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Interaction of Furosemide with Lipid Membranes

Diana Bach, Chana Vinkler, Israel R. Miller, and S. Roy Caplan
Department of Membrane Research, The Weizmann Institute of Science, Rehovot 76100, Israel

Summary. The interaction of furosemide with different phospholipids was investigated. Its influence on the lipid structure was inferred from its effect on the phase transition properties of lipids and on the conductance of planar bilayer membranes. The thermotropic $_{t}$ properties of dipalmitoyl phosphatidylcholine, phosphatidylethanolamine (natural), dipalmitoyl phosphatidylethanolamine, brain sphingomyelin, brain cerebrosides and phosphatidylserine in the presence and absence of furosemide were investigated by differential scanning calorimetry. The modifying effect of furosemide seems to be strongest on phosphatidylethanolamine (natural) and sphingomyelin bilayers. The propensity of furosemide to decrease the electrical resistance of planar lipid membranes was also studied and it is shown that the drug facilitates the transport of ions. Partition coefficients of furosemide between lipid bilayers and water were measured.

Key Words: Furosemide · partition into lipids · differential scanning calorimetry · PLM · conductance

Introduction

The sulfonamide diuretic furosemide (Fig. 1) has proved to be one of the most important additions to the clinical armamentarium of therapeutic drugs of this type. Its predominant and also specific renal effect is the inhibition of sodium, potassium, and chloride reabsorption from the lumen of the thick ascending limb of Henle's loop, which takes place via a three-ion cotransporter (Velazquez & Wright, 1986). In so doing it brings about the excretion of large volumes of relatively dilute urine, and hence is the diuretic of choice for the treatment of severe edematous conditions (Allen, 1983). Like other members of the class of loop, or so-called "high-ceiling" diuretics (e.g. bumetanide, ethacrynic acid), all of which act on the cotransporter from the lumen compartment, furosemide invariably increases renal potassium excretion in parallel with the desired sodium and water excretion. Within the systemic circulation it binds extensively to plasma proteins, and reaches its site of action in the kidney not by glomerular filtration but as a consequence of secretion by cells of the proximal tubule (Velazquez & Wright, 1986). Furosemide-sensitive cotransport systems have also been observed in several epithelial as well as red cells, and have recently been subjected to detailed study in the human red cell (Brugnara et al., 1986; Canessa et al., 1986). However, the interaction of furosemide with lipids has not hitherto been systematically investigated. Since furosemide is not soluble in hydrocarbons, and yet, as will be seen, exerts a profound effect on protein-free membrane permeability, it is clearly desirable to characterize the nonspecific, yet potentially significant, aspects of its properties.

As the detailed mechanism of action of furosemide is not well understood and as the drug has to cross various membrane barriers on the way to its target site, it was of interest to investigate its interaction with the simplest model membranes—phospholipid bilayers.

In the present work two approaches were used to evaluate this problem: (1) to investigate the influence of furosemide on the structure of various lipid bilayers varying in polar head groups or hydrocarbon regions by employing DSC; (2) to investigate the ability of furosemide to decrease the resistance of lipid bilayers to ion transport (Na^+) as evaluated from electrical measurements of PLM. For quantitative interpretation of the data the concentration of the drug in the lipid phase has to be known. As no values of partition coefficient were reported in the literature, partition coefficients of furosemide between solvents and water or lipids and water were also determined.

Abbreviations

DSC—differential scanning calorimetry; PLM—planar lipid membranes; DPPC—dipalmitoyl phosphatidylcholine; DMPC—dimyristoyl phosphatidylcholine; PE—phosphatidyl ethanol-

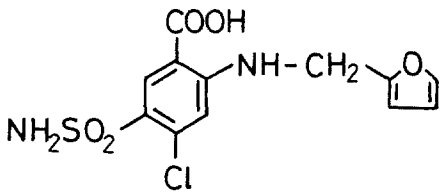

Fig. 1. Chemical formula of furosemide

amine (natural); DPPE—dipalmitoyl phosphatidylethanolamine; Cerebrosides—brain cerebrosides; SP—brain sphingomyelin; PS—phosphatidyl serine; Diphytyl-PC—diphytanoyl phosphatidylcholine; T_m—temperature of the middle of the peak; T_p—temperature of the pretransition; T_c—temperature of the onset of the transition; ΔH—enthalpy of melting.

