Biochimica et Biophysica Acta, 769 (1984) 57–66
Elsevier

BBA 71946

INTERACTION OF A POLYMERIC BIGUANIDE BIOCIDE WITH PHOSPHOLIPID MEMBRANES

T. IKEDA [a,*], A. LEDWITH [a], C.H. BAMFORD [a,**] and R.A. HANN [b]

[a] *Department of Inorganic, Physical and Industrial Chemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX and* [b] *Corporate Laboratory, Imperial Chemical Industry, P.O. Box 11, The Heath, Runcorn, Cheshire (U.K.)*

(Received September 19th, 1983)

Key words: Phospholipid membrane; Biocide-membrane interaction; Biguanide; Phase transition; Differential scanning calorimetry; Fluorescence polarization

Differential scanning calorimetry (DSC) and fluorescence polarization methods have been used to study the interactions between phospholipid membranes and a polymeric biocide, poly(hexamethylene biguanide hydrochloride) (PHMB). It was found that PHMB had very little effect on neutral lipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas it greatly reduced the phase transition temperature of phosphatidylglycerol (PG), an acidic lipid found in bacteria. Although the corresponding monomeric biocide had a similar effect on the PG bilayer, the behaviour towards mixed lipid bilayers of PC and PG has been shown to be completely different for the polymeric and monomeric biocides: viz. the former can induce isothermal phase separation into a PHMB-PG complex domain and a PC-enriched domain, whilst the latter cannot. This may account for the great difference in bactericidal activity between them. It is suggested that PHMB interacts primarily with negatively charged species in the membranes, inducing aggregation of acidic lipids in the vicinity of the adsorption site, where higher fluidity and higher permeability are expected. The results have shown that two factors might be crucial in the cidal activity of such types of cationic disinfectants as biguanides: phase separation and interaction with the hydrocarbon interior of the membranes. Polymeric biocides could be particularly effective by virtue of their ability to combine hydrophobic character and multiple charges within a single molecule.

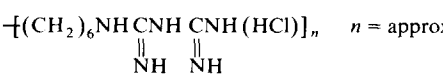
Introduction

Since the early work of Rose and co-workers [1,2] biguanides have been widely used as antimicrobial agents. Among them, chlorhexidine, a bisbiguanide, has been one of the best and most popular antibacterial agents owing to its broad spectrum of activity and low mammalian toxicity. The bactericidal activity of chlorhexidine has been shown to be much greater than that of monomeric biguanides [3]. This fact has stimulated the development of poly(hexamethylene biguanide hydrochloride) (PHMB) [3,4]; bacteriological studies have revealed that PHMB is more active than its

* Present address: Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama, Japan.
** Present address: Bioengineering and Medical Physics Unit, Duncan Building, Royal Liverpool Hospital, P.O. Box 147, Liverpool L69 3BX, U.K.

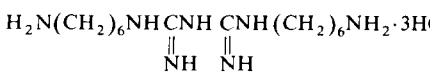
Abbreviations: DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPPE, dipalmitoyl-DL-α-phosphatidylethanolamine; egg PC, egg yolk phosphatidylcholine; egg PE, egg yolk phosphatidylethanolamine; egg PG, phosphatidylglycerol prepared from egg PC; PA, phosphatidic acid; PHMB, poly(hexamethylene biguanide hydrochloride); DAHB, diaminohexyl biguanide; DAH, 1,6-diaminohexane; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimeter; t_m, transition temperature (maximum peak height); P, degree of fluorescence polarization.

corresponding monomer (DAHB) and dimer against a wide range of bacteria.



n = approx. 5

poly(hexamethylenebiguanide hydrochloride) (PHMB)



diaminohexyl biguanide hydrochloride (DAHB)

Although synthetic polymers have long been used as structural replacements for damaged or diseased human bones and tissues, it is only recently that synthetic polymers with biological activity have received attention. Polymeric drugs are expected to show advantages in terms of localization in specific organs or tissues, reduced toxicity and increased duration of action [5,6]. However, very few examples with adequate biological activity have so far been discovered [6,7]. This is partly because bioactive groups often lose their activity when incorporated into a polymer. PHMB is one of the very few examples that has been successfully used in practice. It has a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria, high kill rates and low mammalian toxicity as in the case of chlorhexidine. However, little is known about the details of its mode of action, particularly at molecular level. Hugo and co-workers [8,9] found that chlorhexidine causes disruption of the cytoplasmic membranes of bacteria, followed by the immediate release of the cytoplasmic constituents. It was proposed that the primary action of chlorhexidine was adsorption of the drug onto a surface of the bacterial cell, thus causing disorganization of the membrane structure leading to consequent leakage of the cytoplasmic constituents [8,9]. Later the leakage of K^+ was found to correlate well with the kill, the percentage of dead cells almost equalling the percentage K^+ loss [10]. Furthermore, Davies and coworkers [3,4] observed similarity between the modes of action of chlorhexidine and PHMB, reporting that both cause precipitation of the cytoplasmic constituents. These early results suggest that a crucial step in the bactericidal action of PHMB may be disruption of the cytoplasmic membranes of bacteria, followed by release of K^+ and cytoplasmic constituents. A similar pattern of membrane activity has been observed in PHMB; there are, however, significant differences, which will be reported elsewhere [11].

