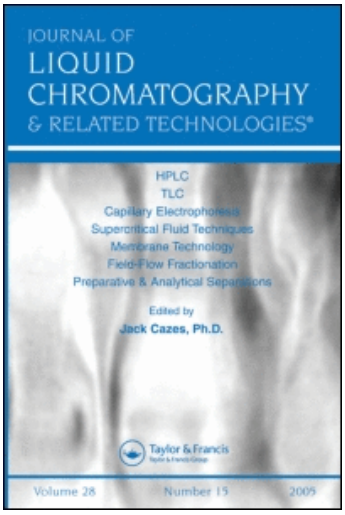

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A Study of Lipid-Lipid and Lipid-Polypeptide Interactions by High Performance Liquid Chromatography

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A STUDY ON LIPID-LIPID AND LIPID-POLYPEPTIDE INTERACTIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Ternary systems containing phosphatidylcholine-cholesterol, phosphatidylcholine-gramicidin A or cholesterol-gramicidin A in tetrahydrofuran have been examined by high performance liquid chromatography. Preferential solvation of cholesterol and especially gramicidin A by phosphatidylcholine is observed. These results are interpreted in terms of hydrophobic interactions between membrane components.

INTRODUCTION

The basic matrix of biomembrane structure consists of a phospholipid bilayer, in which sterols, proteins and occasionally other lipids are embedded. The interaction of the various membrane components is currently a matter of study and discussion (1). The amphiphilic nature of phospholipids determines that the lamellar phase occurs only in the presence of excess water, for the naturally occurring phospholipid mixtures (2). However, phospholipids should interact with other membrane components even in solution, and the study of such interactions in organic solvents could provide a complementary view to the data obtained in aqueous suspensions. In the present paper we propose a novel

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approach to the study of lipid-lipid and lipid-protein interactions using high performance liquid chromatography (HPLC).

The method involves the equilibration of a chromatographic column with a phospholipid in organic solution; cholesterol or any other membrane component is then injected, dissolved in the solution used to equilibrate the column. Elution onto the column with the same equilibrating solution leads to the emergency of cholesterol (polypeptide, etc.) from the column, accompanied by a "trough" in the elution diagram; the trough (or vacant peak) is an indication of preferential solvation (3-7) of the second solute. When this preferential solvation is by phospholipid, it may be interpreted in terms of solute-solute interactions (8-10).

In this paper, we describe our results concerning the preferential solvation parameters in ternary systems containing tetrahydrofuran (THF)(1)/phosphatidylcholine (PC)(2)/cholesterol (CH)(3) and THF(1)/PC(2)/gramicidin A (3). Gramicidin A is used as a model of intrinsic polypeptide. Our results indicate the potential usefulness of HPLC as a tool for the study of the interactions between membrane components in non-aqueous solutions.

EXPERIMENTAL

CH was obtained from Sigma Chemical Co., St. Louis, Mo. USA. Gramicidin A was from Koch Light Lab. Egg yolk PC was purchased from Merck, purified according to Singleton et al. (11) and its purity checked by thin layer chromatography. THF was a Merck spectroscopic reagent.

A M-45 solvent delivery system, a U6K universal injector and a differential refractometer, model R 401, from Waters Assoc. were used in all the experiments. Samples were occasionally monitored with a Varian Varichrom variable wavelenght UV detector. The system was equipped with two μ-styragel columns with 10^4 and

10^2 Å nominal porosities, from Waters Assoc. The flow rate was 1.0 mL/min and the temperature, 28 °C.

Columns were equilibrated with THF/PC solutions, previously filtered through 0.45 μm Millipore filters, at concentrations ranging from 0.97×10^{-2} to 3.11×10^{-2} M. Higher concentrations could not be used because under these conditions the columns did not work adequately. For each eluent system CH or gramicidin A samples at several concentrations were prepared immediately before injection by dissolving them in the corresponding equilibrating solution. The injected volume was always 100 μL.

Before injecting any CH (or gramicidin A) sample, several 100 μL injections of binary solution containing a known excess or defect in weight fraction (Δw_i°) of any of the i components (i=1,2) at a fixed eluent composition were injected. The Δw_i° are related to the areas, A_i°, of the excess or defect peak appearing in the chromatogram. The subsequent injection of the same volume of a CH (or gramicidin A) solution, at a concentration c_3, will cause a peak (vacant peak), of area A_i, with a defect in the component preferentially adsorbed. The Δw_i value corresponding to the area A_i of the vacant peak can be deduced from the calibration Δw_i° vs. A_i°.

