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Spectroscopic Studies on the Interaction of Polymeric In-chain Biguanide Biocide with Phospholipid Membranes as Probed by 8-Anilinonaphthalene-1-sulfonate

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Interaction of a highly effective polymeric biocide poly(hexamethylene biguanide hydrochloride)(PHMB) with phospholipid model membranes was investigated spectroscopically by using 8-anilinonaphthalene-1-sulfonate (ANS) as a fluorescent probe. PHMB enhanced the fluorescence intensity and shifted the maximum wavelength of fluorescence of ANS embedded in the negatively-charged liposome composed of egg PC and dicetyl phosphate, while it had only small effect on the neutral liposome composed of egg PC alone. These results were interpreted in terms of preferred binding of PHMB to negatively-charged species. The Benesi-Hildebrand type plots revealed that PHMB was adsorbed to a greater extent onto the negatively-charged membrane than onto the neutral membrane: the dissociation constants for the liposome–ANS complexes derived from the plots gave quantitative evidence for the enhanced binding of PHMB to the negatively-charged membrane. Diaminohexyl biguanide (DAHB), a corresponding monomer to PHMB, was found to have no significant effect on either the neutral nor the negatively-charged liposome, a finding probably related to the fact that DAHB is practically inactive.

Poly(hexamethylene biguanide hydrochloride)(PHMB) is a polymeric biocide which exhibits highly antimicrobial activity against a wide range of microbes such as bacteria, fungi and yeasts.[1] Its mode of action was reported to be similar to that of monobiguanides and bisbiguanides.[2] The sequence of elementary events in the lethal action of those biguanide compounds can be summarized as follows:[3]

1) Adsorption on to the bacterial cell surface;
2) Diffusion through the cell wall;
3) Binding to the cytoplasmic membrane;
4) Disruption of the cytoplasmic membrane;
5) Release of K^+ ions and other cytoplasmic constituents;
6) Precipitation of cell contents and death of the cell.

Among the elementary processes described above, the binding to the cytoplasmic membrane is a very important process, as the disruption of the membrane is considered to be a crucial step in the cidal action. However, the molecular nature of the interaction of the polymeric biocide with the bacterial cytoplasmic membrane has not been fully understood at molecular level.

Fluorescence spectroscopy has been used extensively to study membrane-associated phenomena. Among the common fluorescent probes, 8-anilinonaphthalene-1-sulfonate (ANS) has been one of the most widely used probes in membrane studies.[4] It has been used as a probe for protein studies[5–7] and to study the energized state of mitochondrial membranes.[8]

In the present study, the interaction of PHMB with phospholipid membranes has been investigated by fluorescence spectroscopy with ANS as a probe to obtain insight into the adsorption behavior of the polymeric biocide on to the bacterial membranes.

Experimental

Materials. Egg yolk phosphatidylcholine (egg PC, chromatographically pure) and dihexadecyl phosphate (DCP) were obtained from Sigma and used without further purification. 8-Anilinonaphthalene-1-sulfonate (ANS, magnesium salt) was purchased from BDH (>98% pure) and used as received. PHMB and its corresponding monomer diaminohexyl biguanide (DAHB) were hydrochloride salts, and PHMB was free from contamination by starting materials, DAHB being > 99% pure. (These biguanide compounds are kind gifts from Dr. D. Pemberton of ICI, UK).

Method. Liposomal solutions were prepared by dispersing lipids in Tris-HCl buffer (20 mM Tris, 100 mM KCl, pH 7.4) with the aid of sonication (a MSE probe-type sonicator). The dispersion was then subjected to ultracentrifugation at 100000×g for 40 min (a MSE Prepspin 50). By this procedure, a transparent liposome solution was obtained. Although the preparation method of the liposome solutions was essentially the same in all experiments, two different ways of addition of ANS were employed: i) ANS was incorporated in the liposomal phase by cosonication. That is, lipid films containing ANS were prepared by mixing the lipid solution in chloroform and the ANS solution in ethanol in a flask and by removing the solvents under dry N_2 and subsequent storage in high vacuum. The Tris-HCl buffer was added to the dried lipid film and the lipid was dispersed by sonication. These liposome solutions were used in the experiments concerning Figs. 1, 2, and 3. The effect of PHMB and DAHB was studied by adding some amounts of concentrate of PHMB and DAHB to the liposome solutions. ii) For measurements of fluorescence of ANS at various ANS concentrations (experiments on Benesi-Hildebrand plots, Figs. 4 and 5), liposome solutions which consisted only of lipids were prepared first, and to 4 ml portion of the liposome solution various amounts of ANS concentrate (1.43 mM in

distilled water) were added by means of a microsyringe (up to 50 μl). The resulting solution was mixed vigorously for 30 s and fluorescence spectra were taken. It was confirmed that the fluorescence spectra did not change with time: after 1 h the same spectra were obtained. In the measurements of the ANS fluorescence in the presence of PHMB (Fig. 4, b-d and Fig. 5, b-d), PHMB concentrate was added to the liposome solution by means of a microsyringe with vigorous mixing before the ANS concentrate was added. It must be mentioned here that ANS incorporated in the liposome by cosonication method (i) followed by addition of PHMB gave similar spectra to those observed when ANS was added after PHMB was added to the liposome solution composed of lipids alone.

