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Electrostatic Interaction between Merocyanine 540 and Liposomal and Mitochondrial Membranes

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Summary. The fluorescence of merocyanine 540 (MC) in liposomal and mitochondrial suspensions was measured under various conditions. Under a given condition, both the amount of dye bound to the membrane and the zeta potential were determined simultaneously. It was found that the fluorescence intensity was proportional to the amount of bound dye and correlated with the zeta potential of particles. The fluorescence intensity was represented quantitatively in terms of the Langmuir adsorption isotherm, when the electrostatic interaction acting between MC and membrane surface was properly taken into account. It was concluded that the changes in MC fluorescence in the liposomal and mitochondrial suspensions are mainly attributed to the changes in the surface potential of the membranes.

Fluorescence probes have been used extensively in studies of the physical properties of macromolecules in solution. Recently, their use has been extended to biological membranes and has provided a variety of information on the underlying molecular mechanism of biological functions (Brand & Gohlke, 1972; Radda & Vanderkooi, 1972; Waggoner, 1976*a*). Cohen *et al.* (1974), using squid axons, screened more than 600 dyes in a search for voltage-sensitive optical probes. Among the dyes exhibiting an optical response, merocyanine 540 (abbreviated hereafter as MC) is one of the most sensitive probes. MC gives a large fluorescence change, easily visible in single sweep, during an action potential in a giant axon (Salzberg, Davila & Cohen, 1973; Cohen *et al.*, 1974). Other biological preparations that have been studied with MC probe are single muscle fibers (Oetliker, Baylor & Chandler, 1975; Vergara & Benzanilla, 1976), heart cells (Salama & Morad, 1976), mitochondria (Chance *et al.*, 1974), and synaptosomes (Kamino & Inouye, 1978).

A class of merocyanines called "Brooker cyanines" exhibit large responses to changes of the dielectric constant of "Z-value" of solvents (Brooker *et al.*, 1965). The fluorescence of MC decreases by a factor of 20 when the dye is transferred from ethanol to water (Cohen *et al.*, 1974). Waggoner (1976*b*) pointed out that the dye is hardly permeable to biological membranes, because the negatively charged group is localized on the MC molecule, like anilinonaphthalene sulfonate.

Investigation of fluorescence changes of MC in artificial membranes such as

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liposomes affords a valuable information on the mechanism of the fluorescence changes in biological membranes (Waggoner & Grinvald, 1978). The present study deals with an analysis of MC fluorescence in liposomal suspensions under the presence of salts of varying concentrations. It is found that the fluorescence intensity is proportional to the amount of the dye bound on the membranes and correlates closely to its surface potential. The binding of MC to the membranes is analyzed with the Langmuir adsorption isotherm which is modified so as to implicate the electrostatic interaction between the MC molecule and membrane surface. Similar analysis is applied to MC fluorescence in mitochondrial suspension. The fluorescence and zeta potential are measured with energized or nonenergized mitochondria under the presence of KCl and MC of varying concentrations. It is shown that changes in MC fluorescence during energization as well as those caused by changes in ionic strength in medium are attributed to changes in the surface potential of mitochondria.

Materials and Methods

Lipid was extracted from *Esherichia coli* with chloroform-methanol (2:1, v/v) and the extracts were washed by the method devised by Folch, Lees & Sloane-Stanley (1957). For preparation of multilamellar liposomes, the lipid obtained was suspended by agitation with a flask mixer in glass-distilled water (Bangham, Standish & Watkins, 1965).

Rat liver mitochondria were prepared by the standard method as described in a previous paper (Aiuchi *et al.*, 1977). Sedimented mitochondria were stocked at 0°C. For measurements of the fluorescence and electrophoresis of mitochondria, the stocked mitochondria were suspended in a solution containing 10 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 0.2 mM EDTA, 250 mM sucrose and 1 μM rotenone. To alter the ionic strength, we substituted KCl for a part of sucrose, keeping the tonicity constant.

