J. Membrane Biol. 101, 113–118 (1988)

The Journal of
Membrane Biology

Penetration of Furosemide into Phospholipid Monolayers

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Summary. Furosemide is a surface-active anion and it tends to displace lipid monolayers from the surface at positive polarizations lowering their potential stability range. The efficiency of the penetration and the displacement increases with decreasing surface pressure of the monolayer. Lower capacitance at a wider potential range corresponds to higher surface pressure. Monolayers with higher capacitances are indeed more readily penetrated and displaced as demonstrated by further increase in their capacitance and increase in their proton conductance. Furosemide raises the capacitance of the monolayer in the stable region due to intercalation between the head groups thus reducing the thickness of the hydrocarbon layer. In pure PC monolayer about 10% increase in capacitance is observed in the presence of 6×10^{-4} M furosemide. The effect of furosemide becomes more pronounced with increasing sphingomyelin content in the mixed monolayers. The monolayer of PE is more condensed and its capacitance is lower ($\sim 1.45\ \mu F/cm^2$) and is stable in a wider potential range than that of PC. It is less affected by furosemide and concentrations higher than 10^{-3} M are required to narrow the stability range and to increase the capacitance.

Key Words furosemide · phospholipid monolayers · capacitance · polarographic current

Introduction

Furosemide is a loop diuretic acting primarily via inhibition of active readsorption of Cl^- in the lumen of the diluting (ascending) segment of the loop of Henle (Allen, 1983). Indirectly it also prevents reabsorption of Na^+ which follows Cl^- passively. It is used to promote Na^+ and water excretion but concomitantly it also increases potassium excretion. Furosemide-sensitive transport systems have also been observed and recently subjected to detailed investigation in human red cells (Canessa et al., 1986). Furosemide also has some adverse side effects (Allen, 1983). On the way to the target sites as well as to the sites where it may have adverse effects, furosemide has to cross various membrane barriers. Moreover, its action on different sites may be influenced by its effect on the phospholipids embedding the site. The understanding of the interaction of furosemide with phospholipids becomes therefore of major importance. The extremely low solubility of furosemide in hydrocarbons excludes the possibility of its action on phospholipid monolayers through the hydrocarbon layer. All its effect on either the structure or the permeability of phospholipid layer may proceed only via its interaction with the polar groups.

In this work we show the effect of furosemide on the structure and the permeability of different phospholipid monolayers.

Materials and Methods

Interaction of furosemide with spread monolayers of phospholipids was investigated on a dropping mercury electrode as described elsewhere (Pagano & Miller, 1973; Miller, Rishpon & Tannenbaum, 1976; Miller & Rishpon, 1977). The procedure can be summarized as follows: the lipids were spread from a 1 mg/ml solution in hexane contained in 1 to 2% ethanol on the surface of a buffered solution (pH 8) containing different concentrations of furosemide in a polarographic cell with a surface area of 35 cm^2 until reaching a surface concentration of 0.5 $\mu g/cm^2$. This surface concentration is equivalent to nearly two fully compressed monolayers (Lecompte & Miller, 1987). This excess is necessary to allow for the depletion by the passing mercury drops which removes from the air/water interface a monolayer area section equal to the area of each passing drop. The dropping mercury electrode was positioned about 0.4 mm above the surface so that after detachment of each drop the contact with the surface was broken for 1 to 2 sec. The growing drop then contacted the monolayer which was transferred to the mercury interface where its capacitance was measured by phase-controlled AC polarography and its permeability to protons by DC polarography measuring oxygen reduction current using a PAR 170 polarograph.

