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EFFECT OF IONIC STRENGTH ON THE MEMBRANE FLUIDITY OF RABBIT INTESTINAL BRUSH-BORDER MEMBRANES

A FLUORESCENCE PROBE STUDY

TAKAO OHYASHIKI and TETSURO MOHRI

Department of Physiological Chemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11 (Japan)

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The effect of ionic strength on the fluidity of rabbit intestinal brush-border membranes has been studied using two fluorescence probes, pyrene and 1-anilino-8-naphthalene sulfonate (ANS). The imposition of a potential gradient on the pyrene-probed membrane vesicles (out > in) with increasing NaCl concentration in the medium resulted in a marked enhancement of the excimer formation efficiency, accompanied by a decrease in the ratio of fluorescence intensities of the probe at 392 and 375 nm. Fluorescence polarization of the pyrene-membrane complex is independent of temperature in the absence of salts, while it is dependent on temperature from 10 to 47°C in the presence of salts, as shown by the thermal Perrin plots of polarization. It has been demonstrated that there is a linear relationship between the changes in the pyrene excimer formation efficiency in the membranes and of the values of the binding parameters of ANS for the membranes. From these results, it is suggested that the lipid phase of the membranes becomes more fluid by shielding negatively charged groups of the membrane surface and that there is a fairly close correlation between the membrane organization and the membrane surface charge density.

Introduction

It has been recently recognized that the electrostatic surface potential of biomembranes has an important role in the context of functions of the membranes, such as passive and energy-linked permeation of ions [1–4] and metabolites [5–7], oxidative phosphorylation [8] and enzyme regulation in glycerol-3-phosphate dehydrogenase of insect thoracic muscle mitochondria and arylsulphatase C of rat liver microsomes [9].

Some investigators [10–13] have also presented several hypotheses postulating that the membrane structure might undergo appreciable modifications under an imposed potential gradient. However, there have been few lines of evidence supporting this proposition.

It is, therefore, of interest to investigate the dependence of membrane structure on the membrane surface charge density and/or transmembrane electrical potential. In the present study, we have used two fluorescent dyes, pyrene and 1-anilino-8-naphthalene sulfonate (ANS), to obtain information on the state of the lipid organization of rabbit intestinal brush-border membranes in association with the membrane surface charge density.

Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate.

ANS is known to be adsorbed to the hydrophobic regions of artificial and biological membranes, and there is a good deal of evidence that ANS fluorescence correlates closely to surface potentials between a membrane and the aqueous bulk phase [14–17]. Therefore, changes in the surface charge density of the membranes can be followed by measuring the fluorescence change of ANS. Kobatake and co-workers [15,17] have analyzed quantitatively the effect of ionic strength on interaction between ANS and liposomal and mitochondrial membranes.

On the other hand, pyrene is an uncharged molecule and forms an excited dimer (excimer) by a diffusion-controlled process during its fluorescence lifetime. Therefore, this probe is an useful tool in the analysis of the structural change in the hydrocarbon regions of biomembranes [18,19].

In this study we present important experimental evidence demonstrating a close relationship between the changes in the membrane surface charge density and the membrane fluidity in rabbit intestinal brush-border membranes.

Materials and Methods

Materials

Pyrene and ANS (magnesium salt) were purchased from Wako Pure Chemicals. Pyrene was recrystallized from ethanol once before use and dissolved in ethanol to make a stock solution. All other materials were reagent grade obtained from commercial sources.

Preparation of membrane vesicles

Brush-border membranes were isolated from rabbit intestine according to the method of Hopfer et al. [20], with a slight modification as described in a previous paper of ours [16]. Isolated membranes were suspended in 10 mM Tris-HCl buffer, pH 7.4. Protein concentration was assayed by the method of Lowry et al. [21], using bovine serum albumin as standard.