A Hitachi 204-R spectrophotometer was used for fluorescent measurement with a mercury lamp at 25°C, and the procedure was essentially the same as in the previous paper (Aiuchi *et al.*, 1977). To compare the fluorescence intensity obtained under various conditions, the fluorescence of a solution of 1 μM rhodamine B in methanol was used as the reference. The fluorescence intensity, f, used in the subsequent arguments is defined as

$$f = f_{MC}/f_{Rho} \tag{1}$$

where f_{MC} and f_{Rho} stand for the fluorescences of MC in a sample and of rhodamine B in 1 μM aqueous solution, respectively.

The concentration of unbound MC in the suspending medium was determined as follows: after centifugation of the suspension (100,000 × g, 60 min for liposomes and 10,000 × g, 5 min for mitochondria) the supernatant was diluted with equal volume of methanol and the fluorescence of the resulting solution was measured. The fluorescence in a water/methanol mixture (1:1, v/v) was linearly proportional to the concentration of MC as high as 3 μM.

Absorbance measurements were carried out with a spectrophotometer, Hitachi type-323.

The Zeta potential, ζ, was calculated from the electrophoretic mobility of liposomes or mitochondria with the aid of the Helmholtz-Smoluchowski equation. The electrophoretic mobility was measured with a microelectrophoretic apparatus (Cytopherometer, Carl

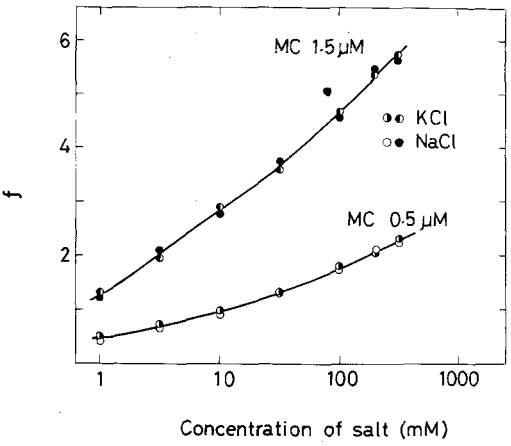

Fig. 1. Fluorescence intensity, *f*, of MC added to liposomal suspension as function of molar concentratin of KCl or NaCl. The "total concentration" of MC was 0.5 μM (◑,KCl;○,NaCl) or 1.5 μM(◑,KCl;●,NaCl). The concentration of lipid in the suspension was 0.5 mg/ml. Fluorescence intensity, *f*, is the ratio to that of a solution of 1 μM rhodamine B in methanol

Zeiss, West Germany) at 25°C as described previously (Aiuchi *et al.*; 1977; Kamo *et al.*, 1978).

Merocyanine 540 (MC) was purchased from Nihon Kankoshikiso Kenkyusho Co., Ltd. (Okayama, Japan).

Results and Discussion

Interaction of MC with Liposomes

The wavelength 546 nm was used for excitation of MC, which is one of line spectra of mercury lamp. As will be shown later, the optimum wavelength for excitation of MC is about 570 nm. However, this wavelength was not used for excitation, because 570 nm was too close to separate the fluorescence emission from the scattered light. The maximum wavelength of emission of MC in liposomal suspension was 582 nm, and this value was independent of the concentration and species of salts in the medium as far as examined. The wavelength of emission of MC in liposomal suspension was nearly equal to that obtained with frog hearts (Salama & Morad, 1976).

Figure 1 shows the fluorescence intensity, *f*, of MC in the liposomal suspension as a function of salt concentration, where the lipid concentration in the suspension is fixed at 0.5 mg/ml. The concentration of the dye in Fig. 1 is the "total" concentration which includes both free and bound MC in the suspension. The fluorescence intensity increased with increase of salt concentration at a given dye concentration, and no appreciable difference in fluorescence intensities was observed in KCl and NaCl solutions. The MC fluorescence attained to a steady value within 10 sec after an addition of concentrated NaCl solution to the lipo-