Emission spectra were recorded on a Perkin-Elmer Hitachi MPF-4 spectrofluorometer and the spectra were uncorrected.

Results

Figure 1 shows the fluorescence spectra of ANS incorporated in egg PC vesicles. As is well known, free ANS emits only very weak fluorescence peaking at around 530 nm in water. In our measurements, the emission spectrum of ANS in buffer alone coincided with the base line at the same sensitivity as used to measure the spectra shown in the figure. Furthermore, addition of 500 μg/ml PHMB to ANS solution (2.2 μM) in buffer alone caused no significant change in spectrum; no detectable emission at the same sensitivity as used for the spectra shown in Fig. 1. This means that increase in the intensity of fluorescence (I_F) observed on addition of PHMB occurs only in the ternary system, egg PC-PHMB-ANS.

Since the first report by Stryer on emission properties of ANS in various solvents,[9] it has been known that both λ_{max} and the fluorescence quantum yield (ϕ_F) are strongly dependent on the polarity of the solvent: with decreasing polarity, λ_{max} shifts to lower wavelengths and ϕ_F increases. Comparison of the fluorescence spectra of ANS embedded in the egg PC liposome and those in various solvents demonstrated that the binding site for ANS in the PC liposome has similar polarity to that of methanol. Similar spectra were observed when ANS molecules were incorporated in negatively-charged liposomes composed of egg PC and DCP (Fig. 2, a), though the intensity was somewhat reduced. These results indicate that the polarity of the binding site for ANS is not so different even when negatively-charged species are present in bilayer. When 210 μg/ml PHMB was added to the egg PC dispersion containing ANS prepared by the method (i), I_F of ANS was slightly increased: Increase of I_F at 460 nm was recognized, though the maximum still remained at 482 nm (Fig. 1, b). Addition of 200 μg/ml DAHB caused no change in I_F and λ_{max}.

Drastic change in spectrum was observed when PHMB was added to egg PC-DCP mixed liposome prepared by the method (i), which was negatively-charged at pH 7.4. Figure 2 shows the fluorescence spectra of ANS embedded in the egg PC-DCP mixed

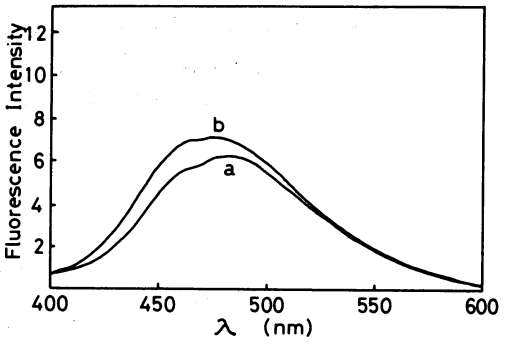

Fig. 1. Fluorescence spectra of ANS embedded in egg PC liposome in the absence and presence of PHMB: a, without PHMB; b, with 210 μg/ml PHMB added. The liposome solution was prepared by dispersing 0.8 mM egg PC in Tris-HCl buffer at pH 7.4; [ANS]= 2.2 μM, λ_{ex}=350 nm.

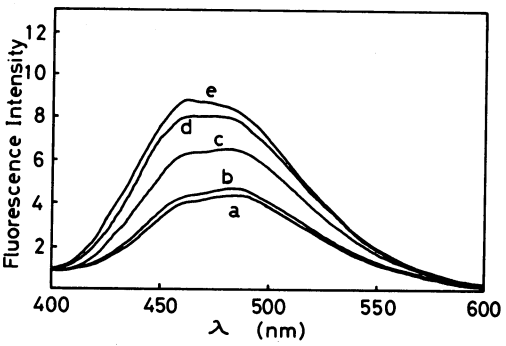

Fig. 2. Fluorescence spectra of ANS embedded in egg PC-DCP liposome in the presence of various concentrations of PHMB: [PHMB]=a, 0; b, 10.1 μg/ml; c, 50.4 μg/ml; d, 111 μg/ml; e, 170 μg/ml, λ_{ex}= 350 nm.
The liposome solution was prepared by dispersing 0.8 mM egg PC and 0.2 mM DCP in Tris-HCl buffer at pH 7.4; [ANS]=2.2 μM.