Materials and Methods

Furosemide (5-[aminosulfonyl])-4-chloro-2[(2-furanylmethyl) amino] benzoic acid), dipalmitoyl phosphatidylethanolamine, dimyristoyl phosphatidylcholine and Trizma Base were purchased from Sigma, St. Louis, Mo. Phosphatidyl ethanolamine (egg yolk) grade I, phosphatidylserine (bovine spinal cord), and sphingomyelin were bought from Lipid Products, South Nutfield, England. Diphytanoyl phosphatidylcholine was obtained from Avanti, Birmingham, Alabama. Dipalmitoyl lecithin was obtained from Dr. Bertchold's Lab., Bern, Switzerland. Brain cerebrosides were purchased from Supelco, Belfonte, Pa. All other reagents were of analytical grade. The furosemide solution was prepared by dissolving the drug in an equivalent and a half or two equivalents of Trizma Base (except for the experiments for determination of the ionization constant) and the final pH was 8.3 to 8.5.

Determination of the Ionization Constant

About 100 μmoles of furosemide were dissolved in an excess of NaOH in the presence of about a 10-fold concentration of NaCl. After complete dissolution of the drug the solution was titrated with 0.1 M HCl with constant stirring and bubbling of nitrogen.

Determination of the Partition Coefficient

For the determination of partition coefficients furosemide was added to 0.15 M NaCl buffered with 2×10^{-2} M Tris · HCl buffer to give a final concentration of 10^{-2} M furosemide. To about 10 mg dry lipids 250 μl of furosemide-salt solution were added. In the case of lipids purchased as solutions in organic solvents, the solvents were driven off by a stream of nitrogen and kept under high vacuum for 3 hr.

The lipid/furosemide dispersions or solvent/furosemide dispersions were shaken on a mechanical shaker for 1 hr. In the case of DPPC, PE and DMPC the samples were also sonicated for 8 min in a water bath sonicator (Laboratory Supplies Co. Inc., Hicksville, N.Y., Model G112SP1G) at the appropriate temperature or freezed-thawed (3×) prior to incubation. The results obtained with or without treatment prior to incubation were similar. Also prolonged incubation up to 4 hr yielded results similar to those obtained after 1-hr incubation.

The dispersions were then centrifuged in an Ependorff table centrifuge at the appropriate temperature for 15 min. Dispersions containing PE or DPPC were centrifuged in a Beckman Airfuge centrifuge at 30 psi for 30 min. Furosemide concentration was then determined in the clear aqueous phase by measuring optical densities at $\lambda = 330$ nm. Optical densities were measured for a control containing furosemide before the equilibration (OD_n) and for the same dilutions of the aqueous phase after equilibration with the lipid (OD). The difference in the two optical density values gives the concentration of furosemide in the lipid phase. For K_p calculations it was assumed that 1 mg lipid is equal to 1 μl.

The partition coefficient of furosemide (lipid/H_2O or solvent/H_2O) is defined in the following way:

$$K_p = \frac{(OD_n - OD)}{OD} \cdot \frac{V_{H_2O}}{V_{lipid}} \quad (1)$$

where V_{H_2O} and V_{lipid} are the volumes of water and lipid in the suspension, respectively.

DSC Measurements

PE and PS were obtained in chloroform/methanol 2:1. The solvents were driven off by a stream of nitrogen and subsequently the phospholipids were kept under high vacuum for 3 hr. All the lipids (in powder form) were weighed (0.8 to 1.1 mg) directly into the aluminum pans of the instrument. The lipids were weighed on a Cahn Balance (Cahn Model 4100 Electrobalance, Cahn/Ventron Corp., Paramount, Calif.) and appropriate volumes of 10^{-1} M NaCl buffered with 2×10^{-2} M Tris · HCl with or without furosemide were added. For the experiments with SP and DPPE 1.5×10^{-1} M NaCl buffered with Tris · HCl was used. The concentration of lipids was 8 to 10%. The pans were sealed, vortexed for 1 min and incubated for 1 hr at 45°C (DPPC), 35°C (PE), 38°C (PS), 80°C (cerebrosides), 80°C (DPPE), or 70°C (SP). The DSC measurements were performed on a DuPont 990 instrument equipped with cell base II. Scan rates of 5°/min were usually employed, and the first and subsequent scans were similar indicating that during incubation equilibration with water was achieved.

PLM

Planar lipid membranes, solvent free, were formed from monolayers by the method of Montal and Mueller (1972). The phospholipids investigated were PE and diphytanoyl phosphatidylcholine. The phospholipids were dissolved at a concentration of 2% in hexane, and the Teflon® septum was pretreated with 2% squalene in hexane. For voltage application and current measurement silver-silver chloride electrodes were used, the current being fed into a Keithley 427 current amplifier (Keithley Instruments, Cleveland, Ohio) and the resulting voltage recorded on a Kipp and Zonen BD8 recorder. Experiments were performed in 10^{-1} M NaCl buffered with 2×10^{-2} M or 5×10^{-2} M Tris · HCl at pH 8.3. Furosemide was always added with stirring to one side of the bilayer (virtual ground).