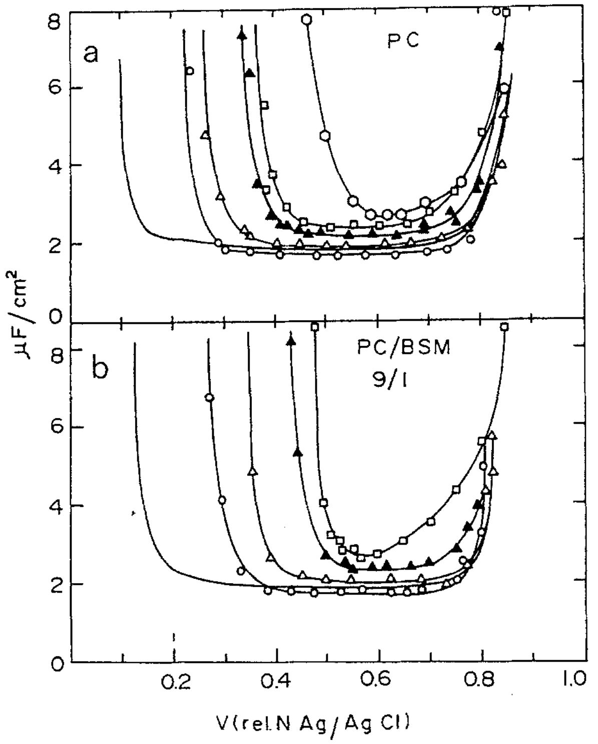

Fig. 1. Differential capacitance of pure PC (*a*) and of 90% PC/10% BSM monolayers (*b*) as a function of potential relative to normal Ag/AgCl electrode in the presence of different concentrations of furosemide at pH 8.3. Furosemide concentrations: 0–without points; 2 × 10^{-4}–○; 6 × 10^{-4}–△; 10^{-3}–▲; 2 × 10^{-3}–□; 4 × 10^{-3}–⬡

Results

MONOLAYER CAPACITANCE

It is important for the capacitance and even more so for the permeability measurements of the monolayers to maintain them in a faultless state. Cracks or voids occupying an area fraction of the monolayer give proportionally increased capacitance values, but may give completely leaky monolayers. Folds have negligible effects either on capacitance or conductance. Since monolayers below the phase transition do not reseal instantaneously when cracks are formed during the growth of the mercury drops, their capacitances and their permeabilities were very unsteady. We restricted therefore our investigation to monolayers of lipids above their phase transition. We studied monolayers of egg PE and egg PC and of mixtures of egg PC with up to 33% bovine brain sphingomyelin (BSM) at 28°C at which temperature all these lipids and even the lipid mixture containing 33% BSM are known to be in the liquid crystalline phase (Untracht & Shipley, 1977).

We also measured the effect of furosemide on the capacitances and on the permeabilities of monolayers of DPPC and BSM at around 50°C.

In Fig. 1(*a*) and (*b*) the capacitance of a condensed monolayer of egg PC and of (1/9) mixture of BSM/PC in the presence of different concentrations of furosemide is presented as a function of potential. In the absence of furosemide the PC monolayer is stable between −120 and −800 mV relative to N Ag/AgCl electrode. This corresponds to the range of +340 to −340 mV relative to the zero charge point (electrocapillary maximum) where the electrical field across the monolayer is zero. The minimal measured capacitance is 1.8 μF/cm² around the zero charge point when the lipids are oriented with their hydrocarbon chairs towards the mercury surface and it increases abruptly at +340 and −340 mV relative to the zero charge point. At the positive polarization the monolayer is displaced from the surface while at the negative polarization it is reoriented (Miller & Rishpon, 1977; Lecompte & Miller, 1980). Increasing concentrations of furosemide narrow the stability range of the monolayers and increase their capacitance. The narrowing of the range proceeds from the positive polarization which is in keeping with the properties of furosemide as a surface-active anion. It adsorbs in the whole potential region but more strongly on the positively charged mercury surface from where it displaces the hydrocarbon chains of the lipids. As a surface-active anion it also shifts the zero charge point (electrocapillary maximum) toward negative potentials. At the same time the capacitance of the monolayer in the stable region increases gradually with the concentration of furosemide up to 2.75 μF/cm² in the presence of 4 × 10^{-3} M furosemide. Addition of sphingomyelin to PC in a mixed monolayer narrows the stability range, until at 33% BSM the stability range is down to 550 mV. Lower concentrations of furosemide are required to disrupt the mixed monolayers. As in pure PC monolayers furosemide affects the mixed monolayer stability mainly at positive polarization. In Fig. 1(*b*) the capacitance curves of a monolayer containing 90% PC and 10% BSM in the presence of different concentration of furosemide are presented. The stability region is narrowed and the effect of furosemide on it is more pronounced than in pure PC. This trend continues with increasing concentration of BSM. In Fig. 2 the potential range of monolayer stability in the presence of different concentrations of furosemide is presented as a function of BSM content.

