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Effect of Polymyxin B on the Structure and the Stability of Lipid Layers

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Summary. Polymyxin B (PX) does not penetrate phospholipid monolayers and bilayers at low field strength across the lipid layers. The degree of penetration of PX is evaluated from its effect on the capacitance of the monolayers and on the conductance of the bilayers. PX added to one side of a bilayer causes its destabilization, it also enhances destabilization of lipid monolayers at positive electric fields across the surface layer in the direction of the adsorbed PX. PX lowers very little the fluorescence polarization of 1,6-diphenyl 1,3,5 hexatriene embedded in phospholipid vesicles. It is suggested that the penetration mechanism of PX into gram-negative bacteria is based on transient local breakdown of the plasma membrane.

Negatively charged lipids act as polymyxin receptors in the membranes of gram-negative bacteria (Teuber, 1974). Fusion of polymyxin-resistent *Acholeplasma laidlawii* B with phosphatidyl glycerol or cardiolipin liposomes induced polymyxin susceptibility (Teuber & Bader, 1976). Replacement of cardiolipin, phosphatidylglycerol, and phosphatidyl ethanolamine by an ornithine-amide lipid converts the highly polymyxine-susceptible *Pseudomonas* fluorescence into a polymyxin-resistant species (Dorrer, 1976). In a publication preceding the present one, it has been shown that the basic antibiotic polymyxin B interacts nearly stoichiometrically (charge per charge) with negatively charged lipids in monolayers (Teuber & Miller, 1977). As from the stoichiometric interaction per se, no conclusion can be derived concerning the penetration of the polymyxin into the lipid layer or other structural changes which could provide a clue for the mechanism of its crossing a bacterial plasma membrane; other experiments had to be attempted to reach this goal.

It has been shown that increase in conductance and in capacitance of lipid bilayers and of monolayers can provide a measure of the extent

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of their penetration by polypeptides and proteins (Bach & Miller, 1973), (Miller & Rishpon, 1977), (Pagano & Miller, 1973). Phase transition in phospholipid bilayer vesicles containing the diphenyl hexatriene (DPH) fluorescence probe, affects its fluorescence polarization (Shinitzky & Barenholz, 1974). Changes in fluorescence and in phase transition occur during interaction of phospholipid vesicles with polypeptides giving information on the structure of the lipid-polypeptide contact site (Träuble, Middelhoff & Brown, 1974).

These were the considerations in the present work for our applying AC polarography on lipid monolayers, DC resistance measurements on planar lipid bilayers, and fluorescence polarization measurements of DPH incorporated in lipid bilayer vesicles while interacting with polymyxin B.

Materials and Methods

Polymyxin B sulphate was a gift from Pfizer GmbH (Karlsruhe, Germany). The pure phospholipids used in this work were purchased from Lipid Products, Nutfield, England. *Escherichia coli* total lipids were extracted from late log phase cells (Ames, 1968). Spectra grade solvents hexane and decane were used for forming monolayers and planar bilayers.

The polarographic investigation of spread lipid monolayers has been described elsewhere (Pagano & Miller, 1973; Miller & Rishpon, 1977). A dropping mercury electrode is located above the monolayer (in the present case at its collapse pressure) so as to contact it within 2 sec after the start of formation of a new drop, while the reference and the auxilliary electrodes are in the solution. Phase dependent AC polarography was measured with a Princeton Applied Research (PAR) Model 170 Electrochemistry System. In every case polymyxin was injected underneath the lipid layer after its formation. Lipid bilayers were formed from phospholipid solutions in decane on a circular hole in a Teflon septum as described elsewhere (Bach & Miller, 1973). Phosphatidyl serine (PS) bilayers were formed and investigated at 37 °C; the other lipid bilayers at 22 °C. Different quantities of aqueous solution of polymyxin (6 mg/ml) were added on one or both sides of the formed black bilayer membranes to reach concentrations between 0.2 and 6 μg/ml. In some experiments the films were generated in solutions containing the polypeptide.

Fluorescence polarization was measured on the Elscint instrument Model MV-1 (Elscint, Haifa, Israel), which measures directly the fluorescence polarization $-p=(I_{\parallel}-I_{\perp})/(I_{\parallel}+I_{\perp})$.