liposome ([egg PC]=0.8 mM, [DCP]=0.2 mM) in the presence of various concentrations of PHMB (0—170 μg/ml). With increasing concentration of PHMB, I_F increased gradually with gradual change in the shape of the spectrum. At 170 μg/ml of PHMB, λ_{max} is clearly changed to 460 nm. Addition of DAHB to the negatively-charged liposome composed of egg PC-DCP ([DAHB]=500 μg/ml) caused a slight increase of I_F, but to an insignificant extent. In Fig. 3 is depicted the increase of I_F as a function of the concentration of PHMB: the ratio $I_F/(I_F)_0$ at 460 nm was plotted against the concentration of PHMB, where I_F and $(I_F)_0$ are the fluorescence intensities at 460 nm in the presence and absence of PHMB, respectively. It can be readily seen from the figure that the increase of I_F at 460 nm is strongly dependent on the negative surface charge of the liposome. In the case of the neutral egg PC liposome, increase of I_F was very small and practically saturated at 50 μg/ml. On the other hand in the negatively-charged liposome composed of egg PC-DCP ([egg PC]=0.8 mM, [DCP]=0.09 mM) the $I_F/(I_F)_0$ increases with the concentration of PHMB added. Furthermore, much greater increase of I_F was ob-

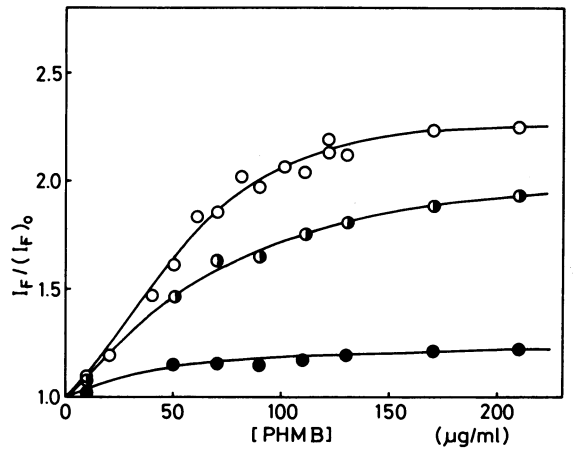

Fig. 3. Effect of PHMB on the fluorescence intensity of ANS embedded in various liposomes: (●), in egg PC liposome ([egg PC]=0.8 mM); (◑), in egg PC-DCP mixed liposome ([egg PC]=0.8 mM, [DCP]=0.09 mM); (○), in egg PC-DCP mixed liposome ([egg PC]=0.8 mM, [DCP]=0.2 mM). [ANS]=2.2 μM.

Table 1. Effect of Mg^{2+} and Ca^{2+} ions on fluorescence intensity of ANS embedded in egg PC liposome and in egg PC-DCP liposome

[ion]		I_F at 485 nm[a]	
	(μM)	egg PC	egg PC-DCP[b]
Mg^{2+}	0	1.00	1.00
	50	0.90	0.92
	99	0.89	0.91
	244	0.87	
Ca^{2+}	0	1.00	1.00
	5	0.96	0.99
	15	0.95	0.97
	49	0.94	0.99
	97	0.93	0.99
	239	0.93	0.97

a) Relative intensity; [ANS]=2.2 μM, λ_{ex}=350 nm. b) [egg PC]=0.8 mM, [DCP]=0.2 mM.

served in the more negatively-charged liposome which contained higher compositional ratio of DCP ([egg PC]=0.8 mM, [DCP]=0.2 mM).

The effects of Mg^{2+} and Ca^{2+} ions on fluorescence intensity of ANS embedded in the egg PC liposome and in the egg PC-DCP liposome were investigated ($MgSO_4$ and $CaSO_4$ were used). As seen in Table 1, unlike PHMB, these divalent cations rather lowered the intensity. These results clearly show that the enhancement of fluorescence intensity observed on adding PHMB is not just a sequence of electrostatic interaction between ANS and positive charges.

In order to investigate adsorption behavior of PHMB onto phospholipid membranes, incorporation of ANS molecules into liposomes without or with PHMB added was studied spectroscopically. In these experiments, addition of ANS was performed by the method (ii) (see Experimental). Higher concentrations of ANS gave rise to increased intensity of fluorescence, but no change in the shape of the spectrum, regardless of the presence of PHMB.

