Fig. 7B and C), although it can move quickly from the outer surface to the bulk aqueous phase, electrophoretically. The decay of carotenoid after the flash excitation (in a time range longer than the release time of ethidium) can be assumed to be mainly due to the movement of ions other than ethidium (Fig. 7D), since addition of ethidium slightly affected the decay rate. During this period, ethidium will move very slowly across the central part of the membrane. The movement of ethidium within the membrane will not be detected in the absorbance change, since the process will not accompany larger absorbance change. Such situations explain the time courses in Fig. 6. Upon the flash-induced change of intramembrane electric field, ethidium will rapidly move from its binding site just beneath the outer surface to the bulk aqueous phase electrophoretically, by dissipating the field in that region until the ratio of its concentration in the binding site to that in the bulk medium equilibrates the difference of electrical potential between these two phases. After the equilibration is attained, movement of ethidium

(mainly re-binding to the surface) can be estimated to be very slow, since there are no electrochemical forces to move ethidium. Non-electrogenic, diffusional movement of ethidium, which mostly depends on the movement of other ions, will re-bind ethidium to the binding sites. However, if the numbers of ethidium moved at the inner and outer surfaces of the membrane are identical, the absorbance change due to ethidium will not be observed. The observation of the flash-induced release of ethidium suggests that the distribution of ethidium (Fig. 7C) may not be symmetrical across the membrane. A smaller amount of ethidium may bind to the inner surface than that released from the outer surface or it may simply reflect the slower rate of binding to the membrane than release from the membrane. The former possibility seems more probable, since (1) only a smaller amount of ethidium-binding to the inner surface will result in a drastic change of dye concentration at the inner bulk aqueous phase, and (2) the surface area of the outer surface of chromatophore is estimated to be about 40% larger than that of the inner surface if we assume one chromatophore vesicle to be a sphere of 25 nm radius with membrane thickness of 6 nm [26]. The amount of ethidium movement observed upon the flash excitation is the difference between the amounts of the release from the outer surface and the binding to the inner surface. If such a release and binding occurs, then the actual number of ethidium released form the outer surface to the bulk aqueous phase can be estimated to be more than three times (about 0.7 molecules of ethidium released per reaction center) than estimated above.

Three types of hydrophobic ion

Previous study by Itoh [17] has shown that the hydrophobic ions such as tetraphenylboron rapidly move inside the hydrophobic central part of the membrane after a flash excitation. Such ion flux will decrease the electrical field in the central part of the membrane. If there is no flux between the surfaces and the bulk aqueous phases, the electrical potential created in the surface regions will not disappear [17]. On the other hand, if ethidium moves more slowly across the central part of the membrane than other ions present in the medium, the decay rate of the electrical potential in this central region will little be affected by the presence of the dye (Fig. 3).

These considerations classify the membrane-binding ions into three types, on a view of limiting factors which determine the permeation rate across membranes: (1) ions with fast movements across the whole part of the membrane (valinomycin-K^+ complex and K^+ (Fig. 6b)); (2) ions with the faster movement inside the hydrophobic central part of membrane (hydrophobic ions with delocalized charge such as tetraphenylboron$^-$ [17] and oxonol$^-$ (data not shown)); (3) ions with the faster movement between the surface regions and the bulk aqueous phases (less hydrophobic ion with localized charges such as ethidium and merocyanine [14,17]. This classification will contribute to resolve the details of movement of charges inside biological membranes. Use of these probes with different characteristics will serve to differentiate the change of membrane potential inside the membrane from that in the surface regions.

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