Results and Discussion

AC Polarography of Lipid Monolayers Interacting with Polymyxin

A lipid monolayer is stable at the mercury/water interface at polarizations (potential difference – either positive or negative – from the zero

charge potential) below 0.5 V, having a constant specific capacitance of about 1.5–1.7 $\mu F/cm^2$, which is in good agreement with the expected value for a 11–12 Å thick hydrocarbon layer with an effective dielectric constant $\varepsilon = 2$. It would correspond to a larger thickness of the layer for larger values of ε. It is also about twice the values obtained for bilayers formed from monolayers (Montal, 1976; Benz et al., 1975). Penetration of the hydrocarbon layer by polypeptides causes an increase in capacitance, while mere adsorption may cause a small decrease in capacitance values (Miller & Rishpon, 1977). As evident from Figs. 1 and 2, polymyxin reduces the lipid capacitance by 0.35 $\mu F/cm^2$ in the stable potential region around the zero charge potential. The zero charge potential is about 0.5 V relative to Ag/AgCl electrode for the pure electrode surface or with a lipid layer without polymyxin, but it is shifted in positive direction due to polymyxin adsorption by up to 100 mV depending on salt concentration (Figs. 1*a* and 2*a*). One can conclude unambigously from here that, at least, at these low polarizations polymyxin is adsorbed but does not penetrate the lipid layer. The decrease in capacitance during adsorption without penetration may be caused by condensation of the lipid layer, and thus by thickening of the hydrocarbon layer, and through the serial contribution of the capacitance of the adsorbed polypeptide layer. Knowing the total amount adsorbed (Teuber & Miller, 1977) and the minimal value of the capacitance of a polypeptide layer of this surface concentration ($\sim 12\ \mu F/cm^2$) (Miller & Rishpon, 1977), the latter contribution to the capacitance lowering cannot exceed 0.1 $\mu F/cm^2$. It is evident from Figs. 1 and 2 that negative polarization of the electrode surface facilitates displacement of the lipid layer by polymyxin. The displacement is accompanied by a sudden increase in the differential capacitance caused by an influx of charges onto the mercury surface in course of the displacement. At low polymyxin concentrations which show, however, full biological activity and complete surface saturation (0.5 μg/ml), the desorption (displacement) potential is shifted by 150–200 mV towards more positive values, depending on concentration and ionic strength. The displacement potential continues to shift with further increase in polymyxin concentration until the displacement at negative polarizations starts very close to the zero charge potential. The specific capacitance value at the displacement potential or in any other potential region is independent of the time of exposure of the monolayer to the mercury electrode surface. In other words, the enhancement of the breakdown of the lipid layer structure by polymyxin is enhanced by an electrical field across lipid layer with the positive direction towards the polymyxin-