Labelling of membranes

Labelling of the membranes with pyrene was usually carried out as follows: suspensions of the membrane vesicles (1.0 mg/ml protein) in 10 mM Tris-HCl buffer, pH 7.4, were mixed with 3.3 μM pyrene. The reaction was carried out for 30 min at 0°C and was terminated by dilution with the same buffer. The mixture was centrifuged at $27000 \times g$ for 20 min. The pellets were washed with the same buffer and the final pellets were suspended in 10 mM Tris-HCl buffer, pH 7.4.

Fluorescence measurements

Fluorescence intensity and polarization measurements were carried out using a Hitachi MPF-4 spectrofluorometer equipped with a rhodamine B quantum counter as previously described [16,22]. The excimer formation efficiency of pyrene was expressed as the value of the excimer-to-monomer fluorescence ratio, I_E/I_M, which was calculated from the fluorescence intensities at 470 nm (for the excimer) and 392 nm (for the monomer), with excitation at 340 nm. The excitation and emission wavelengths used for ANS fluorescence measurements were 340 and 470 nm, respectively.

Determination of ANS-binding parameters

The maximum amount of bound ANS and the apparent dissociation constant (K_d) of the ANS-membrane complex were estimated according to the fluorescence titration procedures proposed by Wang and Edelman [23] as described in a previous paper of ours [16].

Results and Discussion

Fluorescence properties of pyrene-probed membranes

The fluorescence properties of pyrene in the presence and absence of rabbit intestinal brush-border membranes are summarized in Table I.

As can be seen in this table, the monomer fluorescence intensity at 392 nm, the ratio of fluorescence intensities at 375 and 392 nm, I_{392}/I_{375}, and the excimer formation efficiency of pyrene-probed membranes were larger than those of pyrene alone.

Effect of ionic strength on fluorescence properties of pyrene-membrane complex

Fig. 1 shows the variation of the dimer/monomer fluorescence intensity ratio of the pyrene-membrane complex as a function of NaCl concentration. With increasing NaCl concentration, the excimer formation efficiency of the complex

TABLE I

FLUORESCENCE PARAMETERS OF PYRENE AND ITS COMPLEX WITH THE MEMBRANE AT 25°C

10 mM Tris-HCl buffer, pH 7.4. Pyrene, 3.3 μM; membrane protein, 0.150 mg/ml. Excitation wavelength was 340 nm.

System	Monomer fluorescence intensity at 392 nm	I_{392}/I_{375} ratio	I_E/I_M ratio
Pyrene alone	61 (100%)	1.02 (100%)	0.163 (100%)
Pyrene-membrane complex	83 (137%)	1.24 (122%)	0.192 (118%)

