Biochimica et Biophysica Acta, 735 (1983) 380–386
Elsevier

BBA 71674

INTERACTION OF BIOLOGICALLY ACTIVE MOLECULES WITH PHOSPHOLIPID MEMBRANES

I. FLUORESCENCE DEPOLARIZATION STUDIES ON THE EFFECT OF POLYMERIC BIOCIDE BEARING BIGUANIDE GROUPS IN THE MAIN CHAIN

TOMIKI IKEDA [a], SHIGEO TAZUKE [a,*] and MITSUO WATANABE [b]

[a] *Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259, Nagatsuda, Midori-ku, Yokohama and* [b] *Japan Spectroscopic Co. Ltd., Hachioji, Tokyo (Japan)*

(Received December 13th, 1982)
(Revised manuscript received September 19th, 1983)

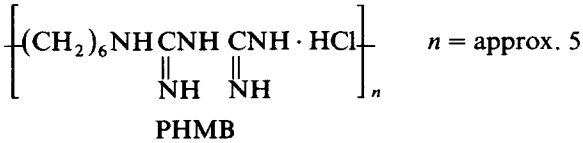
Key words: Membrane-drug interaction; Biguanide biocide; Phospholipid bilayer; Fluorescence polarization

Interaction of poly(hexamethylene biguanide hydrochloride) (PHMB), which is a polymeric biocide bearing biguanide groups in its main chain, with phospholipid bilayers was studied by the fluorescence depolarization method. A strong interaction of PHMB with negatively charged bilayers composed of phosphatidylglycerol(PG) alone or of PG and phosphatidylcholine (PC) was observed, whereas neutral PC bilayers were not affected. On adding PHMB, the fluorescence polarization of diphenylhexatriene embedded in the negatively charged bilayers was reduced to a great extent, especially in the gel phase. This was interpreted in terms of PHMB-induced expansion and fluidization of the bilayer, which enables the probe molecule to undergo less-hindered torsional motion. Similarity between PHMB and polymyxin B in the structure, the mode of action against bacteria and the interaction with lipid membranes is discussed.

Introduction

Synthetic polymers with biological activities have received much attention [1,23]. Much effort has gone into the study of the structure-activity relationship in naturally occurring biopolymers in order to prepare synthetic polymers as effective biologically as the natural ones. However, very few successful examples have been reported so far. This is partly because incorporation of isolated bioactive groups into polymer chains often diminishes the activity [2]. This fact clearly indicates that the microenvironment around the active sites plays a crucial role to exhibit their activities.

Poly(hexamethylene biguanide hydrochloride) (PHMB) is one of the few successful examples and has been used widely in practice.



PHMB

It has high antimicrobial activities against a wide range of microbes, such as bacteria, fungi and

* To whom correspondence should be addressed.

Abbreviations: PHMB, poly (hexamethylene biguanide hydrochloride); PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DPPA, dipalmitoylphosphatidic acid; DPPS, dipalmitoylphosphatidylserine; DSC, differential scanning calorimetry.

yeasts [3,4]. Particularly, it is effective against bacteria: it has a broad spectrum of antibacterial activity against both gram-positive and gram-negative bacteria, high kill rates and low mammalian toxicity [3,4]. Cytological studies have revealed that the target site of this polymeric biocide is the cytoplasmic membranes of microbes. Its adsorption to the membranes results in their disruption, followed by rapid release of K^+ and cytoplasmic constituents [3,4]. The loss of these essential components from the microbial cells leads to the death of microbes. Therefore, the interaction of the biocide with cytoplasmic membranes is a primary process in the cidal action. However, the molecular mechanism of the action still remains obscure. It is quite important to know the mode of action at molecular level, not only from the viewpoint of seeking the optimal condition for this biocide to act but also in order to prepare more successful ones.

