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POLYMYXIN B-PHOSPHATIDYLGLYCEROL INTERACTIONS

A MONOLAYER (π, ΔV) STUDY

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Summary

Through a monolayer investigation (π, ΔV), it is shown that the cationic antibiotic polymyxin B (or E) strongly interacts with films of acidic lipids, namely the didodecanoyl- and dihexadecanoylphosphatidylglycerol. The zwitterionic dihexadecanoylphosphatidylcholine was an unsuitable substrate. Interactions occurred at and above a polymyxin B concentration in the subphase of $2.5 \cdot 10^{-7}$ M, bringing about a considerable increase of both π and ΔV. These interactions proceeded in two steps, as revealed by a biphasic change of ΔV with time. They were independent of the film molecular packing (fluid or gel states) and of the initial film pressure.

Since it was possible to monitor the relative number of polymyxin B and didodecanoyl- or dihexadecanoylphosphatidylglycerol molecules in the monolayer, it is demonstrated that, at saturation, one polymyxin B molecule is bound to five phosphatidylglycerol molecules, a result which accounts for an exact neutralization of the charges.

From competition experiments, it is shown that Na^+ is ineffective in removing polymyxin B from the interface. Ca^{2+} appeared to be a stronger competitor but no complete antibiotic desorption was observed even at a Ca^{2+} concentration of 100 mM.

As a working hypothesis, the antibiotic/lipid (1/5) system was assumed to constitute by itself one molecular species. The mixing of the polymyxin B/didodecanoylphosphatidylglycerol (1/5) system with an excess of lipid molecules in the monolayer was found to be ideal both in terms of π and ΔV. With dihexadecanoylphosphatidylglycerol, a small condensing effect could be

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detected only at intermediate surface pressures, in a region where the lipid phase transition occurred.

The molecular area of polymyxin B interacting with didodecanoylphosphatidylglycerol can be calculated to be 1.23 ± 0.05 nm^2. It is proposed that the whole antibiotic molecule penetrates the film, the five bound lipid molecules being distributed around the peptide structure, at given positions imposed by the five 2,4-diaminobutyric acid residues.

Introduction

Polymyxin is a generic name for a group of chemically related substances isolated from various strains of *Bacillus polymyxa* [1—3]. These compounds are broad-spectrum antibiotics exhibiting activity against Gram-negative bacteria, Gram-positive bacteria, yeasts and protozoa [2,3]. Four polymyxins have been described (polymyxin A, B, D and E) each one consisting of one fatty acid residue attached through an amide bond to a linear tripeptide linked to a heptapeptide ring. The presence of 5—6 2,4-diaminobutyric acid residues confers a net positive charge to these molecules. It is now well recognized that the primary site of action for these antibiotics is the bacterial membrane [2,3]. Most likely through interactions with acidic phospholipids, these peptido-lipids cause a rapid permeability change of the cytoplasmic membrane, resulting in a release of cellular materials [2,3]. Such an effect could be related to a change in the membrane fluidity [4]. As yet, at the molecular level, little is known of the way these molecules interact with lipids and deorganize the membrane assembly. The present investigation was stimulated by the recent review from Storm et al. [3] as well as by our previous interest in the effect of ions [5—9] and amphipathic charged molecules [10] on the phase properties of charged phospholipids.

Moreover, as already emphasized by Hartmann et al. [11], polymyxins can be regarded as very simple models of proteins with amphiphilic properties, well suited for studying some basic aspects of lipid-protein interactions.

The present report deals with a monolayer study (π, ΔV) of the interactions between polymyxin B and phosphatidylglycerol, a widespread lipid in bacteria [12], which in addition has been previously shown to favour the binding of polymyxins to bacterial cells [13].

It is shown that the positively charged polymyxin B (five free amino groups) binds rapidly and strongly to the negatively charged phosphatidylglycerols, but not to the zwitterionic phosphatidylcholine. These interactions, which do not depend on the initial lipid molecular packing, result, at saturation, in the formation of a molecular pattern in which five phosphatidylglycerol molecules are bound to one polymyxin B molecule. Some properties of this system are described.

Materials and Methods

Materials. 1,2-Didodecanoyl-*sn*-glycero-3-phosphoryl-1′-*sn*-glycerol sodium salt and *rac*-1,2-dihexadecanoylglycerol-3-phosphoryl-1′-*rac*-glycerol ammo-

nium salt were both of synthetic origin [9,14]. *rac*-1,2-Dihexadecanoylglycerol-3-phosphorylcholine was obtained from Sigma (U.S.A.). All these compounds were checked for purity by thin-layer chromatography.

Polymyxin B (sulphate) was purchased from Sigma (U.S.A.) Polymyxin E (sulphate) was a generous gift from Laboratories Roger Belon (France).

Methods. The surface potential was measured with an apparatus using two americium electrodes, the principle of which has been already described [7]. Film pressure was measured using a floating barrier (paraffin-coated mica) connected to a torsion balance of our fabrication allowing continuous recording of the film surface pressure. Experimental conditions were identical to those described in a previous report [6]. Ultrapure water from an industrial source (Motorola, Toulouse) was used. The lipids were spread in the form of $CHCl_3/CH_3OH$ (5/1, v/v) solutions. A more polar solvant, $CHCl_3/CH_3OH$ (1/1), was required for dissolving the polymyxins. In this case, good spreading was obtained by depositing small droplets (about 1 μl) of the solutions on the water surface.

