International Journal of Pharmaceutics, 90 (1993) 59–71

IJP 02973

Interaction of aminoglycosides and colistin with model membranes: Liposomes and monolayers

C. Colomé [a], M.A. Alsina [b], M.A. Busquets [b], I. Haro [a] and F. Reig [a]

[a] *Department of Peptides, C.I.D.-C.S.I.C., Jordi Girona 18–26, 08034 Barcelona (Spain)* and [b] *Physicochemical Department, Faculty of Pharmacy, Barcelona (Spain)*

(Received 22 May 1992)
(Accepted 7 July 1992)

Key words: Aminoglycoside; Polymixin; Liposomes; Monolayer

Summary

The interaction of gentamicin (G), kanamycin (K), spectinomycin (Sp), streptomycin (St) and a polymixin, colistin (C), with lipids was studied by using liposomes and monomolecular layers as membrane models. The lipids used were phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylinositol disphosphate (PIP_2), gangliosides (Gan) and cholesterol (Chol). The results show that only colistin is able to induce the leakage of entrapped carboxyfluorescein (CF) from the liposomes after incubation. Moreover, this interaction is not clearly related to a single type of phospholipid, is dependent on the PL/colistin ratio and is slightly modified by the pH or the temperature of the incubation media. The interactions of these molecules with the polar heads of phospholipids were studied using 1-anilino-8-napthalenesulphonic acid (ANS) as fluorescent probe. The polarization values indicate that these antibiotics induce rigidification of the membrane. Monolayers having the same lipid composition of liposomes were spread on aqueous subphases and pressure increases, after injection of drug molecules, were recorded. In this set of experiments all molecules exhibit interaction, colistin and kanamycin being those with the maximum activity. Moreover, no specific differences could be assigned to the different lipids used except for kanamycin and gangliosides. The mathematical equations of the penetration kinetics for all the assays carried out were determined. The Lineweaver-Burk equation gave the best fit to the experimental values, the regression coefficient being higher than 0.98 in all cases. Compression isotherms of the same phospholipid mixtures were recorded, the antibiotic molecules being dissolved in the subphase. The compressibility and area/molecule were slightly affected by the presence of aminoglycosides in the aqueous phase. In contrast, the presence of colistin induced expansion of the monolayer especially at low pressures, thus indicating the existence of interactions. Comparing these results with those in the literature, it appears clear that the membrane model used exerts a strong influence on the results obtained.

Introduction

The mechanism of action of aminoglycosides and polymixins is related to their ability to inhibit protein synthesis and to produce in vivo and in vitro misreading, as well as to destabilize the membrane structure, respectively.

To achieve their targets in the cells, it is necessary for these molecules to cross several barriers. For this reason, the study of the interactions between the lipid components of the membrane and these drug molecules has been the subject of several papers. Nevertheless, the information accumulated to date is in most cases contradictory.

Correspondence to: C. Colomé, Department of Peptides, Faculty of Pharmacy, Barcelona, Spain.

When using the leakage of CF as a parameter of disturbance, interactions are very slowly manifested (Au et al., 1987) whereas when using turbidimetry increases, as a reference for stability, it takes only 4 min to achieve a full interaction (Aramaki and Tsuchiya, 1989).

Some authors have found a specific interaction between gentamicin and phosphatidylinositol diphosphate as far as the disruption of liposome structure is concerned, suggesting that this could be a key step in the toxic action of aminoglycoside antibiotics (Forge et al., 1989). However, using liposomes and diphenylhexatriene as a fluorescent probe, it has been shown that gentamicin does not interact with the hydrophobic domain of the bilayer (Mingeot-Leclercq et al., 1989), thus it appears to be doubtful whether these antibiotics can promote the leakage of an entrapped dye.

The aim of the present study was to detect the effect of aminoglycosides and polymixin E on the ordered structures of several natural phospholipids, when arranged in mono- and bilayers.

Materials and Methods

Chemicals

L-α-Phosphatidylcholine (PC) was purchased from Merck and purified as previously described (Singleton et al., 1965). L-α-Phosphatidylinositol (PI), L-α-phosphatidylinositol disphosphate (PIP_2), L-α-phosphatidylserine (Ps) and cholesterol (Chol) were from Sigma. Gangliosides (Gan) were supplied by Supelco.

The purity of the lipids was greater than 98% as estimated by thin-layer chromatography (Alsina et al., 1989). Sodium phosphate was from Merck. Gentamicin sulphate (Antibioticos S.A. Spain), kanamycin (Meijis K. Ltd, Japan), spectinomycin, streptomycin and colistin (Dumex, Denmark), were kindly supplied by Infavet (Spain). Their purity was checked by HPLC on reverse phase and pre-column OPA derivatization. Standard solution (10^{-4} M) of each of the above were used for the interaction studies.

The water used in monolayer studies was distilled over permanganate in an all-glass aparatus. Chloroform and ethanol (Merck) were of quality grade pro-analysi.

1-Anilino-8-naphthalenesulphonic acid (ANS) was from Sigma; crude carboxyfluorescein (Eastman Kodak) was purified by column chromatography on silica gel eluting with distilled water (30 gas silica/1 g CF). The CF concentration of the samples used, as determined by UV spectroscopy, was 60 mM.

Preparation of liposomes

Dehydration-rehydration vesicles (DRV) were prepared according to the description given by Kirby and Gregoriadis (1986). Non-entrapped CF was removed by centrifugation at $19000 \times g$, followed by column chromatography on Sephadex G-50.

The CF content was quantified by measurement of the absorbance at 490 nm of a diluted fraction ($\times 10^4$) of the vesicle preparation in the presence of 1% Triton X-100. The concentration of phospholipids in the liposomes was 5 mg/ml.

Liposomes were prepared from lipid mixtures of the following compositions: PC/Chol (1:1); PC/Chol/PS (1:2:1); PC/Chol/PI (1:2:1); PC/Chol/Gan (1:2:1); PC/Chol/PIP_2 (1:2:1).

