STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, XLV, 1-2, 2000

INTERACTION OF PROCAINE WITH BIOMEMBRANES

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ABSTRACT. Compression isotherms of stearic acid (SA), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC) and of cholesterol (C) monolayers, as well as of mixed DPPC and C films have been recorded both in the absence and the presence of procaine. The penetration of procaine molecular species from the aqueous subphase into the lipid monolayers has been studied. By using Gibbs' equation, penetration numbers were derived from compression isotherms. Results are discussed in terms of molecular conformations, hydrophilic and hydrophobic interactions, allowing an insight into the molecular mechanism of procaine binding and penetration into the insoluble lipid monolayers studied.

Keywords*. Compression isotherms; lipid monolayers; procaine binding; procaine penetration*

INTRODUCTION

Studies concerning the interaction between anesthetics and natural membranes revealed a direct correlation between the efficiency of local anesthetics and their penetration into the lipid monolayers [1]. Investigations performed by using different experimental techniques showed some anesthetics to increase the fluidity of lipid bilayers [2], to decrease the order in hydrocarbon chains [3,4], to lower the temperature of the gel to liquid crystal phase transition [5,6], to extend the surface area of both monolayer films maintained at constant surface pressure [7.8] and of erythrocyte membranes [7,9] and to increase the surface pressure of lipid films maintained at constant area [1,10-12]. The molecular origin of the effects observed is presumed to be the weakening of the packing of the lipids due to the anesthetic molecules inserted [8]. The penetration has been evidenced by the energies of interaction of anesthetics with phospholipid monolayers spread at the air/water interface [1].

The major part of the experiments was performed by using monolayer membranes of lipids [1,8,11, 12]. The reason for using these model systems is the evidence of a direct correlation between the anesthetic efficiency and the oil/water partition coefficients of anesthetics [1]. Further, the monolayer represents the half of a lipid bilayer, the latter one determining the structure of the biomembrane [30]. The study of monolayers allows us to obtain direct information, at the molecular level, concerning the conformation and packing of molecules having biological significance in natural biomembranes under conditions near to the "*in vivo'* ones. Monomolecular films called also monolayers provide a convenient structural framework for experimental research and allow a quantitative treatment of the physico-chemical interactions between the film forming molecules, between the latter ones and the various subphase components (drugs, electrolytes, soluble proteins etc.). Monolayers spread at the air/water interface are sufficiently stable and suitable for experimental research.

Previously, we studied the influence of procaine (P), dissolved in the bulk subphase, upon stearic acid (SA) monolayers, spread at the air/water interface, by recording surface pressure (π, mN/m) *versus* mean molecular area (A, nm^2/molecule) isotherms, by using acidic, unbuffered and alkaline aqueous subphases [13-20]. Besides SA, also L-α-distearoyl phosphatidylcholine (DSPC), L-α-dipalmitoyl phosphatidylcholine (DPPC) and cholesterol (C) monolayers, as well as mixed DPPC and C containing monolayers have been used as membrane models [21-23].

In this paper we present the maximum binding and the maximum penetration of procaine into various lipid monolayers as a function of the subphase pH, and of the lipid nature, as well as a function of the compactness of the lipid monolayers in the absence and in the presence of cholesterol. The surface solution thermodynamics will be employed to describe the binding and the penetration of procaine into lipid monolayers and we will show that our biophysical approach can be simultaneously fitted to multiple sets of data (e.g., binding at various subphase pH values and at different lipid layers in the absence and in the presence of cholesterol). Finally, we will compare the maximum penetration numbers of procaine into different lipid monolayers at the saturation of lipid layers with procaine molecular species.

The primary purpose of this study is to determine the maximum of procaine penetration into various lipid layers and to evidence the role of electrostatic effects in binding of anesthetics to lipid membranes and the role of cholesterol on the procaine penetration into lipid layers. A secondary purpose is to establish the pH conditions where the most of procaine is bound to the lipid monolayer membranes showing an enhanced stability of the lipid monolayer membranes.

RESULTS AND DISCUSSIONS

In our studies the π *vs.* A compression isotherms have been recorded by using the Wilhelmy method SA has been spread on different aqueous subphases: viz. having pH 2 generated by HCl, bidistilled water with pH 5.6, which will be referred to as unbuffered subphase and phosphate buffer solution with pH 8.

Compression isotherms have been recorded both in the absence of P and by dissolving P in the aqueous subphase ensuring a P concentration of 10^{-3} M or 10^{-2} M. Since both film forming materials SA and P may participate in protolytic

equilibria, it is interesting to see the fraction α of different molecular species as function of pH. In these calculations the surface acidity constant of SA, equal to pK=5.63 [24], as well as the acidity constants of the double protonated PH_2^{2+} and the single protonated PH^+, equal to pK=1.95 and 8.87, respectively [25], have been used. Results are presented in Fig.1.