The molecular nature of the interaction of the polymeric biocide with the cytoplasmic membrane of bacteria has remained obscure so far. There are two possible sites in the cytoplasmic membrane for interaction with PHMB: the membrane-bound proteins and the phospholipids. Relatively little is known about the membrane-bound proteins. On the other hand, the phospholipids have been extensively studied. It appears that the neutral lipid phosphatidylethanolamine (PE) constitutes about 80% of the total lipids in the cytoplasmic membrane of *Escherichia coli* and that the acidic phospholipid phosphatidylglycerol (PG) and a dimer of PG called cardiolipin are each present to the extent of 10% [12]. Furthermore, the previous studies seem to suggest that biguanide biocides interact with the membranes in a non-specific way [10,11], so that a study of the interaction between PHMB and bacterial phospholipids could be a valid experimental approach to elucidation of the mechanism of action of the biocide at the molecular level. We have adopted this approach, but we are aware that specific interaction involving the membrane-bound proteins cannot be excluded.

It has been recognized that liposomes, self-assembling bilayer leaflets of phospholipids, serve as a good model for the cell membrane since lipid bilayers provide a basic structure for biological membranes with which the membrane-bound proteins are associated extrinsically or intrinsically [13]. Therefore, liposomes have been widely used to study the interaction of local anaesthetics [14], proteins and polypeptides [15–19], metal ions [16] and a wide range of drugs [20] with biological membranes. In the present study, the interaction of PHMB with liposomes has been investigated in detail by using differential scanning calorimetry and fluorescence polarization methods to obtain some insight into the mode of action of the polymeric drug at molecular level. Combination of these two methods is expected to provide a deeper understanding of the mechanism.

Materials and Methods

Materials

Dipalmitoyl-DL-α-phosphatidylcholine (DPPC, 99%) and dipalmitoyl-DL-α-phosphatidylethanolamine (DPPE, 98%) were obtained from Sigma, London. Egg yolk phosphatidylcholine (egg PC), egg yolk phosphatidylethanolamine (egg PE) and phosphatidylglycerol prepared from egg PC (egg PG) were purchased from Lipid Products. The purity of these lipids was more than 99%. All the lipids were used without further purification. PHMB and its corresponding monomer DAHB were kindly supplied by Dr. D. Pemberton of ICI, U.K. They were supplied as hydrochloride salts; PHMB was free from contamination by starting materials and DAHB was better than 99% pure. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Aldrich and used as received (98%). All other chemicals are Analytical Grade. Water was first deionized and then distilled.

Differential scanning calorimetry (DSC)

Calorimetric experiments were performed on a Perkin-Elmer DSC-1B calorimeter normally operating at a scan rate of 8 C deg./min. 10 mg of the phospholipid in chloroform/methanol were placed in a 3 ml phial and the solvent was removed under dry N_2 followed by overnight storage in high vacuum. 30 μl of Tris-HCl buffer (20 mM Tris/100 mM KCl, pH 7.4)/ethyleneglycol mixture (1 : 1, v/v) was added to the dried lipid film and then the lipid was dispersed by agitating under a N_2 atmosphere above the phase transition temperature of the lipid. For example, egg PG was dispersed at room temperature, DPPC at 50°C, DPPE at 70°C and DPPC/egg PG mixture at 50°C. The dispersed lipid sample was then transferred to a 20-μl aluminum sample pan and sealed. Additives dissolved in the Tris buffer/ethyleneglycol solution were incorporated during dispersion. A small endothermic peak due to the pretransition of DPPC was not observed in the presence of ethyleneglycol. The pretransition was clearly seen when only the Tris buffer was used as dispersion medium, in agreement with a previous report [21]. For each sample, at least four scans were performed to check reproducibility. The heating curves provide more accurate transition temperatures and are shown in this report. Indium (99.9999%) and benzene (99.9%) were used to calibrate the calorimeter.

Fluorescence polarization measurements

The degree of fluorescence polarization (P) is defined by the following equation:

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

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected through an analyzer oriented parallel and perpendicular, respectively, to the direction of polarization of the excitation light. The apparatus used in this study has been described elsewhere [22]. It enables a direct plot of P against temperature to be obtained with great reproducibility and high S/N ratio. The sample was stirred magnetically and its temperature was varied from 0 to 90°C by means of a water jacket. DPH was used as a probe to estimate the fluidity of the hydrocarbon region of the bilayer and was excited at 366 nm [23]. The apparatus was operated at a scan rate of less than 1 C deg./min both on heating and on cooling. We did not try to convert measured P values into microviscosities or to derive the rate of rotation of the probe molecule [23]. Such conversions can be misleading, particularly in the absence of data on the emission lifetime.