RESULTS AND DISCUSSION

Figure 1A shows a typical chromatogram of CH at a 30 mg/mL concentration with a 3.11×10^{-2} M solution of PC in THF as eluent. The first eluting peak appearing at an elution volume (V_e) of 13.3 mL corresponds to the vacant peak and the second one to the solvated CH (V_e=16.5 mL). As depicted in figure 1B the areas of the vacant peak are proportional to the amount of injected CH. This indicates that CH is preferentially solvated by PC.

The elution volume of the vacant peak depends on the PC concentration in the eluent, increasing as this concentration

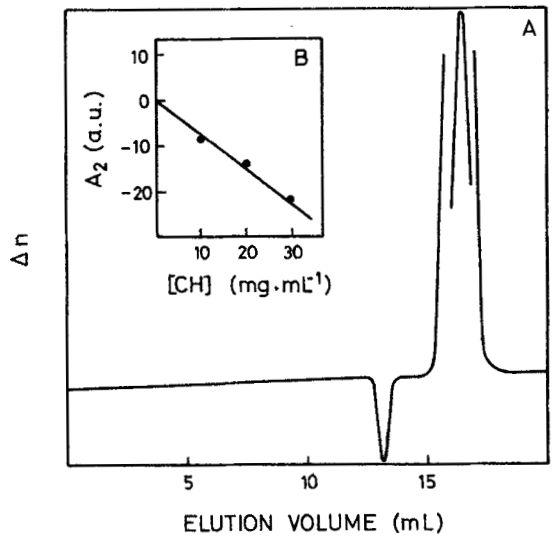

FIGURE 1. A. Chromatogram obtained by injection of 100 µL of a solution of CH (30.0 mg/mL) in the eluting solution. Eluent: 3.11 x 10^{-2} M solution of PC in THF.
B. Dependence of the vacant peak area on CH concentration.

decreases. This phenomenon is more pronounced at low PC concentrations. It seems that the retention of PC in the column is not only due to size-exclusion, and a reversible adsorption may also occur. On the other hand, we have checked that for a given concentration of injected CH, the vacant peak areas increase as PC concentration in the eluent becomes higher.

The preferential solvation coefficient λ has been classically measured by means of techniques such as dialysis equilibrium - differential refractometry (12-15), light scattering (16-18) and more recently gel permeation chromatography. The evaluation of λ by HPLC has been described by several authors (3-5) and by ourselves (6) for ternary systems solvent 1/ solvent 2/ macromolecule 3. When the eluent is composed of two liquids, λ is expressed as the excess or defect

in volume fraction of one of the solvents in the domain of the macromolecule with respect to the bulk solvent, once the thermodynamic equilibrium has been attained. However, in this case our eluent is a mixture of a liquid (THF) and a solid (PC) and it is more convenient to express the excess or defect in weight fraction instead of volume fraction, because in this way no PC density values are needed. According to this, λ may be expressed as:

$$\lambda = \frac{\Delta w_i}{c_3} \qquad (i= 1 \text{ or } 2) \qquad (1)$$

Negative values will be arbitrarily assigned to λs deduced from equation (1) when preferential solvation of component 3 (CH) by component 2 (PC) occurs. Positive λs would imply preferential solvation by component 1 (THF).

Results corresponding to the calibration curves at different eluent compositions are plotted in figure 2. In order not to overcrowd the figure, only two calibration curves have been included, but similar plots have been obtained for all the eluents used.

The results for CH samples injected at three concentrations in eluents with a PC composition ranging from 0.97×10^{-2} to 3.11×10^{-2} M are summarized in table 1; w_2 being the weight fraction of PC in the eluent.

The solutions under study correspond to PC/CH molar ratios in the injected sample between 1:1 and 1:8. A preferential solvation of CH by PC involves a PC defect, expressed as a negative increment of weight fraction for component 2, Δw_2, in the outer binary phase in thermodynamic equilibrium with the ternary phase, and yielding a vacant peak of area A_2. From A_2 and the corresponding calibration curves, Δw_2 values can be obtained. λ values are negative for all the eluent compositions tested (see table). This indicates, as explained above, that CH is preferentially solvated by PC.

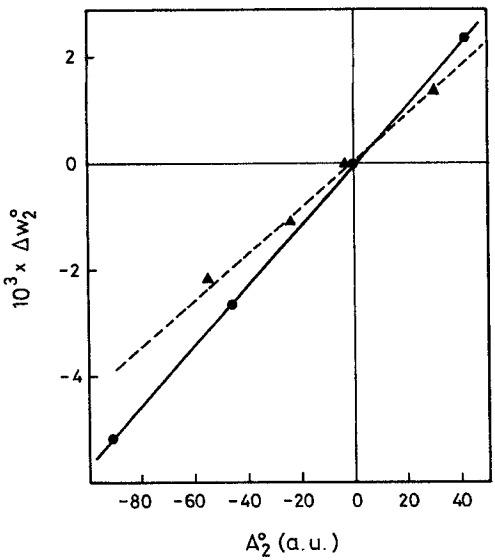

FIGURE 2. Calibration curves of excess and defect increments of weight fraction for PC (Δw_2°) vs. areas, A_2°, at two eluent concentrations. (●) 3.11 x 10^{-2} M; (▲) 1.30 x 10^{-2} M.