Permeability studies

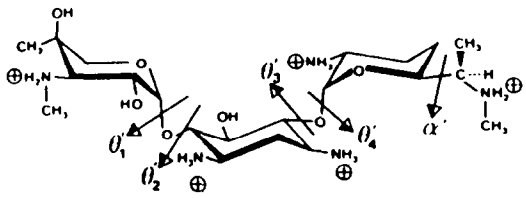
The liposomes were incubated with antibiotic solutions as follows: to 1.3 ml of PBS, 100 μl of 5×10^{-4} M drug solutions and 5 μl of liposomes were added.

After 30 min of incubation at 20 ± 1 or 37 ± 1°C, 10 μl of the mixture was taken and diluted with 2.5 ml of PBS. The fluorescence of this solution was measured before and after addition of 50 μl of 10% Triton X-100 ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm).

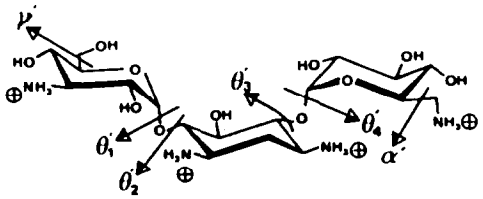
As reference, the same phospholipid mixtures with PBS instead of drug were prepared.

Fluorescence measurements were carried out using a Perkin-Elmer Model LS 50 spectrofluorimeter (widths of excitation and emission slits = 7 nm). All experiments were reformed in triplicate.

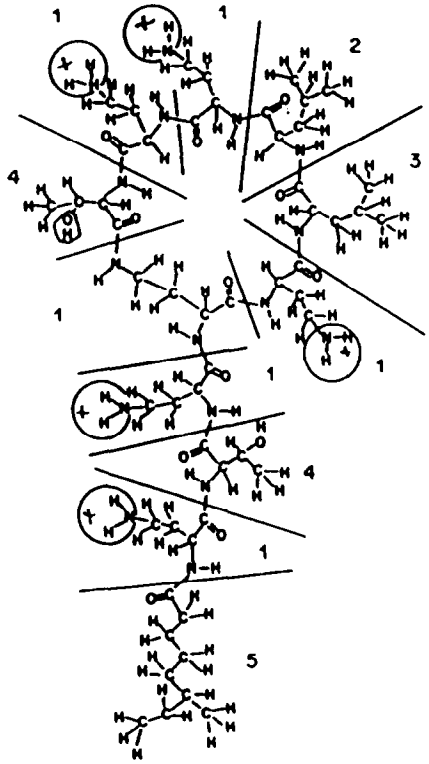
For incubations carried out at 37°C a thermostated cuvette holder connected with a Techne bath was used.



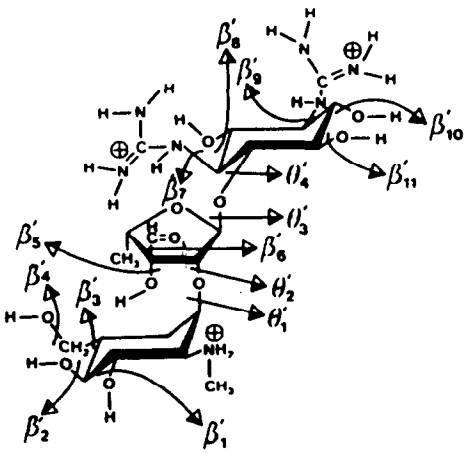
GENTAMICIN



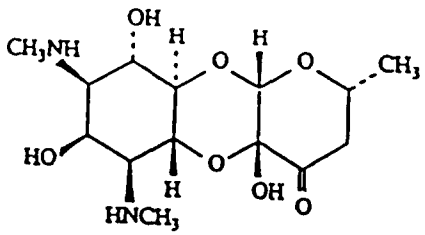
KANAMYCIN



COLISTIN



STREPTOMYCIN



SPECTINOMYCIN

Scheme 1. Molecular structures of antibiotics.

Microviscosity studies

Aliquots of liposomes of different composition were incubated with various concentrations of ANS for 60 min, in order to achieve the saturation level of this marker. Fluorescence was measured at $\lambda_{ex} = 380$ nm and $\lambda_{em} = 480$ nm, the slit width being 10 nm. These values were corrected for the individual fluorescence of the liposomes.

Once the phospholipid/ANS relationship had been selected, the same incubation process (30 min) was repeated but with different concentrations of the antibiotics in the medium. The values assayed were PL/antibiotic: 500 : 1; 250 : 1; 125 : 1; 75 : 1. Fluorescence polarization was determined with the equipment reported previously.

Monolayer studies

Measurements at constant surface area. A mini Teflon trough, cylindrical in shape, with capacity of 50 ml was used. Throughout this study, aqueous subphase was a PBS (pH 7.4, conductivity 15.4 mS cm^{-1}, 313.35 mosm/kg).

The film-forming lipid mixtures and drug solutions had the same compositions and concentrations as those used in liposome-CF studies.

Lipid solutions were spread in chloroform to the required initial surface pressure (5 or 20 mN/m).

Drug 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, with a platinum plate suspended from a Sartorius micro-balance. Surface pressure changes could be determined in the range ± 0.1 mN/m. After spreading the lipid solution, the film was allowed to stabilize for 10 min before injecting the drug 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. The same experiments were carried out without monolayer to ascertain the lack of surface activity of the drug molecules.

Compression isotherms. The compression isotherms were measured on a Langmuir film balance equipped with a Wilhelmy platinum plate, as described by Verger and De Haas (1973). The output of the pressure pick-up (Sartorius microbalance) was calibrated by recording the well-known isotherm of stearic acid. This isotherm is characterised by a sharp phase transition at 25 mN/m for 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 on double-distilled water using a microsyringe, and at least 10 min 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. All the isotherms were run at least three times in the direction of increasing pressure with freshly prepared film. The accuracy of the system under the conditions in which the bulk of the reported measurements were made was ± 0.5 mN/m for surface pressure.

Results and Discussion

Carboxyfluorescein leakage

Yields in CF entrapment for the different lipid compositions were PC/Chol, 12%; PC/PS/Chol, 6%; PC/PI/Chol, 8%; PC/PIP_2/Chol, 3%; and PC/Gan/Chol, 0.4%. The concentration of CF inside the liposomes was 60 mM.

Gentamicin, streptomycin, spectinomycin, kanamycin and colistin (Scheme 1) were incubated with liposomes composed of different lipids and containing entrapped carboxyfluorescein.