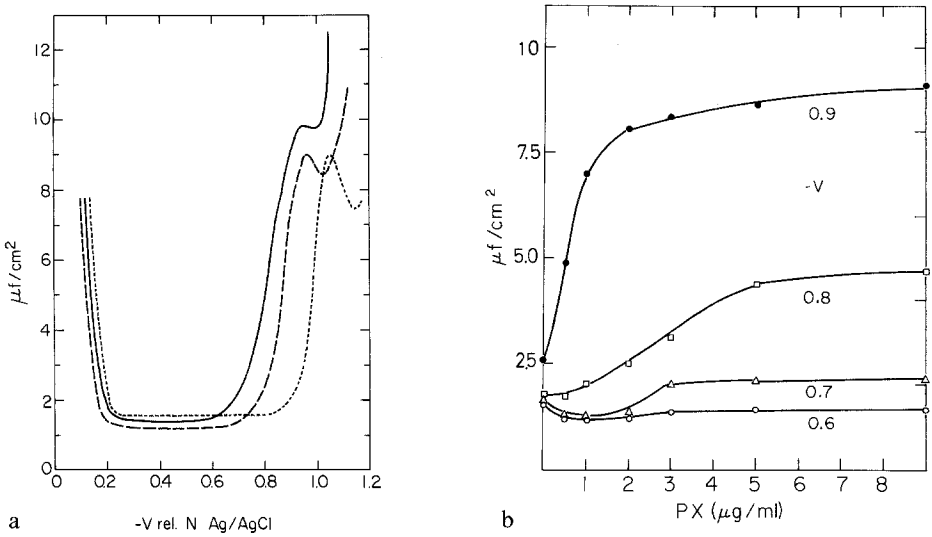

Fig. 1. Effect of polymyxin B (PX) on the differential capacitance of a condensed monolayer of phosphatidyl glycerol subphase pH 7.3, 0.1 N NaCl. (*a*): Dependence of the monolayer capacitance on potential-PX concentration in the subphase: dotted line, 0; dashed line, 2 μg/ml; solid line, 10 μg/ml. (*b*): Dependence of monolayer capacitance on the concentration of added PX at constant potentials as indicated

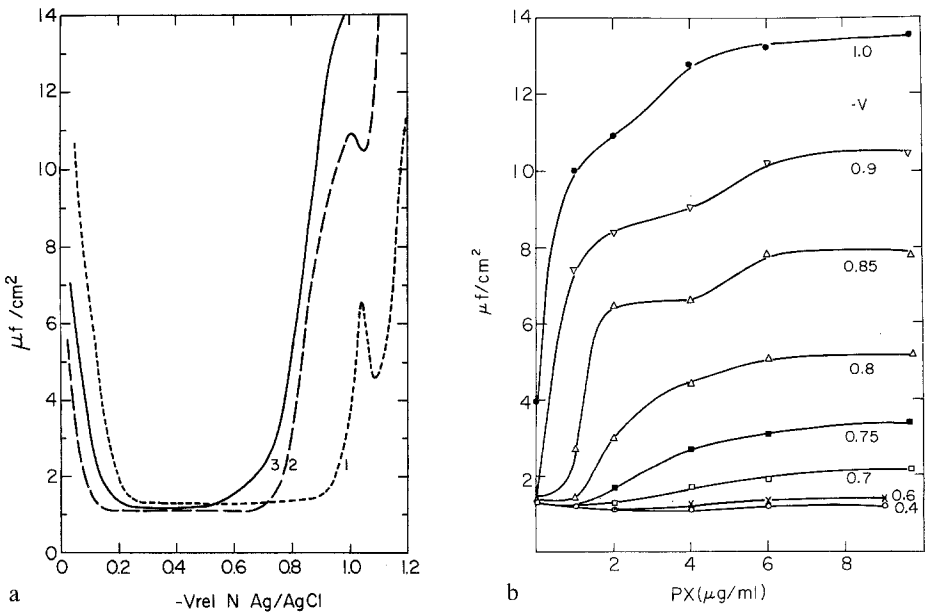

Fig. 2. Effect of PX on the differential capacitance of a condensed monolayer of *E. coli* lipids. Subphase at pH 7.3, 0.01 N NaCl. (*a*): Potential dependent capacitance curves at PX concentrations: dotted line, 0; dashed line, 2 μg/ml; solid line, 10 μg/ml. (*b*): Dependence of the capacitance on PX concentration at the indicated potentials

covered side. The monolayer breakdown does not induce further adsorption of PX. Electrical fields of comparable sizes and directions can be formed across plasma membranes during polymyxin adsorption until local charge reversal, if the net charge of the inner side of the membrane has an adequate negative charge density.

Interaction of PX with Planar Black Lipid Membranes (BLM)

The BLM's were prepared from different lipids or lipid mixtures as to vary their charge density. In PS bilayers the charge density corresponded to about one negative charge per 60 $Å^2$; in those mixtures obtained from the 70% phosphatidyl ethanolamine and 30% phosphatidyl glycerol and from the bacterial lipids, the area per charge is between 200 $Å^2$ and 180 $Å^2$. Bilayers prepared from pure phosphatidyl ethanolamine have at pH 7 less than one charge per 800 $Å^2$ but interaction with polymyxin enhances its negative charge. The resistances of the different BLM's was in every case higher than 4.10^7 ohm cm^2, and in no case was it affected by the added oligopeptide. The only influence the added antibiotic had, when added assymmetrically (to one compartment only), was on the stability of the bilayer membranes, reducing it by an extent increasing with the charge density of the film and with decreasing ionic strength. The effects were strong at 10^{-3} M and 10^{-2} M NaCl and disappeared above 0.1 M NaCl. The decrease in stability became significant only at higher polymyxin concentrations than 0.5 μg/ml, increasing further with concentration until when more than 3 μg/ml PX was added to one side of the BLM it collapsed within 10 min. The same concentration of PX added simultaneously on both sides of the membrane did not affect the stability. At these concentrations of PX effects on bilayer conductance have been observed in the presence of 2.5×10^{-5} M sodium dodecyl sulphate; however, this detergent also affects the conductance without added PX (Antonov, Korepanova & Vladimirov, 1976).