Throughout all experiments, reference surface potentials of aqueous subphases lay around 17—20 mV. Film compressions were reproducible within 1% ($\pm 5 \cdot 10^{-3}$ nm^2) whereas reproducibility of ΔV determination was ±10 mV. The data presented here are the average of 2—3 experiments, carried out at a constant temperature of 20° C.

Results

Interactions between polymyxin B and phosphatidylglycerols

A first set of experiments was performed at a constant molecular area; polymyxin B was added to the subphase at a given concentration under preformed films of didodecanoylphosphatidylglycerol, the changes in π and ΔV then being recorded with time. No interaction between polymyxin B and films of this lipid could be detected for antibiotic concentrations lower than $2.5 \cdot 10^{-7}$ M. At and above this concentration, a strong interaction with the lipid was revealed by large increases in both film surface pressure and surface potential. This interaction proceeded in two steps, as clearly shown in Fig. 1, where π and ΔV changes are plotted against time, for a film of didodecanoylphosphatidylglycerol at the initial pressure of 5 mN $\cdot$ m^{-1} and for a polymyxin B concentration of $2.5 \cdot 10^{-7}$ M. A linear pressure increment with time of 17 mN $\cdot$ m^{-1} was observed within the first 33 min, accompanied by a non-linear ΔV increase of 190 mV (point I), above which a new ΔV jump of 100 mV was detected, for a very small $\Delta\pi$ increase of 1.5 mN $\cdot$ m^{-1} only. The system reached its final equilibrium (point II) after a further 12 min.

Stirring the subphase for longer periods or adding more polymyxin B to the subphase only accelerated the process, without modifying its biphasic character. A linear relationship was observed between the concentration of the antibiotic in the subphase and the half-time necessary to reach the equilibrium (point II). Thereby, in agreement with Teuber and Miller [15], the interaction of polymyxin B with films of didodecanoylphosphatidylglycerol appeared to be partly diffusion controlled. This could explain why at very low antibiotic concentration (less than $2.5 \cdot 10^{-7}$ M) no interaction could be detected within

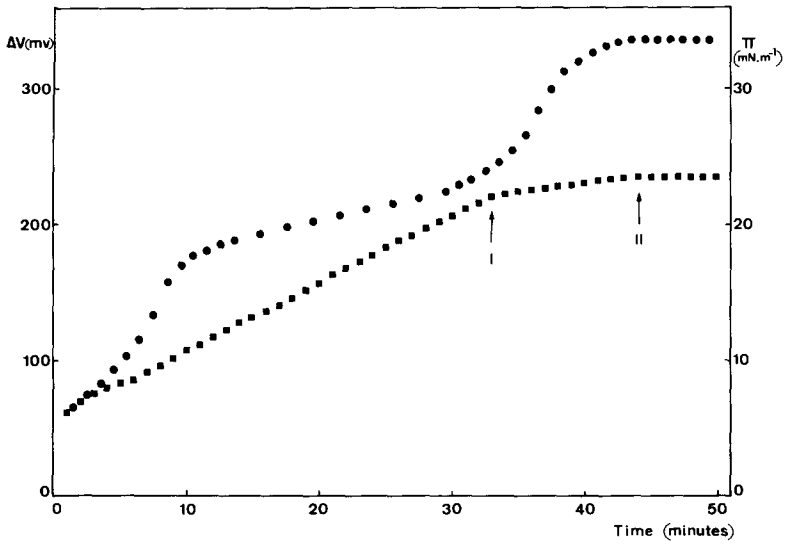

Fig. 1. Time-dependent interaction of polymyxin B with a film of didodecanoylphosphatidylglycerol. The lipid was spread on pure water (pH 6) and compressed to the initial film pressure of 5 mN · m^{-1}. Then polymyxin B was added to the subphase at a concentration of $2.5 \cdot 10^{-7}$ M. π and ΔV were recorded after stirring the subphase for 1 min.

the time of our experiments (1 h). Furthermore, the possibility that the antibiotic partly adsorbs on the Teflon trough cannot be excluded.

At this stage, it is to be noted that upon addition of polymyxin B to the subphase, the pH remained constant at 6, a value at which the molecule is known to be fully protonated [16]. Such a pH lies in the range 6—8 where polymyxins have been shown to exhibit a maximul activity [17,18]. Furthermore, polymyxins are known to be slightly surface active and any possible changes in the surface tension on the reference side of the surface balance should be noted. Nevertheless, increment in surface pressure amounted to only 0.5 mN · m^{-1} at the highest concentration of polymyxin B used in these experiments (10^{-6} M) and after 1 h. For that reason, no correction was applied to π measurements.

Results at equilibrium, for various initial film pressures and polymyxin B concentrations, are schematically presented in Fig. 2. As can be seen, above the concentration of $2.5 \cdot 10^{-7}$ M, the final state of the system was independent of the polymyxin B concentration in the subphase. It only depended on the initial film pressure. This appears to be in contrast with the S-shaped binding curve found by fluorescence for polymyxin B interacting with phosphatidic acid [11]. In any case, the surface potential at equilibrium reached a constant value of 340 mV. From these data, an apparent dissociation constant for polymyxin B interacting with phosphatidylglycerol in monolayers can be estimated to be less than $2.5 \cdot 10^{-7}$ M. This figure is an order of magnitude less than both the value of $2 \cdot 10^{-6}$ M recently reported for polymyxin B binding with vesicles of phosphatidylglycerol [4], and the minimum inhibitory concentration of 2 μg/ml ($1.56 \cdot 10^{-6}$ M) currently reported for this antibiotic [3].