There are two main possible sites in the cytoplasmic membrane of bacteria for interaction with PHMB: membrane proteins and phospholipids. It appears that zwitterionic phosphatidylethanolamine (PE), acidic phosphatidylglycerol (PG) and its dimer cardiolipin are essential components in bacterial cytoplasmic membranes and the outer membranes of their cell walls [5]. For example, in *Escherichia coli*, PE and PG constitutes 75 and 25% of the total lipids, respectively. *Pseudomonas putida*, the same gram-negative bacterium, has a similar composition to that of *E. coli*: PE, 86% and cardiolipin, 14%. In *Staphylococcus aureus*, a typical gram-positive bacterium, the acidic PG and cardiolipin have much higher compositional ratios: PG, 37% and cardiolipin, 24% [5]. There has been much evidence indicating that biguanide biocides interact with the membranes in a non-specific way, suggesting that the interaction with phospholipids may be a crucial step [6].

In a subsequent paper [7], we report that PHMB does not interact with neutral phospholipids, such as PC and PE, whereas it forms complexes with an acidic phospholipid PG. DSC measurements of mixed bilayers consisting of PC and PG have shown that the polymeric biocide induces isothermal phase separation into PG – PHMB complex domain and a remaining PC domain [7].

In this paper, we report the molecular interaction of PHMB with negatively charged membranes, studies further by the fluorescence depolarization method.

Materials and Methods

Materials

Dimyristoyl-L-α-phosphatidylcholine (DMPC, 98%), dipalmitoyl-DL-α-phosphatidylcholine (DPPC, 99%), distearoyl-L-α-phosphatidylcholine (DSPC, 99%), dipalmitoyl-L-α-phosphatidyl-DL-glycerol (DPPG, ammonium salt) were obtained from Sigma and were used without further purification. PHMB was a kind gift from Dr. D. Pemberton of ICI, U.K. 1,6-Diphenyl-1,3,5-hexatriene was purchased from Aldrich and recrystallized from acetone. All other chemicals were of analytical grade.

Fluorescence polarization measurements

The degree of fluorescence polarization (P) defined by the following equation (Eqn. 1) was recorded with apparatus constructed by Japan Spectroscopic Co. Ltd. (JASCO) in the course of a joint research scheme on construction and application of a simple and reliable apparatus for fluorescence polarization measurements.

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected with an analyzer oriented parallel and perpendicular, respectively, to the direction of polarization of the excitation light. The light source was a 500-W xenon lamp and excitation was performed with a conventional optical system composed of a Jobin Yvon H-20 monochrómator, quartz lenses and a polarizer. The linearly polarized light was focused into the center of the sample cell. The emitted light was observed at right angles to the direction of the excitation beam through an interference filter which was used to cut off the strayed excitation light as well as the scattered light from the sample. In the present study, diphenyl hexatriene was used as a probe to estimate the fluidity of the hydrocarbon region in the bilayer. Its fluorescence with the excitation at 360 nm has three apparent maxima, at 404, 427 and

450 nm, when embedded inside the bilayer. The interference filter used in this study is transparent only to light with wavelengths between 420 and 480 nm. Then the emitted light was passed through a polarizer rotating at a rate of 12 Hz, which was used to obtain the values of $(I_{\parallel} + I_{\perp})$ and $(I_{\parallel} - I_{\perp})$ with a single photomultiplier. The P values were obtained by a conventional ratio circuit. Those P values were plotted on the y-axis and the temperature of the sample, measured by means of a thermocouple inserted into the cell, was recorded on the x-axis of a X-Y recorder. Thus, a direct plot of P against temperature was obtained with great accuracy. The temperature of the sample was controlled, from 10 to 70°C, by means of a water jacket around the cell. It was confirmed that at scan rates of less than 1°/min the heating and cooling curves were almost completely superimposable and reproducible.

Preparation of liposome solutions of various composition

Liposome solutions were prepared by dispersing lipids in Tris-HCl buffer (50 mM Tris, $I = 0.1$ (with added NaCl), pH 7.45), followed by sonication and ultracentrifugation. Phospholipids dissolved in chloroform and a diphenyl hexatriene solution in the same solvent were mixed thoroughly in a small-volume phial and the solvent was removed under N_2 stream, and the lipid films containing diphenyl hexatriene were dried by overnight storage under vacuum. The Tris-HCl buffer was added so that the resulting lipid and diphenyl hexatriene concentrations would be 1 mM and 2.6 μM, respectively. Sonication was performed above the phase-transition temperature of each lipid component with a TOMY UR-200P probe-type sonicator. Completely transparent liposome solutions were obtained by subsequent ultracentrifugation at $100000 \times g$ for 40 min (Hitachi 55P-2 Ultracentrifuge). PHMB was added to the liposome solution as the same buffer solution and incubated for 30 min at temperatures higher by at least 10°C than the gel-to-liquid crystalline phase-transition temperatures (T_m) of the samples.