Liposome solutions for the fluorescence polarization experiments were prepared by dispersing lipids in Tris-HCl buffer (20 mM Tris/100 mM KCl, pH 7.4), followed by sonication. Lipid films containing DPH were prepared by the same procedure as described for the DSC experiments. The final concentrations of the lipids and DPH were 1 mM and 1.5 μM, respectively. Sonication was performed above the transition temperature of each lipid with a MSE probe-type sonicator (150 W); the liposome solution was then subjected to ultracentrifugation at $100\,000 \times g$ for 1 h (MSE Prepspin 50). By this procedure, a completely transparent liposome solution was obtained, necessary for minimising depolarization arising from light scattering. Electron microscopy showed that the liposomes thus prepared were almost entirely unilamellar.

Results

Differential scanning calorimetry

The effect of PHMB on neutral phospholipid membranes (PC and PE) was first investigated by DSC. DPPC dispersion gave a very sharp endothermic peak at $t_m = 44°C$ on heating (Fig. 2, curve e). t_m is defined as the temperature at which the maximum peak height occurs. This value (44°C) is in good agreement with the value 43°C previously reported by Boggs and co-workers [17], but a little higher than that reported by Thompson and coworkers [24]. When 20% (w/w) PHMB was added to the DPPC dispersion and incubated at 50°C, no significant change in t_m or the shape of the peak was observed. It is more interesting to study the interaction of PHMB with PE bilayers since 80% of the phospholipids present in the cytoplasmic membrane of *E. coli* are PE molecules as already mentioned. Two types of PE were examined by DSC: DPPE and egg PE. The DPPE dispersion gave a sharp endothermic peak at $t_m = 56°C$. However, the thermotropic behaviour of this dispersion was not as simple as that of DPPC. The DPPE dispersion sometimes gave a second endothermic peak at $t_m = 84°C$ on heating, though on cooling it always gave a single peak at $t_m = 50°C$. This is consistent with the view that PE molecules are not able to form a stable bilayer by themselves [25], and show a metastable phase behaviour [26]. When 20% (w/w) PHMB was added to the DPPE dispersion and incubated at 70°C, the t_m of the main transition was reduced from 56°C to 55°C, but no other change was detected. The behaviour of egg PE dispersion was less complicated than that of the DPPE dispersion. It gave a broad peak at $t_m = 16°C$ (scan rate at 16 C deg./min) since it has a variety of acyl chains. Addition of 20% (w/w) PHMB to the egg PE dispersion induced a shift in t_m to a lower temperature by 2 C degrees, but there was no other significant change.

The solubility of PHMB in water is quite high (approx. 40% w/v), so that it may be expected that when PHMB is added to a lipid dispersion consisting almost entirely of multilamellar liposomes the interaction of PHMB is restricted to the outermost bilayers so that the portion of lipids exposed to PHMB is relatively small and the effects of PHMB on the lipids in DSC thermograms may escape detection. In order to test this possibility, lipids were dispersed in Tris buffer/ethyleneglycol mixture which already contained PHMB. This procedure was expected to produce homogeneous distribution of PHMB molecules among the aqueous phases inside and outside the multilamellar liposomes. Addition of PHMB in this way, however, did not cause any change in the DSC thermograms. From these results, it can be concluded that the effect of PHMB on the bilayers of neutral phospholipids such as PC and PE is small.

Fig. 1 shows the effect of PHMB and other cations on the thermotropic properties of egg PG, which is an acidic phospholipid and is negatively charged at physiological pH. As mentioned before, PG and its dimer cardiolipin are effectively the sole acidic lipid components in the cytoplasmic membrane of *E. coli*. The egg PG dispersion gave a broad endothermic peak at $t_m = -5°C$ (curve a). As with egg PE, heterogeneity in the composition

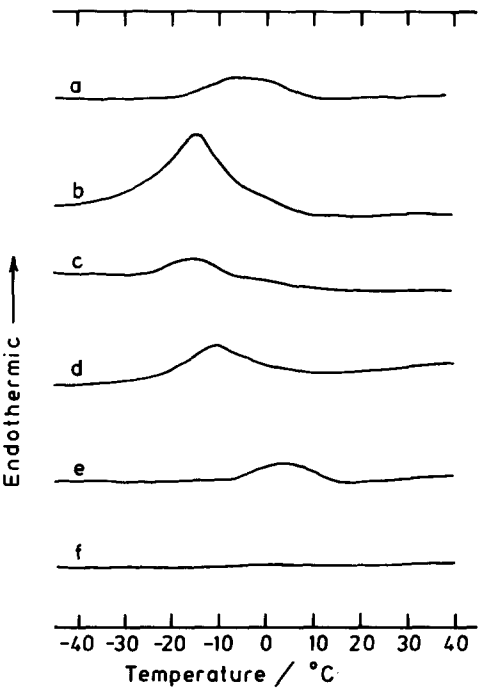


Fig. 1. Effect of various cations on egg PG membrane. a, none; b, 2.5 mg PHMB; c, 2.5 mg DAHB; d, 2.5 mg DAH; e, 0.44 M Mg^{2+}; f, 0.44 M Ca^{2+}. 10 mg of egg PG was dispersed in 30 μl of Tris-HCl buffer/ethyleneglycol mixture (1:1, v/v) at pH 7.4.

of the PG acyl chains produces a broad phase transition. Addition of 20% (w/w) PHMB induced a large shift of the phase transition to a lower temperature ($t_m = -15°C$) (curve b). When PHMB was added to the egg PG dispersion, precipitation of the lipid was observed. A similar thermogram with $t_m = -15°C$ was obtained in the presence of 20% (w/w) of the monomer DAHB (curve c). However, no precipitation was observed when DAHB was added to the egg PG dispersion. 1,6-Diaminohexane dihydrochloride (DAH) was studied to examine the effect of the amine end-groups carried by both PHMB and DAHB. As may be seen from curve d, 20% (w/w) DAH shifted the phase transition to a lower temperature, but the effect was smaller than those of PHMB and DAHB. Addition of DAH did not cause any change in appearance of the egg PG dispersion.