The stability of the monolayers decreases as the temperature increases. We measured the capacitance of pure monolayers of DPPL and of BSM

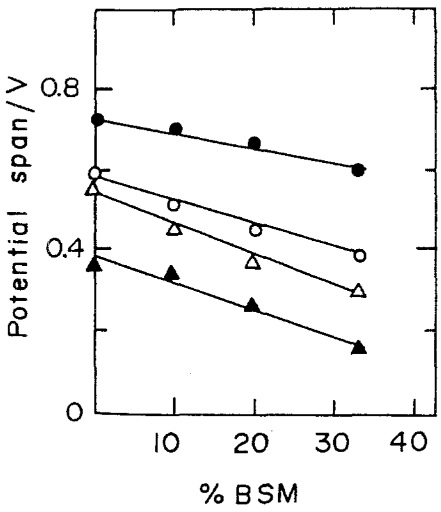

Fig. 2. Potential range stability of mixed PC/BSM monolayers in the presence of different concentrations of furosemide as a function of BSM content of the monolayer. Furosemide concentrations: ●—● 0; ○—○ 2 × 10^{-4}; △—△ 6 × 10^{-4}; ▲—▲ 10^{-3}

above their phase transition at 46 and at 48°C. The capacitance of both monolayers was about 2 μF/cm^2 at the zero charge point. The stability range was found to be between 250 and 600 mV relative to N Ag/AgCl electrode for BSM and between 230 and 720 mV for DPPC. The effect of furosemide parallels the stability range. 10^{-3} M furosemide suffices to displace the BSM monolayer except for a narrow range between −550 and −700 mV relative to N Ag/AgCl electrode. At the same concentration of furosemide a more or less stable DPPC monolayer is retained between −500 and −750 V. The capacitances at the minimum in the presence of 10^{-3} M furosemide are about 4 μF/cm^2 for DPPC and higher for BSM.

Phosphatidylethanolamine (PE) as seen from Fig. 3(*a*) and (*b*) gives more condensed monolayers than PC. The specific capacitance of the very broad minimum of the capacitance versus potential curve is only 1.45 to 1.5 μF/cm^2. Even at potentials more negative than the rearrangement potential (−900 mV relative to N calomel electrode) the capacitances are around 4 μF/cm^2 and they increase sharply at −1.1 V. Furosemide has a very small effect on these monolayers and at pH 8.3 a concentration of 1.2 × 10^{-2} M is required to shift the positive monolayer breakdown potential from −200 to −350 mV relative to 1 N Ag/AgCl electrode. At pH 7 (Fig. 3*b*) 8.6 × 10^{-3} M of furosemide is required to shift the positive breakdown potential from −150 to −325 mV. The PE monolayer is slightly negatively charged at pH 8.3 which shifts the potential of the stability region; however the effect of furosemide is only slightly pH dependent. As in the case of the other lipid monolayers, furosemide increases the monolayer capacitance in the stable region around the zero charge point. In the case of PE monolayer the increase is from 1.45 to 1.85μF/cm^2 in the presence of 0.86 × 10^{-2} M furosemide at pH 7 and from 1.52 to 1.96 μF/cm^2 in the presence of 1.2 × 10^{-2} M at pH 8.3.