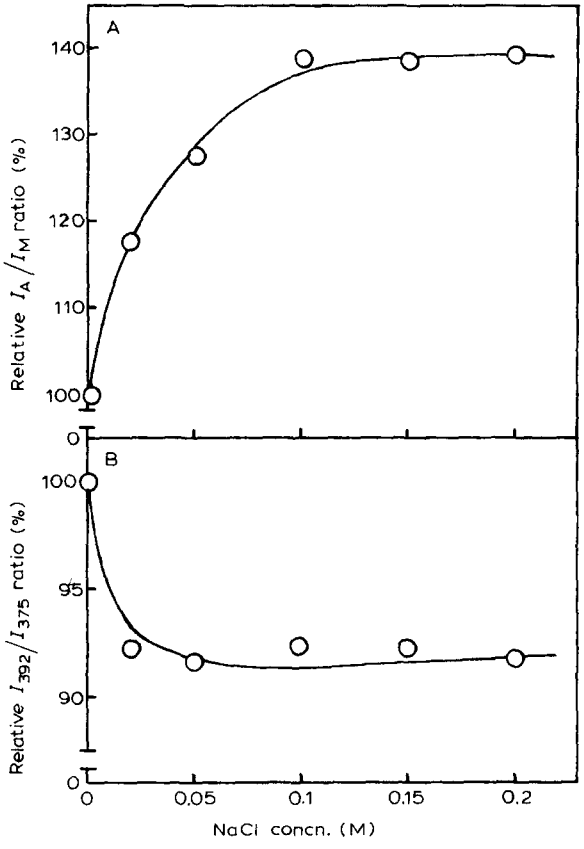

Fig. 1. NaCl concentration dependence of fluorescence properties of the pyrene-membrane complex. A, Relative excimer-to-monomer fluorescence intensity ratio. B, Relative peak ratio of intensities at 375 and 392 nm. Protein concentration of pyrene-labeled membranes was 0.150 mg/ml. NaCl concentration was varied from 0.02 to 0.2 M at 25°C. The values were expressed as the relative for those in the absence of NaCl in each case. Other conditions were the same as described in the legend to Table I.

increased, attaining a maximum at 0.1 M NaCl. In contrast, the peak ratio, I_{392}/I_{375}, exponentially decreased, showing a plateau above 0.05 M NaCl. On the other hand, the spectral profiles of emission were the same, regardless of the presence or absence of NaCl (data not shown). The NaCl concentration required to induce the half-maximal change in the excimer-to-monomer fluorescence ratio was approximately 24 mM.

Since it is established that the excimer formation of pyrene is dependent on a diffusion-controlled process [18,19], the increase in the excimer-to-monomer ratio of the probe in the membranes may be attributed to a decrease in the viscosity of the hydrocarbon regions of the membranes, which produces an arrangement of pyrene molecules that is favorable to excimer formation. A decreased peak ratio of fluorescence intensities at 392 and 375 nm, I_{392}/I_{375}, also suggested salt-induced alteration in the arrangement of the hydrocarbon region of the membranes. In a previous paper [24], we demonstrated, using an ethanol/water binary mixture, that the I_{393}/I_{375} ratio of pyrene decreases with increasing solvent polarity. Accordingly, the decrease in the ratio observed with increasing NaCl concentration of the medium may indicate an increase in proximity of solvent molecules to pyrene molecules located in the hydrocarbon cores of the membranes due to salt-induced increment of the membrane fluidity.

Temperature dependence of the fluorescence parameters of pyrene in the membranes

The temperature dependence of the intensity and polarization of fluorophore in the membranes may serve as an indication of a temperature-dependent rearrangement of membrane structure, hence the effect of temperature on the fluorescence parameters of the pyrene-membrane complexes with and without salt was investigated.

As shown in Fig. 2A, the degree of fluorescence polarization of the complex without salt revealed no temperature dependence over the temperature range tested, suggesting that the rotatory relaxation time of the kinetical unit carrying the dye and/or the dye itself is assumed to be very long, as compared to the lifetime of the probe [25]. In contrast, the degree of the polarization of the complex in the presence of salts decreased with

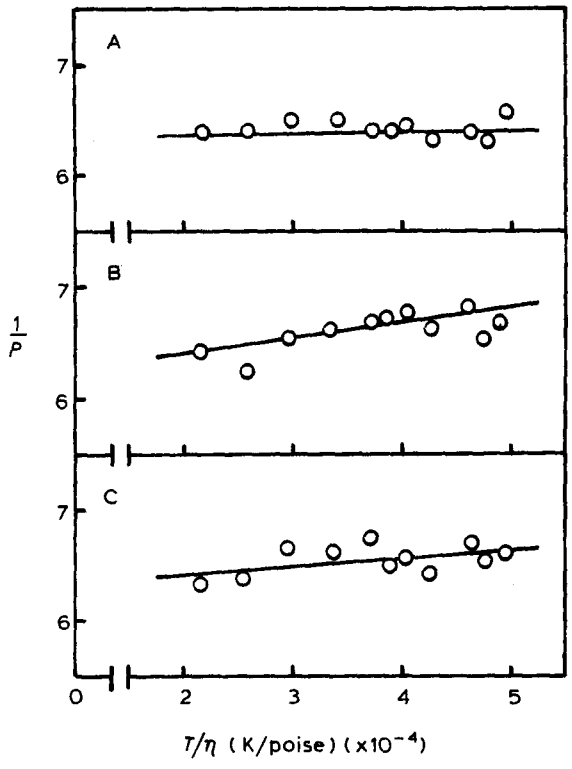

Fig. 2. Temperature dependence of the fluorescence polarization of the pyrene-membrane complex in the presence and absence of salts. Temperature was varied from 10 to 47°C. A, No salt; B, 0.1 M NaCl; C, 0.1 M KCl. Excitation and emission wavelengths were 340 and 392 nm, respectively. Other conditions were the same as described in the legend to Fig. 1.