Results and Discussion

Determination of the Ionization Constant of Furosemide and its Partition Coefficient for the System Solvents/Water and Lipids/Water

Furosemide has two ionizable groups: a carboxylic group and an amino group. Knowledge of their ionization constants is essential for the interpretation of experimental results. Orita et al. (1972) determined the ionization constants for furosemide in dioxane-water mixtures and obtained pK values 7.5 and 3.8. As these pK values are probably too high due to the use of organic solvents we repeated the titration by dissolving furosemide directly in an excess of NaOH (without organic solvents) and titrated back with HCl. The pK values obtained by us are 5.3 and about 2.5 (in the presence of 10^{-2} M NaCl). Furosemide starts to precipitate below pH 6, when the zwitterionic form with a net zero charge is formed, and curiously the precipitate does not dissolve at low pH when the drug becomes positively charged. In the present work all the experiments were performed at pH 8.1 to 8.4 to ensure that we were working with a pure negative form of the molecule. Another important parameter for the interpretation of the experimental results is the concentration of furosemide in the lipid phase. This parameter can be calculated from the known values of the partition coefficients (K_p) of furosemide between the lipid and the salt solution. To our knowledge the partition coefficients of furosemide between lipid and water were not hitherto reported in the literature. The only value reported was the K_p for octanol/H_2O at pH 7.4 (0.4) (Allen, 1983). This value is much higher than the one measured by us at pH 8.2 (0.05). The partition into octane was too low to be measurable. As judged from the K_p values in solvents and lipids the hydrophobicity of furosemide is quite low, as the drug almost does not partition into the simple hydrocarbon solvent octane. Partition of furosemide into octanol is higher than into octane showing that the polar head group is very important for the accumulation of the drug into the lipid phase. The K_p values obtained for the various lipids are presented in Table 1. In the case of DPPC and SP K_p measurements were performed both in the gel and liquid-crystalline states giving rise to comparable values in both phases. To further verify this point, partition coefficients for DMPC in the gel and liquid crystalline phases were measured and gave similar values (Table 1). For cerebrosides and PS the values of K_p are very low, in the case of PS due to the electrostatic repulsion between the negatively charged lipid and the negatively charged furosemide.

Table 1. Partition coefficients of furosemide (lipid/H_2O)

Lipid	Temp. (°C)	K_p
DPPC	22	33.1 ± 3.4 (10)[a]
	54	23.8 ± 4.1 (17)
DMPC	0–2	20.4 ± 2.8 (4)
	36	28.6 ± 5.7 (11)
PE	26	11.9 ± 2.7 (17)
DPPE	72	5.3 ± 3.2 (3)
SP	22	12.0 (2) (14.0,10.4)
	35	14.0 (1)
	52	15.5 ± 2.1 (3)
PS	22	0.5 (2) (0.35,0.56)

[a] Numbers in brackets indicate the number of experiments performed.

Thermotropic Properties of Lipids Interacting with Furosemide

Thermotropic mesomorphism is a feature shared by all membrane lipids. The temperature and enthalpy of the phase transition are functions of the structure of the head groups, hydrocarbon chains, and degree of hydration and are strongly influenced by the interaction with various substances such as proteins, drugs, etc. (Bach, 1983). The thermotropic properties of lipids can be investigated by different biophysical methods; however differential scanning calorimetry (DSC) is the most direct one as it is a thermodynamic technique, which does not need probes. In the present study DSC was employed for the investigation of the influence of furosemide on the thermotropic properties of lipids. Lipids differing in the structure of the polar head and in the hydrocarbon chains were investigated in an attempt to evaluate specific interactions with the drug.

DPPC

Figure 2 presents the thermograms of DPPC alone and in the presence of furosemide at different concentrations in the phospholipid phase. The DSC data are also summarized in Table 2. The thermotropic properties of DPPC alone obtained in this work are as reported in the literature (Bach, 1983), characterized by a pretransition at 35°C and by a main transition at 41.5°C. The nature of the pretransition is not fully understood, but it was suggested (Rand, Chapman & Larsson, 1975; Janiak, Small & Shipley, 1976) that this endotherm stems from the change of the angle of tilt of the hydrocarbon