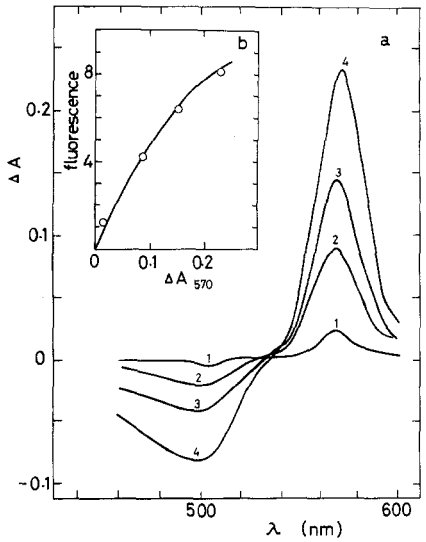

Fig. 2.(*a*): Effect of NaCl on the difference spectrum between MC in liposomal suspension and MC in aqueous solution. The concentration of MC was 2 μM and of lipid in the suspension, 0.16 mg/ml. (*1*): 1 mM NaCl; (*2*): 10 mM NaCl; (*3*): 30 mM NaCl; (*4*): 100 mM NaCl. (*b*): Relation between fluorescence intensity of 582 nm and changes of absorbance of 570 nm. Fluoresence was expressed in arbitrary units

somal suspension, and the steady value stayed constant for more than 20 min. These results suggest that the dye molecules do not penetrate the liposomal membrane and that the change in MC fluorescence may be attributed to the change in bound MC to the membrane surface.

Fig. 2*a* shows the changes of difference spectra, ΔA, between MC in water and in liposomal suspension at various concentrations of NaCl. Note that the absorbance of 570 nm increases with increase of NaCl concentration, in contrast to that of 500 nm. Figure 2*b* illustrates the relationship between the absorbance of 570 nm $(\Delta A)_{570}$, and fluorescence intensity of 582 nm. Figure 3 shows the fluorescence intensity as a function of the amount of bound MC on liposomes in various salt concentrations, where the bound MC was calculated from the measurement of MC concentration in supernatant of liposomal suspension (*see Materials and Methods*) and the total amount of the dye added in the system. Figure 3 indicates that the fluorescence intensity in liposomal suspension is proportional to the amount of MC bound to liposomes and that the quantum yield of bound MC is independent of cation species and of salt concentration in the medium. Waggoner and Grinvald (1978) studied the MC fluorescence with a liposome made of egg phosphatidylcholine (PC) and obtained a result similar to that of Fig. 2*a*. They examined a wider range of dye concentration compared to the present study, and found that two different states of MC exist in the concentration range examined. One absorbs at 570 nm which is a highly fluorescent monomer of MC, and the other absorbs 520 nm which is a slightly fluorescent dimer. When 1.38 μmol of MC adsorb to 0.21 mM PC liposome (corresponding to 0.17 mg/ml)[1], 90% of

[1]Mol wt of egg PC is 790 (Shah, D.O., Schulman, J.H., 1965. *J. Lipid Res.* **6**:341.

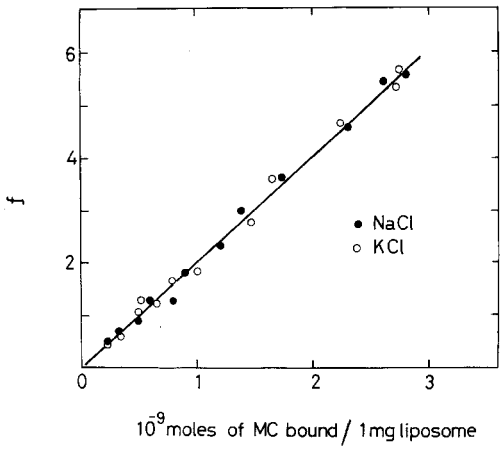

Fig. 3. Relation between fluorescence intensity, f, of MC and amount of MC bound to liposomes. ○,KCl; ●,NaCl

membrane-bound MC exists as monomer. Since the ratio of dye/lipid in the present experiments is much smaller than that of Waggoner and Grinvald, it is not unreasonable to consider that the membrane-bound dye molecules exist mainly in monomer form and there is no aggregation under the present experimental condition. In fact, the fluorescence of MC increases linearly with the concentration of dye bound to the liposomes, as seen in Fig. 3.

