Biochimica et Biophysica Acta, 1026 (1990) 105–112
Elsevier

BBAMEM 74911

Phase separation in phospholipid bilayers induced by biologically active polycations

Tomiki Ikeda, Hideki Yamaguchi and Shigeo Tazuke †

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama (Japan)

(Received 15 December 1989)

Key words Phase separation, Polycation, Phospholipid bilayer, Antimicrobial activity, Antibacterial activity, Minimum inhibitory concentration, DSC

The interaction of various polyionenes with phospholipid bilayer membranes was explored by means of differential scanning calorimetry (DSC) with special reference to their antimicrobial activities. A strong interaction was observed between the polyionenes and acidic phospholipids, whereas zwitterionic phospholipid bilayers were not affected significantly by the polycations. Addition of the polyionenes was found to result in phase separation in mixed bilayer membranes composed of acidic and zwitterionic phospholipids. The ability to induce phase separation strongly depended on the structure of the polyionenes. Polyionenes with rigid spacers were found to be most effective to induce phase separation and to be most active in antimicrobial activity. Polyionenes with rigid and flexible spacers in the alternate fashion exhibited less activities which were similar to those of all flexible spacers. Furthermore, their mode of interaction with bilayers was again similar to those of all flexible spacers. Our results indicate that the rigid spacers are favorable for strong interaction with membranes which are assumed to be the target sites of the polycationic biocides, leading to the higher activity. Other factors affecting both the antimicrobial activity and the mode of interaction with membranes were molecular weight and hydrophobicity. With increasing molecular weight, both the activity and ability to induce phase separation increased. Introduction of hydrophilic groups into the spacers resulted in loss of activity and ability to induce phase separation. The antimicrobial activity and the mode of interaction with membranes were correlated and interpreted on the basis of conformational concept of the polyionenes in solution.

Introduction

Many studies have been done on antibacterial activity of polyionenes, polycations containing quaternary ammonium salts in their main chains, and it was revealed that the polyionenes in general exhibit higher antibacterial activity against various types of bacteria than their monomeric analogues [1–4] As in the case of low molecular weight cationic disinfectants, the target site of the polyionenes is the bacterial cytoplasmic membrane [5] Due to the high positive charge density of the polyionenes, they are easily adsorbed on to the negatively charged cytoplasmic membranes and disorganize the membrane structure with simultaneous loss of the membrane function [5,6] It has been reported that strong interaction of the polyionenes with the membranes results in immediate leakage of K^+ ions from the bacterial cells, which is followed by the loss of the other cytoplasmic constituents such as phosphate ions and materials which absorb light at 260 nm (mainly DNA and RNA 260-nm materials) [5] Although cytological studies revealed that the crucial process in the lethal action of the polycations is their interaction with the cytoplasmic membranes [6,7], it is still not obvious how the polycations interact with the membranes

There are two possible sites in the cytoplasmic membrane of bacteria for the polycations to interact with membrane proteins and phospholipids Although there is no definitive evidence available at present on which is the real target, there is some evidence to support that the polycations interact with matrix phospholipids of the membranes Broxton et al [8] investigated antibacterial activity of poly(hexamethylene biguanide hydrochloride), a similar in-chain polycation with biguanide repeat units, and found that this polycation disrupts the cytoplasmic membranes physically and causes the death of the cells The interaction is non-

† Deceased on July 11, 1989

Correspondence T Ikeda, Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama, Japan

specific, suggesting interaction of the polycation with matrix phospholipids [8] Furthermore, Booij et al [9] studied the interaction of low molecular weight cationic disinfectants with membranes and concluded that the site of their interaction is the phospholipid of the membranes [9]

It is well known that phospholipids present in the bacterial cytoplasmic membranes are classified into two groups zwitterionic (almost neutral) and acidic (negatively charged at physiological pH) lipids For example, in *Escherichia coli* zwitterionic phosphatidylethanolamine (PE) constitutes 70% of the total lipids and acidic phosphatidylglycerol (PG), 10% [10,11] The interaction of polycations with various phospholipids has been explored It has been found that polycations interact with acidic phospholipids strongly and induce isothermal phase separation in mixed bilayers of neutral and acidic phospholipids into a polycation-acidic phospholipid complex domain and a neutral lipid domain [12,13] However, little is known about the efficiency of various polycations to induce phase separation in relation to their antimicrobial activity