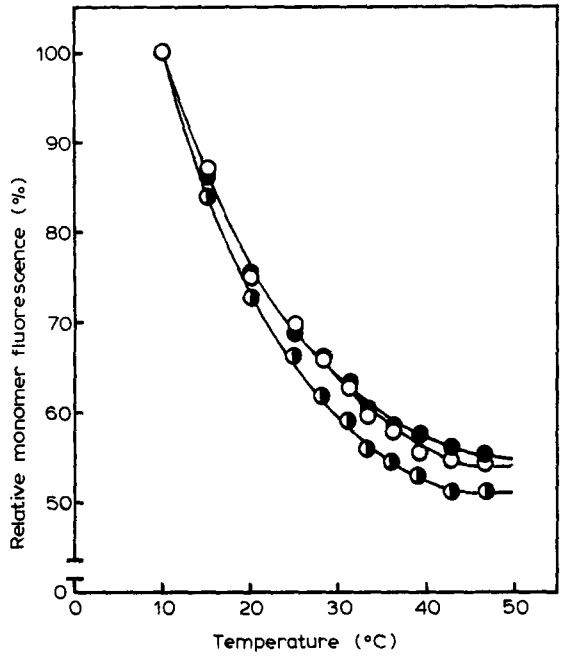

Fig. 3. Temperature dependence of monomer fluorescence intensity of the pyrene-membrane complex. ○, No salt; ●, NaCl; ◑, KCl. Conditions were the same as described in the legend to Fig. 2. Fluorescence intensity is relative to that at 10°C in each system.

increasing temperature (Fig. 2B and C). The slopes of the thermal Perrin plots of the polarization of the systems with KCl and NaCl were estimated to be 0.114 and 0.150, respectively. On the other hand, the values of limiting polarization, P_0, in the absence of rotational motion of the dye were almost the same in all systems ($P_0 = 0.158$).

Fig. 3 shows the monomer fluorescence intensity of the pyrene-membrane complex as a function of temperature. With increasing temperature, the intensity of the monomer fluorescence of the complex decreased monotonically in the same manner, regardless of whether or not salt was present.

Change in the polarization (Fig. 2) may be caused either by a change in the excited-state fluorescence lifetime of the fluorophore or by a change in membrane microviscosity affecting the rotatory movement of it, or by both. As mentioned above, the emission maximum and P_0 of the pyrene-membrane complex did not change upon addition of salt, suggesting that the change of fluorescence intensity of the complex upon addition of salt may be attributed to a change in the transition efficiency without any change in the orientation of the electronic transition moment of the fluorescence emission. In addition, the patterns of the responses of the monomer fluorescence intensity of the complex to varying temperature are almost the same, irrespective of the presence or absence of salts (Fig. 3). Therefore, the appearance of the slope in the Perrin plot of the polarization of the complex by addition of KCl or NaCl is probably due to the thermal activation of some of the membrane components [25] upon interaction of the salt with the membrane surface, although the possibility of the change of the lifetime of fluorophore cannot be ruled out completely. In any case, these results suggest that the nature of the hydrocarbon cores in the membrane interior is

apparently modified by interaction between cations and the membrane surface charged groups.

ANS fluorescence and pyrene excimer formation efficiency

The pyrene excimer formation efficiency and the ANS fluorescence intensity in the membranes in the presence of various salts at a fixed concentration of each (0.1 M) are summarized in Table II.