Similar experiments were carried out using films of dipalmitoylphosphatidyl-

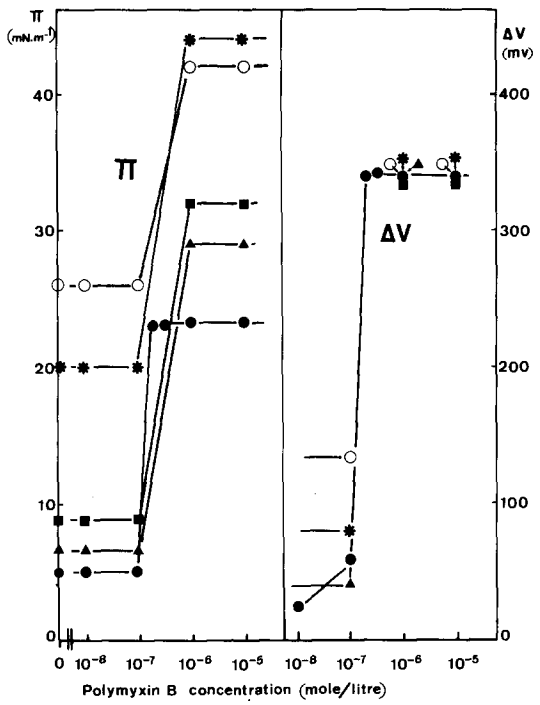

Fig. 2. Changes in surface pressure (left hand) and surface potential (right hand) on addition of polymyxin B below monolayers of didodecanoylphosphatodylglycerol. Increasing amounts of antibiotic were added below films of the lipid at 5 mN · m^{-1} (●), 6.5 mN · m^{-1} (▲), 9 mN · m^{-1} (■), 20 mN · m^{-1} (*) and 26 mN · m^{-1} (○) initial pressures. π and ΔV values presented are those obtained at equilibrium, as point II in Fig. 1.

choline as substrate. In this case, neither the surface pressure nor the surface potential varied, even with polymyxin B concentrations in the subphase as high as 10^{-5} M.

In a second set of experiments, interactions between polymyxin B and didodecanoylphosphatidylglycerol were studied under dynamic conditions, through continuous film compressions.

As previously reported, the lipid on pure water is likely to be protonated [6]. The corresponding compression isotherm, shown in Fig. 3 (curve A), is characteristic of a lipid in the 'liquid-expanded' state. Spreading the lipid on a 10^{-6} M polymyxin B subphase (a concentration which ensures a complete peptide-lipid interaction) and compressing the film (stepwise or continuous compression) resulted in the highly expanded isotherm B, still accounting for a lipid in the liquid-expanded phase. Film collapse occurred at a surface pressure of 43 mN · m^{-1}, well differentiated from the film collapse observed at 39 mN · m^{-1} for didodecanoylphosphatidylglycerol on pure water. The corresponding changes in surface potential are shown in the same figure (curve b).

Results presented in Figs. 2 and 3 are self-consistent and it is clear that regardless of the experimental conditions (static or dynamic), polymyxin B strongly interacts with films of didodecanoylphosphatidylglycerol to achieve, at saturation, a well-defined molecular organization at the interface.

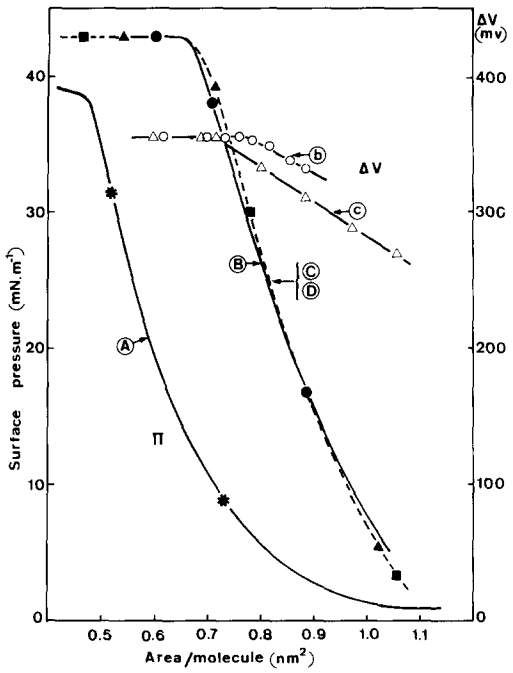

Fig. 3. Compression isotherms and corresponding surface potential for didodecanoylphosphatidylglycerol alone or interacting with polymyxin B. (A) Lipid alone on pure water (*); (B) Lipid on 10^{-6} M polymyxin B (●); (C) Polymyxin B/lipid (1/5) mixture on pure water (■); (D) Polymyxin B/lipid (1/5) mixture on 1 mM NaCl (▲); (b) ΔV changes for the lipid on 10^{-6} M polymyxin B (○); (c): ΔV changes for the polymyxin B/lipid (1/5) mixture on 1 mM NaCl (△). The pH of the subphases remained constant at 6. Curves were calibrated as if the lipid were alone. All these curves are recorder traces, symbols being used for identifying the curves.

