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ORIGINAL CONTRIBUTION

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Interaction of the Hepatitis A peptide VP3(110–121) with lipid mono and bilayer models of cellular membranes

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Abstract The surface activity of HAV-VP3(110–121) peptide was studied at different concentrations in an aqueous solution. Saturation was reached at 0.62 μM concentration. The ability of the peptide to insert into monolayers of CL, SA, DPPC, DPPC/5% CL and DPPC/5% SA was also performed. Mixed monolayers composed of this peptide and the lipid mixtures were also studied as far as the miscibility of the two components is concerned. The mixed monolayers showing small negative deviations from ideality. The values of excess free energy of mixing (ΔG_M^E) suggest that the energy associated to the miscibility process is almost non-significant except for a 0.2 molar fraction of DPPC/SA and 0.6 molar fraction of DPPC/CL. The peptide has an expanding effect upon the monolayers but due to its amphoteric character this interaction is not dependent on the electrical charge of the lipids. In fluorescence studies, the peptide showed some degree of interaction with the lipid polar heads, but no interactions were detected with its alkylchains. This results show that after incubation with DPPC/5% CL and DPPC/5% SA liposomes the peptide remains in the outer part of the bilayers.

Key words Hepatitis A virus, HAV-VP3(110–121) – monolayers – cardiolipin – stearylamine – dipalmitoyl phosphatidylcholine – liposomes

Introduction

Synthetic peptides containing neutralizing epitopes have been investigated in order to find a new generation of vaccines. To achieve this goal the first step to follow is the identification of specific sequence candidates for the neutralizing antigenic determinants in the structural proteins of the viruses. The synthetic HAV-VP3(110–121) peptide [1] (Fig. 1) used in this study was selected according to the hydrophilicity profile generated applying the Hopp and Woods method [2] and the presence of the only RGD sequence existing in the whole HAV capsid primary structure which is responsible for the cell attachment-promoting activity of a variety of proteins [3].

It is widely accepted that specific interactions between bioactive peptides and membrane phospholipids are involved in many important processes in the living organisms as the antigen presenting and recognition processes. The initial binding of an antigen normally occurs on an antibody molecule mounted in a receptor on the cell surface. However, interactions between peptides or proteins and the plasma membrane of macrophages of B-lymphocytes may act as a specific recognition process which precedes endocytosis and antigen presentation. In the present paper we study the physicochemical properties of a Hepatitis A peptide fragment by means of the simplest membrane model to study lipid/peptide interactions, the monomolecular layers of phospholipids. In this sense, we have determined the surface activity of

FWRGDLVFDFQV

Fig. 1 VP3(110–121) structure

HAV-VP3(110–121) peptide via a Wilhelmy surface balance and studied mixed monolayers composed of this peptide and various lipid mixtures as far as the miscibility of the two components is concerned. The interaction between the peptide and the lipid monolayers through compression isotherms and constant area kinetics was also determined. Moreover, the influence of this peptide on the fluidity of lipid bilayers was determined by polarization fluorescence techniques.

Materials and methods

Chemicals

Chloroform pro analysi was from Merck. Dipalmitoyl phosphatidylcholine (DPPC), cardiolipin (CL) and stearylamine (SA) were from Sigma. Monolayers were spread on a subphase of phosphate buffer solution (PBS), pH 7.4 sodium anilinonaphthalene sulfonate (ANS) and diphenylhexatriene (DPH) were from Sigma.

Measurements at constant surface area

A mini Teflon trough, cylindrical in shape, with capacity of 70 ml was used. Throughout this study, the aqueous subphase was PBS (pH 7.4, conductivity 15.4 mS/cm, 313.35 mosm/kg). Lipid solutions were spread from chloroform to the required initial surface pressure (5 or 20 mN/m). Peptide solutions were delivered into the subphase by using a Hamilton syringe. The subphase was stirred continuously by a Teflon-coated magnetic bar at low speed to avoid disturbance of the lipid film.

The surface pressure was measured by the Wilhelmy plate method [4], with a platinum plate suspended from a Sartorious microbalance. The accuracy of the system was in the range of $\pm$ 0.1 mN/m. After spreading the lipid solution, the film was allowed to stabilize for 10 min before injecting the peptide solution into the subphase.