Carboxyfluorescein leakage was expressed as a percentage of the total dye entrapped:

$$\text{dye release: } \%\ \text{CF} = \frac{F - F_0}{F_t - F_0} \cdot 100$$

where F is the fluorescence intensity measured at

a specified time, F_0 at zero time and F_t the total fluorescence after Triton treatment.

The concentration of Triton, time of incubation and relative amounts of drug and phospholipids were determined after selecting the best conditions. Moreover, lipid mixtures containing 50% of cholesterol were chosen in order to avoid marker release due to liposome instability.

Although in the literature (Au et al., 1987) a similar experiment was carried out with an 8 h incubation period, in the present paper incubation times of 30 min were preferred on the assumption that an interaction, if existent, should be readily detected from the outset.

As a reference, liposomes were always incubated under the same conditions with PBS and the latency was used to correct the basal CF leakage.

The concentration of drug in the incubation media was 3.5×10^{-5} M, this level being slightly above those achieved under clinical conditions. The PL/drug ratio was 1:1.

CF leakage measured in triplicate after 30 min of liposome-antibiotic incubation was zero for gentamicin, kanamycin, spectinomycin and streptomycin. By contrast, colistin when incubated under the same conditions promoted the leakage of CF from the liposomes. The values were slightly dependent on the lipid composition of the liposomes as illustrated in Fig. 1.

The influence of the PL/drug ratio on the carboxyfluorescein release was determined by in-

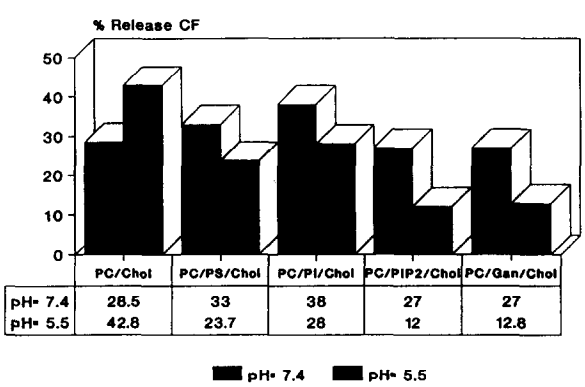


Fig. 1. Release of carboxyfluorescein from liposomes after 30 min treatment with colistin.

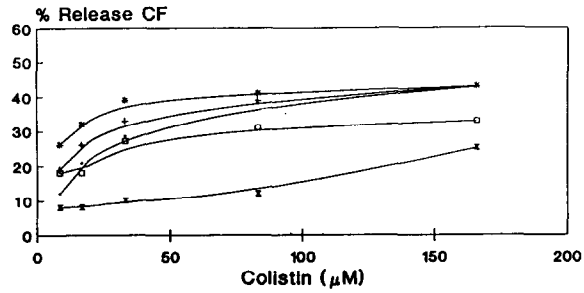


Fig. 2. Effect of colistin concentration on CF leakage from liposomes of different lipid composition (pH 7.4). (●——●) PC/Chol, (+ —— +) PC/PS/Chol, (⋆—— ⋆) PC/PI/Chol, (□—— □) PC/PIP_2/Chol, (×—— ×) PC/Gan/Chol.

cubating the same volume of liposomes with increasing amounts of drug solutions.

The results show that for PL/drug 1:10 no latency decreases could be detected (except for colistin) at any liposomal composition. Moreover, in the case of colistin, fluorescence increases were not linear, showing in constrast a certain tendency to saturation except for PC/Gan/Chol (Fig. 2).

The interaction between acidic phospholipids and these types of antibiotics proceeds very slowly, according to some experiments described in the literature, and for this reason another set of experiments was carried out using longer incubation times (until 20 h) for the liposome-drug mixtures.

As observed before, aminoglycosides do not interact at all with liposomes as far as CF leakage is concerned. We were able to defect a time-dependent leakage only for colistin. These results show that aminoglycoside-phospholipid interactions are not manifested under the conditions employed in our experiments when using the leakage of carboxyfluorescein as a reference parameter.

Nevertheless, trying to better simulate the in vivo behaviour, the same type of experiments were carried out at 37°C. Again in these experiments none of the aminoglycoside molecules produced leakage of the CF after 30 min incubation at this temperature, except colistin. In this case there was a minor increase in leakage of CF, as

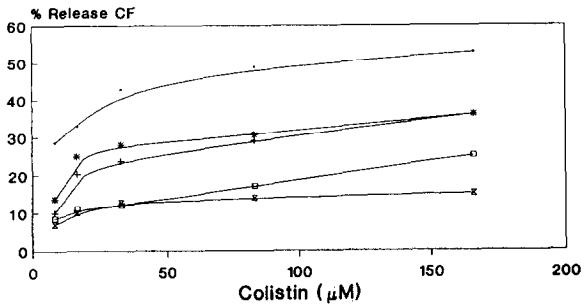


Fig. 3. Effect of colistin concentration on CF leakage from liposomes of different lipid composition (pH 5.5). (●——●) PC/Chol, (+ —— +) PC/PS/Chol, (⋆—— ⋆) PC/PI/Chol, (□ —— □) PC/PIP_2/Chol, (× —— ×) PC/Gan/Chol.

compared to the results obtained working at room temperature.

Due to the ionic character of these molecules the incubations were also performed at pH 5.5. No changes could be detected for aminoglycosides, however, colistin induced CF leakage in a dose-dependent way (Fig. 3). Surprisingly at this acidic pH, neutral liposomes showed the strongest interaction.

This different behaviour can be explained on the basis of the chemical structure of these molecules. At this pH value, aminoglycosides exist as highly hydrophilic cations, and will interact electrostatically with the polar heads of phospholipids. This interaction is probably stronger when the lipids are anionic. Nevertheless, the stability of bilayers is not compromised because the aminoglycosides cannot penetrate into the bilayers. For this reason no release of CF is detected.

The presence of a hydrophobic tail in the molecule of colistin confers to it an amphipathic character. These types of molecules are able to intercalate between the bilayer components creating pores or destabilizing the ordered structure. In this case, the entrapped molecule can leak out from the liposomes. Moreover, the lack of significant differences between acidic and neutral liposomes, or acidic and neutral pH suggests the predominance of hydrophobic interactions in this process.