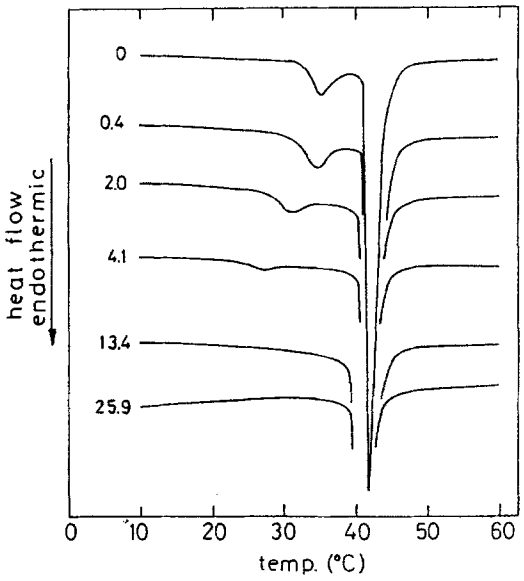

Fig. 2. Thermograms of DPPC alone and interacting with furosemide. Numbers indicate mol % of furosemide in the phospholipid calculated for $K_p = 21$; scan rate 5°/min; sensitivity 0.1 millical/sec · inch

chains. The transition at 41.5°C is due to melting of the hydrocarbon chains (transition from fully extended gel state into kinked structure—liquid crystalline state). As seen from Fig. 2 low concentrations of furosemide decrease the temperature and the size of the pretransition and finally at about 10 mol/100 mol the pretransition disappears completely. At about 4 mol/100 mol the main peak becomes broader and a small shift of T_m is seen. However, within experimental error, no change of ΔH is detected. This effect of furosemide on the pretransition is rather surprising as most other drugs and modifiers cause immediate disappearance of the pretransition at very low concentrations of the added drug (e.g. Bruggeman & Melchior, 1983). To our knowledge only one example of such behavior on the pretransition was published (O'Leary, Ross & Levin, 1984). Furosemide causes a gradual destabilization of the tilted conformation since it intercalates between the polar groups increasing the distances between the hydrocarbon chains. Above 10 mol/100 mol a decrease in the size of the cooperative unit as judged by the broadening of the melting

Table 2. Summary of DSC data

Lipid	Mole % furosemide	T_p (°C)	T_c (°C)	T_m (°C)	Transition half-width (°C)	ΔH (millical/mg)
DPPC:	—	35.7	41.6	42.0	1.5	15.1 ± 0.5[a]
	0.5	34.5	41.5	42.0	1.5	16.7 ± 2.1
	2.0	31.5	41.0	41.5	1.5	15.9 ± 1.5
	4.1	27.5	39.0	41.0	1.7	13.4 ± 1.2
	13.4	—	39.0	39.5	2.0	14.0 ± 0.8
	25.9	—	39.0	40.0	1.5	15.1 ± 0.5
PE:	—	—	—	12.5	—	4.4[b]
	0.4	—	—	12.2	—	3.6
	0.7	—	—	12.0	—	3.2
	1.9	—	—	11.7	—	3.4
	4.1	—	—	9.6	—	2.7
	20	—	—	4.5	—	~2.0
DPPE:	—	—	—	65	1.3	12.9 ± 0.6
	0.2	—	—	64.8	1.5	12.5 ± 1.5
	1.2	—	—	64.5	1.5	11.0 ± 0.7
	2.6	—	—	64.6	3.7	12.2 ± 0.6
	13	—	—	62	4.5	12.0 ± 0.6
SP:	—	—	33[+]	37.4[++]	—	8.3 ± 0.4[c]
	0.5	—	32.8	37.2	—	9.3 ± 0.4
	2.8	—	31.8	36.7	—	10.0 ± 1.2
	7.8	—	31.3	36.3	—	9.5 ± 0.6
	22	—	28.0	33.7	—	5.7 ± 1.6
PS:	—	—	—	13.7	—	5.7[d]
	0.03	—	—	13.2	—	6.4
	0.1	—	—	13	—	5.4
	0.3	—	—	12.6	—	4.7
	1.4	—	—	10.4	—	5.5

[a] Including pretransition.
[b] Due to broadness of the peak in natural lipids neither T_c nor the transition half-width are indicated. Part of ΔH is unaccounted for, due to a shift under the water melting peak.
[c] T_m given for each peak; ΔH calculated over the whole profile. [+] T_{m_1}; [++] T_{m_2}.
[d] Part of ΔH is unaccounted for due to a shift under the water melting peak.

peak and a small decrease of T_m is seen. However, furosemide does not complex with the hydrocarbon chains and does not eliminate freezing as no change in the enthalpy of melting was detected.