Fluorescence spectra were recorded with a Hitachi MPF-4 spectrofluorimeter and absorption spectra were measured with a Shimazu UV-200 spectrometer.

Results

The P vs. temperature curves for single and mixed liposomes composed of various PCs are shown in Fig. 1. All curves shown in this report are those observed on cooling. The phase-transition temperatures derived from Fig. 1 are summarized, and compared with the literature values, in Table I. Because of the ultracentrifugation at such a high g as 100000, multilamellar liposomes with large diameter were removed and the resulting solutions contained almost entirely unilamellar vesicles. Therefore, the values of T_m listed in Table I correspond to those of unilamellar liposomes. Good agreement exists between our values and values reported by Lentz et al. [8] for single liposomes composed of DMPC, DPPC and DSPC.

In mixed liposomes, the composition was always kept at a 1 : 1 molar ratio and the total concentration of the phospholipids was maintained at 1 mM. It is apparent that both DMPC/

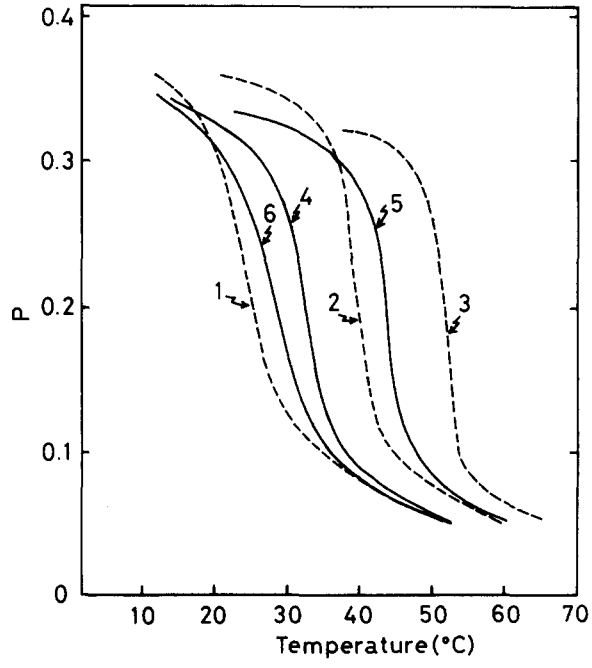


Fig. 1. P vs. temperature plots for single and mixed liposomes of PCs: 1, DMPC; 2, DPPC; 3, DSPC; 4, DMPC/DPPC mixture; 5, DPPC/DSPC mixture; 6, DMPC/DSPC mixture. The total concentration of the lipids was 1 mM in Tris-HCl buffer at pH 7.45 and each component in the mixture was 0.5 mM.

TABLE I

PHASE TRANSITION TEMPERATURES OF SINGLE AND MIXED VESICLES COMPOSED OF PCs

All the samples were prepared by dispersing the lipid(s) containing diphenyl hexatriene in Tris-HCl buffer (50 mM Tris, $I = 0.1$(NaCl), pH 7.45), followed by sonication and ultracentrifugation. The concentrations of the lipid(s) and diphenyl hexatriene were 1 mM and 2.6 μM, respectively.

Lipid	T_m(°C)	
	This work	From Ref. 8
DMPC	22	20.9
DPPC	39	36.4, 36.9, 39
DSPC	51.5	51.3
DPPG	36.5	
DMPC/DPPC(1:1)	31.5	
DPPC/DSPC(1:1)	43.5	
DMPC/DSPC(1:1)	28	

DPPC and DPPC/DSPC mixtures give intermediate curves between pure components, which is consistent with the observations by Lentz et al. [9] and is indicative of complete miscibility between each component. On the other hand, the curve obtained from the DMPC/DSPC mixture was a little peculiar and was shifted toward a lower temperature region than expected from the complete miscibility between DMPC and DSPC. This behavior can be rationalized by the observation that DSC studies on this mixture showed partial phase separation of the lipids [10].