Another apparently inconsistent aspect is that colistin interacts better with weakly acidic phospholipids (PS, PI) than with the strongest ones (PIP_2, gangliosides). A possible explanation for this fact is that the above lipids are not completely ionized at neutral or acidic pH due to the high superficial charge density (Wang et al., 1984); in that sense, other authors have already demonstrated the existence of hydrogen bonding among the hydroxy groups of PIP_2 (Boggs, 1980). Although this phenomenon has not been described for gangliosides one can assume that, due to the similarity in the polar groups of both types of lipids, this hypothesis can be also valid for them. Thus, the presence of polar groups showing strong intermolecular interactions would act as a barrier for colistin molecules to penetrate into more hydrophobic zones of the bilayer, thereby stabilizing the liposome.

On the other hand, the most extensive leakage of carboxyfluorescein, detected at pH 7.4 with reference to pH 5.5, can be explained by differences in the protonation of the colistin molecule at both pH values. Colistin at neutral pH will have its amino groups less extensively ionized than at acid pH, since the whole molecule is slightly more hydrophobic in the first case.

All these results show that the aminoglycoside-phospholipid interaction, if it exists, should be very weak and proceed very slowly, with the result that this behaviour is not likely to correlate with physiological events.

Moreover, the physiochemical behaviour of these molecules is consistent with the main characteristics of both types of antibiotics, since it is well known that polymixins mainly disturb the cellular membranes whereas aminoglycosides require active transport to cross them.

Surface studies

To better ascertain the existence and type of antibiotics/lipids interactions a parallel set of experiments was carried out by using monomolecular layers of lipids. This model of membrane has been used frequently to detect drug/lipid interactions and in some cases can be more sensitive than liposomes by themselves. (Davis and Jones, 1986; Alsina et al., 1990, 1991).

The surface activity of the aminoglycosides molecules was checked at first (without mono-

layer) by their injection into the subphase at different concentrations. No pressure changes were detected for subphase concentrations 100-fold greater than the standard levels. Colistin, in constrast showed superficial activity in accordance with its more hydrophobic character.

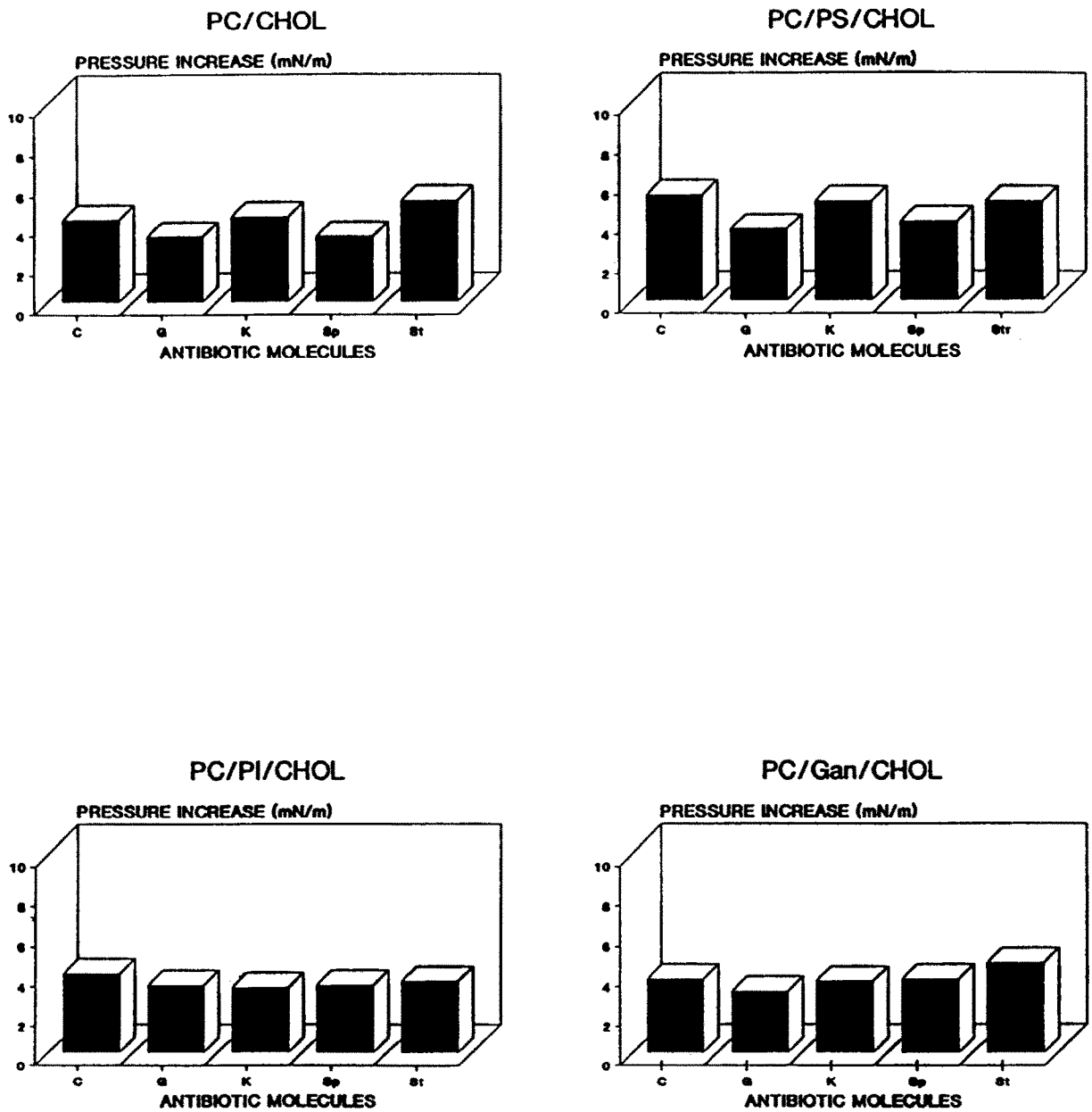


Fig. 4. Pressure increases measured after 30 min of drug injection in the subphase.