Pressure increases were recorded continuously for 30 or 60 min. Each run was carried out in triplicate and reproducibility was usually within 0.1–0.2 mN/m.

Compression isotherms

To carry out these studies the output of the pressure pick-up (Sartorious microbalance) was calibrated by recording the well-known isotherm of stearic acid. This isotherm is characterized by a sharp phase transition at 25 mN/m for a subphase of pure water at 20 °C. The Teflon trough for measuring compression isotherms (surface area 495 cm^2, volume 330 ml) was regularly cleaned with hot chromic acid; moreover, before each experiment it was washed with ethanol and rinsed with double-distilled water. Before each run, the platinum plate was also cleaned with chromic acid and rinsed with double-distilled water. Films were spread using a microsyringe, and at least 10 min were allowed for solvent evaporation. Films were compressed at a rate of 4.2 cm/min; changes in the compression rate did not alter the shape of the isotherms. Standard deviation for these measurements was 3.5%.

All measurements were made at 21 $\pm$ 1 °C.

Fluorescence studies

Fluorescence polarization was determined using a Perkin–Elmer LS 50 spectrofluorometer equipped with a thermostatable cuvette holder and polarizers. Small unilamellar vesicles prepared as described in [5] (105 nm diameter) were incubated with ANS or DPH. Liposomes saturated with ANS or DPH were incubated with the peptide and its fluorescence polarization measured as a function of the temperature. Excitation and monitoring wavelengths were 380 and 480 nm and 365 and 425 nm, for ANS and DPH, respectively. The observed fluorescence intensities and polarization changes were compared with those of probe/liposomes and probe/peptide. Membrane fluidity was estimated on the basis of the reciprocal of polarization. The temperature range was 20–50 °C.

Effect of VP3(110–121) on the fluidity of bilayers

DPH and ANS hydrophobic and hydrophilic molecules, respectively, were used as fluorescence probes. The degree of anisotropy was obtained by the following equation:

$$r = (I_{\parallel} - I_{\perp} g)/(I_{\parallel} + 2I_{\perp} g) ,$$

where g is an instrumental correction factor, $I_{\parallel}$ and $I_{\perp}$ are, respectively, the emission intensities polarized vertically and horizontally to the direction of the polarized light. Fluorescence anisotropy has been widely used to monitor fluidity [6]. Experiments were carried out in duplicate.

Results and discussion

Mixed monolayers

Interactions between the VP3(110–121) peptide and the mixed monolayers were studied, taking the lipid mixture

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as one component and changing the molar relationship of it respect to the peptide. The pure peptide is able to form a stable monolayer as already described [7]. The composition of mixed monolayers was DPPC/5% SA and DPPC/5% CL (in order to check for electrostatic as well as hydrophobic interactions).

Compression isotherms of mixed monolayers obtained are represented in Figs. 2 and 3. Comparing the isotherms it can be appreciated that the end of the compression process is achieved without passing through solid state. Moreover, the slope of mixed monolayer isotherms is similar to that of pure-lipid components. This suggests that the presence of peptide in the mixture does not affect the ordered state of lipid molecules in the monolayer.

Values represented in insets of Figs. 2 and 3 show small deviations from ideality at almost any molar fraction. The areas/molecule of mixed monolayers are, in general, close to ideality or slightly lower, thus indicating a condensation effect. For those containing SA, maximal deviation from ideality appears as 0.8 molar fraction of peptide. On the contrary, mixed monolayers of DPPC/CL/VP3 have negative deviations from ideality all over the molar compositions studied. From these values the excess free energy of mixing (ΔG_M^E), (Tables 1 and 2) was calculated by applying Eq. (1). This equation was obtained following the Goodrich [8] and Pagano [9] approaches. Numerical values were calculated according the mathematical method of Simpson, as follows:

$$\Delta G_M^E = \int_0^{\pi} A_{12}\,d\Pi - N_1 \int_0^{\pi} A_1\,d\Pi - N_2 \int_0^{\pi} A_2\,d\Pi\,, \qquad (1)$$

components, N_1 and N_2 are the molar fractions of the monolayer components 1 and 2.