Phosphatidylethanolamine

As it was reported recently that phosphatidylethanolamine is the second most abundant phospholipid in the plasma membrane of dog kidney (Carmel et al., 1985), it was of interest to investigate the influence of furosemide on the thermotropic properties of phosphatidylethanolamine bilayers. In phosphatidylethanolamine the head groups are hydrogen bonded, as expressed by the higher stability and high melting temperature of the lipid. The interaction of furosemide with two types of phosphatidylethanolamines was investigated: natural (egg yolk) PE, which is a mixture of acyl chains, one at least of which is unsaturated, and disaturated lipid-dipalmitoyl phosphatidylethanolamine, which has the same acyl chains as the DPPC previously investigated, but differs from it in the head group. Figure 3(*a*) presents the thermograms of PE alone and interacting with furosemide and in Table 2 the DSC data are summarized. As PE is a natural lipid with a mixture of acyl chains its melting peak is very broad, and its T_m is 12.5°C. It has recently been shown (I. R. Miller, accompanying paper) that in phospholipid monolayers in the liquid crystalline phase the effect of furosemide in raising the electrical capacitance (i.e. modifying the hydrocarbon layer) is least in the case of PE. In contrast to its effect on monolayers, addition of furosemide causes a gradual decrease of T_m amounting to about 8°C at 20 mol/100 mol furosemide, and decrease of the enthalpy of melting. The decrease of ΔH, which depends on the hydrocarbon chains, is clearly much smaller than that reported in Table 2 and may well be artifactual, as part of the melting peak of PE modified by furosemide shifts under the water melting peak and is not taken into account in the calculations of ΔH. As the PE peak is broad, changes in half-width with interaction cannot be detected. In an attempt to overcome the interference from water melting, experiments of PE with furosemide were repeated in 30% ethylene glycol/70% salt solution. In this case the effect of furosemide on T_m was about half of that in the presence of salt solution only. Ethylene glycol might affect the distribution coefficient and the interaction of the drug with PE. The partition coeffi-

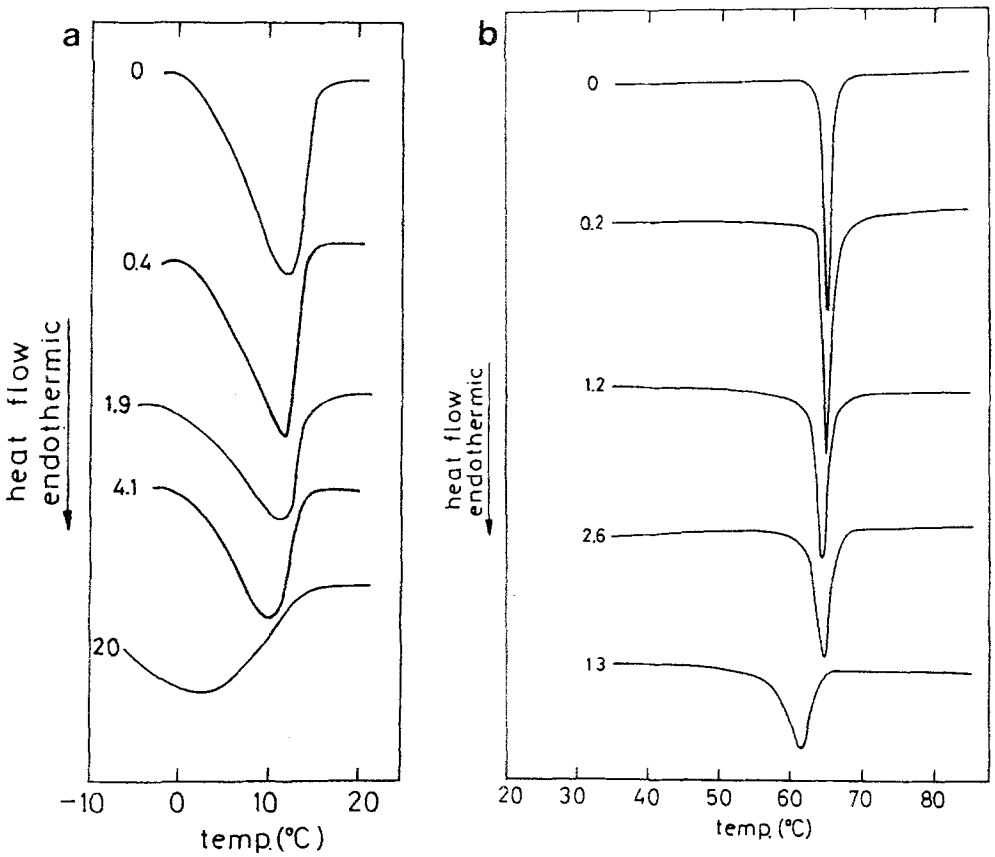

Fig. 3. Thermograms of phosphatidyl ethanolamine alone and interacting with furosemide. Numbers indicate mol % furosemide in the phospholipid. (*a*) PE: K_p = 12; scan rate 5°/min; sensitivity 0.02 millical/sec · inch. (*b*) DPPE: K_p = 5; scan rate 5°/min; sensitivity 0.1 millical/sec · inch