In this paper, we report interaction of polyionenes having various spacer structures between positively charged N atoms with mixed bilayers of neutral and acidic phospholipids We further report the antimicrobial activities of the polyionenes and discuss the structure-activity relationship in connection with the ability of the polyionenes to interact phospholipid membranes

TABLE I

Molecular weights of polyionenes

	Molecular weight	DP [a]
5	40000	170
10	30000	140
11	30000	140
12	23000	110
13	–	–
14	24000	100
15	19000	100
16	7000	40

[a] Degree of polymerization

Materials and Methods

Materials

Structures of the ionenes used in this study are shown in Fig 1 The ionenes were prepared by the Menschutkin reaction between diamines and dibromides in dimethylformamide and purified by repeated precipitations [5] Molecular weights of the polyionenes were measured with a Chromatix KMX-6 low-angle laser light scattering photometer in phosphate

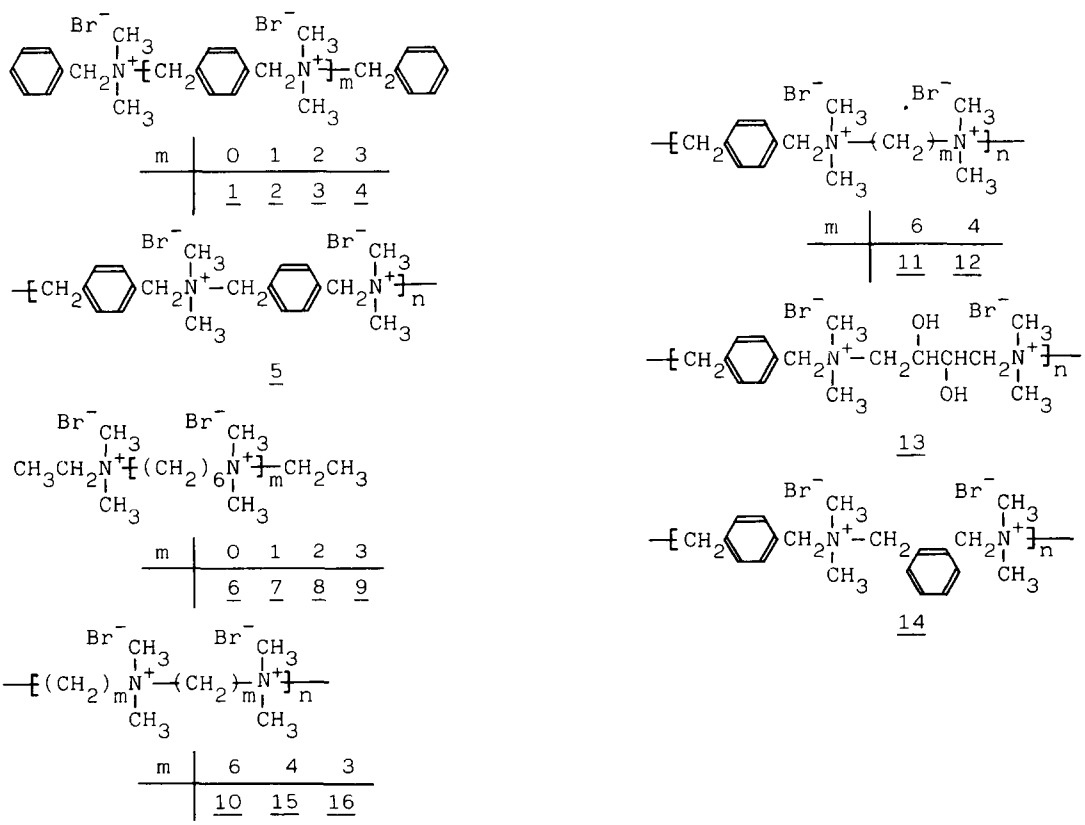

Fig 1 Structures of oligo- and polyionenes used in this work and their abbreviations

buffer (pH 7 4) and their values as well as the degree of polymerization are listed in Table I