Chance *et al.* (1974) considered that the fluorescence enhancement of bound MC is attributable to the isomerization of the dipolar form of MC molecule caused by the local field in the membrane. However, the physicochemical mechanism of fluorescence enhancement of MC induced by binding to membrane is still not clear at the present.

The Langmuir adsorption isotherm can be applied to the binding of dye molecules to the membrane surface. Since a MC molecule bears a negative charge, we must take the electrostatic interaction between MC and liposomal membrane into consideration. The fluorescence intensity is linearly proportional to the number of bound MC, as illustrated in Fig. 3, and hence the Langmuir equation can be modified as follows (Kamo *et al.*, 1978).

$$f = qN\left[\frac{KC\exp(F\psi/RT)}{1 + KC\exp(F\psi/RT)}\right]. \tag{2}$$

Here, q is a proportionality constant related to quantum yield of the dye, N the maximum number of binding sites on the membrane surface, and C the molar concentration of unbound dye. F, R and T have their usual thermodynamic meanings. In Eq. (2), K is given by $K = (1/55.5)\exp(-\Delta G/RT)$, where ΔG represents the nonelectrical part of the free energy change due to binding, and ψ is the electrostatic potential at the membrane surface measured from that in the bulk solution, which is, however, not accessible experimentally. In the present paper, the zeta potential, ζ, is used as an experimental approximation to ψ. Putting ζ in place of ψ and rewriting Eq.(2), we obtain Eq.(3).

$$1/f = (1/qN)\,[1 + (1/KC)\exp(-F\zeta/RT)]\,. \tag{3}$$

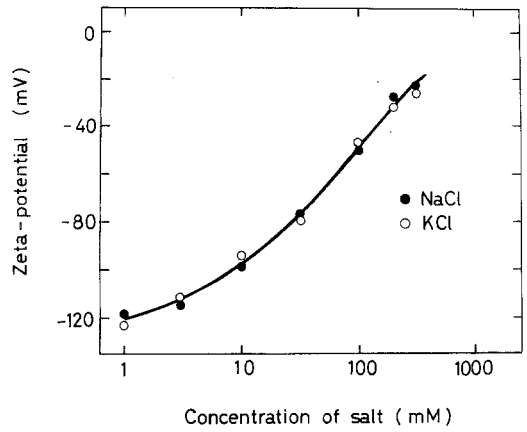

Fig. 4. Zeta potential of liposomes as a function of molar concentration of KCl or NaCl

According to Eq.(3), the plot of $1/f$ against $[(1/C)\exp(-F\zeta/RT)]$ gives a straight line and values of qN and K can be determined from the ordinate intercept and the slope of the straight line.

In Fig. 4, the zeta potential of liposomes is plotted as a function of the molar concentration of KCl or NaCl. The zeta potential of liposomes shown in Fig. 4 was measured in a medium containing 1.5 μM MC, but the data in the presence of the dye were practically identical with those in the absence of the dye. The lipids extracted from *E. coli* consist mainly of phosphatidylethanolamine (PE) (Kaneshiro & Marr, 1962), and the liposomes made of PE have a relatively large negative charge on the membrane surface (Papahadjopoulos & Watkins, 1967). The amount of adsorption of MC to liposomes may be too small to change the zeta potential, because the concentration of the dye is low enough in the present experiment (1.5 μM).

In Fig. 5, $1/f$ is plotted against $[(1/C)\exp(-F\zeta/RT)]$ according to Eq. (3), where the data shown in Figs. 1 and 4 are used. As seen in Fig. 5, a straight line is obtained in all cases examined. This result indicates that the binding of MC to liposomes follows Eq.(3) and that qN and K stay constant in the whole range of salt concentrations studied. In other words, the changes in the fluorescence intensity of MC in liposomal suspension are mainly attributed to changes in the surface potential of liposomes. From the straight line in Fig. 5, the value of ΔG and N were calculated to be -50 kJ/mol and 4.2 nmol/mg lipid, respectively. These values depended on species of constituent lipid of the membrane and of dyes used as shown in a previous paper (Kamo *et al.*, 1978).