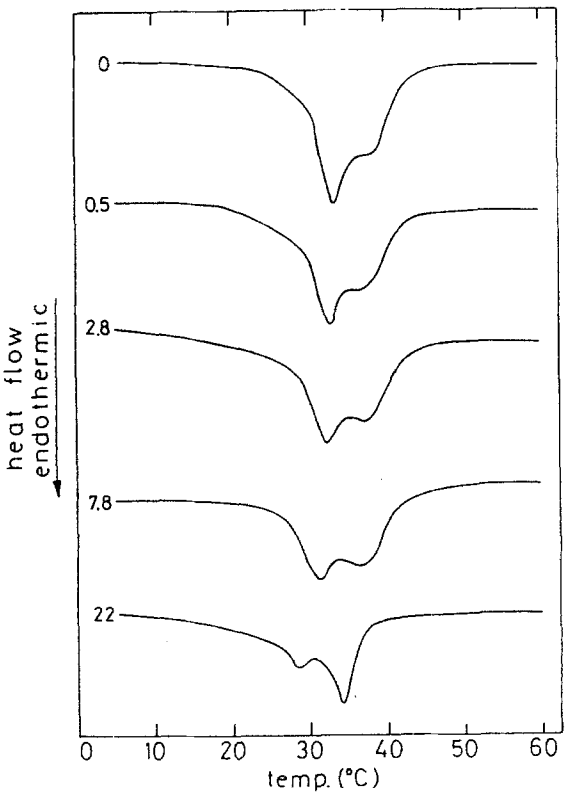

Fig. 4. Thermograms of SP alone and interacting with furosemide. Numbers indicate mol % of furosemide in the lipid-calculated for K_p = 15; scan rate 5°/min; sensitivity 0.04 millical/sec · inch

cient of furosemide from ethylene glycol/water might be lower than from water alone, giving effectively lower concentrations of the drug in the lipid phase, and ethylene glycol might compete with furosemide for the binding sites in the head group region. To evaluate the influence of the head group on the interaction, the effect of furosemide on synthetic dipalmitoyl phosphatidylethanolamine (DPPE) was investigated and compared to the data reported for DPPC (Fig. 3*b* & Fig. 2 and Table 2). As seen from the Table and the Figures, the effect of furosemide on both lipids is similar and a decrease of a few degrees in T_m is obtained without change in ΔH. However, it seems that furosemide has a much stronger effect on DPPE bilayers than on DPPC. In spite of lower concentrations of furosemide in the DPPE phase than in DPPC, the broadening of the peak and the decrease of T_m is higher in the case of DPPE than in the case of DPPC. As both lipids have the same acyl chain residues and differ only in the head groups, it can be concluded that the stronger effect on DPPE stems from the ability of furosemide to break the hydrogen bonds in DPPE in a fashion similar to its effect on the natural PE.

SP

Sphingomyelin is the most abundant phospholipid in the kidney plasma membranes (Carmel et al., 1985). In contrast to other lipids investigated in the present work, SP is a derivative of sphingosine with one acyl residue coming from the sphingosine base and the second one bound through an amide bond. The most abundant sphingosine has an 18-carbon-long chain with one double bond, but other sphingosine bases also exist (Calhoun & Shipley, 1979). The second acyl chain is of varying length and varying degree of unsaturation. Usually the two acyl residues are of unequal length allowing some degree of interdigitation of the hydrocarbon chains in the two monolayers. The high melting temperature of SP is due to hydrogen bonding in the head group region. The SP employed in the present work is from bovine spinal cord and it is heterogenous with respect to the acyl residues, probably containing high amounts of 18 : 0 and 24 : 1 chains similar to SP from bovine brain (Calhoun & Shipley, 1979). As seen from Fig. 4 the thermotropic behavior of SP is very complex, with two maxima at 33 and 37.5°C. This thermotropic behavior is characteristic of natural sphingomyelins and was reported by several groups (Shipley, Avecilla & Small, 1974; Barenholz et al., 1976; Calhoun & Shipley, 1979). It was even claimed that internal phase separation of the two species with different chain length takes place producing two distinct maxima, but no solid proof for such phase separation exists (Shipley et al., 1974; Barenholz et al., 1976). As seen from Fig. 4 and Table 2, the effect of furosemide seems to be stronger on the T_m of the lower temperature peak than on the higher one. Not only is the temperature of the lower peak shifted downward, but its enthalpy of melting also seems to decrease. As it is not possible to separate the whole profile into its components to obtain the enthalpy of each peak, the height of each peak was measured, and is shown as the ratio of the two peak heights. For SP alone the ratio of the two is 1.455, decreasing with the concentration of furosemide added, reaching a value of 0.605 at 22 mol/100 mol furosemide in the lipid. The total enthalpy of melting (over the two peaks) stays almost constant, decreasing by about 30% at the highest concentration of the drug employed. The experimental data reveal that furosemide has quite a strong effect on the structure of SP bilayers, with possible relevance to its influence on the structure of the kidney plasma membranes that are particularly rich in SP (Carmel et al., 1985).