As reference, values of ΔG_M^E lower than RT (2474 J/mol) can be considered as non-significant. In this sense, only the interactions of peptide/SA : DPPC (0.8 : 0.2) and interactions of peptide/CL : DPPC (from 0.4 to 0.8 peptide molar fraction) at 20 mN/m can be taken as representative of a predominance of attractive forces among the molecules. Where experimental values do not differ significantly from theoretical ones, both a lack of miscibility or either an ideal miscibility could be possible, but as all the compression isotherms do not collapse, it is not possible to discard any of these interpretations [10].

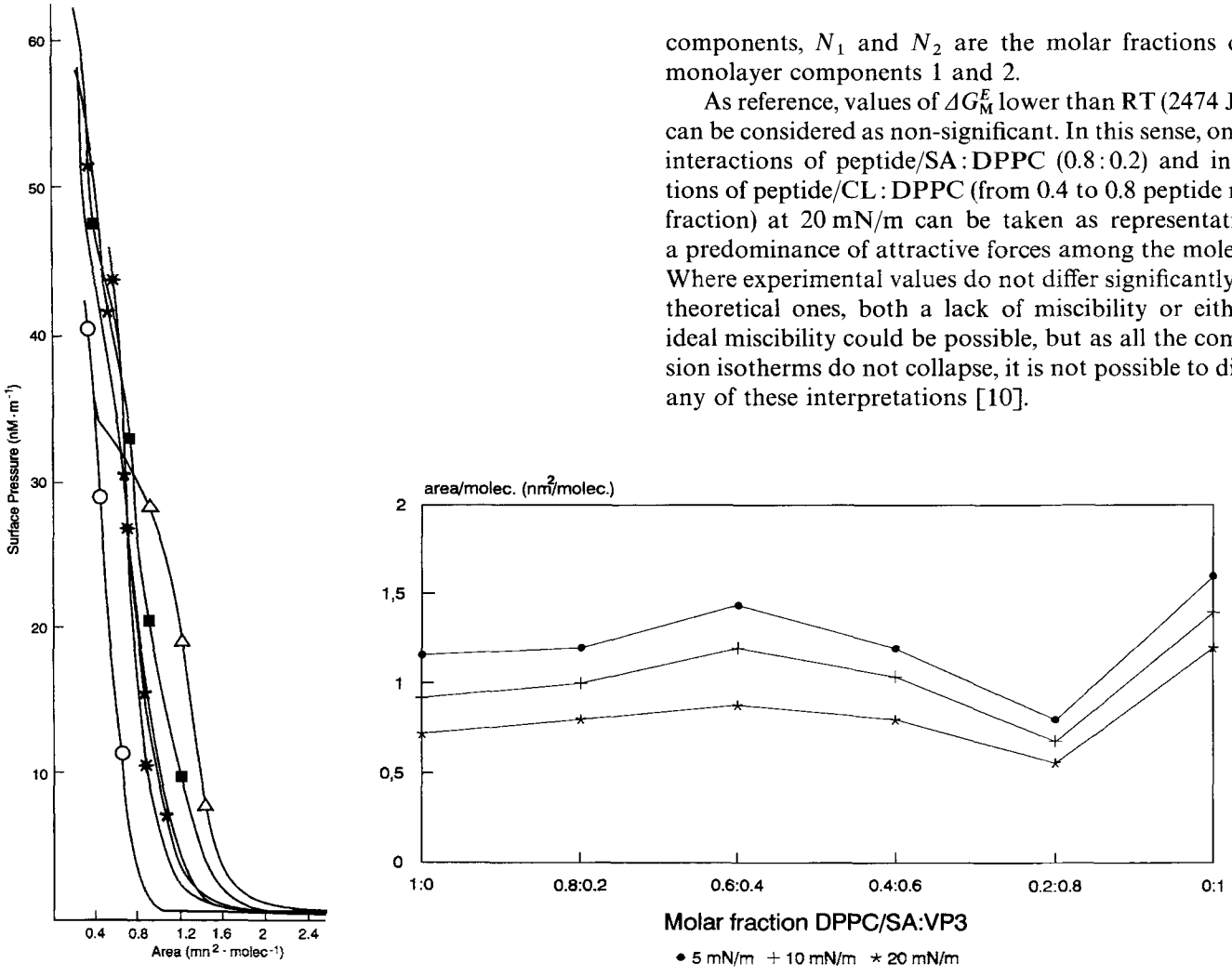

Fig. 2 Compression isotherms of mixed monolayers of DPPC/SA:VP3 on PBS subphase: DPPC/5% SA: (—★—); (DPPC/5% SA)0.8:(VP3)0.2: (—■—); (DPPC/5% SA)0.6:(VP3)0.4: (—•—); (DPPC/5% SA)0.4:(VP3)0.6: (– – –); (DPPC/5% SA)0.2:(VP3)0.8: (—○—); VP3: (—△—)

Comparing ΔG_M^E for both positively and negatively charged monolayers, no conclusion can be drawn about the influence of the charge in this system.

Monolayer binding properties

The insertion of the peptide into lipid monolayers was studied for a peptide concentration 0.31 μM, lower than that of saturation (3.7 μM). These experiments were carried out on monolayers of DPPC, SA, CL and mixtures of DPPC with 5% of CL or SA spread at 5, 10 and 20 mN/m. After injection of the peptide under the monolayer, surface pressure increased very quickly for pure SA and CL monolayers. On the contrary, the increases were slower in mixed monolayers. These trends can be noticed in Fig. 4.