Interaction of MC with Mitochondria.

An application of respiratory chain substrates or ATP to mitochondria leads to a decrease in MC fluorescence and that of uncoupler reverses this effect. In Fig. 6*a*, fluorescence changes in nonenergized mitochondria in the presence of rote-

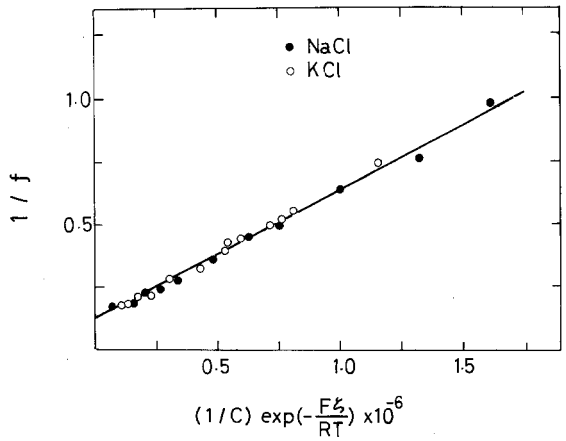

Fig. 5. Plots of $1/f$ against $[(1/C)\exp(-F\zeta/RT)]$ according to Eq. (3)

none are plotted as a function of MC concentration in media of different ionic strengths. The sample contained 0.3 mg mitochondrial protein per ml. Curves 1, 2 and 3 represent fluorescence changes in the buffer solution, the buffer plus 25 mM KCl and the buffer plus 100 mM KCl, respectively. Fluorescence intensity becomes larger with an increase of ionic strength at a fixed concentration of the dye. Curve 4 represents the fluorescence in the buffer plus 1 mM succinate (compare curve 1 with curve 4). Fig. 6*b* represents fluorescence changes as a function of MC concentration in the presence and absence of 1 mM ATP. Comparing each pair of curves (curve 5 for nonenergized and 6 for energized mitochondria in the buffer solution, curve 7 for nonenergized and 8 for energized mitochondria in the buffer plus 100 mM KCl), it is evident that the fluorescence intensity decreases during energization in both cases.

Chance *et al.* (1974) reported a shift in the wavelength of absorbance of ca. 5 nm upon addition of succinate. However, we do not observe a shift in the wavelength of emission by an addition of succinate or ATP. The widths of slit used for emission and for excitation may be too wide (10 nm) to observe a shift of wavelength.

In Fig. 7, the fluorescence intensity of MC, f, is plotted as a function of dye bound to nonenergized and energized mitochondrial membranes. When the dye concentration was below 1.2 μM, a linear relationship was obtained between f and the amount of bound dye. It is reasonable to consider that the fluorescence of MC in the mitochondrial suspension is attributed mainly to the dye molecule adsorbed on the hydrophobic region of the membrane. Chance *et al.* (1974) described that an aliphatic chain of MC allows the molecule to reside in the membrane. The linear relation given in Fig. 7 implies that the quantum yield is scarcely changed due to energization of mitochondria and that the fluorescence responses of MC in mitochondrial suspension are attributed to the change in the amount of bound dye.