Cerebrosides are also derivatives of sphingosine, so it was of interest to investigate their interaction with furosemide and to compare the effect of

the drug on them with its effect on SP. The difference between SP and brain cerebrosides is that the former are zwitterionic phospholipids, the head group being phosphocholine, whereas cerebrosides are neutral compounds and the head group is sugar-galactose. The effect of furosemide on the T_m (at the same bulk concentration) is smaller than in the case of SP. This stems from the much lower concentrations of furosemide in the lipid phase due to the very low K_p (0.6).

PS

As all the experiments in the present work were performed at pH 8.2 to 8.4 under conditions that furosemide is negatively charged, it was of interest to investigate how a negative charge on the lipid would influence the interaction. For this purpose phosphatidylserine, which has one negative charge in this range of pH's, was chosen. The phospholipid used in the present work is from bovine spinal cord, it is heterogenous with respect to the acyl chains, containing both saturated and unsaturated acyl chains. As seen in Table 1 the effect of furosemide on the thermotropic properties of PS is quite small. The maximal decrease of T_m is about 2°C and no change in ΔH is detected. The small effect stems from the low concentration of furosemide in the lipid phase ($K_p \cong 0.4$) probably due to repulsive interactions of the equal charges of the phospholipid and of the drug. However, in this case also the decrease of T_m is partially due to breakage of the hydrogen bonding of the lipid by the furosemide. The experiments with PS were repeated with a synthetic disaturated lipid (dimyristoyl phosphatidylserine), and in this case furosemide had no effect at all. Due to higher cohesive forces (two saturated chains) the concentration of furosemide in the lipid is even lower than that in the case of natural PS resulting in no change in the thermotropic properties.

Planar Lipid Membranes (PLM)

The technique of forming planar lipid membranes (PLM) from monolayers is especially suitable for measuring the effect of various modifiers on the electrical properties of the films. The PLM's are almost solvent free and their electrical resistance is very high. In the present work the interaction of furosemide with PLM formed either from PE or from diphytanoyl phosphatidylcholine monolayers was investigated. Figure 5 presents the conductance of a PLM formed from PE monolayers interacting with furosemide. The base line conductance

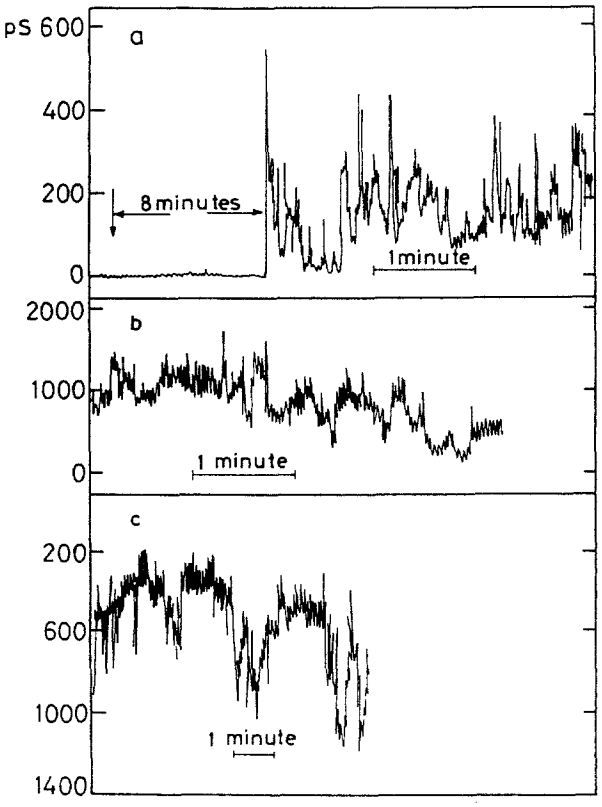

Fig. 5. Planar lipid membrane formed from PE monolayers, medium 2.5×10^{-1} M NaCl buffered with Tris · HCl 5×10^{-2} M, pH 8.1. (*a*) applied potential − 20 mV, base line conductance less than 5 pS; arrow indicates time of addition of 5×10^{-3} M furosemide to one side of the bilayer. (*b*) 40 min after addition of furosemide applied potential − 20 mV. (*c*) 50 min after addition of furosemide applied potential + 100 mV