Pressure increases at 5 mN/m were higher for pure SA and CL monolayers, than for the mixed ones; pure DPPC gave intermediate values. It is difficult to explain why positively and negatively charged monolayers show the same type of interaction with a negatively charged peptide. One can assume that the strong repulsive forces among CL and SA polar heads as well as the solvatation shell around them, left big gaps between molecules and so there is more room available for peptide molecules to insert between them. Mixed monolayers and neutral ones have a lower surface represented in the histogram of Fig. 5. Taken as reference area/molecule values for DPPC, the corresponding ones for CL should be twice because of the presence of

Table 1 Excess free energy of mixing for mixed monolayers of DPPC/5% SA:VP3 at 10 and 20 mN/m (ΔG_M^E, in J/mol). Molar fraction (DPPC/5% SA:VP3)

Tension [mN/m]	0.8/0.2	0.6/0.4	0.4/0.6	0.2/0.8
10	− 597.11	678.95	− 1112.33	− 3650.05
20	− 183.00	910.11	− 2330.64	− 7208.57

Table 2 Excess free energy of mixing for mixed monolayers of DPPC/5% CL:VP3 at 10 and 20 mN/m (ΔG_M^E, in J/mol). Molar fraction (DPPC/5% CL:VP3)

Tension [mN/m]	0.8/0.2	0.6/0.4	0.4/0.6	0.2/0.8
10	− 674.15	− 2215.06	− 1564.99	− 1805.76
20	− 1294.71	− 4386.78	− 3438.17	− 3741.51