The zeta potential of mitochondria was shown to decrease by an addition of

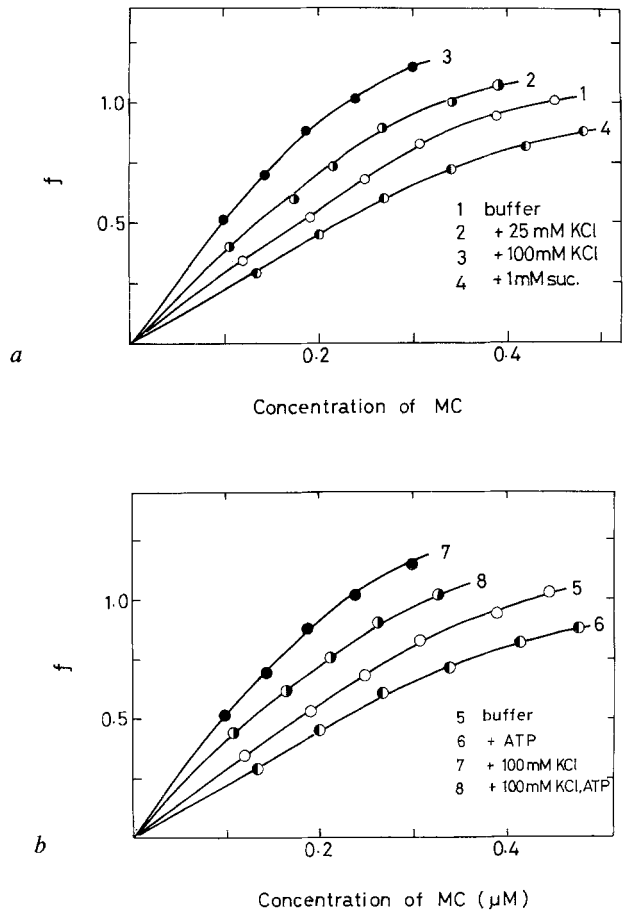

Fig. 6.(*a*): Relation between fluorescence intensity, *f*, and MC concentration for nonenergized and energized mitochondria in the various media. (*1*): the buffer; (*2*): the buffer + 25 mM KCl; (*3*): the buffer + 100 mM KCl; (*4*): the buffer + 1 mM succinate; (*b*): (*5*): the buffer; (*6*): the buffer + 1 mM ATP; (*7*): the buffer + 100 mM KCl; (*8*): the buffer + 100 mM KCl + 1 mM ATP. The concentration of mitochondrial protein was 0.4 mg/ml. The buffer contained 10 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 0.2 mM EDTA, 250 mM sucrose, 1 μM rotenone, and 1.5 μM MC

ATP or succinate as described previously (Kamo *et al.*, 1976; Aiuchi *et al.*, 1977). The data obtained in the presence of 1.5 μM MC were practically equal to those in the absence of the dye as in the case of liposomes. That is, the zeta potential of mitochondria was not altered by an adsorption of the dye. This, presumably, stems from high inherent charge density (negative) on the mitochondrial membrane surface, as in the case of PE liposomes described above.

In Fig. 8, $1/f$ is plotted against $[(1/C) \exp(-F\zeta/RT)]$ according to Eq.(3), where the data shown in Figs. 6 and 7 are utilized. As seen in Fig. 8, all data obtained with nonenergized and energized mitochondria in the media of different

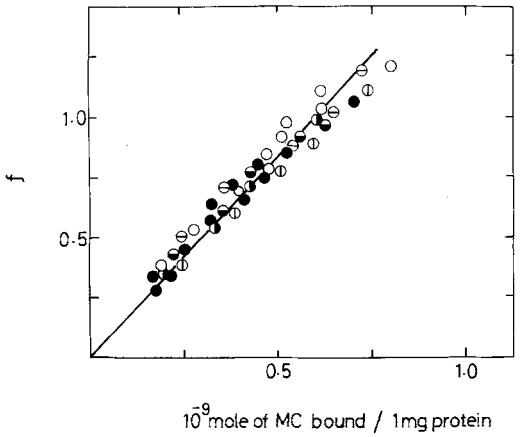

Fig. 7. Relation between fluorescence intensity, f, of MC and amount of MC bound to mitochondria. ⊖, the buffer; ◒, the buffer + 1 mM ATP; ○, the buffer + 25 mM KCl; ◑, the buffer + 25 mM KCl + 1 mM succinate; ○, the buffer + 100 mM KCl; ●, the buffer + 100 mM KCl + mM ATP