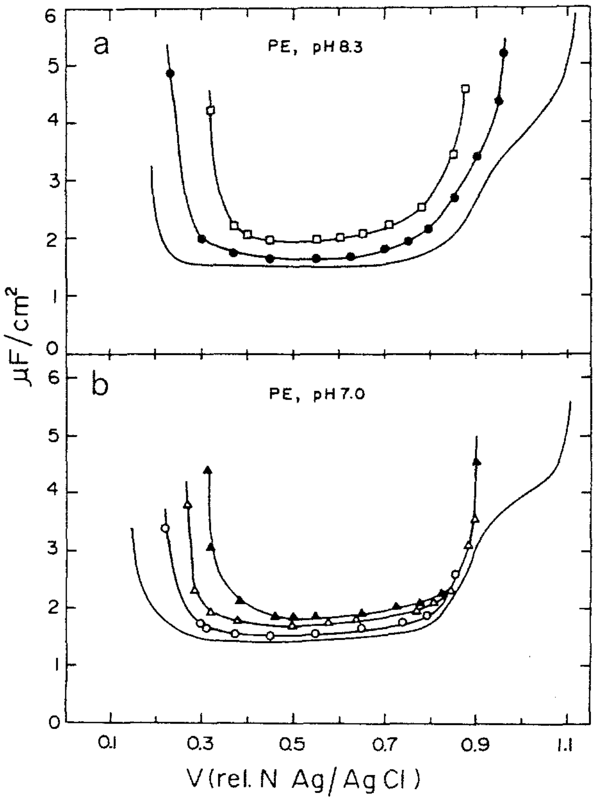

Fig. 3. Differential capacitance of PE monolayers at pH 8.3 (*a*) and at pH 7 (*b*) as a function of potential relative to N Ag/AgCl electrode. Furosemide concentrations: 0–without points; ○—○ 2.15 × 10^{-3}; ●—● 4 × 10^{-3}; △△ *r*.3 × 10^{-3}; ▲▲ 8.6 × 10^{-3}; □ 1.2 × 10^{-2}

PROTON PERMEABILITY

One way to measure proton permeability is to measure reduction currents of O_2. The lipid layers are completely permeable to oxygen and its reduction is controlled by the access of proton required for the reduction to hydrogen peroxide (Bach & Miller, 1970). The modulation of proton permeability by furosemide is bound to express itself in the reduction current of O_2. In Fig. 4 the reduced current i/i_0 of oxygen reduction through a DC monolayer in the

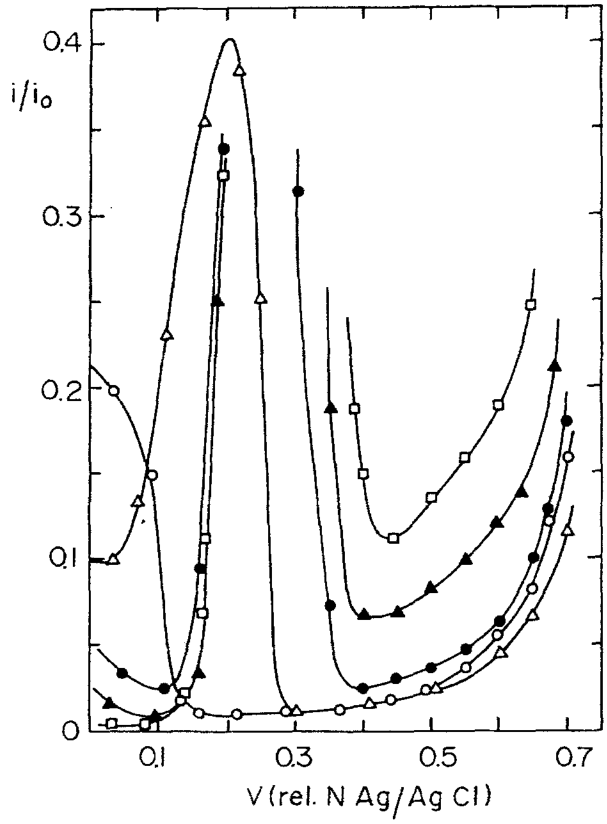

Fig. 4. Reduced oxygen reduction current i/i_0 (I_0 = the current in the absence of monolayers as a function of potential relative to N Ag/AgCl electrode in the presence of a condensed monolayer of PC and different concentrations of furosemide: ○—○ 0; △ 4 × 10^{-4}; ● 1 × 10^{-3}; ▲ 2 × 10^{-3} and □ 3 × 10^{-3} furosemide

presence of different concentrations of furosemide is presented. i_0 is the current in the absence of the lipid monolayer and of furosemide under otherwise identical conditions.