Stoicheiometry of the polymyxin B-phosphatidylglycerol interactions

Experiments consisted of spreading various mixtures of phospholipid progressively enriched in polymyxin B, the corresponding compression isotherms being calibrated as if the lipid were alone. By this means, the normal compression curves of the lipid are to be expected if the added molecules do not interact with the lipid within the film but dissolve in the subphase. On the other hand, any interaction between the two species would result in film expansion. It is clear that such experiments are significant only in so far as the added molecules do not form a film by themselves. Polymyxins are only slightly surface active [19,20] and no film could be detected after spreading polymyxin B on pure water. On the other hand, spreading polymyxin B/didodecanoylphosphatidylglycerol mixtures progressively enriched in polymyxin B did result in more and more expanded films, until the 1/5 molar ratio was reached. The compression curve relative to this ratio is shown in Fig. 3 (curve C). The stabilization of the system in this ratio is clearly illustrated by Fig. 4, where changes in the molecular area calculated for didodecanoylphosphatidylglycerol, as well as changes in surface potential, are plotted versus the peptide/lipid molar ratio, at a constant surface pressure of 20 mN · m^{-1}. As can be seen, further addition of polymyxin B above the 1/5 ratio had no effect, neither on the film molecular packing nor on the surface potential. Such a result clearly demonstrates that

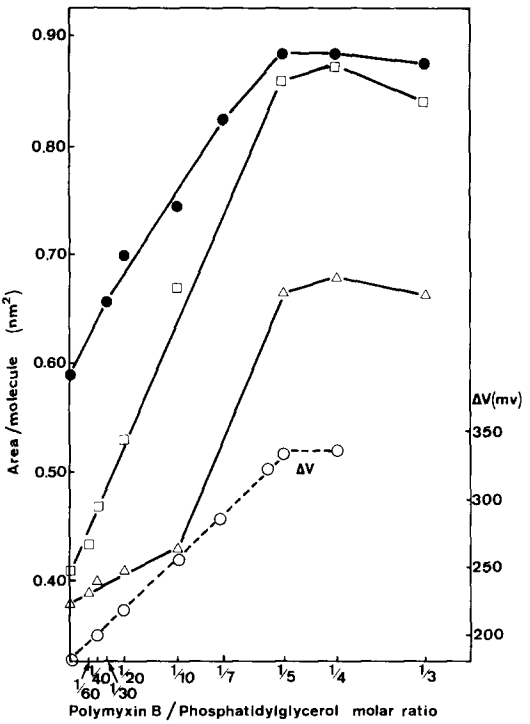

Fig. 4. Changes in molecular area (———) and surface potential (- - - - -) resulting from the addition of polymyxin B to phosphatidylglycerol in the monolayer, plotted as a function of the antibiotic/lipid molar ratio. Antibiotic/lipid mixtures were spread and compressed on pure water (pH 6). From the corresponding compression isotherms (calibrated as if the lipid were alone) the lipid molecular area were calculated at constant surface pressures of $\pi = 20$ mN · m^{-1} (●———●) for didodecanoylphosphatidylglycerol and $\pi = 8$ (△———△) and 25 mN · m^{-1} (□———□) for dihexadecanoylphosphatidylglycerol. ΔV values refer to films of polymyxin B/didodecanoylphosphatidylglycerol mixtures spread on a 1 mM NaCl subphase (pH 6) and compressed at a surface pressure of $\pi = 20$ mN · m^{-1} (○- - - - -○).

above this ratio, corresponding to an exact charge neutralization between the peptide and the lipid molecules, the excess antibiotic dissolves into the subphase.

Keeping this in mind, it is to be noted that curves B and C in Fig. 3 coincide almost perfectly: curve B was recorded after spreading the didodecanoylphosphatidylglycerol alone on a 10^{-6} M polymyxin B subphase; curve C was obtained after spreading the polymyxin B/didodecanoylphosphatidylglycerol (1/5) mixture on pure water. In terms of surface potential, it should be remembered that ΔV cannot be measured accurately on pure water [7,10]. Anticipating on the next section, addition of NaCl at a 1 mM concentration does not perturb the interactions between the antibiotic and the lipid but allows a good ΔV recording. Spreading the polymyxin B/didodecanoylphosphatidylglycerol (1/5) mixture on 1 mM NaCl (pH 6) resulted in the compression isotherm D, identical to curve C obtained on pure water. The corresponding ΔV values (curve d) were very similar to those recorded for films of the same lipid spread on 10^{-6} M polymyxin B (curve b). Altogether, it is clear that polymyxin B strongly interacts with films of didodecanoylphosphatidylglycerol to achieve, at saturation, an intermolecular association with the lipid in the 1—5 molar ratio.

The peptide-lipid interactions still took place at high pressure in the region where the film collapsed. The same curves were obtained with or without ions in the subphase. Any desorption of polymyxin B would likely bring about considerable changes in the surface potential. This is not the case. All along the plateau, the film exhibited a remarkable stability, with a constant surface potential of 340 mV which can be considered as characteristic of the polymyxin B/didodecanoylphosphatidylglycerol (1/5) system.