Dimyristoyl-L-α-phosphatidylcholine (DMPC) and phosphatidic acid prepared from egg PC (egg PA) were obtained from Sigma and used without further purification

Differential scanning calorimetry (DSC)

DSC measurements were performed on a SEIKO I&E SSC-5000 calorimeter equipped with a DSC-100 terminal operating at a scan rate of 1 C°/min Phospholipid dispersions in phosphate buffer (50 mM Na_2HPO_4 + 50 mM $Na_2H_2PO_4$, $\mu = 0\ 15$ by NaCl, pH 7 4) were prepared by vortex agitation above the phase transition temperature of the lipid (T_m) (lipid concentration, 2 5 wt%) [12] After aging, the ionene solution in the same buffer (10 μl) was added to the phospholipid dispersion (40 μl) with vigorous agitation and the mixture was left above T_m for 2 h The sample solution was then transferred to a 70-μl aluminum sample pan and sealed For each sample, at least four scans were performed to check the reproducibility Since heating curves provided more accurate transition temperatures, only heating curves are shown here

Antimicrobial assessment

Minimum inhibitory concentration (MIC) was evaluated by the conventional spread plate method as described previously [6] The period of lag phase in the growth curve in bacterial suspension was measured with an Ohtake BioScanner OT-BS-48 The overnight culture of *Staphylococcus aureus* was washed with sterile distilled water, collected by centrifugation and then suspended in the sterile distilled water so as to give a cell concentration of approx 3 10^6 cells/ml In a 5-ml cell, 1 0 ml of nutrient broth (peptone, 10 g, beef extract, 5 0 g, NaCl, 5 0 g in 1000 ml of sterile distilled water, pH 6 8) and 0 5 ml of the ionene solution in sterile distilled water were placed and equilibrated at 37°C in the BioScanner Measurement was started when 3 5 ml of the bacterial suspension preequilibrated at 37°C were added to the cell The absorbance at 650 nm of the resulting solution was measured in situ periodically and the time required to reach an absorbance > 0 1 was taken as the period of the lag phase During measurements, the sample solution was rotated at 15 rpm and the temperature was kept at 37°C

Results and Discussion

Interaction of ionenes with phospholipid membranes

Addition of **5** to the neutral bilayer of DMPC alone caused no significant change in its thermotropic properties In order to explore the polyionene-induced phase separation behavior in mixed membranes of neutral and acidic phospholipids, a mixture of DMPC and egg PA

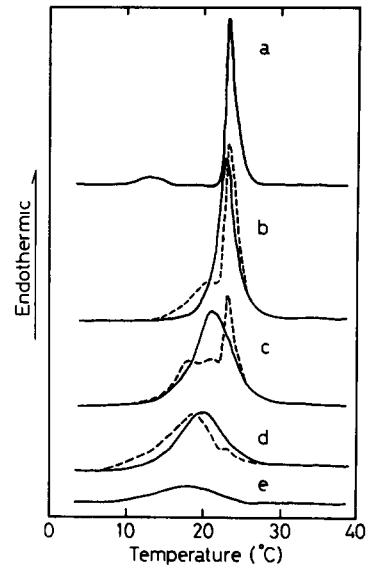

Fig 2 Thermotropic properties of the mixed bilayer of DMPC and egg PA in various proportions of the two components and the effect of polycation **5** on the mixed membranes a, pure DMPC, b, DMPC/egg PA = 9 1 (w/w), c, DMPC/egg PA = 8 2 (w/w), d, DMPC/egg PA = 5 5 (w/w), e, pure egg PA ———, in the absence of **5**, ------, in the presence of 5 mg/ml of **5** Phospholipid dispersions were prepared in phosphate buffer (pH 7 4), and 40 μl of the lipid dispersion (2 5 wt%) and 10 μl of the ionene soltion in the same buffer was mixed, aged and then measured on DSC at the scan rate of 1 C°/min Only the heating curves are shown