In the absence of furosemide the reduced current reaches a minimum of about 0.01 at around −0.2 V. At −0.15 V the monolayer becomes unstable and the current increases abruptly when we proceed towards positive potentials. The reduced current increases mildly with the negatively of the potential up to −0.5 V when its increase with potential becomes successively steeper until at −0.7 V where the monolayer stability decreases the current picks up abruptly. The gradual increase in current can be attributed in part to the increase in the rate of the electrode process. Furosemide at a concentration of 4 × 10^{-4} M displaces the monolayer at anodic potentials and a stable monolayer is obtained only at −0.28 V relative to N Ag/AgCl electrode. The permeability of the monolayer in the potential region between −0.3 to −0.7 V in the presence of 2 × 10^{-4} M furosemide is little lower than in its absence, which is in keeping with the lower capacitance values (Fig. 1*a*). At higher concentrations of furosemide the instability region shifts to more negative potentials and the permeability of the stable monolayer increases with increasing concentration of furosemide. At the same time the current at positive polarizations up to −0.125 V is strongly reduced. In this potential region the anionic furosemide adsorbs on the mercury surface and impedes the electron transfer from the electron.

The effect of furosemide on the proton permeability of PE monolayers and of mixed monolayers of PC with BSM was also measured but is not shown. Furosemide up to 2 × 10^{-3} M affects very little the permeability of the PE monolayer in the stable region even though, and as evident also from the capacitance curves, it shifts the anodic displacement potential to more negative values. It affects the proton permeability appreciably above 5 × 10^{-3} M. At this concentration it also increases the conductance of PE bilayers (Bach et al., 1988). Furosemide, already at low concentrations makes the mixed monolayers of PC with BSM extremely leaky. In every case, including in the absence of lipid monolayer, furosemide reduces the current at polarizations more positive than −0.125 V relative to 1 N Ag/AgCl electrode.

Discussion

The effect of furosemide on lipid monolayers at the mercury/water interface is through competition with the lipid molecules for surface sites and through interaction with the head groups. Furosemide can penetrate the lipid layer and compete with the lipid molecules for surface sites and eventually may displace them from the surface. Being a surface-active anion, it adsorbs and exerts this effect mainly at positive polarizations. As a result, the anodic breakdown of the lipid monolayer shifts towards more negative potentials as the furosemide concentration increases. With the head groups it interacts at all polarizations. At low concentrations it interacts with the head groups aligning them and inducing stronger attractive intermolecular interaction resulting in decrease in capacitance. At higher concentration it intercalates between the head groups increasing the mean area per liquid molecule and thus raising the capacitance.

Absorption of furosemide and its effect on the surface concentration of lipids Γ_L determines the change in surface tension in the following way:

$$-d\gamma = \Gamma_F d\mu_F + \Gamma_L d\mu_L + \Gamma_{H_2O} d\mu_{H_2O}. \qquad (1)$$

The last term in Eq. (1) can be neglected since the molar fraction of furosemide is always below 2×10^{-4} and

$$\frac{d\mu_F}{dX_F} = \frac{RT}{X_F} \gg -\frac{d\mu_{\mathrm{H_2O}}}{dX_F} = \frac{RT}{1 - X_F}. \qquad (2)$$

The total variation of the surface tension $-\Delta\gamma$, after increasing the molar fraction of furosemide from 0 to X_F, is

$$\begin{aligned} -\Delta\gamma &= RT \int_{\Gamma_{L(X_F=0)}}^{\Gamma_{L(X_F)}} \Gamma_L d \ln \Gamma_L + \int_0^{X_F} \Gamma_F d \ln X_F] \\ &= RT[\Gamma_{L(X_F)} - \Gamma_{L(X_F=0)} + (K'_m + K_m^{\mathrm{Hg}}) X_F]. \end{aligned} \qquad (3)$$

K'_m is related to the distribution coefficient measured between water and liposomes (K_p)

$$K'_m = \frac{K_p}{V_{\mathrm{H_2O}}} \delta \approx 10^{-8} K_p$$

where δ is the monolayer thickness ≈ 18 to 20 Å. K_m^{Hg} is the adsorption coefficient at a site between the hydrocarbon chains on the mercury surface. It is usually low since it involves penetration between hydrocarbon chains. We imply here two populations of furosemide molecules, one in contact with the mercury surface and the other adsorbed in the polar region. The furosemide in contact with the mercury surface occupies usually very small area fraction θ with a specific capacitance C_F. The measured capacitance is then a sum of the capacitances of the furosemide and of the liquid domains C_L.