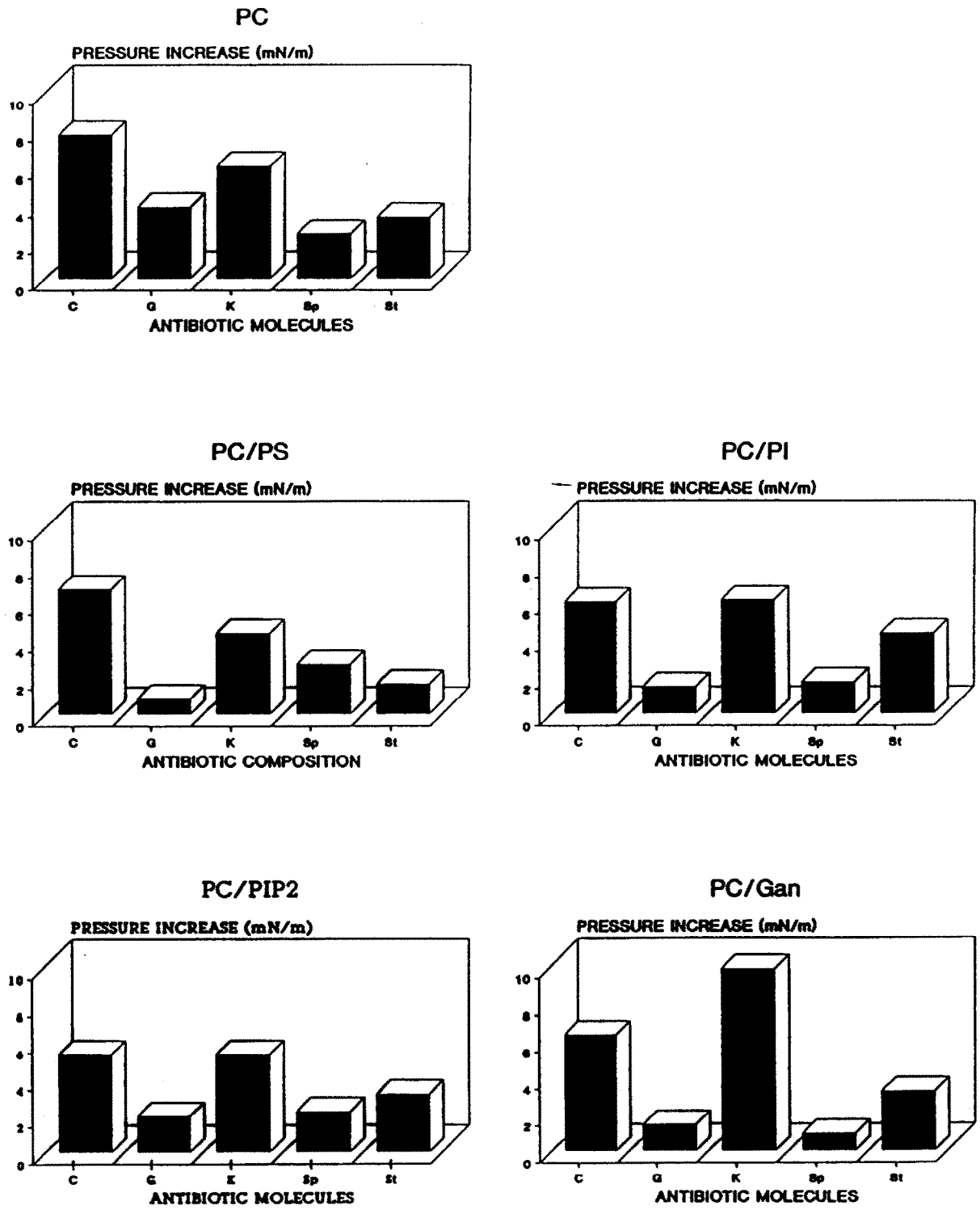


Fig. 5. Pressure increases measured after 30 min of drug injection in the subphase.

To reproduce the experiments carried out with bilayers, the same mixtures of lipids as used to prepare the liposomes were dissolved in chloroform and spread on aqueous subphases (PBS), at different surface pressures.

After injecting the antibiotics into the subphase the pressure increases were recorded. A series of preliminary experiments assaying different initial surface pressures of the monolayers were carried out and due to the low interaction level detected, the initial pressure of the monolayers of lipids in all experiments being 5 mN/m.

The pressure increases achieved after 30 min of drug injection were recorded and are plotted in Fig. 4. In these experiments the PL/ drug ratio was 1:5. The concentration of drug in the subphase was always 2.5×10^{-7} M.

It is evident that the $\Delta\Pi$ values are low and comparable for all the molecules. Moreover, no marked differences associated with the different lipids used could be detected. Gentamicin is the drug that generally shows, less extensive interaction with the lipids.

The influence of drug concentration on the penetration process was also studied in two ways: injecting the drug into the subphase in an additive, continuous manner described by Reig et al. (1988) or injecting, in separate experiments, drug solutions of different concentrations.

The results were the same and confirmed the low interaction level of these molecules with the phospholipids, thus suggesting that the extent of interaction reaches saturation, especially when one takes into account that there was a molar excess of drug over phospholipid in the system.

On consideration of the literature data, the small pressure increases detected in our experiments could be due to the presence in the lipid mixtures of 50% cholesterol. Cholesterol is well known to stabilize mono- and bilayers, decreasing the fluidity of the system, for this reason a new set of experiments was carried out working with monolayer lacking cholesterol.

The results were similar to those above but in this case differences in pressure increases were evident among the different molecules and phospholipids assayed.

The values recorded after 30 min of interaction are depicted in Fig. 5. Moreover in this second set of experiments, a new monolayer composition containing PIP_2 was included.

Colistin and kanamycin resulted in the largest increases in pressure. However, no specific interactions could be clearly detected with any phospholipid except for kanamycin with gangliosides.

The pressure increases measured using this membrane model are, in fact, representative of the organization of the phospholipids in the monolayer. This effect can be the result of either the penetration of a hydrophobic molecule or the insertion of a polar molecule between the polar heads of the lipids forcing them apart. Colistin can behave as in the first case and the aminoglycosides as in the latter.

In fact, Mingeot-Leclercq et al. (1989) have shown that binding of gentamicin to a membrane requires negative charges and that the interaction takes place in the hydrophilic domain. This causes a significant restriction in the movement of the phosphate heads of the phospholipids.