was employed Thermotropic properties of the mixed bilayer of DMPC/egg PA in various proportions of the two components are shown in Fig 2 In any proportion, the mixture exhibited a single endothermic peak at an intermediate temperature between T_m values of the component phospholipids Since the egg PA dispersion gave a broad endothermic peak (Fig 2, e) due to heterogeneity in the acyl chain composition, the mixture with higher compositional ratio of egg PA showed a broader peak Effect of polyionene **5** to the mixed bilayer is also included in Fig 2 Addition of 5 mg/ml of **5** to the mixed bilayer resulted in appearance of two endothermic peaks, one occurring at a higher temperature corresponds to T_m of DMPC Two endothermic peaks were observed in all mixed bilayers of different composition, and these results clearly indicate that the polyionene **5** can induce phase separation in the mixture of neutral and acidic phospholipids into a polycation-acidic lipid complex domain and a neutral lipid domain

Effect of concentration of two types of polyionenes on the phase separation behavior in the DMPC/egg PA mixed membrane was examined (Fig 3) When 0 5 mg/ml of polyionene **5** with rigid *p*-xylylene spacer was added to 20 mg/ml of the DMPC/egg PA mixture (DMPC/egg PA = 8 2, w/w), a peak maximum was shifted to a high temperature and a shoulder was observed at a temperature which corresponds to T_m of DMPC (A-c) With increasing concentration of the

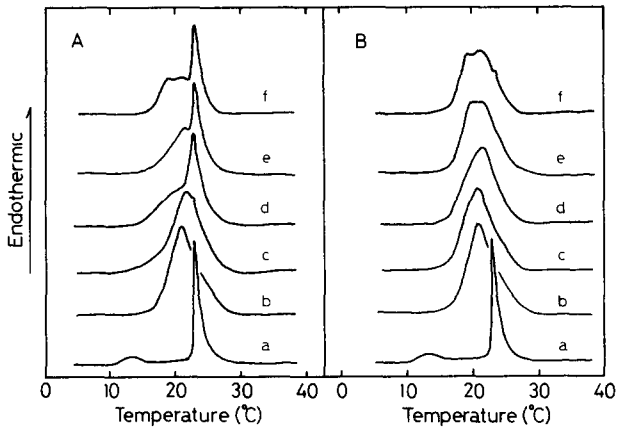

Fig 3 Effect of various amounts of polycations on DMPC/egg PA mixed bilayer (8 2, w/w) (A) Effect of **5** a, pure DMPC, b, DMPC/egg PA (8 2, w/w), c, DMPC/egg PA+0 5 mg/ml of **5**, d, DMPC/egg PA+1 0 mg/ml of **5**, e, DMPC/egg PA+2 5 mg/ml of **5**, f, DMPC/egg PA+5 0 mg/ml of **5** (B) Effect of **10** a, pure DMPC, b, DMPC/egg PA (8 2, w/w), c, DMPC/egg PA+0 5 mg/ml of **10**, d, DMPC/egg PA+1 0 mg/ml of **10**, e, DMPC/egg PA+2 5 mg/ml of **10**, f, DMPC/egg PA+5 0 mg/ml of **10** Phospholipid dispersions (20 mg/ml) were prepared as described in the legend to Fig 2

polycation **5**, phase separation took place more clearly In the presence of 1 0 mg/ml of **5**, the peak corresponding to T_m of DMPC still remained broad (A-d), while at higher concentrations of **5** (2 5 mg/ml or 5 0 mg/ml) this peak became narrower (A-e and f) In the presence of 5 0 mg/ml of **5**, FWHM (full width at half maximum) of the endothermic peak corresponding to T_m of DMPC was nearly equal to that of pure DMPC membrane ($\approx$ 1 C°) (A-a and f) This means that at this concentration of **5**, complete phase separation occurred and a domain composed of DMPC alone was formed