$$C = \theta C_F + (1 - \theta) C_L$$

and

$$\theta/(1 - \theta) = K_m^{\mathrm{Hg}} X_F. \qquad (4)$$

The capacitance of the lipid domain depends on the thickness of the lipid layer and assuming incompressibility.

$$\Gamma'_L C_L = \text{constant}$$

and

$$(\Gamma_L C_L)_{(F=0)} = \Gamma'_L C_L \qquad \Gamma_L = (1 - \theta)\Gamma'_L \qquad (5)$$

where Γ'_L is defined as the surface concentration in the lipid domain. Hence

$$\Gamma_L = (1 - \theta)\Gamma_{L(F=0)} \left(1 + \frac{C_{L(F=0)} - C_L}{C_L}\right). \qquad (6)$$

The distribution coefficient of furosemide between liposomes of different kinds and water, K_p, is around 20, which makes $K'_m \approx 2 \times 10^{-7}$ mole · cm^{-2}. When varying the furosemide concentration between 2×10^{-4} M to 10^{-2} M ($X_F = 3.6 \times 10^{-6}$ to 1.8×10^{-4}) its surface concentration varies between $\sim 7 \times 10^{-13}$ and 3.6×10^{-11} moles/cm^2 or 43×10^{10} and 22×10^{12} molecules/cm^2. This corresponds to one furosemide per 400 to 8 molecules of lipid. One furosemide per 200 or more lipid molecules causes their alignment resulting in thickening of the lipid layer and in lowering its capacitance. Only at higher surface concentrations the furosemide molecules intercalating between the polar residues of the lipids, expand the monolayer reducing the thickness of its hydrocarbon layer and increasing the capacitance. If the cross-sectional areas of the furosemide and of the polar group of the lipid are a_F and a_L, respectively, then in the lipid domain

$$\begin{aligned} \Delta\Gamma &= \Gamma_{L(F=0)} - \Gamma_L^{(1-\theta)} = \Gamma_F \frac{a_F}{a_L} \\ &= \Gamma_{L(F=0)} \frac{C_L - C_{L(F=0)}}{C_L} \end{aligned} \qquad (7)$$

where $\Gamma_L^{(1-\theta)}$ is the surface concentration of the lipid in the not displaced lipid domain

$$\Gamma_L^{(1-\theta)} = \frac{\Gamma_L}{1 - \theta}$$

and

$$C_L = \frac{\theta_{L(F=0)} C_{L(F=0)}}{\Gamma_{L(F=0)} - K'_m X_F \frac{a_F}{a_L}}. \qquad (8)$$

C in Eq. (4) becomes then

$$C = \theta C_F + (1 - \theta) \frac{C_{L(F=0)} \Gamma_{L(F=0)}}{\Gamma_{L(F=0)} - K'_m X_F \frac{a_F}{a_L}}. \qquad (9)$$

Knowing or assuming a value for C_F, we can calculate θ, which represents the degree of penetration of furosemide into the hydrocarbon layer. We assumed that C_F equals the capacitance of a condensed monolayer of furosemide measured in solution in the presence of a high concentration of furosemide. In the Table the calculated values of θ, of the hydrocarbon penetration and binding constant to the mercury K_m^{Hg} of furosemide as well as $\Delta\gamma$ calculated by Eq. (3) are presented. K_m^{Hg} was obtained from the calculated values of θ. In the case

Table. Effect of furosemide on the capacitance of monolayers of PE, PC and of a mixture of 20% SM and 80% PC at −0.5 V relative to N calomel electrode

a. PE

X_F	$C(\mu F/cm^2)$	$K'_m 10^7$	$\Delta\gamma$(dyn/cm)
2×10^{-5}	1.45	2.9×10^{-7}	0.17
7.75×10^{-5}	1.6	3.45×10^{-7}	0.8
1.55×10^{-4}	1.75	2.6×10^{-7}	1.25

b. PC ($K'_m = 2.2 \times 10^{-7}$ mole/cm²)