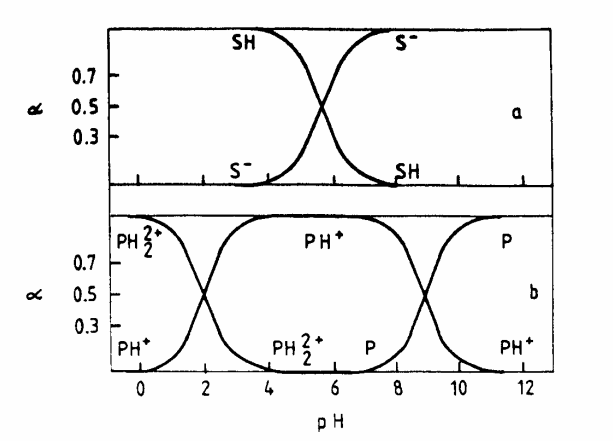

Figure 1. Fraction of molecular species as function of pH;
a – for stearic acid (SA or SH); b – for procaine (P).

As seen from Fig.1 at pH 2 the SA film is completely unionized. On unbuffered water of pH 5.6 besides the neutral SA molecules an important amount of stearate anions is present. At pH 8 stearic acid is completely ionized and gives a charged film.

Procaine is a tertiary amine compound (anesthetic), containing also a primary amine group bound to the aromatic ring and may exist as neutral molecules (P), monocations (PH^+) or dications (PH_2^{2+}). At pH 2, the PH_2^{2+} amounts to 47%, the remaining part of 53% being PH^+. On pure unbuffered water the only species is PH^+. At pH 8 the predominant species is PH^+, but one has also 12% of neutral molecules.

The effect of t9he subphase procaine upon the compression isotherms can be seen from Fig. 2. Obviously, procaine molecular species (P) have an expanding effect upon the SA monolayer, i.e. at a given π, value A is higher in the presence of P, which shows the penetration of P molecules into the SA monolayer, in substantial agreement with literature data on simplified membrane models [26-28]. In the same time an important increase of the collapse pressure is observed, *i.e.* in the presence of P the lipid monolayer becomes more stable.

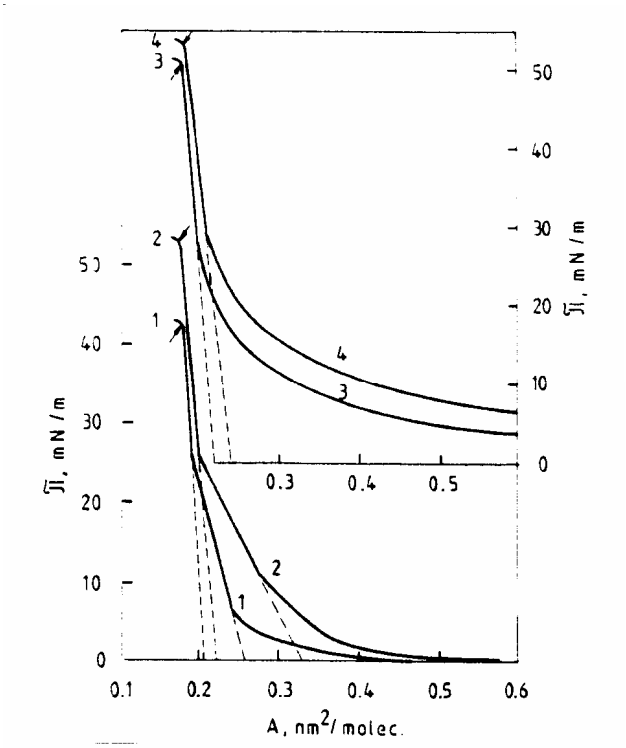

Figure 2. Compression isotherms of SA monolayers; curves (1) and (2) – unbuffered subphase of pH 5.6 and pH 5.2, respectively; curves (3) and (4) for pH 8; (1) and (3) for [P] = 0; (2) and (4) for [P] = 10^{-3} M.