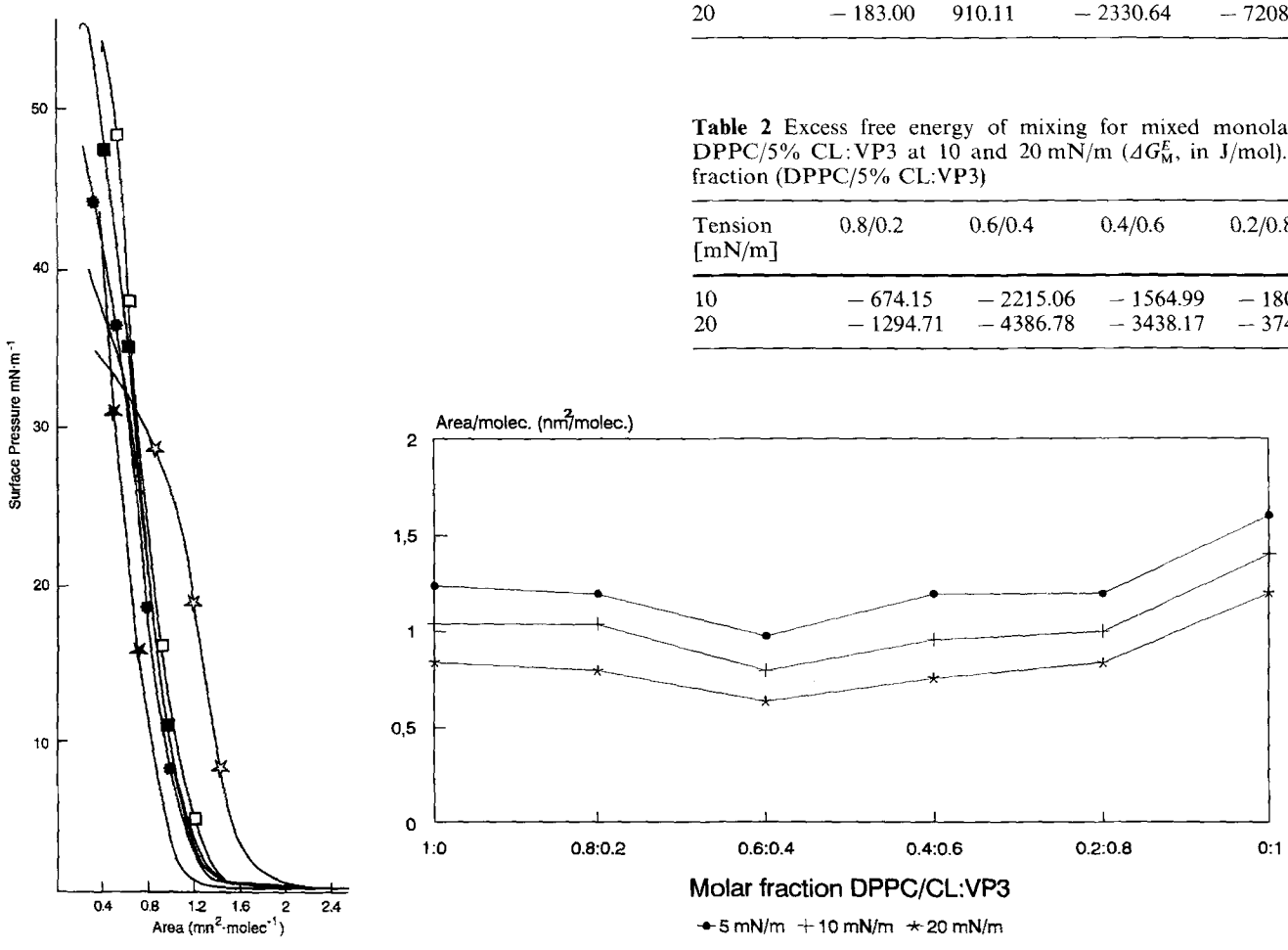

Fig. 3 Compression isotherms of mixed monolayers of DPPC/ CL:VP3 on PBS subphase: DPPC/5% CL: (—□—); (DPPC/5% CL)0.8:(VP3)0.2: (—■—); (DPPC/5% CL)0.6:(VP3)0.4: (—★—); (DPPC/5% CL)0.4:(VP3)0.6: (—•—); (DPPC/5% CL)0.2:(VP3)0.8: (– – – –); VP3: (—☆—)

four alkylchains in the molecule, and those of SA should be half due to the presence of only one alkyl chain. As can be seen CL as well as SA show higher area/molecule values than those expected thus indicating the presence of repulsive forces among the charged polar heads. Moreover, the lack of differences between peptide-CL and peptide-SA interactions can also be due to the asymmetric distribution of charges in the peptide chain. According to its sequence there is an accumulation of negative and positive charges at the terminal carboxyl and amino ends, respectively. This fact would favor the interaction between different parts of the peptide molecule and the monolayer with opposite charge; the final result being independent of the electrical charge of the monolayer.

The same type of interactions was studied in other experiments, spreading lipid monolayers on VP3(110–121) containing subphases and compressing at 4.2 cm/min. The peptide concentration in the subphase was chosen so that no surface pressure changes were detected after spreading the monolayer, before compression. Initial surface pressures were lower than 1 mN/m. A 15 min period was left for interaction before the compression started. Five sets of two monolayers were obtained for the following compositions: DPPC; DPPC/5% SA; DPPC/5% CL; SA and CL.