Effect of a polyionene with flexible spacer (**10**) on the mixed bilayer of DMPC/egg PA was quite different from that of the polyionene with rigid spacers (**5**) Addition of 0 5 mg/ml of **10** caused little significant change in the phase transition behavior of the mixed membrane (Fig 3, B-c) At higher concentrations (2 5 mg/ml and 5 0 mg/ml), the endothermic peak was split mainly into two maxima, indicative of the presence of two different domains (B-e and f) At the highest concentration examined (5 0 mg/ml), a shoulder was observed in the high temperature region, which corresponds to the T_m of DMPC These results indicate that although the polyionene **10** can induce phase separation in the mixed bilayer, a pure DMPC domain is scarcely formed, but rather are formed two domains One corresponds to a polycation-acidic lipid complex domain and the other to a DMPC-egg PA domain with higher compositional ratio of DMPC than the original mixture

Effect of molecular weight of ionenes on the phase transition behavior of the mixed bilayer of DMPC/egg PA is shown in Fig 4 Two series of ionenes with different spacer structure were examined one with *p*-xylylene spacer and the other with hexamethylene spacer When a monomer of the homologues having the xylylene spacer was added to the mixed membrane of DMPC/egg PA (8 2, w/w), the endothermic peak was shifted to a lower temperature by 2 C°, but the peak was still single and no sign of phase separation was observed (curve c) Addition of a dimeric homologue (**2**) produced two maxima in the endothermic peak (curve d) In the dimer, there are two positively charged N atoms in the molecule, thus aggregation of acidic lipids in the vicinity of the adsorption site takes place to some extent Addition of a tetrameric homologue (**4**) induced phase separation more clearly than the lower molecular weight homologues Two domains were clearly formed as seen in the two maxima of the endothermic peak (curve e) Phase separation was observed to most extent when the polymeric homologue (**5**) was added (curve f)

Effect of molecular weight was much less obvious in the homologues having hexamethylene spacers Addition of the monomeric (**6**) or the dimeric (**7**) cations to the same mixed bilayer caused a shift of the original endothermic peak to a lower temperature only by 0 5 C° without any other significant change (curves g and h) When a tetrameric homologue (**9**) was added, a shoulder was observed at a high temperature, indicative of phase separation only to some extent (curve i) As was described already, the polymeric homologue (**10**) induced phase separation, but to a lesser extent than **5** (curve j)

Phase separation behavior of the mixed bilayer of DMPC/egg PA induced by various polyionenes is

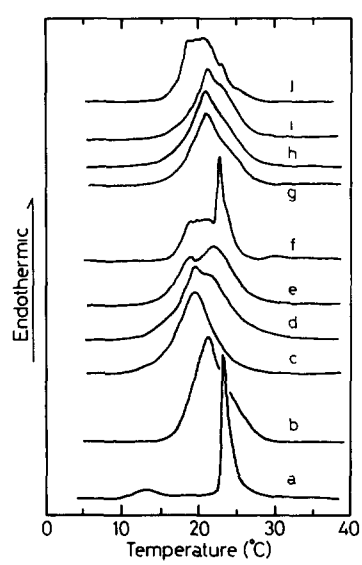

Fig 4 Effect of oligo- and polycations on mixed bilayer of DMPC/egg PA (8 2, w/w) a, pure DMPC, b, DMPC/egg PA (8 2, w/w), c, **1**, d, **2**, e, **4**, f, **5**, g, **6**, h, **7**, i, **9**, j, **10** The mixed phospholipid dispersions (20 mg/ml) were prepared as described in the legend to Fig 2 and the concentrations of the ionenes were 5 mg/ml