Let us introduce some notations. The water will be considered as being component 1, the soluble surfactant as component 2 and the insoluble surfactant as component 3. The number of molecules of these components at the air/water interface will be denoted as N_1, N_2 and N_3, respectively. The mean molecular area of the insoluble surfactant will be denoted with A_3. Nevertheless, this magnitude is correlated to A_t, the whole area of the interface, according to the relation

$$A_3 = A_t / N_3 \qquad (1)$$

By denoting the partial molecular areas (cross-section areas), *i.e*. the actual area necessities of the molecules, by $\overline{A}_1$, $\overline{A}_2$ and $\overline{A}_3$, one has

$$A_t = N_1\overline{A}_1 + N_2\overline{A}_2 + N_3\overline{A}_3 \qquad (2)$$

Since the procaine substrate has an expanding effect upon the SA monolayers, it is useful to define an area increment $\Delta A = A_3 - A_{30}$, representing the difference between the mean molecular area of the insoluble surfactant (A_3), at a given surface pressure π in the presence of component 2, and the mean molecular area of component 3, at the same surface pressure π, but in the absence of the

soluble surfactant 2 (A_{30}). Since in the absence of the soluble surfactant, the interface is a binary system, instead of Eq.(2) one has

$$A_t' = N_1' \overline{A}_1 + N_3' \overline{A}_3 \quad (3)$$

and the significance of A_{30} will be:

$$A_{30} = (N_1' \overline{A}_1 + N_3' \overline{A}_3) / N_3' \quad (4)$$

By constructing the area increment (ΔA) vs. π curves given in Fig. 3, one may observe that at compression ΔA and consequently the P penetration increases, but at the highest π values ΔA vanishes, *i.e.* the penetrated P is squeezed out from the monolayer, probably by forming a monolayer of procaine cations, leading to the stabilization of the SA monolayer, and to its enhanced collapse pressure.

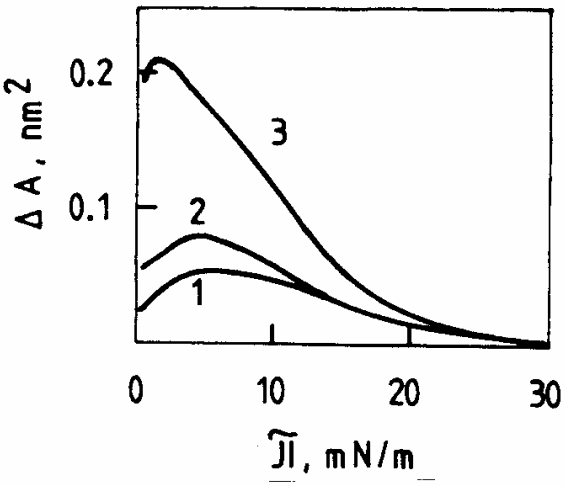

Figure 3. Molecular area increment (ΔA) due to the subphase P: curve (1) pH 2; (2) for pH 5.2; (3) for pH 8

One may see further that the expanding effect of P increases with increasing pH and becomes very important in alkaline media.

The penetration of P into the SA monolayer can be characterized by the penetration number n_p, which can be defined as the number of drug molecules (N_2) divided by the number of SA molecules (N_3)

$$n_p = N_2 / N_3 \quad (5)$$

It can be shown [14], that the penetration number may be correlated to the area increment ΔA, *viz.*

$$n_p = \Delta A / (A_{30} - \overline{A}_3 + \overline{A}_2) \quad (6)$$

Since the SA molecules are vertically oriented in the interface even at low surface pressures, for $\overline{A}_3$, the cross sectional area of the COOH group may be

taken, $\overline{A}_3$ = 0.18 nm^2. Concerning the area requirement $\overline{A}_2$ of procaine, molecular model calculations have been performed for different conformations of the molecule [14] and the results were compared to the adsorption data for pure procaine monolayers [20, 29]. Eq. (6) describes very well the experimental n_p *vs*. A_3 curves if for the variation of A_2 one presumes that at the spreading of the SA monolayer the P
molecules are adsorbed at the interface in a horizontal, lying down position (A_2 = 1.50 nm^2/molecule). At compression of the procaine monolayer the P molecules gradually adopt a vertical position and this process is completely achieved (A_2 = 0.40 nm^2/molecule) at π about 26 mN/m, that corresponds to the liquid condensed to solid phase transition of the SA pure monolayer.

Penetration number values have been derived from the compression isotherms, by using Gibbs' equation. The adsorption of the subphase component 2 at the air/water interface in the presence of an insoluble lipid monolayer forming component 3, obeys the following relation:

$$\Gamma_2 = \frac{1}{kT}\left(\frac{\partial \pi}{\partial \ln c_2}\right)_{T,A_3} \qquad (7)$$

at constant T and for constant mean molecular area A_3 of the insoluble surfactant. The penetration number n_p is equal to:

$$n_p = \frac{A_3 - \overline{A}_3}{kT}\left(\frac{\partial \pi}{\partial \ln c_2}\right)_{T,A_3} \qquad (8)$$

where $\overline{A}_3$ stands for the partial molecular area of component 3. By taking in Eq.(8) for $\overline{A}_3$ the collapse area A_{3c} of SA, n_p values were calculated as function of A_3.

The n_p *vs.* A_3 curves allowed us to have an insight into the conformational changes of P during the compression of the SA monolayer. Upon compression of the SA monolayer spread on subphases of pH = 2, n_p increases at the beginning and it attains a maximum value at about 10 mN/m. Further on, it begins to decrease, indicating a squeezing out of the P molecules from the SA monolayer.