Benesi-Hildebrand type plots have been commonly used to estimate dissociation constants of complexes in many systems.[10,11] This type of plots is applicable to our system since only ANS molecules bound to liposomes contribute to the observed fluorescence intensity.[11]

$$\text{liposome}\cdot\text{ANS} \overset{K_D}{\rightleftharpoons} \text{liposome} + \text{ANS}$$

$$\frac{1}{I_F} = \frac{1}{(I_F)_\infty} + \frac{K_D}{(I_F)_\infty}\cdot\frac{1}{[\text{ANS}]} \quad (1)$$

where [ANS] is the initial concentration of ANS (M); I_F, the fluorescence intensity of ANS associated with liposomes; $(I_F)_\infty$, I_F at the infinite concentration of ANS; K_D, the dissociation constant for liposome-ANS complex (M). The concentration of liposomes can be estimated from the concentration of phospholipids employed (1 mM) and the aggregation number of egg PC vesicles reported (N=2335)[12] as 0.4 μM. The concentrations of ANS used for this study were 3.6—18 μM, so that the concentration change in ANS due to association can by neglected. Thus Eq. 1 suffices for the present study. In Fig. 4 are shown $1/I_F$ *vs.* 1/[ANS] plots for the egg PC liposome-ANS system in the presence of various concentrations of PHMB. It is evident from the figure that with increasing concentration of PHMB the slope of the plots is reduced, whilst the intercept remains practically identical. Similar plots for the egg PC-DCP mixed liposome-ANS system are shown in Fig. 5. The same trend with respect to the PHMB concentration may be seen. From these plots, the dissociation constants were calculated and listed in Table 2.

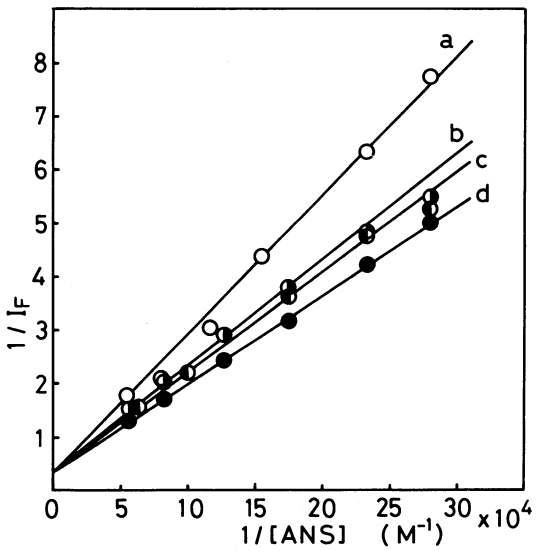

Fig. 4. $1/I_F$ *vs.* 1/[ANS] plots for egg PC-ANS complex in the presence of various concentrations of PHMB:
[PHMB]=a, 0; b, 20.3 μg/ml; c, 50.8 μg/ml; d, 102 μg/ml.
The liposome solution was prepared by dispersing 0.8 mM egg PC in Tris-HCl buffer at pH 7.4.

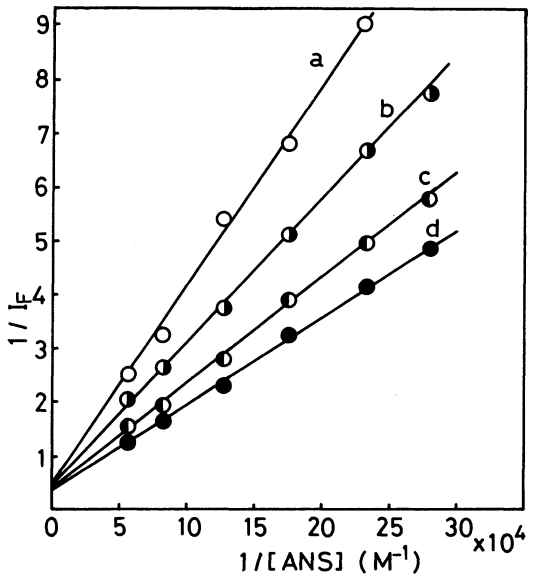

Fig. 5. $1/I_F$ *vs.* 1/[ANS] plots for egg PC-ANS complex in the presence of various concentrations of PHMB:
[PHMB]=a, 0; b, 20.3 μg/ml; c, 50.8 μg/ml; d, 102 μg/ml.
The liposome solution was prepared by dispersing 0.8 mM egg PC and 0.2 mM DCP in Tris-HCl buffer at pH 7.4.