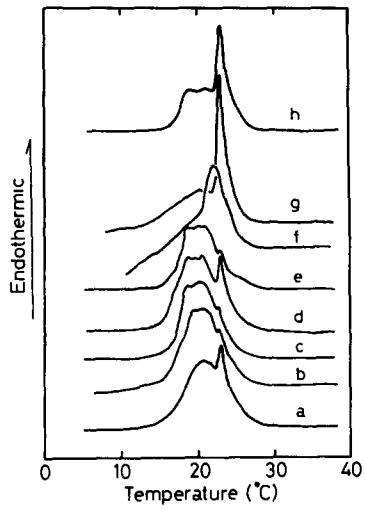

Fig 5 Effect of various polycations on mixed bilayer of DMPC/egg PA (8 2, w/w) a, **16**, b, **15**, c, **10**, d, **12**, e, **11**, f, **13**, g, **14**, h, **5** The mixed phospholipid dispersions (20 mg/ml) were prepared as described in the legend to Fig 2 and the concentrations of the ionenes were 5 mg/ml

shown in Fig 5, which contains the thermograms of the DMPC/egg PA/**5** and DMPC/egg PA/**10** mixtures for comparison Among the polyionenes having methylene spacers (**10**, **15** and **16**), one with the shortest spacer length (trimethylene, **16**) seems to be most effective in inducing phase separation (curve a) With increasing spacer length, the peak due to the pure DMPC domain became less obvious (curves b and c) However, interaction of the polycation with the bilayer in the polycation-acidic lipid complex domain seems to be enhanced with increasing spacer length The endothermic peak observed when the polyionene with hexamethylene spacer (**10**) was added (curve c) is clearly shifted to a lower temperature than those of DMPC/egg PA/**15** (curve b) and DMPC/egg PA/**16** mixtures (curve a) This stronger interaction of **10** with egg PA is most probably produced by the wedge effect exhibited by the longer, more hydrophobic hexamethylene spacer of **10** Comparison of the thermograms of curve b–e reveals an interesting effect of rigid spacer on the phase transition behavior of the DMPC/egg PA mixed bilayer Introduction of the rigid *p*-xylylene spacer into one of the two successive hexamethylene spacers (**11**) did not cause any significant change in the phase transition behavior as is seen in curves c and e This result suggests that even though rigid spacer is incorporated in the alternate manner into the main chain the overall mode of the interaction of the resulting polyionene with the bilayer is scarcely affected and in fact is governed by the flexible hexamethylene spacer On the other hand, replacement of one of the two successive tetramethylene spacers in **15** by the *p*-xylylene spacer (**12**) enhanced phase separation and the peak due to the pure DMPC domain was more clearly observed than that in the DMPC/egg PA/**15** mixture (curves b and d) This means that in the polyionene having short flexible spacers the introduction of rigid spacers in the alternate fashion caused a significant effect on its mode of interaction with the mixed bilayers However, in the polycation-egg PA complex domain, the mode of interaction seems to be unaffected by the introduction of the xylylene spacer since the peak at a lower temperature oc-

TABLE II

Antimicrobial activities of various polyionenes [a]

	Bacillus subtilis	*S aureus*	*E coli*	*Aerobacter aerogenes*	*Pseudomonas aeruginosa*
5	5– 10	5–10	100–330	≈100	100–330
14	100–330	33–66	100–330	100–330	330–660
11	33– 66	10–33	100–330	100–330	100–330
12	33– 66	33–66	100–330	100–330	100–330
13	100–330	33–66	>1000	>1000	>1000
10	33– 66	33–66	100–330	100–330	100–330
15	33– 66	33–66	100–330	100–330	100–330
16	≈66	33–66	100–330	100–330	100–330
	Aspergillus niger	*Penicillium citrinum*	*Trichoderma* T-1	*Torulopsis candida*	*Geotrichum candidum*
5	100– 330	100– 330	330– 660	10– 33	100–330
14	660–1000	660–1000	660–1000	100–330	100–330
11	330– 660	100– 330	330– 660	10– 33	100–330
12	660–1000	100– 330	330– 660	33– 66	100–330
13	>1000	>1000	>1000	100–330	>1000
10	660–1000	100– 330	660–1000	10– 33	100–330
15	>1000	330– 660	>1000	33– 66	100–330
16	>1000	>1000	>1000	100–330	100–330

[a] MIC (μg/ml) determined by the spread plate method

curred nearly at the same temperature Introduction of hydrophilic OH groups into the flexible tetramethylene parts of **12** drastically changed the mode of interaction of the resulting polyionene with the mixed bilayer As is seen in curve f, the resulting polyionene (**13**) did scarcely cause change in the endothermic peak of the mixed bilayer