is very low, less than 5 pS. After determining the base line conductance, 5×10^{-3} M furosemide was added to one side of the film (virtual ground) (the addition is marked by an arrow on the graph) and the solution was stirred. After a few minutes (Fig. 5*a*) the conductance increased strongly, reaching values of a few hundred pS. However, the increase of conductance is not smooth, and it fluctuates between high and low values. With time the resistance drops further but the current still fluctuates (Fig. 5*b*). Finally after about 50 min from the time of the addition of the furosemide (the solution is stirred from time to time), the conductance increased to over 1000 pS (Fig. 5*c*), but fluctuations are still seen. In some cases even higher values of the conductance were reached. The *I-V* curves seem to be ohmic within ±100 mV as measured with ramp potentials (±100 mV in 100 sec); however random fluctuations are also obtained here. Similar behavior was obtained with a PLM formed from diphytanoyl phosphatdylcholine. No quantitative difference

with respect to the action of furosemide on the two types of PLM was detected. In an attempt to evaluate the number of furosemide molecules forming the conducting unit, the conductance of PLM (formed from PE or from diphytanoyl PC) as a function of the furosemide concentration was investigated. Plots of the log conductance of the PLM as a function of the modifier concentration usually yield the number of molecules forming the conducting unit. The conductance of PLM formed from monolayers of PE as a function of the furosemide concentration added was measured. The base line conductance was low (~5 pS). Addition of very low concentrations of furosemide, less than 10^{-4} M, did not influence the conductance of the PLM. This finding is in agreement with the results of Vodyanov, Vodyanov and Murphy (1985) who reported that furosemide blocks apomorphine-dopamine receptor channels in PLM, but has no effect at a concentration of 10^{-5} M on pure protein-free bilayers. Addition of 10^{-4} M furosemide causes a transient increase of the conductance up to 50 pS, but with time the conductance drops to the initial value and stays at this low value until another portion (final concentration 4×10^{-4} M furosemide) is added. This second addition caused another transient followed by a decrease of the conductance to a low value. Stable high values of the conductance not decreasing to the base line are reached only at much higher concentrations of furosemide (about 5×10^{-3} M) (Fig. 5) and then the conductance fluctuates. This behavior precludes the calculation of the size of the conducting unit. However, from the data of Fig. 5 and the solubility properties of furosemide in lipid bilayers, some speculations about its propensity to increase the conductance can be made. Furosemide is a small charged molecule and probably quite hydrophilic. It is unlikely that it will act as an ion carrier by itself. Based on the data of Fig. 5 it seems that in bilayers conducting structures, formed from several furosemide molecules, are built. The size of the conducting unit is probably not constant; it seems that it aggregates and disaggregates within the bilayer causing random fluctuations of the conductance. Formation of the conducting unit takes place only at high concentrations of furosemide. It is also possible that with time furosemide crosses the bilayer to the other side (zero concentration of furosemide) and desorbs there. The transient nature of conductance at low concentrations of furosemide might be due to several causes: (i) The local concentration of furosemide reaching the bilayer is high causing a transient, and as this high concentration dissipates with time the conductance drops to a low value. (ii) Some critical concentration of furosemide is needed for sustaining the open conducting unit. Below this concentration the conformation of the conducting unit is unstable, and it either closes creating a nonconducting entity or the furosemide diffuses to the other side of the bilayer.

Summary and Conclusions

Furosemide interacting with lipid bilayers modifies their thermotropic properties. The effect of the drug is highest in SP and natural PE bilayers. As these two phospholipids are important constituents of kidney plasma membranes this effect of furosemide might have relevance to its action in vivo.

Furosemide also causes a decrease of the electrical resistance of PLM. The effect is obtained at high concentrations of the drug; however, it is possible that in vivo such local high drug concentrations could be reached by binding to certain proteins or lipid receptors as a primary event.

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Note Added in Proof

Recently it was drawn to our attention that M.A. Singer [*Can. J. Physiol. Pharmacol.* **52**:930–941 (1974)] reported a small efflux of cations from liposomes induced by furosemide.