

Binding of a Neuropeptide, Substance P, to Neutral and Negatively Charged Lipids[†]

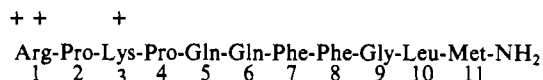
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ABSTRACT: The binding of substance P (SP), a positively charged neurotransmitter peptide, to neutral and to negatively charged phospholipids has been investigated by means of a monolayer technique. Monolayers formed at room temperature from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), or mixtures of the two, were maintained throughout the course of a binding experiment at a constant surface pressure while the monolayer surface area was monitored. Injection of SP into the aqueous subphase (154 mM NaCl, 10 mM Tris adjusted to pH 7.4) led to an expansion of the monolayer surface area that was attributed to a spontaneous insertion of SP between the lipid molecules. A quantitative evaluation of the area increase at constant pressure yielded SP insertion isotherms that showed that levels of SP insertion increased directly with the monolayer POPG content and decreased to negligible levels at surface pressures above 35 ± 1 mN/m. If electrostatic effects were ignored, these data showed biphasic behavior in Scatchard plots. The apparent binding constants ranged, at 20 mN/m, from $(3.2 \pm 0.3) \times 10^4$ M⁻¹ for 100% POPG monolayers to $(2.0 \pm 0.05) \times 10^3$ M⁻¹ for 25% POPG/75% POPC monolayers. At 32 mN/m, a monolayer surface pressure that mimics bilayer conditions, the apparent binding constant for a 100% POPG monolayer was measured to be $(1.1 \pm 0.05) \times 10^3$ M⁻¹. However, for a monolayer containing only 25% charged lipids, corresponding to a natural membrane composition, K_{app} at 32 mN/m was estimated to be at most 41 M⁻¹. When electrostatic effects were taken into account by means of the Gouy-Chapman theory, these same data yielded linear Scatchard plots that were best described in terms of a straightforward partitioning of SP into the monolayer lipids. The average value of the partition coefficient was 29 ± 2 M⁻¹ at 20 mN/m and ~ 1 M⁻¹ at 32 mN/m. Thus, the binding of SP to negatively charged lipid surfaces is dominated by electrostatic attraction, while hydrophobic interactions play only a secondary role.

Substance P (SP)¹ is a positively charged undecapeptide belonging to the tachykinins, a family of peptides sharing a common C-terminal amino acid sequence and a similar broad range of biological activities (Buck & Burcher, 1986). SP has aroused special interest as a putative neurotransmitter or neuromodulator in the transmission of pain information and has the amino acid sequence



It has been proposed on the basis of NMR measurements, conformational energy calculations, and structure-activity studies that, upon binding to a membrane-anchored receptor, SP assumes a U-shaped conformation via a hydrogen bond between Gln-6 and Met-11 (Sandberg & Iversen, 1982).

On the other hand, the amphiphilic nature of SP is evident from the segregation of charged and hydrophobic amino acids toward the amino- and carboxy-terminal ends, respectively, of the polypeptide chain. This has suggested the possibility that SP receptor binding is membrane assisted (Schwyzer, 1986, 1987) and has provoked studies of the interaction of SP with lipids and detergents. Circular dichroism measurements

in particular indicate that SP assumes a partially α -helical conformation in the presence of trifluoroethanol (Rolka et al., 1986), sodium dodecyl sulfate (Wu et al., 1982; Wu & Yang, 1983), lysophosphatidylglycerol micelles (Woolley & Deber, 1987), or negatively charged lipid vesicles (Rolka et al., 1986; Wu & Yang, 1983). The formation of a partial α -helix in SP is much more pronounced in the presence of detergent micelles than in the presence of negatively charged lipid vesicles. The α -helical region in SP is centered about Pro⁴-Gln⁵-Gln⁶-Phe⁷-Phe⁸ and probably comprises the eight to nine carboxy-terminal amino acids (Rolka et al., 1986; Erne et al., 1986; Woolley & Deber, 1987). When SP was covalently linked to poly(ethylene glycol) the addition of trifluoroethanol induced no helical structure (Koziej et al., 1985). However, this compound was shown to be physiologically inactive.

Binding constants of SP to lipid membranes have been estimated by using thermodynamic calculations (Schwyzer et al., 1986) but have not been determined experimentally. Nevertheless, it has been proposed that, under physiological conditions, SP inserts into the hydrophobic core of lipid membranes with its carboxy-terminus arranged in an α -helical conformation, the latter being oriented essentially parallel to the long axis of the lipid fatty acyl chains.

The purpose of this study is to visualize directly the possible insertion of SP into the hydrophobic core of a lipid membrane by using a monolayer technique and in so doing to obtain

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¹Abbreviations: SP, substance P; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; CD, circular dichroism.

experimental measures of SP-lipid binding constants. Lipid monolayers at an appropriate lateral pressure (around 32 mN/m; Seelig, 1987) are well suited to mimic bilayer membranes. Provided that the length of the long molecular axis of a molecule inserting into the monolayer does not exceed that of the lipid molecules, the resulting steric arrangement should be very similar to that found in a bilayer. The intercalation of a molecule between the lipids of a half-membrane can be quantitated directly by monitoring the consequent area increase, ΔA , of the monolayer (initial area A) at constant surface pressure. The relative area increase, $\Delta A/A$, allows a molecular interpretation of the binding process.