Biological activities of polyionenes

Table II shows antimicrobial activities of polyionenes against various microbes such as bacteria (*B subtilis, S aureus, E coli, A Aerogenes* and *P aeruginosa*) and fungi (*A niger, P citrum, T* T-1, *T candida* and *G candium*) Figures in the table indicate the range of minimum inhibitory concentration (MIC, μg/ml) General trend can be seen in the table that polyionenes are more active against Gram-positive bacteria (*B subtilis, S aureus*) than against Gram-negative species (*E coli, A aerogenes* and *P aeruginosa*) and fungi Among the polyionenes examined **5** is most active and has a wide spectrum of antimicrobial activity Replacement of one of the two successive *p*-xylylene spacers in **5** by *o*-xylylene spacer lowered the activity of the resulting polyionene **14** Furthermore, introduction of the hydrophilic OH groups in **12** also led to loss of the activity as seen for MIC values of **13** Among the polyionenes having alkyl spacers (**10**, **15** and **16**), effect of spacer length on antibacterial activities could not be clarly recognized, although on antifungal activities it is evident the activities increased with increasing spacer length (**16** < **15** < **10**) except *G candium* The rigid *p*-xylylene spacer present in the main chain in the alternate manner as in **11** and **12** caused no significant change in antimicrobial activities in comparison with their reference polyionenes **10** and **15**, but from another point of view replacement of rigid spacers in **5** by flexible alkyl spacers in the alternate fashion results in loss of the activities against Gram-positive species

In order to see the difference in activity more quantiatively, antibacterial activity against *S aureus* was evaluated by the period of the lag phase in growth curve of the *S aureus* suspension in the presence of various concentrations of the polyionenes and the results are shown in Table III In this table figures indicate the period of the lag phase expressed in h In the absence of the polyionenes, the lag phase was 3 0 h Among the polyionenes having both the *p*-xylylene spacer and the alkyl spacer in the alternate manner (**11**, **12** and **13**), **11** was most active and **13** was least active Furthermore, among the polyionenes having all alkyl spacers (**10**, **15** and **16**), **10** was most active and the order of activity is quite clearly seen as **16** < **15** < **10**

TABLE III

Antibacterial activities of polyionenes against S aureus [a]

	Concentration (μg/ml)			
	1 0	3 0	5 0	10 0
11	10 5	>18 0	>18 0	>18 0
12	7 5	15 0	>18 0	>18 0
13	4 0	4 5	6 0	9 0
10	12 0	>18 0	>18 0	>18 0
15	9 0	13 5	>18 0	>18 0
16	7 5	10 5	16 5	>18 0

[a] Period of lag phase expressed in h which was determined by measurement of absorbance at 650 nm Blank, 3 0 h

Mode of interaction of ionenes with bilayer membranes in relation to their antibacterial activity

The results obtained in this work clearly demonstrate that both the antimicrobial activity and the mode of interaction with the bilayer membranes are strongly affected by the spacer structures of the polyionenes which link the positively charged N atoms in the main chain The significant findings on the antimicrobial activity are (1) rigid spacer is most favorable for high activity, (2) the activity is governed by flexible parts when both the rigid and flexible spacers are present, (3) among the polyionenes with all flexible (alkyl) spacers, those with longer chains exhibit higher activity, (4) introduction of hydrophilic groups into the spacers reduces the activity In relation to the polyionene–membrane interaction, the following results are significant (1) the polyionenes with rigid spacers induce phase separation in the mixed bilayer of neutral and acidic phospholipids most effectively, (2) the ability to induce phase separation is mainly governed by the flexible parts when both the rigid and flexible spacers coexist, (3) hydrophilic groups lower the ability to induce phase separation From these significant findings, correlation between the antimicrobial activity and the ability to induce phase separation is clear The most active polyionene was **5** and this polycation induced phase separation most effectively In general, polycations which interact with bilayer membranes of neutral and acidic phospholipids more strongly in terms of phase separation seem to show higher antimicrobial activity