Comparing the area/molecule for monolayers spread on PBS or on peptide solutions, the interaction of the peptide with all the monolayers was evident. The presence of peptide in the subphase has a soft expanding effect. This behavior was general, irrespective of the electrical charge of the monolayer, obviously area increases become smaller at higher pressures and isotherms fuse at surface pressures around 35 mN/m. Only DPPC/SA isotherms run parallel with a constant area difference of 0.16 nm^2/mol. This value although very small to correspond to a peptide β-sheet section suggests the presence of a small percentage of VP3(110–121) molecules that remain attached to the monolayer (Table 3).

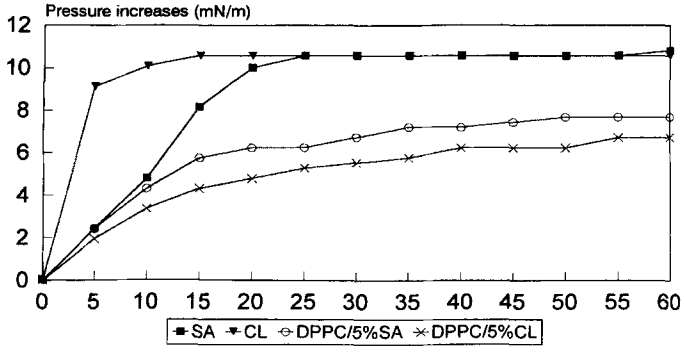

Fig. 4 Pressure increases after injecting VP3 under monolayers of different lipid composition. Initial monolayer pressure: 5 mN/m

Effect of VP3(110–121) on the fluidity of bilayers

It is well known that the packing of phospholipid in bilayers can be modified by the insertion of any molecule

Table 3 Lipid area/molecule increases in nm^2/molecule, produced by the presence of VP3 in the subphase

Pressure [mN/m]	DPPC	CL	SA	DPPC/CL	DPPC/SA
5	0.12	0.08	0.56	0.12	0.08
10	0.16	0.08	0.16	0.12	0.16
15	0.08	0.05	0.08	0.12	0.16
20	0.08	0	0.06	0.06	0.16

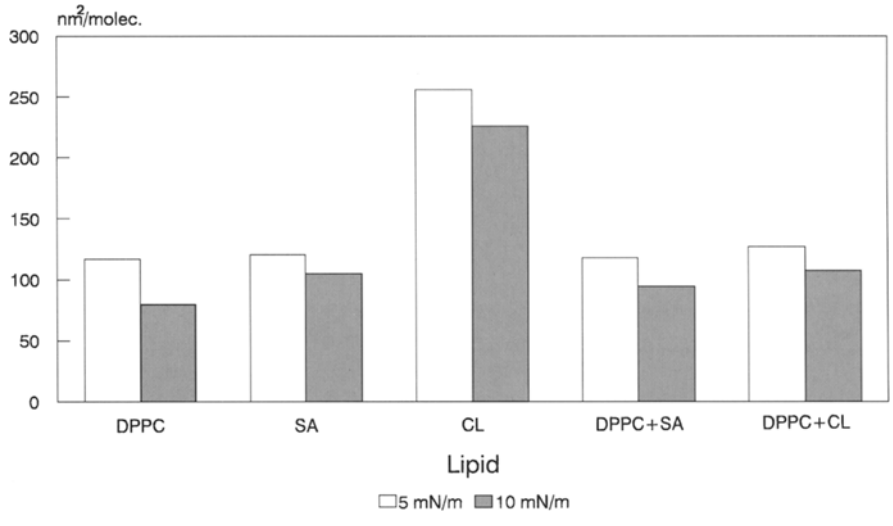

Fig. 5 Area/molecule (nm^2/mol) of the isotherms measured at 5 and 10 mN/m

in them. According to this if a fluorophore is located in the bilayer its motion can be restricted or increased by the presence of an added compound. Changes in microviscosity can then be measured through fluorescence polarisation. Nevertheless, as fluorophores can in some cases experiment quenching effects or/and transference of energy between them, a previous study was carried out in our system in order to rule out this possibility. No interactions among the peptide and ANS were detected when working in the absence of lipids. This was indicative of a lack of interactions and at the same time allows to rule out the presence of peptide micelles or aggregates.