The P vs. temperature curves for mixed liposomes composed of PC and DPPG are shown in Fig. 2. It can be seen that all the mixtures give intermediate curves between each component. This fact clearly indicates that the DPPG is completely miscible with the three kinds of PCs employed, which differ from DPPG in hydrocarbon chain length by two or less carbon atoms.

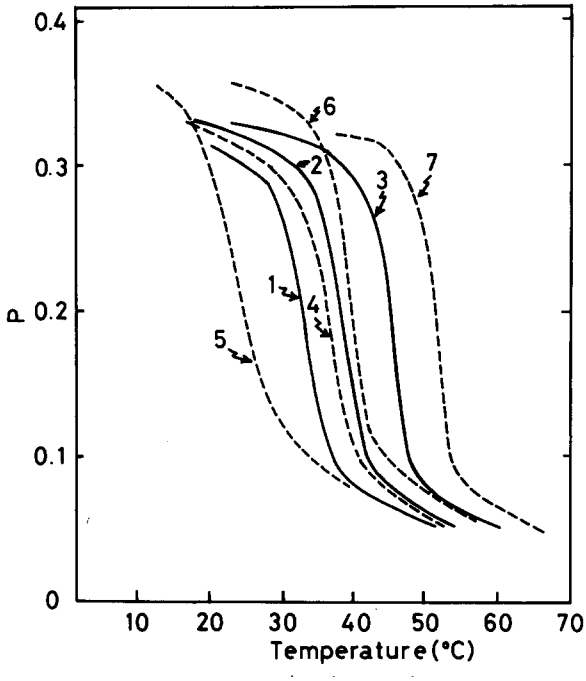


Fig. 2. P vs. temperature plots for mixed liposomes of DPPG and various PCs: 1, DPPG/DMPC mixture; 2, DPPG/DPPC mixture; 3, DPPG/DSPC mixture; 4, DPPG; 5, DMPC; 6, DPPC; 7, DSPC. The total concentration of the lipids was 1 mM in Tris-HCl buffer at pH 7.45 and each component in the mixture was 0.5 mM/

Effect of PHMB

The effect of PHMB on neutral bilayers was studied first. It is noted that PHMB has no significant interaction with DPPC and DPPE bilayers [7]. In this study, the effect on other PC bilayers of single and mixed compositions was examined. As

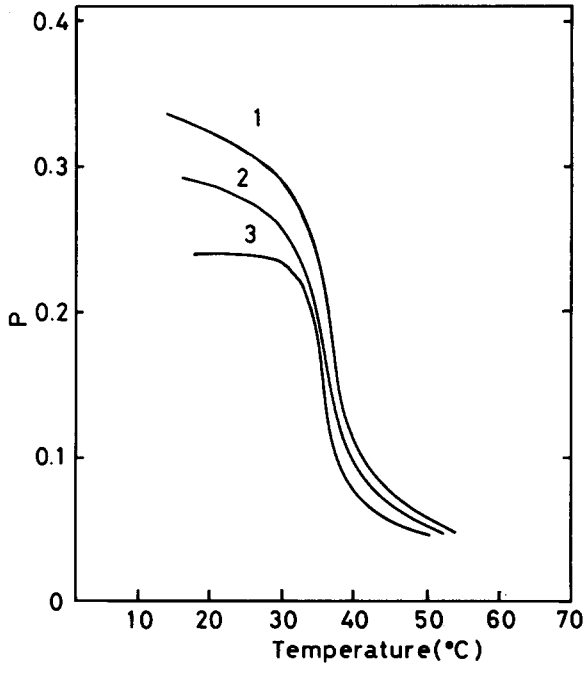


Fig. 3. Effect of PHMB on P of DPPG single liposome: 1, without PHMB; 2, 50 μg/ml PHMB; 3, 100 μg/ml PHMB.

expected, no significant effect was observed on DMPC and DSPC single bilayers, nor on mixed bilayers of any combination of the three PCs.

On the other hand, the effect of PHMB on negatively charged liposomes was drastic. As shown in Fig. 3, addition of 50 μg/ml of PHMB to the DPPG liposome solution produced a shift in T_m to a lower temperature and reduced the P values to a great extent, particularly in the gel phase. A more remarkable change in P was observed when 100 μg/ml of PHMB was present in the solution. Further addition of PHMB caused precipitation and made it impossible to obtain reliable P values.