X_F	$C(\mu F/cm^2)$	θ	K_m^{Hg}(mole/cm²)	$\Delta\gamma$
1.1×10^{-5}	2	0.0061	2.54×10^{-7}	0.5
1.8×10^{-5}	2.15	0.0172	3.05×10^{-7}	0.4
3.6×10^{-5}	2.4	0.0215	2.7×10^{-7}	1.4
7.2×10^{-5}	2.75	0.035	2.2×10^{-7}	2.0

c. 20% SM 80% PC ($K'_m = 2.2 \times 10^{-7}$ mole/cm²)

3.6×10^{-6}	2.2	0.0076	9.5×10^{-7}	0.475
1.1×10^{-5}	2.5	0.0186	7.75×10^{-7}	1.05
1.8×10^{-5}	2.8	0.0314	7.85×10^{-7}	1.5

of PE, θ was assumed to be zero and K'_m was calculated for this condition.

The values for the interaction coefficient of furosemide for K'_m with PE monolayers in the Table correspond to distribution coefficients of K_p between 23 and 31 which is about equal to those measured with PE suspensions (Bach et al., 1987). There is evidently no tendency for furosemide to penetrate the dense hydrocarbon layer of the PE monolayers. The tendency of furosemide to penetrate increases with decreasing density of the lipid monolayer. The hydrocarbon penetration mercury adsorption coefficient K_m^{Hg} through the PC monolayer is about equal to the adsorption coefficient to the polar region while in the even more expanded mixed monolayers of 20% SM 80% PC it is about three times larger. The measured proton transport across the monolayers confirms at least qualitatively the calculated values of θ in the different monolayers. In the PE monolayers where the area fraction θ of the hydrocarbon layer penetrated by furosemide approaches zero, the conductance is also less affected by furosemide. Only at relatively high concentrations (>2×10^{-3} M) the conductance increases appreciably. Penetration in the present case also reflects interaction with the memory surface and it probably does not occur in lipid bilayers. The penetration of the head group region and subsequent expansion of the bilayer is a general phenomenon.

I wish to thank Mr. Haim Graet for his technical help.

References

Allen, R.C. 1983. Sulfonamide diuretics. *In:* Deuretics. E.J. Gragoe, editor. pp. 119–200. John Wiley, New York

Bach, D., Miller, I.R. 1970. The inhibition of oxygen reduction by adsorbed monolayers of phospholipids, proteins and synthetic polybases. *Electrochim. Acta* **15**:533–543

Bach, D., Vinkler, C., Miller, I.R., Caplan, S.R. 1988. Interaction of furosemide with lipid membranes. *J. Membrane Biol.* **101**:103–111

Canessa, M., Brugnara, C., Cusi, D., Tosteson, D.C. 1986. Modes of operation and variable stoichiometry of the furosemide sensitive Na and K fluxes in human red cells. *J. Gen. Physiol.* **87**:113–142

Lecompte, M.F., Miller, I.R. 1980. Interaction of prothrombin and its fragments with monolayers containing phosphatidylserine 2. Electrochemical determination of lipid layer perturbation. *Biochemistry* **19**:3439–3446

Lecompte, M.F., Miller, I.R. 1987. Reversibility of prothorombin adsorption on lipid monolayers. *J. Colloid Interface Sci.* (*in press*)

Miller, I.R., Rishpon, Y., Tennenbaum, A. 1976. Electrochemical determination of structure and interactions in spread lipid monolayers. *Bioelectrochem. Bioenerg.* **3**:528–542

Miller, I.R., Rishpon, Y. 1977. Structure and permeability of lipid monolayers interacting with proteins and polypeptides. *In:* Electrical Phenomena at the Biological Membrane Level. E. Roux, editor. Elsevier, Amsterdam.

Pagano, R.E., Miller, I.R. 1973. Transport of ions across lipid monolayer: IV. Reduction of polarographic currents by spread monolayers. *J. Colloid Interface Sci.* **45**:126–137

Untracht, S.H., Shipley, G.G. 1977. Molecular interactions between lecithin and sphingomyelin: Temperature and composition dependent phase separation. *J. Biol. Chem.* **252**:4449–4457

Received 8 June 1987; revised 13 October 1987