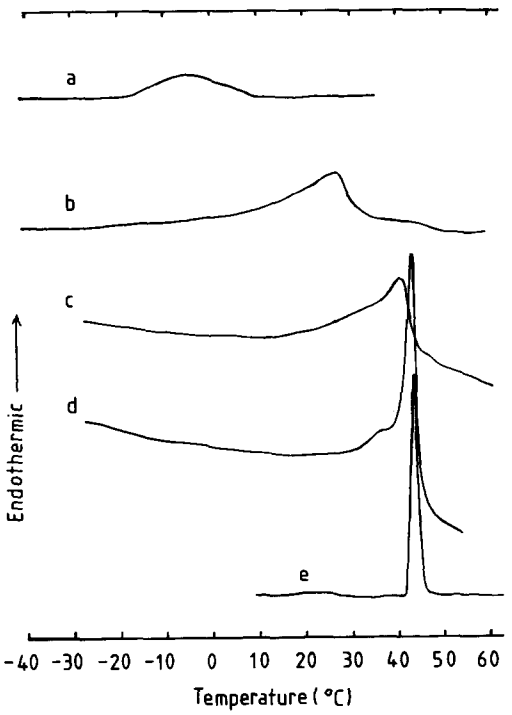


Fig. 2. Differential scanning calorimetry thermograms on mixed lipid membranes of DPPC and egg PG. a, pure egg PG; b, 50:50 (w/w) DPPC/egg PG mixture; c, 65:35 (w/w) DPPC/egg PG mixture; d, 84:16 (w/w) DPPC/egg PG mixture; e, pure DPPC. All the samples were dispersed in Tris-HCl buffer/ethyleneglycol mixture (1:1, v/v) at pH 7.4 and incubated at 50°C for 2 h.

The effect of inorganic divalent cations was also studied. Consistent with previous findings [16], equimolar quantities of Mg^{2+} shifted the t_m to higher temperature from $-5°C$ to 5°C (curve e) and equimolar quantities of Ca^{2+} led to the disappearance of the endothermic peak (curve f).

Fig. 2 shows the thermotropic properties of DPPC-egg PG mixtures with various compositions. Curves (a) and (e) refer to pure egg PG and DPPC, respectively. Mixtures gave a single endothermic peak at an intermediate temperature depending on the proportions of the two lipids. At 50:50 (w/w) (DPPC/egg PG), a peak was observed at $t_m = 27°C$ (curve b). Since this system (50:50) gave a t_m which was well separated from those of the pure lipids, it was chosen as a model membrane system for the study of the effect of cations on the mixed lipid membrane.

When 17% (w/w) PHMB was added to the DPPC/egg PG dispersion, the t_m was found to shift to a higher temperature from 27°C to 32°C and a second endothermic peak appeared at $t_m =$

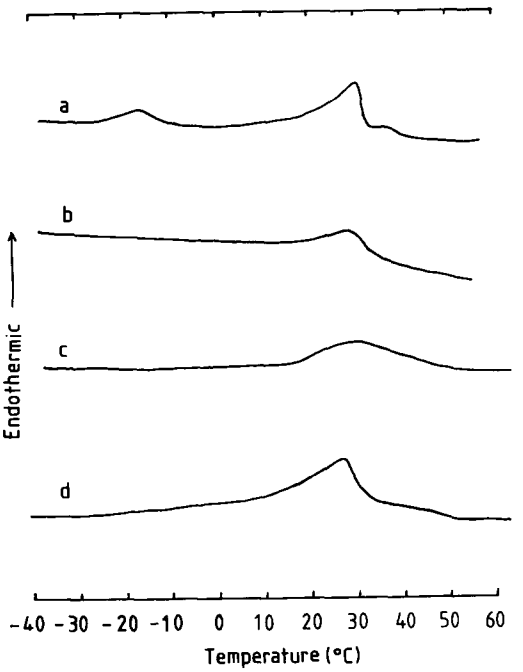


Fig. 3. Effect of various cations on a mixed lipid membrane of DPPC and egg PG (50:50, w/w). a, 2 mg PHMB; b, 2 mg DAHB; c, 2 mg $MgCl_2 \cdot 6H_2O$; d, none. 5 mg DPPC and 5 mg egg PG were dispersed in 30 µl of Tris-HCl buffer/ethyleneglycol mixture (1:1, w/v) at pH 7.4.