Penetration number values increase with increasing pH, especially in the alkaline region, as seen from the maximum n_p values given in Table 1 for 10^{-3} M and 10^{-2} M of P solutions.

This is due to the protolytic equilibria in which participate both P and SA. Increasing pH entails gradual deprotonation of PH_2^{2+} into PH^+ and P and consequently increases the surface activity of the soluble surfactant. On the other hand the deprotonation of the SA molecules makes easier the penetration of the P cations into the negatively charged SA monolayer.

Table 1.

Influence of pH upon the maximum penetration number values of P into SA monolayers

Subphase	Maximum n_p	
	$c_2 = 10^{-3}$ M	$c_2 = 10^{-2}$ M
pH 2	0.040	0.051
Unbuffered	0.059	0.089
pH 8	0.113	0.130

We have performed a similar study by using, instead of SA, L-α-dipalmitoyl phosphatidyl choline (DPPC) and L-α-distearoyl phosphatidylcholine (DSPC) as insoluble film forming surfactant. The subphase was a solution of procaine in twice distilled water, the pH being of 5.6. The maximum penetration numbers are given in Table 2.

Table 2.

Maximum penetration number values of P into SA, DSPC and DPPC monolayers

Insoluble surfactant	Maximum n_p	
	$c_2 = 10^{-3}$ M	$c_2 = 10^{-2}$ M
SA	0.059	0.089
DSPC	0.075	0.091
DPPC	0.127	0.170

As seen, at the same P concentration n_p increases in the order SA < DSPC < DPPC. The effect of the insoluble monolayer to enhance the P adsorption is due to the hydrophobic interactions between the molecules of both surfactants, but it is strongly influenced also by the compactness of the film.

The hydrophobic moiety of the P molecule is of small dimensions and, consequently, in its interaction with the long hydrocarbon chains of the insoluble surfactants no important differences may arise. Thus, the different behaviour might be assigned to the hydrophobic interactions between the molecules of the insoluble surfactant. The attracting forces between the neighbouring molecules will be the most important in the case of SA, having a small polar group and a single saturated hydrocarbon chain and thus SA will give the most compact film, being the most disadvantageous for the P penetration.

DSPC and DPPC have identical polar headgroups and two saturated hydrocarbon chains each one. These chains are longer with DSPC as compared to DPPC and therefore the former gives more compact films than the latter. Both films are however looser than the SA one and consequently the above order of penetration numbers is quite reasonable.

As lipid membrane models also cholesterol (C) monolayers and mixed monolayers containing C and DPPC in the molar ratio 1:1 have been used. The pure C monolayer at the air/water interface is very condensed even at low surface pressures, characterized by close packing of vertically oriented molecules.

The maximum n_p values observed at 10^{-2} and 10^{-3} M subphase P concentration are presented for C, DPPC, and mixed C and DPPC (1:1) films.

Results are compared with the arithmetical mean of n_p values for C and DPPC films, noted (C + DPPC) / 2, given in the last column of Table 3.

Table 3.

Maximum penetration number values of P into C, DPPC, and mixed C and DPPC monolayers

[P], M	Maximum n_p for			
	C Experimental	C and DPPC Experimental	DPPC Experimental	(C + DPPC)/2 Calculated
10^{-2}	0.032	0.058	0.170	0.101
10^{-3}	0.023	0.031	0.127	0.077

The n_p values reveal that procaine has affinity to the DPPC domain interfaces that are probably dominated by lipid chain conformations in substantial agreement with epifluorescent microscopy data [23].

It is interesting to observe that the maximum n_p values of the mixed monolayer are much more closer to the n_p values for C monolayers, that the ones obtained with DPPC. Due to the hydrophobic interactions between the air phase hydrocarbon moieties of C and DPPC, the mixed monolayer is almost as condensed as the pure C one. Thus, cholesterol being a membrane stabilizer in the same time it is found to diminish the membrane permeability and the adsorption of procaine on lipid monolayers. It is also shown that cholesterol appears to squeeze out procaine from the interfaces, especially near the collapse of lipid monolayers. These results are in substantial agreement with findings on the general model study of anesthetics, like halothane and cocaine-derivatives, and lipid membrane interactions [28].

CONCLUSIONS

Our results show that the compression isotherms of insoluble monolayers are a very useful tool in investigating membrane models. It allows to observe the insertion and penetration of soluble surfactants from the subphase into the insoluble lipid monolayer and to have an insight into the molecular mechanism of this penetration, which may play an important part in the physiological effects of anesthetics. In the same time one may observe the effect of cholesterol upon the compactness of phospholipid monolayers and upon the membrane permeability.

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