KCl and NH_4Cl were efficient for excimer formation of pyrene, as were NaCl and NaSCN, whereas LiCl was the least efficient. On the other hand, the order of effectiveness in increasing ANS fluorescence was LiCl > NaCl = NH_4Cl > KCl > NaSCN > choline chloride > control (no salt). A very similar order of effectiveness in enhancing ANS fluorescence has been reported for these salts in rat brain microsomal membranes [26], monkey brain plasma membranes [27], sheep erythrocyte membranes [27], and dipalmitoylphosphatidylcholine liposome [27,28]. It is deduced from the values for NaCl and NaSCN that the change of anion species produces a distinct difference in the effectiveness in ANS fluorescence enhancement. This fact is probably explained as due to a difference in the degree of anion-generated membrane potential change, as reported with the membrane of *Physarum polycephalum* [29].

In a separate experiment, we investigated the relation between the changes in the ANS-binding parameters and in the pyrene excimer formation efficiency with increasing ionic strength, because it is assumed that the change of the dye binding parameters of the membrane reflects directly the change in the nature of the membrane surface [15,17].

As shown in Fig. 4, the plots of the amount of bound ANS and the K_d value of the dye for the membranes against the pyrene excimer formation efficiency show linear (proportional or inversely proportional) correlations between them.

Kobatake and coworkers [15,17] have demonstrated that the change in ANS fluorescence in liposomal and mitochondrial suspensions associated with changes in ionic strength of the medium is proportional to the amount of bound dye and correlates closely to the surface potential of their membranes. As is well known, the membrane surface potential, ζ-potential, is a function of surface charge density [30]; a decrease in surface charge density as a result of binding of ions to the membrane surface will lead to a decrease in magnitude of the potential. The decrease of the poten-

TABLE II

EFFECTS OF SALTS ON PYRENE EXCIMER FORMATION EFFICIENCY AND ANS FLUORESCENCE IN THE MEMBRANES

10 mM Tris-HCl buffer, pH 7.4. Salt concentration was 0.1 M. The membrane protein concentration of pyrene-labeled membranes was 0.150 mg/ml. Fluorescence intensity of ANS-membrane complex was followed at 470 nm after 10 min addition of 3.33 μM ANS to the membrane suspensions (0.150 mg/ml protein) with 340 nm for the excitation wavelength. Other conditions were the same as described in the legend to Fig. 1.

Salts	Relative excimer formation efficiency (%)	Relative ANS fluorescence intensity (%)
No additions	100	100
NaCl	112	120
NaSCN	113	111
KCl	111	116
LiCl	104	125
NH_4Cl	108	120
Choline chloride	106	105

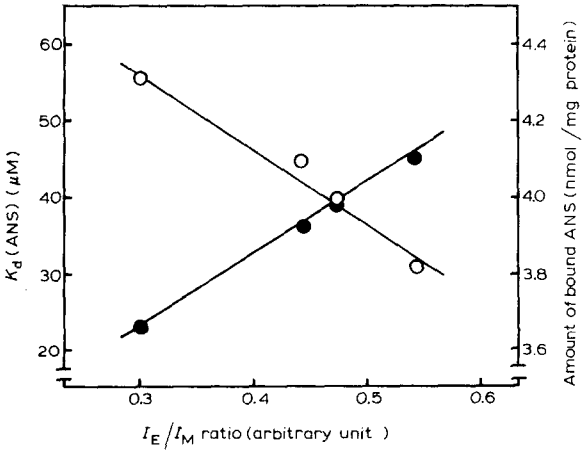

Fig. 4. Correlation between pyrene excimer formation efficiency and ANS-binding parameters for the membranes. NaCl concentration was varied from 0.02 to 0.1 M. ○, Dissociation constant of the ANS-membrane complex; ●, amount of bound ANS for the membranes. Other conditions were as described in the legend to Table II.

tial in turn reduces the electrostatic repulsion force between ANS and the membranes, leading to increase of the amount of ANS bound to the membranes, and consequently resulting in an increase of the fluorescence of the ANS-membrane system.