These interactions between polymyxin B and phosphatidylglycerol proved to be independent of the film molecular packing. Indeed, some experiments carried out with dihexadecanoylphosphatidylglycerol gave results identical to those obtained with its shorter analogue. As can be seen in Fig. 5, the lipid alone on pure water exists only in the gel state [6]. Spreading polymyxin B/lipid mixtures progressively enriched in the peptide resulted in film expansion with the triggering of the phase transition. Stabilization of the system in the 1/5 molar ratio is shown in Fig. 4, at the reference surface pressures of $\pi = 8$ and 25 $mN \cdot m^{-1}$. As a control, spreading the lipid alone on a 10^{-6} M polymyxin B subphase resulted in a compression isotherm superimposable on curve 5 (Fig. 5) which was obtained after spreading the 1/5 polymyxin B/lipid mixture. At saturation (curves 5—7 in Fig. 5) a phase transition occurred at the relatively high surface pressure of 32 $mN \cdot m^{-1}$. Similar results were obtained with polymyxin E (curves not shown).

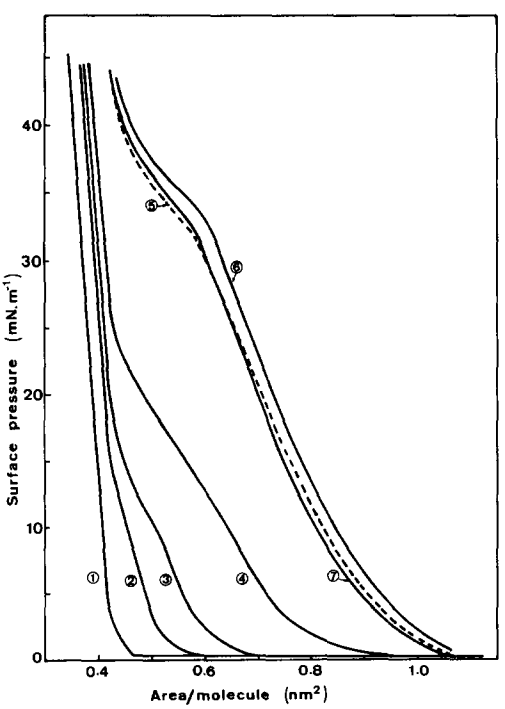

Fig. 5. Compression isotherms (recorder traces) for polymyxin B/dihexadecanoylphosphatidylglycerol mixtures in the molar ratios 1, 0/1; 2, 1/40; 3, 1/20; 4, 1/10; 5, 1/5; 6, 1/4; 7, 1/3. The subphase was pure water (pH 6). Curves were calibrated as if the lipid were alone.

Competition experiments between polymyxin B and ions

Experiments were carried out at constant molecular area by recording π and ΔV with time, first for films of didodecanoylphosphatidylglycerol spread on subphases at a given salt concentration, then after addition of polymyxin B into the same subphase, to achieve a 10^{-6} M concentration.

It is worthwile noting that the biphasic process previously observed on pure water turned out to be monophasic above concentrations of 10 mM NaCl or 1 mM $CaCl_2$, as revealed by the disappearance of the second ΔV increment seen after point I in Fig. 1.

At equilibrium, and as can be seen in Fig. 6, Na^+ seems rather ineffective in competing with polymyxin B. A NaCl concentration of 10 mM was required for detecting a significant ΔV variation. At a concentration of 100 mM NaCl, the ΔV measured in the presence of the antibiotic (310 mV) was still higher than in its absence (230 mV). With respect to the surface pressure, the $\Delta\pi$ between the two states (presence or absence of polymyxin B) was found independent of the salt concentration. In spite of a little change in ΔV, probably originating from dipole reorientations, it is suggested that, at least up to a concentration of 100 mM NaCl, the antibiotic does not leave the film but still interacts with didodecanoylphosphatidylglycerol. This appears to be in contrast with the report by Teuber and Miller that a concentration of 10 mM NaCl in the subphase was enough to remove it from the interface [15]. Our results are, however, consistent with the findings of Newton, i.e. that monovalent cations are ineffective in competing with polymyxin B in living cells [21] and also with the results of Feingold et al., who demonstrated the activity of the antibiotic against phosphatidylglycerol-containing liposomes even with high NaCl concentrations [17].

The picture, in the presence of Ca^{2+}, is somewhat different. This cation is

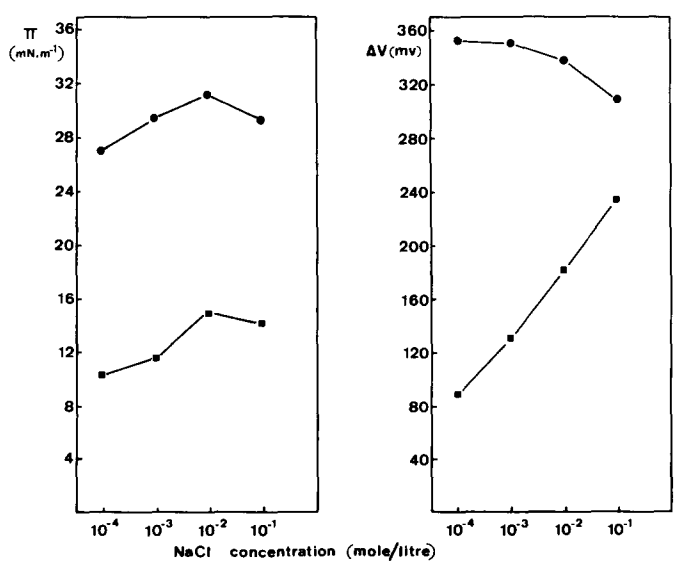

Fig. 6. Changes in π and ΔV for films of didodecanoylphosphatidylglycerol against the NaCl concentration in the subphase (pH 6), in the presence (10^{-6} M) (●) or absence (■) of polymyxin B.