Such ionic interactions have been suggested by Brasseur et al. (1985), who by means of theoretical calculations, showed that gentamicin and streptomycin adapt in different ways between the polar heads of PI depending mainly on its geometry and not on the number of amino groups. This model would explain the differences found in the monolayers without cholesterol.

In a previous paper (Alsina et al., 1991) we have shown that PC/PS/Chol monolayers do not exhibit ideal miscibility behaviour, and mixed monolayers are more condensed than those of the pure components. Moreover, the influence of cholesterol appears to be related to a dilution effect that, by isolating the ionic groups of the phospholipids, diminishes the repulsive forces among them acting as a buffer.

On the basis of the above assumptions, the interactions among phospholipids and antibiotics molecules would be more freely manifested in the absence of cholesterol, as occurred in the present experiments.

The discrepancy between the results obtained in the present paper and literature data can be due to the different pH values (Brasseur et al., 1984), different buffer (Aramaki and Tsuchiya,

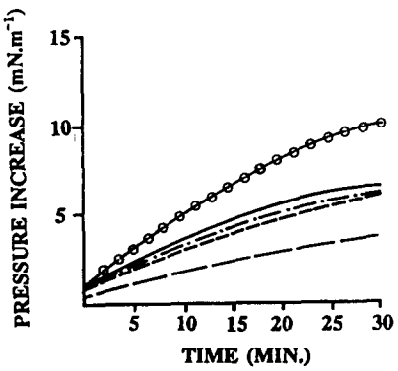


Fig. 6. Pressure increases achieved after 30 min of kanamycin injection under monolayers of different composition. (———) PC, (— — —) PC/PS, (-·-) PC/PI, (— — —) PC/PIP_2, (○———○) PC/Gan.

1989, Forge et al., 1989) and different membrane models. These factors strongly influence the interaction of organic molecules with phospholipids (Alsina et al., 1990).

Fig. 6 shows the time course of such pressure increases for kanamicin in several monolayers. The penetration kinetics is typical for a highly hydrophilic molecule, in the sense that the pressure increases very slowly and, apparently, saturation occurs after a long period of time.

Although colistin has surface activity itself, the pressure increases measured for the same molecule in the presence of monolayers are similar to those found for aminoglycosides that lack any surface activity.

This behaviour is not anomalous, since, in general, the presence of a monolayer inhibits the activity of hydrophobic molecules and increases the apparent surface activity of those that are hydrophilic (Reig et al., 1992).

In the search for specific differences in the interaction between antibiotics and lipids, a mathematical study of the penetration kinetics was performed.

From a thermodynamic viewpoint, the interaction between drug molecules and monomolecular layers of phospholipids can be likened with the process of drug–receptor interaction in binding assays. Following this assumption, the affinity of the molecules under study to phospholipids could fit a Lineweaver-Burk or exponential equation. To verify this hypothesis two mathematical expressions were applied:

$$\frac{1}{P} = \frac{K}{P_m} \cdot \frac{1}{t} + \frac{1}{P_m} \quad (1)$$

$$P = axe^{bt}$$

$$\ln P = \ln a + bt \quad (2)$$

The regression coefficients were higher than 0.98 with both equations but slighly better for Eqn 1. Moreover, the results were not dependent on the presence of cholesterol in the monolayer. P_m represents the maximum pressure achieved and K corresponds to the time taken to achieve $P_m/2$.

The calculated values of K and P_m are listed in Table 1 and 2. Here, a strong dependence on monolayer composition and antibiotic can be observed, but no specific relationships can be determined.

Compression isotherms

To complement the above-described studies with phospholipid monolayers, a new set of experiments was designed. In this case the monomolecular layers of phospholipids were spread on PBS subphases, containing aminoglycoside solutions (10^{-4} M), and compressed at 4.2 cm/min. The lipid monolayers were composed of PC/PS, PC/PI, PC/Gan and pure PC. Choles-

TABLE 1

Maximal pressures increases (mN/m) calculated according to the Lineweaver-Burk equation

Lipid composition	P_m				
	C	G	K	SP	ST
PC/Chol	7.0	5.57	13.8	16.8	8.59
PC/PS/Chol	9.48	7.28	40.0	10.4	10.0
PC/PI/Chol	10.04	4.91	6.70	10.0	6.08
PC/Gan/Chol	6.52	4.16	5.13	8.76	8.13
PC	13.37	4.73	11.89	4.69	7.56
PC/PS	10.98	1.06	8.45	4.48	10.0
PC/PI	11.90	2.88	9.88	2.84	17.4
PC/PI/Gan	10.5	1.66	16.6	2.68	4.03
PC/PIP_2	16.3	2.79	13.3	3.18	13.9

TABLE 2

K values (min) calculated according to the Lineweaver-Burk equation

Lipid composition	K				
	C	G	K	SP	ST
PC/Chol	17.9	22.9	67.7	93.2	20.9
PC/PS/Chol	27.9	29.3	98.0	49.5	33.4
PC/PI/Chol	54.2	15.0	29.3	46.7	29.5
PC/Gan/Chol	18.5	9.8	22.5	41.3	23.1
PC	23.39	7.41	25.7	26.7	34.3
PC/PS	18.1	6.82	39.6	19.0	97.8
PC/PI	37.0	24.0	21.0	24.4	84.9
PC/Gan	22.7	4.97	23.9	51.0	9.37
PC/PIP_2	53.5	11.6	32.2	11.8	94.9

terol was not included in the composition as a consequence of the above results obtained in the penetration experiments.

No differences in the compressibility and collapse pressures could be detected among the four monolayers when compressed on PBS. Moreover, the presence of aminoglycosides in the subphase has no effect on the above-described parameters irrespective of the composition of the lipid monolayer.

Only colistin had a clear expanding effect on the monolayers. This behaviour is illustrated in Fig. 7.

Nevertheless, the collapse pressure was the same as for aminoglycosides (around 50 mN/m).

Measurements of microviscosity

Although aminoglycosides were unable to induce leakage of CF from inside the liposomes, the results of monolayer studies showed the existence of weak interactions between lipids and antibiotics.

Asuming that these interactions are most probably electrostatic in nature, a final set of experiments was carried out with ANS. This marker exhibits sensitivity to membrane potential and surface charge and is a useful tool to study hydrophilic interactions.