It has been reported that diphenyl hexatriene is only fluorescent in more or less hydrophobic environments and partitions into both the gel and liquid crystalline phases of phospholipid bilayers in equal proportions [11]. Therefore, the P values of diphenyl hexatriene embedded in the bilayer are a measure for the fluidity of the hydrocarbon region of the bilayer. However, a simple interpretation of the results obtained from steady-state measurements will possibly lead to a wrong conclusion [12,13]. As the Perrin equation predicts, P is intimately correlated with both the fluorescence lifetime and the rotational relaxation time of the probe [14]. Thus, we are allowed to derive significant conclusions from the steady-state measurements only with the aid of information on the lifetime. Under the experimental conditions employed in this study, the fluorescence intensity of diphenyl hexatriene can be taken as a convenient measure for the lifetime of the probe [15]. Addition of PHMB to the DPPG liposome containing diphenyl hexatriene caused a slight decrease in intensity, by about 6% at the concentration range of 12–50 μg/ml, and further addition did not produce any significant change in the fluorescence intensity. Thus, we concluded that the fluorescence lifetime of the probe molecule is not affected significantly by the addition of PHMB. This means that the change in P observed when PHMB was added to the DPPG bilayer can be ascribed to the

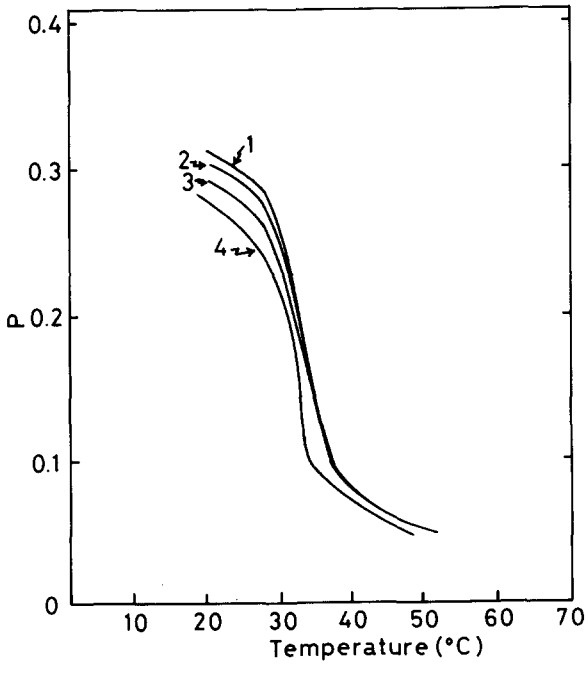


Fig. 4. Effect of PHMB on P of the DPPG/DMPC mixed liposome: [PHMB]: 1, 0; 2, 50 μg/ml; 3, 75 μg/ml; 4, 100 μg/ml. The composition of the mixed liposome was 1 : 1 by molar ratio, and the liposome was prepared in Tris-HCl buffer at pH 7.45.

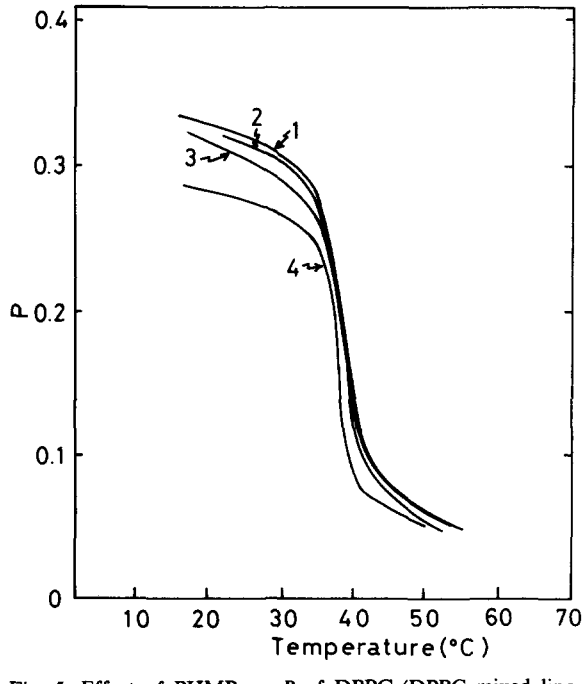


Fig. 5. Effect of PHMB on P of DPPG/DPPC mixed liposome: [PHMB]: 1, 0; 2, 25 μg/ml; 3, 50 μg/ml; 4, 100 μg/ml. The total concentration of the lipids was 1 mM in Tris-HCl buffer at pH 7.45 and each component in the mixture was 0.5 mM.