known to strongly interact with phosphatidylglycerols [5,7–9]. As can be inferred from data gathered in Fig. 7, Ca^{2+} competes with polymyxin B for the phosphate groups in the film, a net π decrease being observed at concentrations of 10 and 100 mM $CaCl_2$. Although our results appear to be in agreement with previous findings [15,21], they do not account for a complete antibiotic desorption, even at a concentration of 100 mM $CaCl_2$. From π data in Fig. 7, and from compression curves supporting the data in Fig. 4, it can be easily calculated that there is still one antibiotic molecule for about six and ten didodecanoylphosphatidylglycerol molecules at concentrations of 10 and 100 mM $CaCl_2$, respectively.

Similar competition experiments carried out by spreading first the lipid on a 10^{-6} M polymyxin B subphase, then stepwise increasing the salt (NaCl, $CaCl_2$) concentration gave identical results.

Influence of polymyxin B on the phase properties of phosphatidylglycerol

Since it was possible to monitor the relative number of polymyxin B and phosphatidylglycerol molecules in the monolayer, it was tempting to study the influence of the antibiotic on the phase properties of the lipid. It should be remembered that polymyxin B does not form any film by itself. To resolve this difficulty, and since the antibiotic/lipid (1/5) association found at saturation could be used as a reference, experiments were carried out by spreading and compressing various peptide/lipid mixtures in which the molar ratio was varied from 0/1 to 1/5. Compression isotherms were calibrated assuming, as a working hypothesis, that the peptide/lipid (1/5) system constituted a single molecular species. The compression curves were analyzed by plotting the mean area per molecule versus the peptide/lipid (1/5) system mol fraction for various

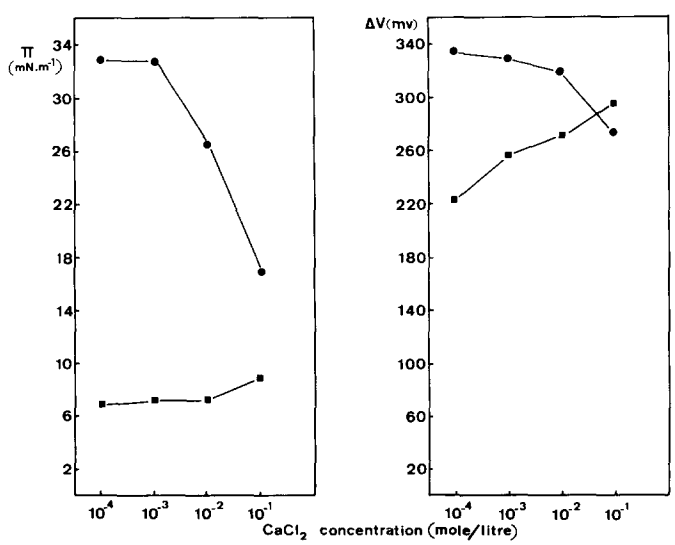

Fig. 7. Changes in π and ΔV for films of didodecanoylphosphatidylglycerol against the $CaCl_2$ concentration in the subphase (pH 6), in the presence (10^{-6} M) (●) or absence (■) of polymyxin B.