MATERIALS AND METHODS

Substance P (SP) was a gift from Merck (Darmstadt). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Birmingham, AL). The water used for buffer and solutions was double-ion-exchanged and glass-distilled. A 10 mM Tris buffer adjusted to pH 7.4 with HCl, containing 154 mM NaCl, was used for all experiments. This buffer has been chosen because it is close to a physiological medium.

The monolayer apparatus consisted of a round Teflon trough designed by Fromherz (Fromherz, 1975) with a total area of 362 cm² divided into eight compartments (Type RMC 2-T, Mayer Feinttechnik, Göttingen, FRG). The surface pressure was measured by the Wilhelmy method using plates cut from filter paper (Whatman, No. 1). Before each measurement the trough and the filter paper were thoroughly cleaned with methanol and distilled water. For binding studies two segments of the trough were each filled with 20 mL of buffer solution. POPC, POPG, or mixtures of the two lipids were dissolved in quantities of 4 mg/mL in hexane/ethanol (9:1 v/v). A drop of the lipid solution on the needle tip of a Hamilton syringe was carefully deposited directly at the air-water interface to form a monolayer. Monolayers spread in this way were immediately stable. They were used for incorporation studies, however, only after a constant pressure had been monitored for a period of 15 min. The surface pressure was brought to the desired value by adjusting the barriers to an appropriate area (generally between 50 and 60 cm²). Small amounts of a 0.025 M stock solution of SP were then injected with a Hamilton syringe into the buffer subphase of each compartment to attain the desired SP concentration. These concentrations were restricted to the micromolar range (2.5–125 μ M) to avoid problems caused by SP self-aggregation that occurs in the millimolar concentration range (Rueger et al., 1984). The buffer phases in each segment were stirred by tiny magnets to assure a homogeneous distribution of SP. The instrument maintained a constant lateral surface pressure throughout the course of a binding experiment by means of an electronic feedback system controlling the position of a moveable barrier. Alterations in the monolayer surface area were then recorded as a function of time. All measurements were done at room temperature (22 \pm 1 $^{\circ}$ C).

RESULTS

Figure 1 shows a typical time course for the penetration of SP into a POPG monolayer as monitored by an area increase ΔA at constant surface pressure (20 mN/m). The insertion of SP into POPG monolayers is relatively fast, a stable equilibrium situation being obtained after 5–10 min.

The dependence of the level of SP insertion on the lateral pressure of the POPG monolayer is summarized in Figure 2, where the relative area increase $\Delta A/A$ was plotted as a

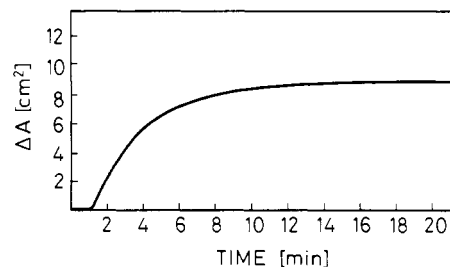


FIGURE 1: Area increase ΔA of a POPG monolayer at 24.5 mN/m induced by insertion of SP (12.5 μ M) as a function of time. The POPC monolayer was spread on buffer solution (10 mM Tris buffer adjusted to pH 7.4, 154 mM NaCl) at 22 \pm 1 $^{\circ}$ C.

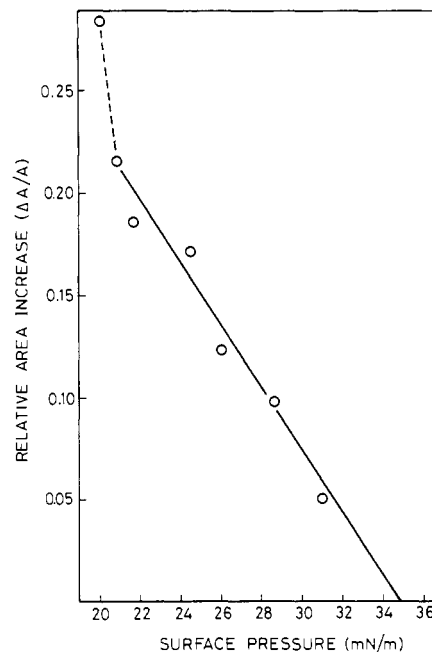


FIGURE 2: Relative area increase $\Delta A/A$ of a POPG monolayer due to insertion of SP (12.5 μ M) as a function of surface pressure.

function of the preset surface pressure of POPG monolayers. For each surface pressure value a new POPG monolayer was prepared. The relative area increase $\Delta A/A$ was highly pressure dependent, and the insertion became increasingly difficult with increasing packing density of the lipid molecules. In the pressure range 21–31 mN/m the relative area increase was a linear function of the surface pressure. Extrapolation to zero area change gives a surface pressure above which no penetration should occur. This cutoff pressure was 35 \pm 1 mN/m for a pure POPG monolayer. At surface pressures below 21 mN/m the relative area increase $\Delta A/A$ as a function of pressure deviated from linearity toward higher values of $\Delta A/A$. An analogous situation has been observed for charged dibucaine partitioning into POPC monolayers, where linearity was observed in the pressure range 25–36 mN/m, and the relative area increase as a function of pressure deviated toward larger values at pressures below 25 mN/m (Seelig, 1987).