VP3(110–121) interacts with DPPC/5% SA liposomes saturated with ANS giving an increase in the polarization values all over the range of temperatures studied (Fig. 6).

It is difficult to find specific significance for the high increase of polarization near the transition temperature. This behavior suggests some reorganization of the system that is associated with an increase of microviscosity of the bilayer due to the presence of the peptide. In the fluorescence values the presence of peptide reduces the fluorescence of ANS at temperatures between 23 °C and 50 °C in a lineal study.

A similar behavior was observed with liposomes of DPPC/5% CL. Polarization increases slightly, the values in the samples containing the peptide being higher than those without it (Fig. 7).

As a common trend the presence of peptide has a soft rigidifying effect all over the range of temperatures studied. This behavior had already been observed in other peptides.

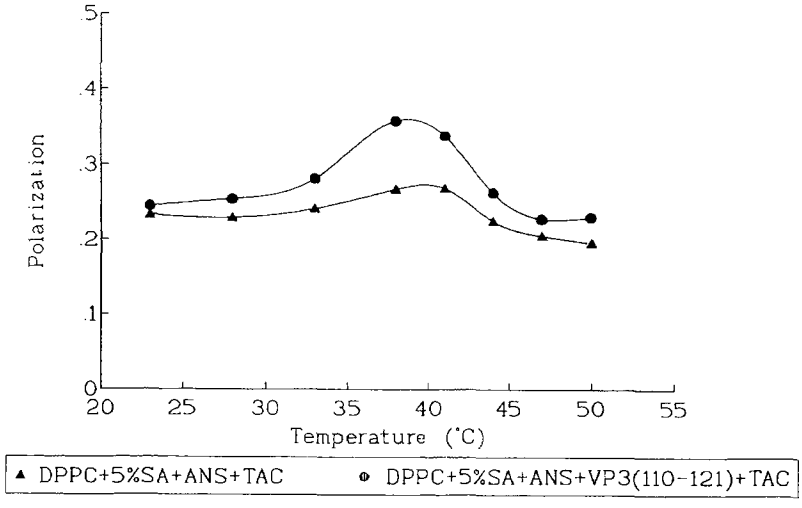

Fig. 6 Influence of VP3 on the polarization values of DPPC/5% SA saturated with ANS

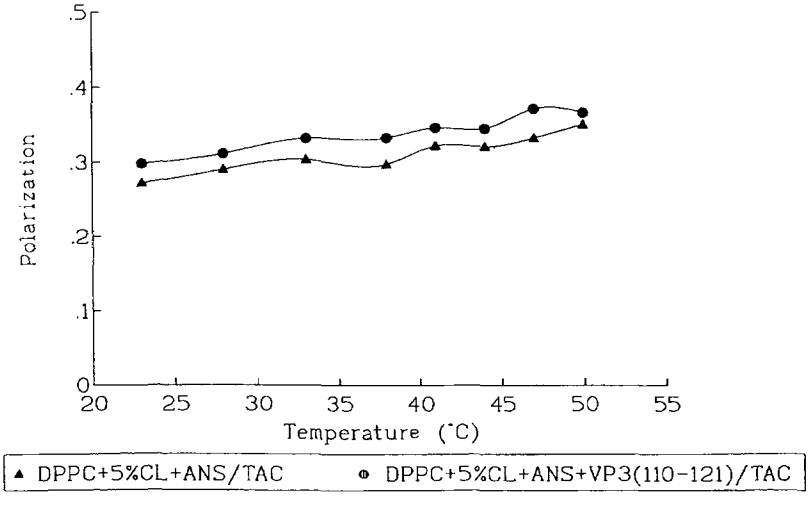

Fig. 7 Influence of VP3 on the polarization values of DPPC/5% CL saturated with ANS

On the contrary, when using DPH as anisotropy marker, no interaction was found for both lipid compositions. This suggests that the peptide remains at the outer part of bilayers and is not able to induce any change in the ordered state of the internal alkylchains.

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