−15.5°C (Fig. 3, curve a). The new peak clearly corresponds to that observed when PHMB is added to the pure egg PG dispersion (Fig. 1, curve b). This result is consistent with the view that PHMB induces isothermal phase separation of the mixture into a PHMB-PG complex domain (t_m = −15.5°C) and a DPPC-enriched domain. From the position of the former peak, it appears that DPPC molecules are efficiently excluded from the PHMB-PG complex domain. When 17% (w/w) DAHB was added to the mixture, the t_m was found to shift to a slightly higher temperature, but unlike PHMB, DAHB did not produce any new peaks (Fig. 3, curve b). A thermogram somewhat similar to that found with DAHB was obtained when 17% (w/w) $MgCl_2 \cdot 6H_2O$ was added to the mixture (Fig. 3, curve c). Thus neither DAHB nor Mg^{2+} can induce phase separation in the DPPC/egg PG mixture. These experiments with the mixtures of DPPC/egg PG therefore reveal an outstanding property of PHMB, namely that only the polyelectrolyte can induce phase separation in the mixture.

Fluorescence polarization measurements

Pure DPPC liposomes gave a very sharp change in P with temperature (Fig. 4, curve a). P is a useful indicator of the fluidity of the hydrocarbon interior of the bilayer in which the probe (DPH) is located [23]. A decrease in P indicates greater freedom of rotation of the probe, and higher fluidity of the hydrocarbon region [23,24]. Therefore the abrupt drop in P with temperature in pure DPPC liposomes demonstrates that the phase transition takes place over a narrow range of temperature, in good agreement with the DSC results (Fig. 2, curve e). The midpoint temperature for the change in P can reasonably be correlated with t_m as measured in DSC thermograms. Pure DPPC liposomes gave t_m = 40.5°C on heating, somewhat lower than that observed in DSC experiments (t_m = 44°C). The difference may arise from variations in the scan rates employed; the higher scan rate (8 C deg./min) used for the DSC measurements is likely to increase the apparent transition temperatures.

In contrast to DPPC liposomes both egg PG (Fig. 4, curve d) and egg PC (Fig. 5, curve c) liposomes exhibited a gradual decrease in P with

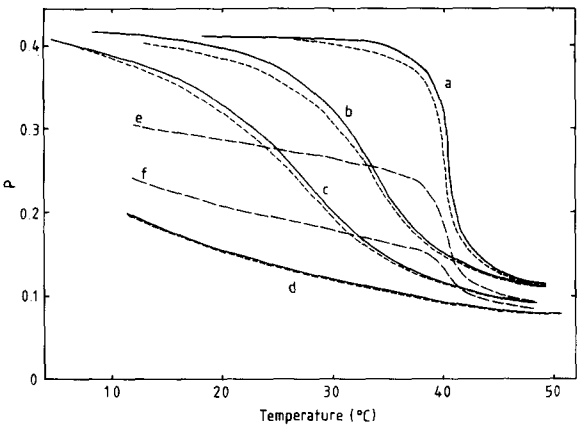


Fig. 4. Fluorescence polarization vs. temperature curves for DPPC/egg PG mixed membranes. a, pure DPPC; b, 75:25 (mol/mol) DPPC/egg PG mixture; c, 50:50 (mol/mol) DPPC/egg PG mixture; d, pure egg PG. The total concentration of the lipids was 1 mM in Tris-HCl buffer at pH 7.4, and the solid line indicates a heating curve in each set. Curves e and f are theoretical curves calculated from the cooling curves of a and d (see Discussion).

temperature consistent with the broad endothermic peaks observed by DSC. Unfortunately, for technical reasons, measurements could not be made below 0°C, so that the maximum P values which correspond to solidified liposomes were not obtained in our experiments. Although both lipo-

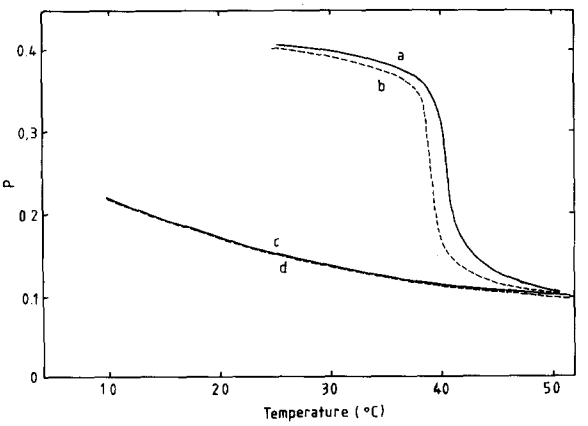


Fig. 5. Effect of PHMB on fluorescence polarization of DPH embedded in lipid membranes. a, DPPC; b, DPPC in the presence of 200 μg/ml PHMB; c, egg PC; d, egg PC in the presence of 200 μg/ml PHMB. The concentration of the lipids was 1 mM in Tris-HCl buffer at pH 7.4, and cooling curves are shown in all cases.

somes gave similar curves, the egg PC showed higher values of P over the whole range of temperature examined.

DPPC/egg PG mixtures gave intermediate curves (Fig. 4, curves b, c). It is clear that as the proportion of PG in the mixture increases, the P vs. temperature curve approaches monotonically that of pure egg PG. In the 50:50 (mol/mol) DPPC/PG mixture, the change in P was smooth and the t_m was observed as 27°C (curve c). This value is close to that obtained from DSC measurements on the similar system (Fig. 2, curve b).