Figure 3 shows the relative area increase $\Delta A/A$ as a function of the mole percent of POPG in mixed POPG/POPC monolayers at 20 mN/m. A linear increase of the relative surface area $\Delta A/A$ with increasing percentage of POPG was observed for all concentrations of SP measured in the range 6.25–125 μ M. This demonstrates that the addition of negatively charged lipid enhances the adsorption and penetration of SP.

At a pressure of 20 mN/m no penetration of SP was observed for a neutral lipid monolayer, i.e., 100% POPC, not even at the highest concentration measured (125 μ M). However,

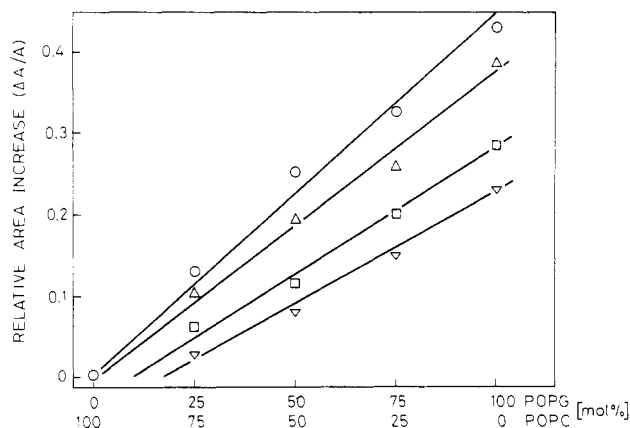


FIGURE 3: Relative area increase $\Delta A/A$ as a function of mole percent of POPG in mixed POPG/POPC monolayers at 20 mN/m measured at different SP concentrations: (∇) 6.25 μM ; (\square) 25 μM ; (Δ) 75 μM ; (\circ) 125 μM .

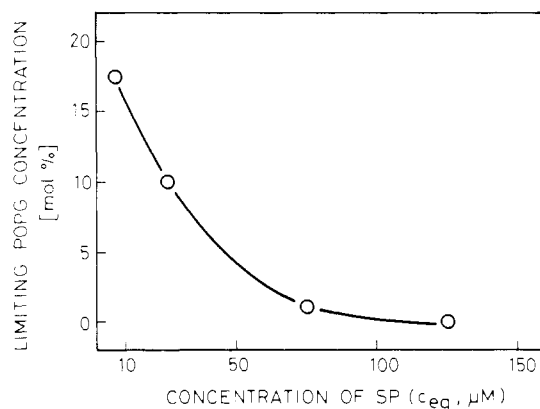


FIGURE 4: Limiting POPG content (mole percent) needed to induce SP insertion as a function of SP concentration. The data refer to a monolayer pressure of 20 mN/m.

the addition of even a small percentage of POPG led immediately to a distinct area increase $\Delta A/A$. Extrapolation of the straight lines in Figure 3 to zero area increase gives the limiting POPG content of monolayers at which for a given SP concentration penetration starts to occur. Increasing the POPG content (mole percent) beyond this limiting concentration linearly enhances SP binding. In Figure 4 the limiting POPG concentration (mole percent) that is necessary to induce SP penetration is plotted as a function of SP concentration. This curve directly illustrates the strong dependence of SP penetration on the POPG content of monolayers. At low concentrations of SP the negative charge in the monolayer must be increased to achieve penetration.

The relative area increase $\Delta A/A$ as a function of the equilibrium concentration of SP ($c_{\text{eq}} = 2.5\text{--}125 \mu\text{M}$) is plotted in Figure 5. The upper four insertion isotherms refer to a surface pressure of 20 mN/m, whereas the lowest curve represents the insertion into a pure POPG monolayer at 32 mN/m. At 20 mN/m all curves showed a pronounced increase of the relative area change $\Delta A/A$ with increasing concentration of SP in the low concentration range, up to about 12.5 μM , and a leveling off at higher concentrations. At 32 mN/m penetration of SP into POPG monolayers was rather weak. The transformation of these curves into true insertion isotherms will be discussed below.

SP dissolved in buffer solution shows some surface activity due to its amphiphilic nature. The maximal surface pressure measured as a function of concentration is only 3.5 mN/m, well below the surface pressures of the lipid monolayers.