The variation of the preferential solvation parameter λ is plotted in figure 3 as a function of w_2; it can be observed that the absolute value of λ increases gradually as the eluent PC concentration increases. We have not found data in the literature about these systems for comparison. However λ values are of the same order of magnitude than those described for two solvents/one polymer (4-6) or one solvent/two polymers (7) systems, when a similar range of weight (or volume) fraction is used.

When gramicidin A is used as component 3 in a similar range of eluent PC concentrations varying from 0.97 x 10^{-2} to 2.59 x 10^{-2} M, a slight overlapping of the vacant peak with gramicidin A peak occurs (figure 4) in all the range of injected gramicidin A concentrations (from 0.5 to 16.0 mg/mL).

TABLE 1

Values Obtained for THF/PC/CH System from HPLC Chromatograms at Different Eluent Compositions.

Eluent PC concentration $.10^2$ (mole.L^{-1})	$w_2.10^2$	c_3 (mg.mL^{-1})	$\Delta w_2.10^4$	V_e (mL)	$\lambda^*.10^2$ (mL.g^{-1})
3.11	2.63	10.0 20.0 30.0	- 4.8 - 7.7 -12.1	13.3	-4.0±0.4
2.59	2.20	10.0 20.0 30.0	- 3.2 - 5.7 - 7.4	13.3	-2.9±0.3
2.15	1.83	10.0 20.0 30.0	- 2.4 - 4.7 - 8.3	13.5	-2.4±0.2
1.72	1.47	10.0 20.0 30.0	- 1.3 - 3.8 - 5.4	13.6	-1.8±0.3
1.30	1.11	10.0 20.0 30.0	- 1.8 - 2.8 - 4.1	13.7	-1.4±0.2
0.97	0.84	10.0 20.0 30.0	- 0.5 - 1.5 - 2.2	13.8	-0.7±0.1

* λ parameter for each eluent is the median of the values obtained for the three injected CH concentrations ± their average deviation.

The PC defect peak increases with gramicidin A concentration, the injected volume and the eluent PC concentration. Therefore, even an accurate quantitative evaluation of λ parameter being not possible in this system, a qualitative estimation of λ allows the comparison with the PC (2)/ CH (3) system. So, similarly to CH in this latter system, gramicidin A is preferentially solvated by PC, but with λ values in the gramicidin A case up to 50 fold higher than those obtained for CH.

The gramicidin A/ PC molar ratios in all the experiments with this polypeptide (from 1:1 to 1:50) are within the range

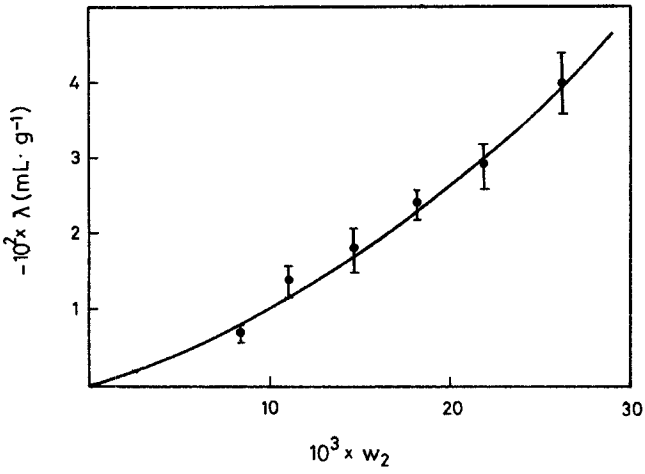

FIGURE 3. Preferential solvation parameter λ vs. weight fraction of PC in eluent, w_2 , for the THF/PC/CH system. Each point is the median value $\pm$ its average deviation.

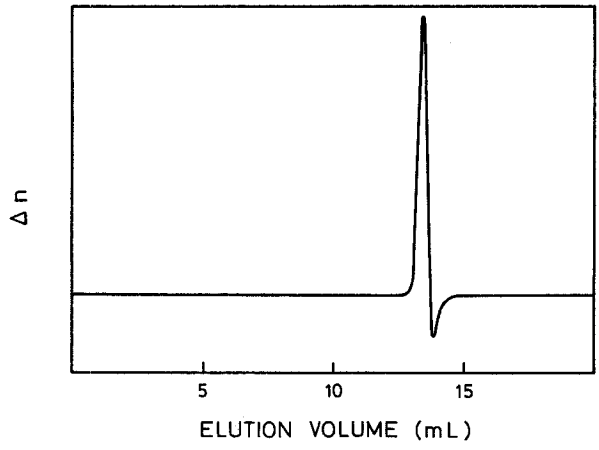

FIGURE 4. Chromatogram obtained by injection of 100 µL of a solution of gramicidin A (1.5 mg/mL) in the eluting solution. Eluent: 1.30 x 10^{-2} M solution of PC in THF corresponding to a PC/gramicidin A molar ratio of 16:1.