The effect of PHMB on the fluidities of various liposomes was investigated. Addition of 200 μg/ml PHMB to the 1 mM DPPC dispersion produced a shift in t_m to a lower temperature by 1.2 degrees C (Fig. 5, curves a, b). No significant change in P for the egg PC liposome was observed on adding 200 μg/ml PHMB over the whole temperature range examined (10–52°C) (Fig. 5, curves c, d). Since the egg PC is entirely in the liquid-crystalline state in this temperature range, this result is consistent with the very small effects observed in DPPC above the phase transition temperature, 200 μg/ml PHMB caused precipitation of the egg PG, so that the fluorescence polarization could not be measured in these mixtures.

Much more interesting phenomena were observed when PHMB was added to DPPC/egg PG mixed liposomes. Fig. 6 shows changes in P before and after addition of PHMB to the mixture. Curve a represents P vs. temperature plots for 50:50 (mol/mol) DPPC/egg PG mixed liposomes in the presence of 100 μg/ml PHMB: comparison with curve c shows that above 27°C PHMB produced higher, and below 27°C much lower, values of P. (Below 16°C, the solution became irreversibly turbid and the P values obtained were not reliable. Consequently, PHMB was added at 50°C and the solution was cooled.) Curve b shows that the effect of 50 μg/ml PHMB on 50:50 DPPC/egg PG liposomes is similar. At this concentration of PHMB, no change in the appearance of the liposome solution was observed on cooling to 5°C. Liposomes with a different ratio of DPPC/egg PG were also examined. Curve d shows P vs. temperature plots for 75:25 (mol/mol) DPPC/egg PG liposomes with 100 μg/ml PHMB added. Comparison with curve e shows that in this system

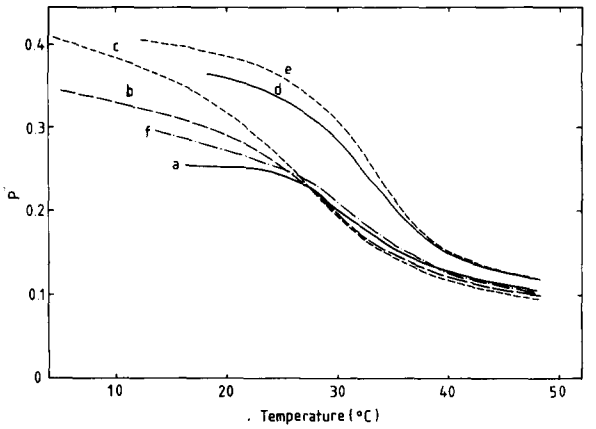


Fig. 6. Effect of PHMB on fluorescence polarization of DPH embedded in mixed lipid membranes of DPPC/egg PG: a, 100 μg/ml PHMB in 50:50 (mol/mol) DPPC/egg PG mixture; b, 50 μg/ml PHMB in 50:50 (mol/mol) DPPC/egg PG mixture; c, 50:50 (mol/mol) DPPC/egg PG mixture; d, 100 μg/ml PHMB in 75:25 (mol/mol) DPPC/egg PG mixture; e, 75:25 (mol/mol) DPPC/egg PG mixture. The total concentration of the lipids was 1 mM in Tris-HCl buffer at pH 7.4, and all the curves shown are cooling curves. Curve f is a theoretical curve (see Discussion).

also PHMB produces lower values of P at lower temperatures, though at higher temperatures no significant change in P was observed. In the presence of 100 μg/ml PHMB, precipitation occurred when the solution was cooled below 18°C and data for lower temperatures are not shown in the figure.

Discussion

The results obtained in this study demonstrate that the effect of PHMB on negatively charged lipid bilayers is very large compared to that on neutral lipid bilayers (PC, PE). It can be reasonably concluded that the target sites for PHMB interaction are the negatively charged species in the mixed bilayer of neutral and acidic phospholipids. This is quite reasonable since biguanide groups are strong bases, for which pK_1 lies in the range of 10.5–11.5 and pK_2 in the range of 2–3 [27]. Consequently, at physiological pH the biguanide groups are entirely monoprotonated. Actually biguanide biocides are usually used in the form of salts such as chloride (PHMB, DAHB),

acetate and gluconate (chlorhexidine). This monoprotonated form of biguanides is assumed to be responsible for the cidal action of the biocides. PHMB, being a polyelectrolyte (polycation), has a much higher positive charge density in its vicinity than do single electrolytes. Thus the relatively strong interaction of PHMB with negatively charged species is to be expected.

The effect of Mg^{2+} observed in the DSC experiments has been successfully interpreted in terms of surface adsorption of the cations with resulting partial neutralization of the negative charges of the lipids [16]; this has been assumed to produce tighter packing of lipid molecules, leading to a higher transition temperature [16]. The effect of Ca^{2+} is not well understood, but is believed to be related to the specific effect of Ca^{2+} in many physiological events such as cell fusion. Comparison of the effects of the organic cations with those of the inorganic cations in our experiments has clearly demonstrated that hydrophilic cations such as Mg^{2+}, which are believed to interact only with the polar headgroups of the lipids, can raise the phase transition temperature, whereas amphipathic cations such as PHMB and DAHB which contain lipophilic moieties in their molecules can induce phase transition to occur at lower temperatures.