'fluid' environment induced by PHMB.

The effects of PHMB on the mixed liposomes composed of DPPG and PCs are shown in Figs. 4 and 5. Effects were observed, in all cases, which were similar to those observed in the DPPG liposome. With increasing concentration of PHMB, the P value was reduced gradually.

Discussion

The results obtained in this study support the view that PHMB interacts only with negatively charged bilayers in such a way that the interior hydrocarbon region where diphenyl hexatriene is located becomes more 'fluid'. In stricter sense, the probe molecule in the bilayer is allowed to undergo less-hindered torsional motion in the presence of PHMB [16]. DSC studies have shown that PHMB forms a complex with egg PG molecules which has a lower T_m, by about 10°C, than that of the pure PG bilayer [7]. This result was interpreted in terms of fluidization of the bilayer by complexing with PHMB. The monolayer experiments have demonstrated that PHMB expands greatly the PG monolayer formed at the water/air interface under a constant pressure and increases the monolayer pressure under a constant area (Eyres, B.L. and Brown, J.A., unpublished data). Coupled with these observations, it seems certain that PHMB increases the lateral distance between PG molecules arranged in bilayer and produces more room for the probe molecule to undergo the less-hindered movement.

The biguanide group in PHMB is a strong base, for which pK_1 lies in the range of 10.5–11.5 and pK_2 in the range of 2–3, so that at physiological pH it is completely monoprotonated [17]. Therefore, PHMB behaves as a polycation. Electrostatic attraction between positive charges of biguanide groups and negative charges on PG molecules is most probably the driving force for the complex formation between PHMB and PG molecules. The hydrophobic hexamethylene groups which link biguanide groups may also play a very important role in expanding the PG bilayer. They seem to act as wedges which are inserted into more hydrophobic sites in the bilayer (Eyres, B.L. and Brown, J.A., unpublished data). This would produce a larger distance between PG molecules.

The decrease in T_m observed when 100 μg/ml of PHMB was added to the PG bilayer (2°C) is small compared to that observed in the DSC studies (10°C) [7]. The ratio of PHMB added to PG molecules was 0.14 in the former and 0.25 in the latter. Therefore, the small decrease in T_m in the present study can be ascribed in part to the lower concentration of PHMB. However, it cannot wholly explain the observation. It seemed that 100 μg/ml PHMB in 1 mM DPPG dispersion was a somewhat critical concentration for PHMB to partition into the DPPG bilayer, and above this concentration precipitation of large particles was observed. Those particles seemed to be PHMB-DPPG complexes which were separated out of the bilayer. Below the critical concentration, adsorption of PHMB onto the surface of the PG bilayer does not seem to reduce the stability of the bilayer greatly, and the lipid molecules are preferably arranged in bilayer. However, an increasing amount of PHMB would decrease the stability by disrupting the integrity of the bilayer structure. Above the critical concentration, the lipid molecules complexed with PHMB can no longer retain the bilayer structure and are freed from the liposomal surface as PHMB-DPPG complexes. At 100 μg/ml PHMB, it is inferred that the liposomal bilayer is composed of patches of PHMB-DPPG complexes and unperturbed lipid domain. This means that the lateral phase separation has been induced by PHMB. The coexistence of the two phases may explain the small decrease in T_m observed at 100 μg/ml PHMB; that is, the observed change in P is an 'overall' change arising from the probes located at the different phases. The PHMB-DPPG complex domain itself would have T_m much lower than the observed one.