Fluorescence Polarization

In Fig. 3 the fluorescence polarization of 1.6-diphenyl 1.3.5 hexatriene (DPH) in bilayer vesicles obtained by sonication of *E. coli* lipids is given as a function of temperature in the presence and in the absence of PX.

The concentration of added PX seems to be relatively high (11 μg/ml), but it was actually too low to saturate all the surface charges of the lipid

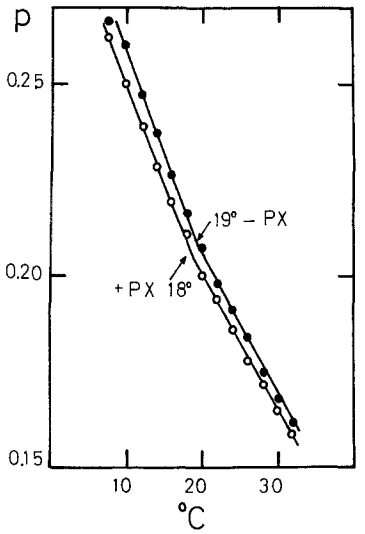

Fig. 3. Temperature dependence of fluorescence polarization (P) of DPH in vesicles of *E. coli* lipids, 600 μg/ml, with 11 μg/ml PX and without added PX

vesicles at a concentration of 600 μg/ml. The 600 μg *E. coli* lipids contain 150 μg phosphatidyl glycerol and 30 μg cardiolipin. If 75% of these negatively charged lipids are on the outer surface of the vesicle, they can bind about 45–50 μg PX. Hence, 11 μg if bound quantitively block less than 25% of the outer negative charge. Under these conditions the micro emulsion is still completely clear. The solution becomes increasingly turbid upon addition of PX until precipitation starts at around 50 μg/ml and is completed above 60 μg/ml. The precipitate does not dissolve upon addition of excess PX. The polymyxin at this partial surface coverage lowers slightly the fluorescence polarization; it also lowers the apparent phase transition from 19 °C to 18 °C. This small but reproducible effect on the fluorescence polarization suggests some weakening of interactions between the hydrocarbon chains, which seem to contradict the assumption that the lowering of the differential capacitances by PX binding results from a condensing effect. It is, however, in qualitative agreement with the differential scanning calorimetry results obtained by Pache, Chapma & Hillaby, 1972, who showed that PX lowers the phase transition temperature of dipalmitoyl lecithin. It is possible that the larger distance between the head-groups on the outer surface of a vesicle of a small radius of curvature allows better penetration than into the planar lipid layers. Alternatively, the adsorption on the outer surface of the vesicle may introduce stresses causing distortion and eventually break-down of

the vesicle structure. This is in keeping with the effect of PX on potassium and glucose permeability in lipid bilayer vesicles (Feingold, Hsu-Chen & Sud, 1974). It is also in agreement with our findings that maximal precipitation is reached at a PX concentration equivalent to all the charged lipid present rather than those on the outer surface of the vesicle only.

Polymyxin does not tend to penetrate planar lipid layers. However, it may cause local or total destabilization by adsorbing to one side of the lipid membrane. This destabilizing effect can be enhanced by electrical fields across the membranes, positive in direction of the adsorption side. In vesicles or in bacterial membranes supported by the cytoskeleton, the lipid bilayer breakdown may be local and transient, followed by a repair process upon penetration of the PX through the leak until nearly equal concentrations of PX on both sides of the membrane are reached.

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