Many studies have been made on the interaction between phospholipid bilayers and proteins which are useful to consider the PHMB-bilayer interaction. Papahadjopoulos and co-workers [15,16] proposed that interaction between proteins and lipid bilayers can be classified into three categories; (1) surface adsorption only, (2) surface adsorption followed by partial penetration of the hydrophobic part into the hydrocarbon interior and deformation of the bilayer, (3) complete penetration into the bilayer. They found that the proteins which belong to category 2 exhibit common features: a large shift in the phase transition temperature of the lipid bilayer to lower temperatures, a great increase in vesicle permeability and a large expansion of acidic phospholipid monolayers at the water/air interface [15]. In connection with our work, two important studies have recently been done: (1) Eyres and Brown observed that PHMB induced an expansion of PG monolayers at the water/air interface (Eyres, B.L. and Brown, J.A., unpublished results), (2) Walker and Jones found that PHMB caused leakage of entrapped molecules from phospholipid liposomes, which was ascribed to the disruption of the membrane (Walker, M. and Jones, M.E.B., unpublished results). Our results coupled with these findings suggest strongly that PHMB acts quite similarly to category 2 proteins. Thus, PHMB is adsorbed onto the surface of the PG bilayer, interacting with the polar headgroups of the lipids through its biguanide groups and with the hydrophobic interior through the hexamethylene groups linking biguanide groups. There follows disorganization of the PG bilayer, leading to higher fluidity, lateral expansion and a higher permeability of the bilayer. Our DSC experiments suggest that DAHB may interact with the PG bilayer in the same way, although there is no supporting evidence available from other methods. However, the effect of DAHB on the mixed lipid bilayer is completely different from that of PHMB. The most remarkable difference between PHMB and DAHB is that the polymeric biocide can induce phase separation in the mixed bilayer of PC and PG, whilst the monomeric one cannot. It is noteworthy that several polyelectrolytes reportedly induce isothermal phase separation in the mixed lipid bilayers of neutral and acidic phospholipids by binding to the acid components: polylysine in PA/DPPC [18], cytochrome *c* in cardiolipin/steroid and cardiolipin/egg PC/steroid [19] and human myelin basic protein in PC/acidic phospholipids [17].

The results on fluorescence polarization support the conclusion from the DSC measurements that PHMB has little effect on neutral lipid membranes. The observed *P* vs. Temperature curves for DPPC/egg PG mixed liposome systems give evidence for complete miscibility of DPPC and egg PG. According to Weber [28,29], the degree of polarization due to several species, each of which is emitting light of different polarization, can be calculated by the following equation:

$$\frac{1}{P} - \frac{1}{3} = \frac{1}{\sum_i \left(f_i \Big/ \left(\frac{1}{P_i} - \frac{1}{3} \right) \right)}$$

where f_i is the fraction of the total intensity emitted by the ith component and therefore bears a direct

relation to the proportion of the ith component present in the solution. In Fig. 4, theoretical curves thus calculated for the 50 : 50 DPPC/egg PG mixture in which complete phase separation is assumed are shown. The calculation was done using the P values experimentally obtained for pure DPPC liposomes (curve a) and pure egg PG liposomes (curve d). Recently site heterogeneity of DPH in lipid bilayers has been reported [30], and it therefore seems unrealistic to assume homogeneous distribution of DPH between rigid and fluid domains. The DPH molecules would be expected to partition preferentially into the most fluid domain available. With this in mind, two cases were considered; viz. $f_{DPPC} = f_{PG} = 0.5$ (curve e), and $f_{DPPC} = 0.2$, $f_{PG} = 0.8$ (curve f). The latter case is probably more realistic, but in both the theoretical curve is far from the observed curve c. If phase separation, even though incomplete, is assumed in the mixture of DPPC and egg PG, the observed high P values in the temperature range of 10 to 20°C can by no means be explained.

On the other hand, the P vs. temperature curves observed in the presence of PHMB (Fig. 6), can be explained in terms of the induced phase separation. The most remarkable effect of PHMB is its diminution of P at lower temperatures and increase of P at higher temperatures. The DSC results indicate that the PHMB-PG complex domains still contain a reduced proportion of PG molecules. It has been shown that in the presence of 100 μg/ml PHMB about 75% of the PHMB molecules are adsorbed on the PG liposomes. If we consider an ideal case where 100 μg/ml PHMB is added to a DPPC (0.5 mM)/egg PG (0.5 mM) mixture and each biguanide group in PHMB binds one PG molecule, the induced PHMB-PG complex domain contains 0.33 mM PG and the DPPC-enriched domain consists of 0.5 mM DPPC plus 0.17 mM PG (75% DPPC, 25% PG). A theoretical curve can be drawn for this case using P values experimentally obtained for the 75 : 25 DPPC/egg PG mixture (Fig. 4, curve b) and pure egg PG liposomes (Fig. 4, curve d), assuming $f_{PHBM\text{-}PG} = 0.33$ and $f_{DPPC\ rich} = 0.67$. This curve is shown in Fig. 6 as curve f. The similarity between the observed curve in the presence of 100 μg/ml PHMB (Fig. 6, curve a) and the theoretical curve is evident. The theoretical curve is based on the assumption that the number of DPH molecules in a domain is proportional to the number of lipid molecules present in the domain. As pointed out earlier, this assumption does not seem realistic if the fluidities of the domains are very different. However, it must be emphasized that the theoretical curve thus obtained fits the observed one relatively well. For a more realistic model with a greater proportion of DPH in the PHMB-PG complex domain, the calculated values of P would be smaller than those shown in Fig. 6, curve f, particularly at lower temperatures, and the agreement between observed and theoretical results would be improved.