Polymyxin B, a polypeptide antibiotic, has been reported to exhibit the same effects on bacteria as do biguanide biocides [18]. It is regarded as owing its primary antibacterial action to binding to the cytoplasmic membrane of bacteria, with subsequent disruption [18]. It is quite interesting to compare the mode of interaction of polymyxin B with phospholipid membranes to that of PHMB. A strong interaction was observed between polymyxin B and negatively charged phosphatidic acid [19]. Binding of polymyxin B to mixed membranes composed of DPPA and DSPC caused phase sep-

aration into a polymyxin-DPPA complex domain and a DSPC domain [20]. Furthermore, the binding of polymyxin B to DPPG bilayers was reported to lower the T_m of the bilayer by 10°C [20], A monolayer study revealed that polymyxin interacts strongly with PG and expands the PG monolayer, whereas zwitterionic PCs are not affected [21]. Polymyxin B is an amphipathic molecule with a cyclic heptapeptide attached to a tripeptide chain terminating with a branched 8- or 9-carbon fatty acid residue [19]. It has five free amino groups associated with diaminobutyric acid units, which are positively charged at physiological pH. Therefore, polymyxin B is quite similar to PHMB in that it behaves like a polycation. Similarity between PHMB and polymyxin B in their structure, mode of action against bacteria and interaction with lipid membranes is quite suggestive. The positively charged parts may be essential for binding to negatively charged parts in cytoplasmic membranes and the hydrophobic parts seem to be indispensable for the interaction with the hydrophobic inner parts of the membranes.

It is worthy mentioning here that melittin interacts quite strongly with negatively charged lipid bilayers [22]. It is also an amphipathic, basic polypeptide of 26 residues and its main physiological effect is lytic action against living cells and lipid bilayers. Fluorescence depolarization studies have shown that it lowers the P values of diphenyl hexatriene embedded in bilayers of acidic phospholipids, such as DPPA, DPPS and DPPG, particularly in the gel phase [22]. This observation seems to give further support to the view described above.

References

1 Donaruma, L.G. and Vogl, O. (eds.) (1978) Polymeric Drugs, Academic Press, New York
2 Batz, H.G. (1977) Adv. Polym. Sci. 23, 26–56
3 Davies, A. and Field, B.S. (1969) J. Appl. Bact. 32, 233–243
4 Davies, A., Bentley, M. and Field, B.S. (1968) J. Appl. Bact. 31, 448–461
5 Gel'man, N.S., Lukoyanova, M.A. and Ostrouskii, D.N. (1975) Biomembranes 6, 35
6 Dlferink, J.G.R. and Booij, H.L. (1974) Biochem. Pharmacol. 23, 1413–1419
7 Ikeda, T., Ledwith, A., Bamford, C.H. and Hann, R.A. (1984) Biochim. Biophys. Acta 769, in the press
8 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521–4528
9 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4529–4537
10 De Kruyff, B., Van Dijck, P.W.M., Demel, R.A., Schuijff, A. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta, 356, 1–7
11 Johnson, S.M. (1981) in Fluorescence Probes (Beddard, G.S. and West, M.A., eds), p. 143 Academic Press, London
12 Jähnig, F. (1979) Proc. Natl. Acad. Sci. 76, 6361–6365
13 Kawato, S., Kinoshita, K., Jr. and Ikegami, A. (1977) Biochemistry 16, 2319–2324
14 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
15 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657
16 Lakowicz, J.R., Prendergast, F.G. and Hogen, D. (1979) Biochemistry 18, 508–519
17 Kurzer, F. and Pitchfork, E.D. (1968) Fortschr. Chem. Forsch. 10, 375–472
18 Franklin, T.J. and Snow, G.A. (1981) Biochemistry of Antimicrobial Action, Ch. 3, Chapman and Hall, London
19 Hartmann, W., Galla, H.J. and Sackmann, E. (1978) Biochim. Biophys. Acta 510, 124–139
20 Sixl, F. and Galla, H.J. (1981) Biochim. Biophys. Acta 643, 626–635
21 El Mashak, E.M. and Tocanna, J.F. (1980) Biochim. Biophys. Acta 596, 165–179
22 Bernard, E., Faucon, J.F. and Dufourcq, J. (1982) Biochim. Biophys. Acta 688, 152–162
23 Carraher, C.E., Jr. and Gebelein, C.G. (eds.) (1982) Biological Activities of Polymers, ACS Symposium Series 186