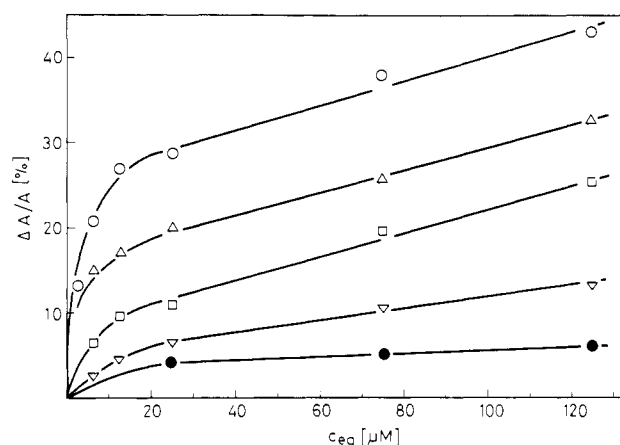


FIGURE 5: Relative area increase $\Delta A/A$ of various lipid monolayers as a function of the equilibrium concentration of SP: at 20 mN/m (\circ) 100% POPG, (Δ) 75% POPG, (\square) 50% POPG, (∇) 25% POPG; at 32 mN/m (\bullet) 100% POPG.

DISCUSSION

When SP is injected underneath a negatively charged POPG monolayer kept at constant surface pressure during the course of an experiment, a film expansion is observed. This area increase might be explained by the insertion of the hydrophobic C-terminal end of SP molecules into the hydrophobic core of the lipids. The extent of the monolayer expansion is highly pressure dependent, as seen in Figure 2, decreasing with increasing surface pressure of the monolayer. An extrapolation to zero area increase leads to a surface pressure of 35 ± 1 mN/m. This limiting pressure may be denoted a "cutoff" pressure since SP should no longer insert into the POPG monolayer at pressures above this value.

In contrast, when SP was injected underneath an electrically neutral POPC monolayer, no surface expansion was observed in the concentration range 2.5–125 μM , not even at low surface pressure. This result provides evidence for a rather low affinity of SP for neutral lipids. Indirect support for this contention comes from CD measurements of SP in the presence of neutral and negatively charged phospholipid bilayer vesicles (Rolka et al., 1986). In the presence of neutral phospholipids SP remained in a disordered conformation, indicating little or no interaction with these lipids. Addition of negatively charged lipids, however, led to a partial α -helix formation of SP. The following quantitative analysis of the monolayer–SP insertion isotherms is limited, therefore, to POPG-containing monolayers.

Insertion of SP into POPG Monolayers. A pure lipid monolayer having a surface area A is composed of n_1 POPG and n_2 POPC molecules. To a first approximation, the surface areas, A_L , of POPG and POPC are assumed to be equal such that

$$A = (n_1 + n_2)A_L \quad (1)$$

The insertion of n_p protein molecules of molecular area A_p leads to an area increase of

$$\Delta A = n_p A_p \quad (2)$$

The relative area increase is hence

$$\Delta A/A = (n_p/(n_1 + n_2))(A_p/A_L) \quad (3)$$

The surface area of POPC is known from monolayer experiments to be 68 \AA^2 at 32 mN/m and 80 \AA^2 at 20 mN/m (Evans et al., 1987). The mean area requirement of peptide α -helices without bulky side groups can be estimated from crystallographic data as 125 \AA^2 (Banner et al., 1987). The

Table I: Gouy-Chapman Analysis: Insertion of SP into Lipid Monolayers with Varying POPG Content [22 ± 1 °C, 0.154 M NaCl, 10 mM Tris, pH 7.4, $z_p = 2.4$ (Effective Charge of SP)]

π (mN/m)	C_{eq} (μ M)	$\Delta A/A$ (%)	X_b (mmol/mol)	X_b/C_{eq} (M ⁻¹)	K_{app} (M ⁻¹)	σ (mcb/m ²)	ψ (mV)	C_M (mM)	X_b/C_M (M ⁻¹)	K_p (M ⁻¹)
20	POPG 25%/POPC 75%									
	6.25	2.8	9.3	1492	$(2 \pm 0.05) \times 10^3$	-44.33	-42.19	0.336	27.73	29 ± 2
	12.5	4.7	15.6	1252		-40.62	-39.23	0.508	30.78	
	25.0	6.6	22.0	880		-37.04	-36.26	0.768	28.62	
	75.0	10.4	34.6	462		-30.25	-30.33	1.315	26.3	
	125.0	13.2	44.6	357		-25.21	-25.68	1.41	31.59	
20	POPG 50%/POPC 50%									
	6.25	7.0	23.3	3727	$(7.5 \pm 0.1) \times 10^3$	-83.11	-67.13	3.54	6.57	
	12.5	9.8	32.6	2607		-76.93	-63.73	5.14	6.33	
	25.0	11.7	39.0	1560		-72.85	-61.35	8.21	4.74	
	75.0	19.6	65.3	870		-57.48	-51.58	9.79	6.6	
	125.0	25.4	84.6	676		-47.42	-44.35	8.25	10.25	
20	POPG 75%/POPC 25%									
	6.25	15	50.0	7999	$(2.3 \pm 0.1) \times 10^4$	-109.70	-79.70	11.62	4.30	
	12.5	17.6	58.6	4687		-103.7	-77.07	18.13	3.23	
	25.0	20.1	67.0	2680		-98.24	-74.45	28.31	2.36	
	75.0	25.9	86.3	1150		-86.35	-68.37	47.84	1.80	
	125.0	32.7	109.0	871		-73.70	-61.39	41.24	2.64	
20	POPG 100%									
	2.5	13.2	44.0	17600	$(3.2 \pm 0.3) \times 10^4$	-158.20	-97.18	24.22	1.80	
	6.25	20.8	69.3	11088		-138.21	-90.58	32.47	2.13	
	12.5	27.2	90.6	7247		-123.2	-85.03	38.45	2.35	
	25.0	28.57	95.2	3807		-120.17	-83.67	67.64	1.40	
	75.0	38.16	127.2	1695		-100.69	-75.10	90.33	1.41	
	125.0	43.24	144.1	1152		-90.45	-70.51	97.59	1.48	
32	POPG 100%									
					$(1.1 \pm 0.05) \times 10^3$				0.015 ± 0.002	1.0^a