In conclusion, our work has provided reason for believing that effective biocides should contain both hydrophilic and hydrophobic parts which interact effectively with the membranes. The great difference in bactericidal activity between PHMB and DAHB is most probably due to the difference in their abilities to induce phase separation, since DAHB has been shown to interact with the pure PG bilayer in the same manner as PHMB. From these arguments, it seems that two factors are crucial in determining the cidal activity of such types of cationic disinfectants as biguanides: ability to induce phase separation and ability to interact effectively with the hydrocarbon interior of the membranes. We believe that polymeric biocides are one of the best candidates to fulfil these two conditions, since the high charge densities available in polyelectrolytes are capable of interacting strongly with negative membrane surfaces, while suitable hydrophobic groups in their structure interact with the membrane interior.

Acknowledgements

The authors wish to thank Dr. C. Green of the University of Liverpool for helpful discussion and Drs. D. Pemberton, P.M. Woodcock, M.E.B. Jones, Mrs. M.E. Walker and Mr. R.M. Swart of Imperial Chemical Industries for many stimulating discussions and providing many valuable chemicals. One of the authors (T.I.) is grateful to ICI for its financial support.

References

1 Curd, F.H.S. and Rose, F.L. (1946) J. Chem. Soc. 729–737
2 Rose, F.L. and Swain, G. (1956) J. Chem. Soc., 4422–4425
3 Davies, A. and Field, B.S. (1969) J. Appl. Bacteriol. 32, 233–243
4 Davies, A., Bentley, M. and Field, B.S. (1968) J. Appl. Bacteriol. 31, 448–461
5 Donaruma, L.G. and Vogl, O. (eds.) (1978) Polymeric Drugs, Academic press, New York
6 Vogl, O. and Tirrell, D. (1979) J. Macromol. Sci. Chem. A13, 415–439
7 Verlander, M.S., Venter, J.C., Goodman, M., Kaplan, N.O. and Saks, B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1009–1013
8 Hugo, W.B. and Longworth, A.R. (1966) J. Pharm. Pharmacol. 17, 28–32
9 Hugo, W.B. and Longworth, A.R. (1966) J. Pharm. Pharmacol. 18, 569–578
10 Elferink, J.G.R. and Booij, H.L. (1974) Biochem. Pharmacol. 23, 1413–1419
11 Broxton, P., Woodcock, P. and Gilbert, P. (1983) J. Appl. Bacteriol. 54, 345–353
12 White, D.A., Lennarz, W.J. and Schnaitman, C.A. (1972) J. Bacteriol. 109, 686–690
13 Quinn, P.J. (1976) The Molecular Biology of Cell Membranes, Macmillan Press, London
14 Papahadjopoulos, D., Jacobson, K., Poste, G. and Shepherd, G. (1975) Biochim. Biophys. Acta 394, 504–519
15 Papahadjopoulos, D., Moscarello, M., Eylar, E.H. and Isac, T. (1975) Biochim. Biophys. Acta 401, 317–335
16 Papahadjopoulos, D. (1977) J. Colloid Interface Sci. 58, 459–470
17 Boggs, J.M., Moscarello, M.A. and Papahadjopoulos, D. (1977) Biochemistry 16, 5420–5426
18 Galla, H.-J. and Sackmann, E. (1975) Biochim. Biophys. Acta 401, 509–529
19 Birrell, G.B. and Griffith, O.H. (1976) Biochemistry 15, 2925–2929
20 Juliano, R. and Stamp, D. (1979) Biochim. Biophys. Acta 586, 137–145
21 De Kruijff, B., Van Dijck, P.W.M., Demel, R.A., Schuijff, A., Brants, F. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 1–7
22 Hann, R.A. (1981) J. Phys. E: Sci. Instrum. 14, 152–153
23 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
24 Thompson, T.E., Lentz, B.R. and Barenholz, Y. (1977) FEBS Symp. 42, 47–71
25 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
26 Van Dijck, P.W.M., De Kruijff, B., Van Deenen, L.L.M., De Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576–587
27 Kurzer, F. and Pitchfork, E.D. (1968) Fortschr. Chem. Forsch. 10, 375–472
28 Weber, G. (1952) Biochem. J. 51, 145–155
29 Weber, G. (1953) Advan. Protein Chem. 8, 415–459
30 Kleusner, R.D., Kleinfeld, A.M., Hoover, R.L. and Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286–1295