All the following experiments were performed using liposomes without cholesterol, since the presence of this component markedly reduces the fluorescence quantum yield of the preparations.

The optimal ratio of ANS to lipid was determined beforehead for each liposomal composition and found to be 1:545 (PC), 1:365 (PC/PS) and 1:135 (PC/PI and $PC/PI/PIP_2$). These differences are due to the different polar heads of the lipids forming the liposomes (Slavik, 1982). Liposomes of all lipid compositions under study showed varying degrees of reactivity towards the aminoglycosides.

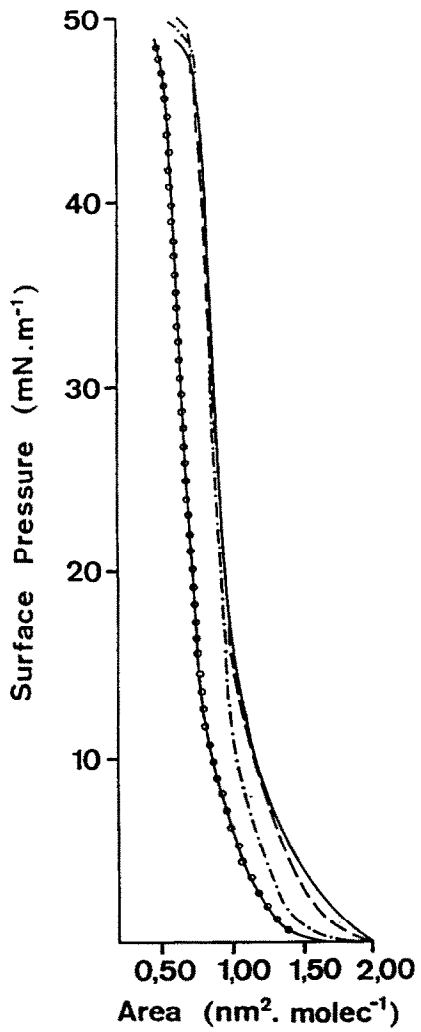


Fig. 7. Compression isotherms of PC, PC/PS, PC/PI and PC/Gan monolayers spread on a subphase containing 10^{-4} M colistin in PBS (pH 7.4). PC (———); PC/PS (— — —); PC/PI (– · –); PC/Gan (○———○).

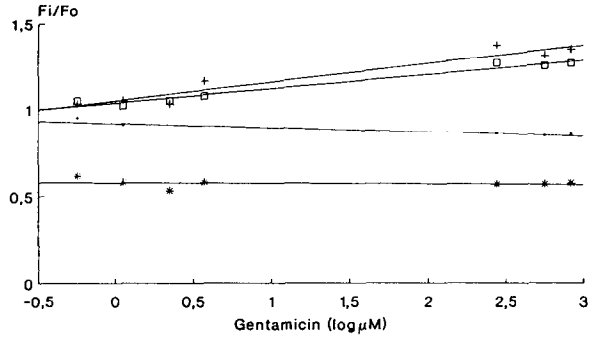


Fig. 8. Relative fluorescence intensity of liposomes incubated with ANS and gentamicin (●——●), (+——+) PC/PS, (★——★) PC/PI, (□——□) PC/PI/PIP_2.

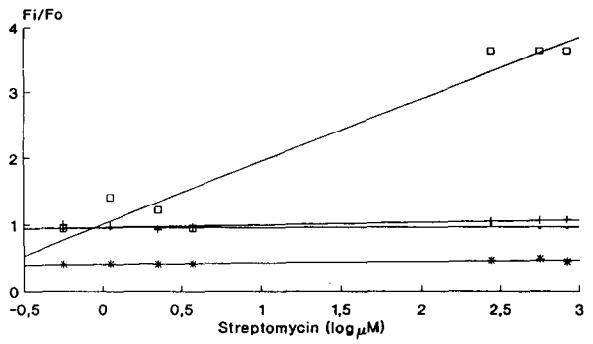


Fig. 10. Relative fluorescence intensity of liposomes incubated with ANS and streptomycin (●——●), (+——+) PC/PS, (★——★) PC/PI, (□——□) PC/PI/PIP_2.

Concentrations of antibiotics lower than 10^{-5} M had very little effect on the fluorescence of ANS in liposomes. However, for concentrations 100-fold higher and acidic phospholipids the lipid-aminoglycoside interactions were clearly manifested, as demonstrated by Figs 8–11, where F_i denotes the fluorescence intensity in the presence of drug and F_0 is the corresponding measurement in its absence.

The main common trend in Figs 8–11 is that when liposomes contain PI, the presence of aminoglycosides promotes a reduction in the fluorescence of the system.

In contrast liposomes composed of PS or PIP_2 display an increase in fluorescence after incubation with aminoglycosides. This point should be studied more carefully in the future.

Nevertheless, none of these molecules induced changes in the λ_{max} of the fluorescence spectra.

Polarization studies carried out under the same conditions as above showed that only liposomes containing PI exhibited an increase in the polarization index, thus suggesting that the presence of antibiotics induces rigidification of the bilayers. As an example, the values obtained for streptomycin are plotted in Fig. 12.

On consideration of the results obtained using the different techniques described above, it appears to be difficult to assign a specific interaction between any of these molecules and phospholipids. In contrast, it appears that the model used to measure interactions and the combination of several properties, for instance, geometry

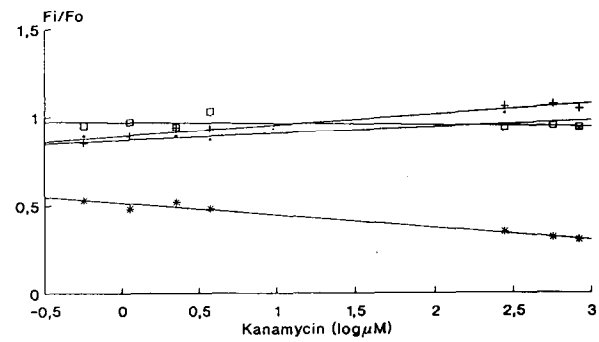


Fig. 9. Relative fluorescence intensity of liposomes incubated with ANS and kanamycin. (●——●), (+——+) PC/PS, (★——★) PC/PI, (□——□) PC/PI/PIP_2.