^a Estimated in analogy to monolayer at 20 mN/m.

α -helical region of SP comprises two phenylalanines and has therefore a larger area. To get an upper limit, the area of a polyphenylalanine α -helix has been determined by computer modeling as 250 \AA^2 . The area of SP in a SP monolayer has been measured to be $240 \pm 5 \text{ \AA}^2$, which is in close agreement with the computed area. The structure of SP in its aggregated form is, however, not yet fully understood (Seelig, unpublished data). An area of 240 \AA^2 will be used in the following. Numerical calculations have also been performed for other A_p values (200 – 250 \AA^2), and it has been found that the exact value of A_p is not so critical in this range of low X_b values. At membrane conditions, 32 mN/m, the results obtained by using the two extreme areas are within given error limits. The area of SP is assumed to be pressure independent in the range 20–32 mN/m. When the area requirements of the lipid and protein molecules are known, eq 3 allows a determination of the degree of binding, X_b

$$X_b = n_p / (n_1 + n_2) \quad (4)$$

from the monolayer expansion measurements. Therefore, the relative area increase

$$\Delta A/A = X_b(A_p/A_L) \quad (5)$$

can be converted directly into binding data.

The results of the numerical evaluation are summarized in the third and fourth columns of Table I. These data on SP insertion into lipid monolayers have been further analyzed in terms of two different models, i.e., a Langmuir adsorption isotherm without electrostatic interaction (equivalent to a conventional Scatchard analysis) and a Langmuir adsorption isotherm with electrostatic interactions, applying the Gouy-Chapman theory in the latter case.

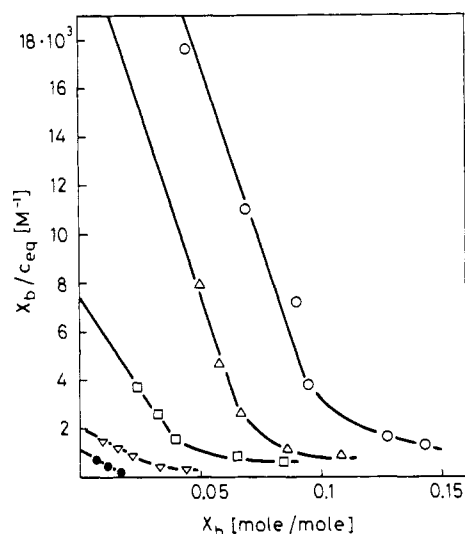


FIGURE 6: Scatchard analysis prior to consideration of charge effects (X_b/C_{eq} as a function of X_b) for SP insertion into monolayers containing (●) 100% POPG at 32 mN/m and (○) 100%, (Δ) 75%, (□) 50%, and (▽) 25% POPG at 20 mN/m.

Insertion Isotherms without Electrostatic Interaction. In a first step, the insertion data were analyzed empirically in terms of a Langmuir adsorption isotherm of the form

$$X_b / (1 - nX_b) = K_{app} C_{eq} \quad (6)$$

where n is the number of lipids bound per molecule of SP and C_{eq} is the bulk equilibrium concentration of SP. We have assumed $C_{eq} \sim C_{tot}$ since X_b is at most about 1% of the total concentration, C_{tot} , of SP. Here K_{app} denotes an apparent binding constant to emphasize that electrostatic interactions are not specifically taken into account. In terms of a Scatchard plot eq 6 may be rewritten as

$$X_b / C_{eq} = K_{app} (1 - nX_b) \quad (7)$$

² We are indebted to Dr. G. Hölzemann for the computer modeling of a polyphenylalanine α -helix.

and numerical data for X_b/C_{eq} are given in the fifth column of Table I. Figure 6 then summarizes Scatchard plots for the various monolayers investigated. At a surface pressure of 20 mN/m all four lipid monolayers (25%, 50%, 75%, and 100% POPG) gave rise to nonlinear Scatchard plots exhibiting an apparently biphasic behavior. From the high-affinity branch of these plots K_{app} was evaluated, and the data are also listed in Table I. The extent of the biphasic behavior and the apparent binding constant are both strongly dependent on the negative surface charge density of the monolayer and increase with the monolayer content of POPG. This Scatchard analysis is, however, only meant to facilitate a comparison with other literature data, since nonlinear Scatchard plots have also been observed for other charged peptides binding to lipids [cf. Tamm (1986)]. Curved Scatchard plots can indicate either multiple binding sites of different affinity or negative cooperativity (Schwarz, 1976). Analyzing the insertion isotherms in terms of the Gouy–Chapman theory (McLaughlin, 1977) will show that the nonlinear character of the isotherms is due to negative cooperativity. The strong electrostatic attraction of SP molecules to the aqueous diffuse layer adjacent to the monolayer surface facilitates the penetration of SP into monolayers containing negatively charged phospholipids, up to the point where the amount of inserted charged SP molecules is large enough to impede further insertion.