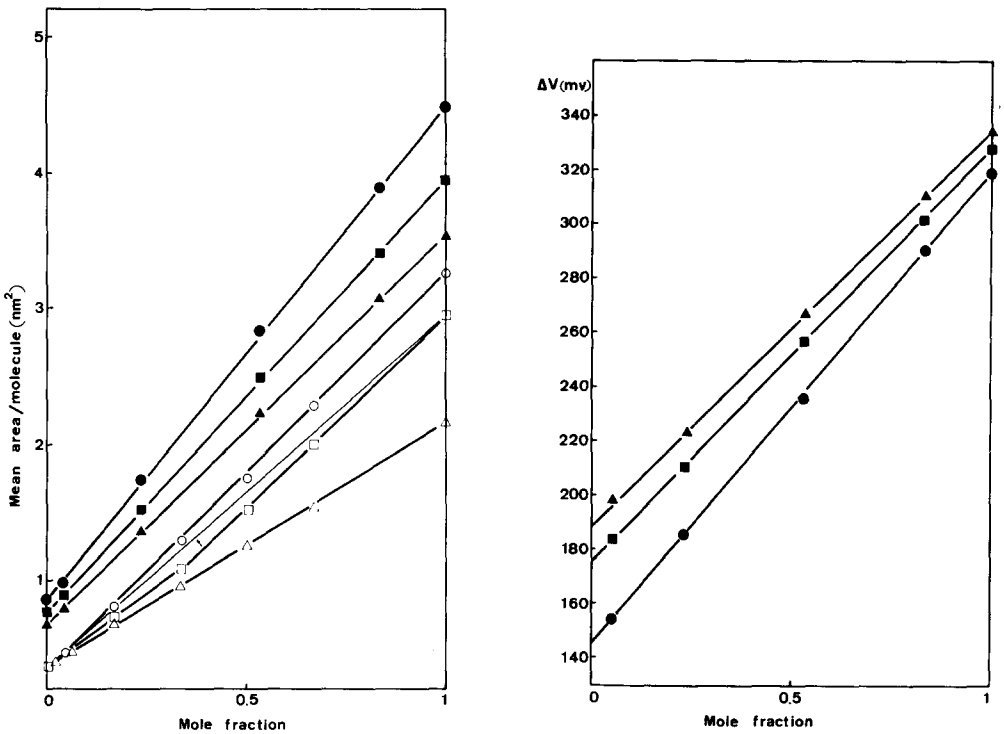

Fig. 8. Mean area per molecule for mixtures of didodecanoylphosphatidylglycerol (●, ■, ▲) and dihexadecanoylphosphatidylglycerol (○, □, △) with polymyxin/phospholipid (1/5) system against the mol fraction of the system at different surface pressures π: ●, 10 mN · m^{-1}; ■, 20 mN · m^{-1}; ▲, 30 mN · m^{-1}; ○, 18 mN · m^{-1}; □, 25 mN · m^{-1}; △, 40 mN · m^{-1}. The subphase was 1 mM NaCl (pH 6).

Fig. 9. Surface potential for mixtures of didodecanoylphosphatidylglycerol with polymyxin B/lipid (1/5) system, against the mol fraction of the system at different surface pressures π: ●, 10 mN · m^{-1}; ■, 20 mN · m^{-1}; ▲, 30 mN · m^{-1}. The subphase was 1 mM NaCl (pH 6).

surface pressures (Fig. 8). The corresponding changes in ΔV for didodecanoylphosphatidylglycerol are given in Fig. 9.

With respect to didodecanoylphosphatidylglycerol and at any surface pressure, it can be observed that no deviation from linearity occurred, either in terms of π or in terms of ΔV. A very small negative deviation (condensation) was detected for dihexadecanoylphosphatidylglycerol, only at intermediate surface pressures ($\pi \approx 25$ mN · m^{-1}), in a region where the compression isotherms all exhibited a phase transition.

Discussion

From the above, it is clear that polymyxin B rapidly and strongly binds to phosphatidylglycerols, a result which confirms previous observations of the high affinity of the antibiotic for acidic lipids [13,15,17]. In contrast, as already reported [15,17,19], the zwitterionic phosphatidylcholine seems unsuitable as a substrate.

As already suggested [3], both electrostatic and hydrophobic forces partici-

pate in stabilizing polymyxin B at the phosphatidylglycerol/water interface. The importance of the electrostatic interactions stems from the very existence in the monolayer and at saturation of one antibiotic molecule for every five lipid molecules, a stoicheiometry which accounts for an exact neutralization of the charges. A similar result was found by Teuber and Miller for labelled polymyxin B interacting with cardiolipin [15].

It is most probable that the hydrophobic tail of the antibiotic penetrates the monolayer and interacts with the phospholipid acyl chains. This is supported by NMR experiments (Barrett-Bee et al., cited in Ref. 3) and by the fact that deacylated polymyxins are less active against bacteria [22]. The importance of the hydrophobic interactions is revealed here by the possibility to bring polymyxin B in the monolayer by spreading of peptide/lipid mixtures at the air/water interface, without desorption and dissolution of the antibiotic into the subphase. It has been suggested that changes in the membrane fluidity due to differences in the chemical structure of the fatty acids in phospholipids could play a role in modulating the antibiotic activity against cell membranes [23]. Actually, such a hypothesis seems irrelevant since polymyxin B is found to bind similarly to didodecanoyl- or to dihexadecanoylphosphatidylglycerol, these compounds providing examples of lipids in the fluid and gel phase, respectively.

Hartmann et al. proposed a model for polymyxin-lipid association [11]. According to these authors, when the paraffin tail has penetrated the hydrophobic part of the membrane, the charged linear peptide chain anchors within the polar head group region, while the peptide ring, considered as an elliptical and flat disc, lies upon the membrane surface. In fact, it is clear from Figs. 2—4 that large film expansions arise from interactions between polymyxin and phosphatidylglycerol. The cross-sectional area of branched fatty acids in monolayers can be estimated around 0.25 nm^2. Therefore, the penetration of the fatty acid moiety of the antibiotic between the polymethylenic chains of didodecanoylphosphatidylglycerol could only contribute to the film expansion by about $0.25/5 = 0.05$ nm^2. Even taking into account a penetration of the hydrophobic residues of leucine and phenylalanine this cannot account for the 0.25 nm^2 film expansion found experimentally (Fig. 3).

The apparent molecular area of polymyxin B at the interface can be easily calculated on the basis of the molecular area found for the peptide/lipid (1/5) system (see Fig. 8), after deduction of the contribution of the five associated lipid molecules. Phosphatidylglycerols, when bound to polymyxin B, are likely to be ionized. However, the repulsive forces which would result are presumably canceled out because of the neutralization of each phosphate group by one amine function. Under this assumption, the molecular area allowed for each linked phospholipid molecule in the peptide/lipid system must be very close to that found for the lipid when non-ionized, i.e. on pure water (curve A, Fig. 3). In these conditions, it is of interest to observe that over a large surface pressure range ($8 < \pi < 39$ $mN \cdot m^{-1}$), the molecular area calculated for polymyxin B associated with didodecanoylphosphatidylglycerol is remarkably constant, 1.23 ± 0.05 nm^2, and strinkingly comparable to the limiting molecular area of 1.23 nm^2 found by Few and Schulman [20] for polymyxin B alone spread as a monolayer over a 70% $(NH_4)_2SO_4$ subphase, just before film

collapse. Calculations are more difficult to carry out in the case of dihexadecanoylphosphatidylglycerol since interactions with the antibiotic trigger the phase transition from the gel to the fluid state. Nevertheless, correction for this phase change leads to a calculated molecular area of about 1.10 nm^2 ($10 < \pi < 25$ $mN \cdot m^{-1}$) still in good agreement with the above values. These values appear to be quite different from the figure of 2.32 nm^2 estimated by Teuber and Miller using radiolabelled polymyxin B [15].