ionic strength fell on a single straight line. These results indicate that Eq.(3) holds in all cases examined where MC concentration, ionic strength in media, and the energized state are changed. Furthermore, the ordinate intercept and the slope of the straight line were almost equal to all cases in Fig. 7, that is, qN and ΔG stayed constant irrespective of difference in energized states of mitochondria and also in ionic strengths in medium. The above results indicate that fluorescence responses of MC under various conditions reflect rather faithfully the changes in the electrostatic interaction between MC and mitochondria, rather than change in quantum yield or N. From the straight line shown in Fig. 8, the values of ΔG and N were calculated to be −41 kJ/mol and 2.8 nmol/mg protein. This value of ΔG

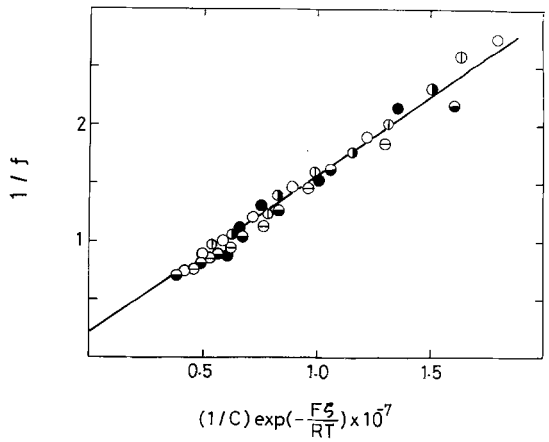

Fig. 8. Plots of $1/f$ against $[(1/C)\ \exp(-F\zeta/RT)]$ according to Eq. (3). Notations are the same as in Fig. 7

was slightly smaller than that for liposomes (−50 kJ/mol) and larger than that of ANS for mitochondria (−30 kJ/mol) (Aiuchi *et al.*, 1977).

The energization of mitochondria causes an increase of the negative zeta potential of the outer membrane. The changes in zeta potential at the outer membrane of mitochondria must be a consequence of energization that occurs at the inner membrane, since the energization of mitochondria is known to occur at the inner membrane. This similar fact was pointed out by Kamo *et al.* (1976) and Shimbo *et al.* (1978) by comparing the zeta potential of mitochondria with the membrane potential determined from the distribution of lipid-soluble cations, e.g., dibenzyl dimethyl ammonium (DDA^+). How is the density of fixed charges at the outer membrane surface altered by the energization occurring in the inner membrane? The detailed mechanism is not known at present. This, however, may be compared to the electrophoretic behavior of microcapsules containing aqueous solution of polyions (Shiba *et al.*, 1971; 1973), where the encapsuled polyelectrolyte can alter the zeta potential of microcapsules made of either charged or uncharged membrane matrix. Note that the polyions are impermeable to the membrane of microcapsules.

Concluding Remarks

In the previous papers (Aiuchi *et al.*, 1977; Kamo *et al.*, 1978) it was shown that the changes in ANS (1-anilinonaphthalene-8-sulfonate) fluorescence in liposomal and mitochondrial suspensions are attributed to changes in amount of ANS bound to the membranes and that the most effective factor in the binding is the change in the surface potential. From the results presented here, together with those in the previous articles, it may be concluded that fluorescence of MC or ANS is an indicator of the surface or zeta potential of the membrane.

Zeta potential is a function of the ionic strength in medium at a given charge density fixed on the membrane surface (Davies & Rideal, 1961). Thus an increase of the ionic strength in medium for a fixed energy state of mitochondria leads to a decrease in zeta or surface potential of mitochondria, which in turn leads to a decrease of the fluorescence intensity of MC (compare curves 5 and 7, or curves 1, 2 and 3 in Fig. 5). On the other hand, it is supposed that the membrane potential of a mitochondrion is not affected significantly by the ionic strength of the medium. From the experimental results presented here, it appears that there is an intimate correspondence between the fluorescence of MC and the change in surface potential and that the fluorescence might not monitor the membrane potential, at least in the experimental conditions studied here. Further study on the relation between the membrane potential and the surface potential may be necessary.

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