Insertion Isotherm with Electrostatic Interaction. The interaction of positively charged SP with a negatively charged lipid was analyzed next in terms of a modified Langmuir adsorption isotherm of the form

$$X_b/(1 - nX_b) = K_p C_{eq} e^{-z_p \psi_0 F_0 / RT} \quad (8)$$

Here ψ_0 is the membrane surface potential that, depending on its sign and size, determines the effective electrostatic attraction or repulsion of SP from the monolayer. The effective charge of SP is denoted z_p . The problem then is to calculate the surface charge density σ and the surface potential ψ_0 .

The insertion of the SP molecules induces not only an area increase in the monolayer but also a change in the surface charge density σ . The total surface area of the monolayer is given by

$$A_T = (n_1 + n_2)A_L + n_p A_p \quad (9)$$

The total surface charge Q_T is

$$Q_T = (-z_1 n_1 + z_p n_p) e_0 \quad (10)$$

where e_0 is the elementary charge, $z_1 = 1$ the valency of the negative lipid, and z_p the valency of SP. SP bears three positive charges at its N-terminal end, but as will be discussed below, the best fit to the experimental data was obtained by assuming a slightly smaller effective charge of $z_p = 2.4$. Equations 9 and 10 may be combined to calculate the surface charge density according to

$$\sigma = Q_T / A_T = (e_0 / A_L) (-X_1 + z_p X_b) / (1 + X_b (A_p / A_L)) \quad (11)$$

where X_b is the mole fraction of inserted SP determined according to eq 5 and X_1 is the mole fraction of negatively charged lipid [cf. Seelig et al. (1988)]. The surface charge density σ can thus be calculated from experimentally determined X_b values. The surface charge density σ gives rise to a surface potential ψ_0 , which may be approximated by means of the Gouy–Chapman theory [cf. McLaughlin (1977)]

$$\sigma = [2000 \epsilon_r \epsilon_0 RT \sum_i c_{i,eq} (e^{-z_i F_0 \psi_0 / RT} - 1)]^{1/2} \quad (12)$$

where $\epsilon_r = 78$ is the dielectric constant of water (at 25 °C),

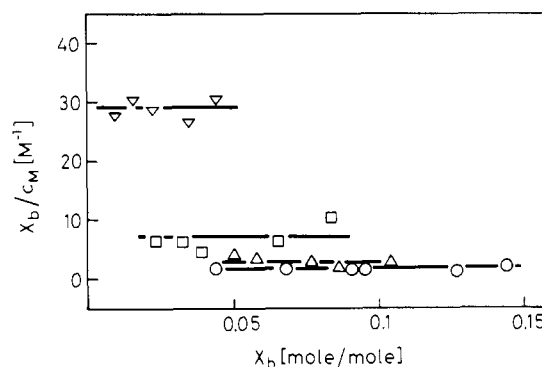


FIGURE 7: Scatchard analysis after consideration of charge effects (X_b/C_M as a function of X_b) for SP insertion into monolayers containing (○) 100%, (Δ) 75%, (□) 50%, and (▽) 25% POPG at 20 mN/m.

ϵ_0 is the permittivity of free space, R is the gas constant, F_0 is the Faraday constant, $c_{i,eq}$ is the concentration of the i th electrolyte in the bulk aqueous phase (in moles per liter), and z_i is the signed charge of the i th species. The effect of the negative surface potential of POPG membranes is to attract positively charged SP molecules to the membrane surface. The concentration of charged SP molecules, C_M , immediately adjacent to the membrane surface is related to its equilibrium concentration, C_{eq} , in the bulk solution according to the Boltzmann equation

$$C_M = C_{eq} e^{-z_p \psi_0 F_0 / RT} \quad (13)$$

Replacing C_{eq} in eq 6 by C_M in eq 13 thus leads to eq 8. Numerical data for σ , ψ_0 , C_M , and X_b/C_M are found in Table I. Note that the surface concentration C_M (mM) of SP is several orders of magnitude larger than the bulk concentration C_{eq} (μ M). This demonstrates the considerable electrostatic attraction of positively charged SP toward the negatively charged membrane surface. This high accumulation of SP in solution close to the membrane surface might eventually lead to the formation of SP aggregates.