From these data, it is suggested that the whole polymyxin B molecule penetrates the monolayer. We recently showed that the methyl ester of lysine can adsorb at water/phosphatidylglycerol interfaces in such a way that the two amino groups interact with two phosphate groups, the hydrophobic chain $-(CH_2)_4-$ lying parallel to an interacting with the non-acylated glycerol residue of the lipid [10]. Although a more complicated situation is obviously to be expected with a decapeptide like polymyxin B, it is believed that the same kind of electrostatic and hydrophobic forces basically still hold in the stabilization of the antibiotic at the lipid interface.

Attempts have been made to determine the conformation of polymyxins in water [24—27] but the conformation of the antibiotics at lipid interfaces is still not known. However, the molecular area of 1.23 nm^2 we have found for polymyxin B bound with phosphatidylglycerol would correspond to a rather compact structure.

Data presented in Figs. 8 and 9 were obtained with the assumption that the polymyxin B/phosphatidylglycerol (1/5) system constituted a single molecular species. In fact, the strength and the relative weight of the electrostatic and hydrophobic forces involved in the stabilization of this system, as well as its life time, are still unknown and the proposal of the existence of a real complex in which the peptide and lipid molecules would be firmly bound cannot be made with certainty. However, as mentioned above, electrostatic interactions are very important for stabilizing the antibiotic at the lipid surface. It is likely that as soon as one polymyxin B molecule reaches the lipid monolayer, it interacts with five lipid molecules to fulfill the charge neutralization requirement. One could argue that polymyxin B might be surrounded by more than five phosphatidylglycerol molecules. In fact, it is easily calculated, on the basis of the surface area of 1.23 nm^2 found for the antibiotic in the monolayer, that it can be in contact with five lipid molecules only. Therefore, considering the peptide/lipid (1/5) system as a whole (and as a reference) for studying the influence of the antibiotic on the phase properties of the lipid seems to be a reasonable working hypothesis. Under these conditions, the straight lines shown in Figs. 8 and 9 must be accounted for either by a phase separation of the 1/5 system from the excess lipids or by the ideal mixing of this system with the extra lipid molecules. The first interpretation might be relevant in the case of dihexadecanoylphosphatidylglycerol since a phase separation has been reported for polymyxin B interacting with dipalmitoylphosphatidic acid liposomes [11]. Furthermore, the association of the antibiotic with the last lipid has been shown to decrease its phase transition temperature. The corresponding phenomenon is observed with dihexadecanoylphosphatidylglycerol since, in the monolayer, its phase transition in the presence of polymyxin B occurs at a higher surface pressure than in the absence of the antibiotic (Fig. 5). A

decrease in the transition temperature is likely to be associated with a loosening of the lipid molecular packing [11]. Such an effect is to be expected with our model in which the lipid molecules are forced to separate from each other to give space to polymyxin B, the five bond phosphatidylglycerols being distributed around the peptide at given places imposed by the position of the five 2,4-diaminobutyric acid residues.

With respect to didodecanoylphosphatidylglycerol, it is worthwhile emphasizing that absolutely no deviation from linearity could be observed, both in terms of π and ΔV (Figs. 8 and 9). In this case, the possibility of a random distribution of the peptide within the lipid monolayer, with a fast exchange between bound and free lipid molecules cannot be excluded. Such a behaviour has been recently suggested for lipids interacting with glycophorin, a major protein from the human erythrocyte membrane [28].

Finally, in the view of the proposed association model between polymyxin B and phosphatidylglycerol, the biphasic kinetic curves shown in Fig. 1 might be explained as follows: the first π and ΔV increases could account for the penetration of the peptide within the lipid monolayer. At or near saturation (π is nearly at its maximum value), the second ΔV jump would correspond to a reorganization of the whole system, and maybe in a cooperative manner. The strong dipoles originating in the phosphate-amino group interactions might be involved in this last step. In this respect, the importance of anionic binding to the properties of polymyxin B has been recently shown by Perkins et al. [27].

To conclude, it is worthwhile emphasizing the considerable lateral film expansion which arises from the interaction of polymyxin B with phosphatidylglycerol, whatever the initial lipid molecular packing may be. Lateral expansion in biomembranes is likely to be limited. Therefore, the biocidal activity of the peptide could simply originate from its ability to interact (in a nonspecific way) with acidic phospholipids, the resulting lateral expansion bringing about a complete disorganization of the membrane assembly and consequently, a release of the cell material [3,4,29]. Severe competition between the antibiotic and intrinsic proteins for their annulus lipids, or competitions with Ca^{2+} for the anionic phosphate site for the acidic lipids can also be envisaged.

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