TABLE 2. DISSOCIATION CONSTANTS FOR EGG PC-ANS AND EGG PC-DCP-ANS COMPLEXES IN THE ABSENCE AND PRESENCE OF PHMB[a)]

	[PHMB] added (μg/ml)			
	0	20.3	50.8	102
egg PC-ANS	6.7×10^{-5}	5.3×10^{-5}	5.0×10^{-5}	5.3×10^{-5}
egg PC-DCP-ANS	8.3×10^{-5}	6.3×10^{-5}	4.5×10^{-5}	3.8×10^{-5}

a) K_D (M) values obtained from the $1/I_F$ *vs.* 1/[ANS] plots shown in Figs. 4 and 5.

Discussion

The results obtained in this study clearly indicate that PHMB interacts more strongly with negatively-charged membranes than with a neutral membrane. This is quite reasonable from the fact that biguanides are strong bases (pK_1=10.5—11.5, pK_2=2—3) and are entirely monoprotonated at physiological pH.[13] Thus PHMB acts as a polyelectrolyte (polycation), and on account of the high positive charge density in its vicinity stronger interaction of PHMB with negatively-charged membranes is expected. The large difference between PHMB and DAHB in their interaction with membranes is most probably related to the great difference in their biocidal activity, DAHB being practically inactive.

NMR studies on the egg PC-ANS system have revealed that the amphipathic probe molecules are located at the interface region of the lipid dispersions where they interact with the head-groups of the lipids.[14] In the absence of PHMB, the lower intensity of fluorescence of ANS observed in the egg PC-DCP liposomes than that observed in the egg PC liposomes is evidently attributable to limited incorporation of the ANS molecules into the negatively-charged liposomes due to electrostatic repulsion. The larger K_D value estimated for the egg PC-DCP-ANS complex (at [PHMB]=0) is in support of this view quantitatively. The shift of λ_{max} observed on addition of PHMB to the egg PC-DCP liposomes can by no means be explained by simple increase in incorporation of ANS molecules in the presence of increased amount of PHMB, because the shape of the spectra remains unchanged when the concentration of ANS is varied at the fixed concentration of PHMB. Furthermore, it is evident from the control experiment already mentioned that direct interaction of ANS with PHMB does not contribute to the shift in λ_{max} and the increase in I_F under the experimental conditions employed to measure the spectra. A probable interpretation is as follows. PHMB is adsorbed on to the surface of the membranes where the sulfonato group of ANS is located. Then binding of a biguanide moiety to ANS as a counterion may take place. As a result, a less soluble complex of PHMB-ANS will be formed. This was evidenced by the formation of insoluble PHMB-ANS complex observed when 1% aqueous solutions of PHMB and ANS were mixed. The complex formation between PHMB and ANS will certainly produce less polar microenvironment around the bound ANS molecule in the membrane. The hydrophobic hexamethylene groups which link biguanide moiety in PHMB may contribute to give more hydrophobic environment to the bound ANS molecule. The effect of negatively-charged species (DCP) may be explained by two factors: (1) enhancement of surface adsorption of PHMB by electrostatic attraction and (2) endowment of additional "less polar" nature to the PHMB-ANS complex by participating in the complex as additional counterions of PHMB. These PHMB-ANS complexes are supposed to be solubilized in phospholipid bilayers.

The K_D values for the negatively-charged liposomes provide further support of the above view. The K_D values for the egg PC-DCP-ANS complex decrease with increasing concentration of PHMB added, whilst those for the egg PC-ANS complex remain nearly constant at the concentrations of PHMB higher than 20 μg/ml. These results seem to indicate that the quantity of PHMB adsorbed on to the neutral egg PC liposome is no longer increased above [PHMB]=20 μg/ml, whereas it increases with increasing concentration of PHMB added for the negatively-charged egg PC-DCP liposome. Actually, the lower values of K_D were obtained for the egg PC-DCP liposomes above [PHMB]=50 μg/ml, although below this concentration the K_D values for the negatively-charged liposomes were higher. Thus, the K_D value is a good indicator of adsorption behavior of PHMB onto the phospholipid bilayers. These results are consistent with preliminary results on electrophoretic mobility of the liposomes in

the presence of PHMB: The egg PC-DCP dispersion gave negative value of mobility and the addition of PHMB brought about reversal of the surface charge, giving positive values of mobility.[15]

In conclusion, our work has demonstrated that adsorption of PHMB takes place to a greater extent on to a negatively-charged membrane than on to a neutral membrane. This indicates that the adsorption site for PHMB is mainly negatively-charged species. In bacterial cytoplasmic membranes, there have been found many negatively-charged species such as membrane-bound proteins and acidic phospholipids.[16] Thus our work has provided reason for believing that the target sites for PHMB interaction are these negatively-charged species in intact bacterial membranes.

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