Inspection of Table I further reveals that ratios of X_b/C_M are constant over the whole concentration range measured. When plotted in terms of a Scatchard plot (Figure 7) straight lines parallel to the abscissa are obtained. In molecular terms this result means that the experimental data are sufficiently described by a simple surface partition equilibrium

$$X_b = K_p C_{eq} e^{-z_p F_0 \psi_0 / RT} \quad (14)$$

The results in Table I were calculated by using a net charge of $Z_p = 2.4$ for SP. If a charge of $Z_p = 3$ is used, the value of X_b/C_M increases with concentration. This is expected neither for a Langmuir adsorption nor for a partitioning process. Decreasing the charge of SP to an effective charge of 2.4 gives constant values of X_b/C_M , within the accuracy of the measurements, for all monolayers measured. One explanation for this charge reduction could be related to the pK_a value of the N-terminus of SP. It has been variously reported between 6.5 and 7.0 (Mehlis et al., 1980; Woolley & Derber, 1987). Thus, if the pH of the system is 7.4, the effective charge of SP would be closer to +2 than to +3. An alternative explanation for a charge reduction on SP could come from experimental results obtained with hexamethonium, which also exerts a smaller effect on the potential ψ than predicted by the classic screening theory (Alvarez et al., 1983). The reason for this effect is the large separation of the charges in hexamethonium (about 10 Å), a distance comparable with the Debye length in a physiological solution (Carnie &

McLaughlin, 1983). In SP a similar situation prevails since the three charged NH_3 groups are also distinctly separated from each other.

From Table I and Figure 7 it is further seen that the values of K_p decrease with increasing POPG content of the monolayer, although, theoretically, K_p should be independent of the surface charge density. The reason for this discrepancy is presumably an overestimation of the monolayer surface charge at high POPG content. It is known from studies of smectic liquid crystals that only 20–30% of the charged constituents are fully dissociated (Jönsson & Wennerström, 1980). A similar counterion condensation could occur in monolayers with high POPG content. Evidence for such an effect was observed recently in Ca^{2+} binding studies to mixed POPG/POPC (Macdonald & Seelig, 1987a) and cardiolipin/POPC bilayers (Macdonald & Seelig, 1987b). In these latter studies all data at POPG concentrations >30% could best be explained by assuming a charge density that was smaller than that predicted for a fully dissociated POPG monolayer. Knowing the binding constant K_p for a monolayer at low POPG content (25 mol %) allows one to estimate the effective surface potential for monolayers at high POPG content. For a 100% POPG monolayer at 20 mN/m the effective surface potential was estimated to be ~ -75 mV instead of -110 mV.

At 32 mN/m SP insertion into a monolayer with low POPG content cannot be measured. The partition coefficient, K_p , has therefore to be determined from insertion measurements into a pure POPG monolayer. The partition coefficient K_p determined by means of the Gouy–Chapman theory, assuming full dissociation of the POPG monolayer, gives a value of $K_p = 0.015 \text{ M}^{-1}$. If a reduction of the surface potential from -120 to -75 mV is assumed in analogy to the monolayer at 20 mN/m, the value of the partition coefficient increases to $K_p = 1.0 \text{ M}^{-1}$. This low K_p value shows that hydrophobic interactions of SP with a monolayer/bilayer consisting of tightly packed lipids is hindered compared to those with a loosely packed monolayer. The dependence of SP insertion on the lateral packing density of fatty acyl chains might explain the fact that SP partitions more easily into lysolipid micelles (Woolley & Derber, 1987) than into lipid bilayers.

The application of the Gouy–Chapman theory has thus a number of favorable consequences. First, the biphasic behavior of the conventional Scatchard plot is eliminated. In hindsight, this biphasic behavior appears to be the result of strong electrostatic attraction at low X_b (high membrane surface charge) and weak attraction at large X_b (low surface charge). Second, the best model for a quantitative interpretation appears to be a simple partition equilibrium. A similar model has been obtained for the binding of charged local anesthetics to bilayer surfaces (Seelig et al., 1988) and appears to be consistent with the amphiphilic structure of SP. The partition coefficient K_p (Table I), after electrostatic effects are corrected for, is rather small, indicating that hydrophobic interactions between SP and the lipid membrane are limited. This is supported by the experimental observation that SP insertion has not been observed with neutral monolayers and bilayers. In the presence of negatively charged lipids, however, SP concentration increases in the immediate vicinity of the membrane surface due to electrostatic interaction, thus rendering insertion more probable.

A comparison can now be made between the binding constants measured here and those estimated by Schwyzer et al. (1986) using thermodynamic calculations. The latter authors reported theoretical binding constants of 21.7 , 2.3×10^3 , and $2.8 \times 10^7 \text{ M}^{-1}$ for SP binding to bilayers having membrane

surface potentials of 0 , -40 , and -120 mV, respectively. Previous studies have shown that the best correspondence in the physical properties of monolayers and bilayers occurs at a monolayer surface pressure of 32 mN/m (Demel et al., 1975; Seelig, 1987). The best comparison then is with our values for 100% POPG monolayers at 32 mN/m, where $K_p \sim 1.0 \text{ M}^{-1}$ and $K_{\text{app}} = (1.1 \pm 0.05) \times 10^3 \text{ M}^{-1}$. Thus, we observe apparent binding constants that are 4 orders of magnitude lower than those calculated theoretically. This large discrepancy is due mainly to an overestimation of the surface charge, as discussed above, which leads to an overestimation of the binding constant.