Although it is difficult at present to characterize exactly how the imposition of salt gradient on the membrane vesicles (out > in) influences the organization of the hydrocarbon regions in the membranes, it is likely from the results described above that the change of the membrane surface charge is one of the important elements controlling membrane fluidity. This idea is strongly supported by the results of Barber and Chow [31], who have investigated the relation between the salt-induced stacking to thylakoid membranes and a change in distribution of the surface charge of the membranes.

References

1 Papa, S., Guerrieri, F., Simone, S. and Lorusso, M. (1972) Bioenergetics 3, 553–568
2 Haydon, D.A. and Hladky, S.B. (1972) Q. Rev. Biophys. 5, 187–282
3 Schäfer, G. and Rowohl-Quinsthoudt, G. (1976) Bioenergetics 8, 73–81
4 Theuvenet, A.P.R. and Borst-Pauwels, G.W.F.H. (1976) Biochim. Biophys. Acta 426, 745–757
5 Meisner, H., Palmieri, F. and Quagliariello, E. (1972) Biochemistry 11, 949–955
6 Palmieri, F., Quagliariello, E. and Klingenberg, M. (1972) Eur. J. Biochem. 29, 408–416
7 Duszyński, J. and Wojtçzak, L. (1972) FEBS Lett. 40, 72–76
8 Schäfer, G. and Rowohl-Quinsthoudt, G. (1975) FEBS Lett. 59, 48–51
9 Wojtçzak, L. and Natecz, M.J. (1979) Eur. J. Biochem. 94, 99–107
10 Papakostidis, G., Zundel, G. and Mehl, E. (1972) Biochim. Biophys. Acta 288, 277–281
11 Coster, H.G.L. (1975) Biochim. Biophys. Acta 382, 142–146
12 Coster, H.G.L. and Zimmermann, U. (1975) J. Membr. Biol. 22, 73–90
13 Lelkes, P.I. (1979) Biochem. Biophys. Res. Commun. 90, 656–662
14 Haynes, D.H. (1974) J. Membr. Biol. 17, 341–366
15 Kamo, N., Aiuchi, T., Kurihara, K. and Kobatake, Y. (1978) Colloid Polym. Sci. 256, 31–36
16 Ohyashiki, T. and Mohri, T. (1979) J. Biochem. 85, 857–863
17 Aiuchi, A., Kamo, N., Kurihara, K. and Kobatake, Y. (1977) Biochemistry 16, 1626–1630
18 Galla, H.-J. and Sackmann, E. (1974) Biochim. Biophys. Acta 339, 103–115
19 Galla, H.-J., Hartmann, W., Theileu, U. and Sackmann, E. (1979) J. Membr. Biol. 48, 215–236
20 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
22 Ohyashiki, T. and Sekine, T. (1979) J. Biochem. 85, 575–580
23 Wang, J.L. and Edelman, G.M. (1971) J. Biol. Chem. 246, 1185–1191
24 Ohyashiki, T. and Mohri, T. (1978) Chem. Pharm. Bull. 26, 3161–3166
25 Weber, G. (1952) Biochem. J. 51, 145–155
26 Gomperts, B., Lantelme, F. and Stock, R. (1970) J. Membr. Biol. 3, 241–266
27 Kirshnan, K.S. and Balaram, P. (1976) Arch. Biochem. Biophys. 174, 420–430
28 Trauble, H. (1971) Naturwissenschaften 58, 277–284
29 Ueda, T., Terayama, K., Kurihara, K. and Kobatake, Y. (1975) J. Gen. Physiol. 65, 223–234
30 Verwey, E.J. and Overbeek, J.T.G. (1948) Theory of Stability of Lipophobic Colloids, Elsevier Publishing Co., Amsterdam
31 Barber, J. and Chow, W.S. (1979) FEBS Lett. 105, 5–10