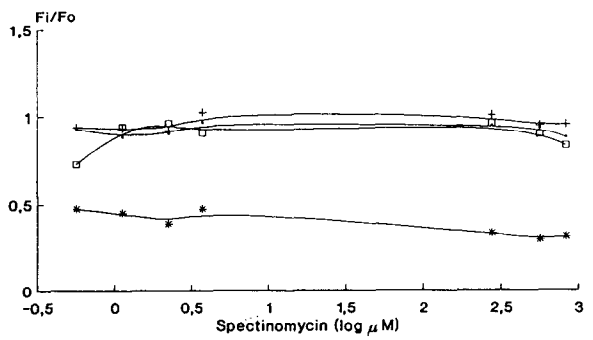


Fig. 11. Relative fluorescence intensity of liposomes incubated with ANS and spectinomycin. (●——●), (+——+) PC/PS, (★——★) PC/PI, (□——□) PC/PI/PIP_2.

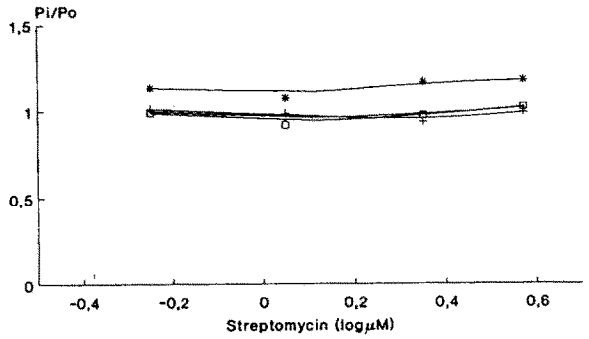


Fig. 12. Relative fluorescence polarization of ANS-liposomes as a function of the streptomycin content (●———●), (+———+) PC/PS, (★———★) PC/PI, (□———□) PC/PI/PIP_2.

of the molecule, ionic charge, basicity, are the main factors affecting the final results.

References

Alsina, M.A., García Antón, J.M., Busquets, M.A., Arnaiz, A. and Reig, F., Modification of phosphatidylcholine-opiates interaction by different buffers. *Colloid Polym. Sci.*, 268 (1990) 163–166.

Alsina, M.A., Mestres, C., García Antón, J.M., Espina, M., Haro, I. and Reig, F., The interaction energies of cholesterol, phosphatidylcholine in spread mixed monolayers at the air water interface. *Langmuir*, 7 (1991) 975–977.

Alsina, M.A., Mestres, C., Valencia, G., García Antón, J.M. and Reig, F., Interaction energies of mixing phosphatidylserine and phosphatidylcholine monolayers. *Colloids Surfaces*, 34 (1989) 151–158.

Aramaki, Y. and Tsuchiya, S., Interactions between Aminoglycosides and phospholipids using liposome: a possible mechanism of nephrotoxicity. *Pharm. Res.*, 6 (1989) 362–366.

Au, S., Weiner, N.D. and Schacht, J., Aminoglycoside antibiotics preferentially increase permeability in phosphoinositide-containing membranes: a study with carboxyfluorescein in liposomes. *Biochim. Biophys. Acta*, 902 (1987) 80–86.

Boggs, J.M., Intermolecular hydrogen bonding between lipids: influence on organization and function of lipids in membranes. *Can. J. Biochem.*, 58 (1980) 755.

Brasseur, R., Carlier, M.B., Laurent, G., Claes, P.J., Vanderhaegue, M.J., Tulkens, P.M. and Ruysschaert, J.M., Interaction of streptomycin and streptomycylamine derivatives with negatively charged lipid layers. *Biochem. Pharmacol.*, 34 (1985) 1035–1047.

Brasseur, R., Laurent, G., Ruysschaert, J.M. and Tulkens, P., Interactions of aminoglycoside antibiotics with negatively charged lipids layers. *Biochem. Pharmacol.*, 33 (1984) 629–637.

Davis, R.J. and Jones, M.N., The interaction of liposomes containing intrinsec erythrocyte membrane proteins with lipid monolayers at air/water and oil/water interfaces. *Biophys. Acta*, 858 (1986) 135–144.

Forge, A., Zajic, G., Davies, S., Weiner, N. and Schacht, J., Gentamicin alters membrane structure as shown by freeze-fracture of liposomes. *Hearing Res.*, 37 (1989) 129–140.

Kirby, C. and Gregoriadis, G., A simple procedure for preparing liposomes capable of high encapsulation efficiency under mild conditions. *Liposome Technol.*, 1 (1986) 19–27.

Mingeot-Leclercq, M.P., Schanck, A., Roveaux-Dupal, M.F., Deleers, M., Brasseur, R., Ruyschaert, J.M., Laurent, G. and Tulkens, P.M., Ultrastructural, physico-chemical and conformational study of the interactions of gentamicin and bis(beta-diethylaminoethilether)hexestrol with negatively-charged phospholipid layers. *Biochem. Pharmacol.*, 38 (1989) 729–741.

Reig, F., Busquets, M.A., García Antón, J.M., Valencia, G. and Alsina, M.A., Interaction of analgesics with lecithin and ganglioside monolayers. *Int. J. Pharm.*, 44 (1988) 257–260.

Reig, F., Busquets, M.A., Haro, I., Rabanal, F. and Alsina, M.A., Interaction of opiate molecules with lipid monolayers and liposomes. *J. Pharm. Sci.*, 81 (1992) 1–5.

Singleton, W.S., Gray, M.S., Brows, M.L. and White, J.L., Chromatographically homogeneous lecithin from egg phospholipids. *J. Am. Oil Chem. Soc.*, 42 (1965) 53–56.

Slavik, J., Anilinonaphthalene sulfonate as a probe of membrane composition and function. *Biochim. Biophys. Acta*, 694 (1982) 1–25.

Verger, R. and De Haas, G.H., Enzyme reactions in a membrane model. *Chem. Phys. Lipids*, 10 (1973) 127–135.

Wang, B.M., Weiner, N.D., Ganesan, M.G. and Schacht, J., Interaction of calcium and neomycin with anionic phospholipid-lecithin liposomes. *Biochem. Pharmacol.*, 33 (1984) 3787–3791.