Only if patches of high local concentrations of negatively charged lipid occurred in nature would our apparent binding constant of $1100 \pm 50 \text{ M}^{-1}$ be of relevance. Although this may be the case in brain tissue, most natural membranes are viewed as containing about 25% of evenly distributed negatively charged lipids having, therefore, a surface potential of about -40 mV. On the basis of the experiments reported here, an apparent binding constant K_{app} of at most 41 M^{-1} can be expected for such a membrane. This is also lower than the value of 2325 M^{-1} estimated earlier (Schwyzer et al., 1986).

CONCLUSIONS

(1) SP does not insert into uncharged monolayers, neither at 20 mN/m nor at 32 mN/m in the concentration range measured (6.25 – $125 \mu\text{M}$). These data strongly suggest that SP does not penetrate into neutral bilayer membranes in this concentration range.

(2) At 20 mN/m SP does insert into monolayers containing negatively charged lipids, whereby the insertion is proportional to the POPG content of the monolayer. Analysis of the insertion isotherms by means of Scatchard plots yields apparent binding constants, K_{app} , varying from $(3.2 \pm 0.3) \times 10^4 \text{ M}^{-1}$ for a monolayer containing 100% POPG to $(2 \pm 0.05) \times 10^3 \text{ M}^{-1}$ for a monolayer containing only 25% POPG. These Scatchard plots are nonlinear, exhibiting an apparently biphasic behavior. It was shown, however, that by taking into account charge effects by means of the Gouy–Chapman theory, the insertion process was described by a partition equilibrium with only one partition coefficient, $K_p = 29 \pm 2 \text{ M}^{-1}$.

(3) Insertion of SP into negatively charged monolayers at surface pressures mimicking bilayer conditions (32–35 mN/m) is weak. At pressures of 35 ± 1 mN/m insertion into POPG monolayers no longer occurs. At 32 mN/m insertion could only be observed for monolayers containing 100% POPG. The apparent binding constant K_{app} was measured to be $(1.1 \pm 0.05) \times 10^3 \text{ M}^{-1}$, and the partition coefficient K_p was estimated to be 1.0 M^{-1} . Insertion of SP into those monolayers best representing natural membranes (32 mN/m, 25% POPG) was too weak to be measured under the above experimental conditions. The binding constants K_{app} and K_p could be estimated, therefore, only by analogy with the results at 20 mN/m, being 41 M^{-1} and 1.0 M^{-1} , respectively.

(4) The Gouy–Chapman analysis further suggested that at high POPG content (>30%) counterion condensation might be occurring. This phenomenon would explain, in part, the large discrepancies between the binding constants calculated by Schwyzer et al. (1986) and the binding constants determined by means of the monolayer technique.

In summary, we conclude that the binding of SP to negatively charged biological membranes will be dominated by electrostatic attraction to the membrane surface due to the positive charges borne by SP. Hydrophobic interactions appear to play only a minor role.

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Registry No. SP, 33507-63-0; POPG, 81490-05-3; POPC, 26853-31-6; POPG-POPC mixture, 118460-95-0.

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Effects of Oxygen on the Metabolism of Nitroxide Spin Labels in Cells[†]

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ABSTRACT: The products of the reduction of nitroxides in cells are the corresponding hydroxylamines, which cells can oxidize back to the nitroxides in the presence of oxygen. Both the reduction of nitroxides and the oxidation of hydroxylamines are enzyme-mediated processes. For lipid-soluble nitroxides, the rates of reduction are strongly dependent on the intracellular concentration of oxygen; severely hypoxic cells reduce nitroxides more rapidly than cells supplied with oxygen. In contrast, the rates of oxidation of hydroxylamines increase smoothly with increasing intracellular oxygen concentration up to 150 μ M. In order to separate the effects on the rates of metabolism of nitroxides due directly to oxygen from effects due to the redox state of enzymes, we studied the cells under conditions in which each of these variables could be changed independently. Oxygen affects the metabolism of these nitroxides primarily by interacting with cytochrome *c* oxidase to change the redox state of the enzymes in the respiratory chain. Our results are consistent with the conclusions that in these cells reduction of lipophilic nitroxides occurs at the level of ubiquinone in the respiratory chain in mitochondria, and oxidation of the corresponding hydroxylamines occurs at the level of cytochrome *c* oxidase.

Nitroxides, in addition to their role as probes of molecular dynamics of membranes and cells (Berliner, 1976, 1979; Swartz & Swartz, 1983; Morse, 1985), might also serve as

effective contrast or imaging agents for NMR (Brasch et al., 1983; Brasch, 1983; Swartz et al., 1986a,b; Swartz, 1988) or ESR (Berliner & Fujii, 1985; Bacic et al., 1988) imaging and in vivo spectroscopy. An attractive feature of this idea is that the reduction of nitroxides, heretofore considered a liability, can be used to advantage, since the reduction of nitroxides and oxidation of hydroxylamines by cells are strongly dependent on the concentration of oxygen (Swartz et al., 1986a,b; Swartz,

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