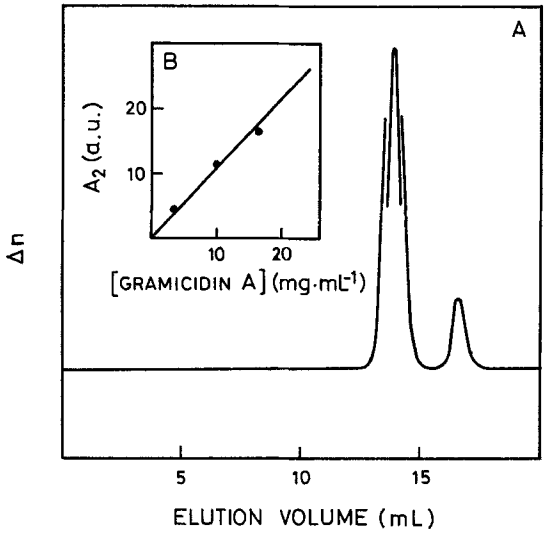

FIGURE 5. A. Chromatogram obtained by injection of 100 µL of a solution of gramicidin A (10.0 mg/mL) in the eluting solution. Eluent: 5.17 x 10^{-2} M solution of CH in THF.
B. Dependence of the peak of CH excess on gramicidin A concentration.

used in studies with liposomes, whereas for the THF(1)/PC(2)/CH(3) system it was necessary to increase the CH/PC molar ratio up to 8:1 in order to obtain significant vacant peaks.

Similar experiments with CH as component 2 and gramicidin A as component 3 have been performed in a range of eluent compositions varying from 2.32 x 10^{-2} to 5.17 x 10^{-2} M. Figure 5A shows a typical chromatogram for this system. The first peak eluting at 14.0 mL corresponds to gramicidin A and the second peak (V_e=16.5 mL) to an excess of CH. As depicted in figure 5B, the peak area of CH excess is proportional to the injected gramicidin A amount. This means, in contrast with the above results, that gramicidin A is preferentially solvated by THF.

TABLE 2

Values Obtained for THF/CH/gramicidin A System from HPLC Chromatograms at Different Eluent Compositions.

Eluent CH concentration $.10^2$ (mole.L^{-1})	$w_2.10^2$	c_3 (mg.mL^{-1})	$\Delta w_2.10^4$	V_e (mL)	$\lambda^*.10^2$ (mL.g^{-1})
5.17	2.20	3.1 10.0 16.6	1.4 4.3 6.3	16.5	4.2±0.4
4.33	1.85	3.1 10.0 16.6	0.8 2.6 3.7	16.5	2.5±0.2
3.62	1.55	3.1 10.0 16.6	0.4 1.5 1.8	16.5	1.3±0.2
2.32	1.00	3.1 10.0 16.6	0.2 0.9 1.3	16.5	0.8±0.1

* λ parameter for each eluent is the mean of the values obtained for the three injected gramicidin A concentrations ± their average deviation.

In table 2, the results corresponding to this system are summarized. Δw_2 values are obtained from the corresponding calibration curves (not shown). Although λ parameter is now positive, its absolute value remains close to that for the THF(1)/PC(2)/CH(3) system.

In summary, using THF as a solvent, gramicidin A-PC and CH-PC interactions are stronger than gramicidin A-CH ones. On the other hand, PC solvates better gramicidin A than CH. These results indicate the possibility of studying lipid-lipid and lipid-protein interactions by HPLC. Preliminary experiments using less polar

solvents, such as dichloromethane, failed to show any preferential solvation of gramicidin A or CH by PC; the requirement of a relatively polar solvent is an indication that the observed interactions are mainly hydrophobic in nature, such as they occur in the lipid matrix of biomembranes (studies using a variety of solvents are presently being carried out by us). The fact that PC interacts with both CH and gramicidin A, whereas these two do not show any interaction, suggests that the long, flexible hydrocarbon chains of the phospholipid are essential for establishing the hydrophobic bonds leading to preferential solvation of the other components. Also, the stronger PC-gramicidin A than PC-sterol interactions may be related to the different effects of proteins and CH, respectively disordering and ordering, on the static order of phospholipid chains, as observed by ^{2}H-NMR (19).

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