It is well known that conformation of polyelectrolytes in aqueous solutions is strongly dependent on spacer structure between charged atoms and ionic strength (μ) of the solution [14] In the absence of added single electrolytes ($\mu \approx 0$), the polyelectrolytes tend to take the most expanded form of conformation due to electrostatic repulsion between the same charges on the polymer chains [14] With increasing μ, the electrostatic repulsion is weakened by shielding effect of the added ions with opposite charge, thus at $\mu \approx 0\,01$ the polyelectrolytes usually take a random and entangled conformation in dilute solution [14] This is true for polyelectrolytes with flexible spacers However, the conformation of polyelectrolytes with rigid spacers is

not determined by μ alone, but it depends on stiffness of the spacers Thus, in the case of **5**, it is reasonably expected that this polycation has rather an expanded form even under high μ conditions In the present study, both DSC measurements and antimicrobial assessment were performed under such conditions as to keep the μ of the samples high enough for polycations with flexible spacers to adopt the entangled conformation Therefore, the difference in the mode of interaction of various polycations with bilayer membranes may be ascribed to the difference in their conformation in the sample solutions It is assumed that **5** in the expanded form behaves like a rod whereas the polycations with flexible spacers act like spheres, thus **5** has the most preferable shape for the interaction with planar electric double layers (charged bilayers) In the entangled form of the polycations with flexible spacers, positively charged moieties are expected to be distributed in a relatively uniform manner on the surface of the sphere in aqueous solutions, but some of the charged moieties may reside inside the polymer coil [14] These circumstances reduce drastically the effective charges of the polycations in their electrostatic interaction with negatively charged bilayers In the phase separation events, negatively charged lipids must gather in the vicinity of the adsorption site of the polycation Efficiency of phase separation is solely a function of the number of negatively charged lipid molecules that are gathered around the absorbed polycation, thus it is evident that the rod-like polycation is much more favorable in inducing phase separation than those with the entangled, spherical shape

It must be mentioend here that preliminary results on the binding isotherms of the oligo- and polycations used in this study toward intact bacterial cells which are negatively charged have shown that the oligo- and polycations with a degree of polymerization (DP) larger than 3 exhibited similar patterns of binding isotherms and in the oligocations with DP less than 3 the degree of binding increased with increasing DP It seems most likely that these results are applicable to the present cation–bilayer membrane interactions Namely, in the homologues with *p*-xylylene spacers the degree of phase separation increased with increasing molecular weight (Fig 4), and this phenomenon seems to be explicable in terms of the increasing degree of binding of the oligocations to the negatively charged bilayer membranes It is of particular interest that in the low molecular weight homologues of **5** MIC against *B subtilis* and *S aureus* was found to decrease with increasing molecular weight for example, against *S aureus,* MIC >1000 μg/ml (**1**, **2**), 100–330 μg/ml (**3**) and 66–100 μg/ml (**4**) Together with the MIC for **5**, it is clear that the antibacterial activity increases with molecular weight This is exactly the order of ability to induce phase separation as shown in Fig 4 In contrast, in the case of the low molecular weight homologues of **10**, MIC against *B subtilis* and *S aureus* did not change with molecular weight (MIC >1000 μg/ml for **6–9**), which is well related to their ability to induce phase separation

When flexible and rigid spacers coexist in the alternate manner in the main chain, the resulting polyionenes became less active and their ability to induce phase separation was lowered These phenomena can again be understood on the basis of the conformational concept Even if polyionenes possess rigid parts, the overall conformation can be assumed to be controlled by flexible parts The mode of interaction of these polycations with bilayer membranes, therefore, is expected to be similar to that of polycations with all flexible spacers, which is just what we observed for **11** and **12** However, electrostatic repulsion operates more effectively in shorter distance, thus it is expected that polyionenes with shorter alkyl chains take a more expanded form and are more favorable in interaction with bilayer membranes This prediction is rationalized by the findings that the polyionene with the shortest alkyl spacer (**16**) showed the most effective phase separation among those of all alkyl spacers (**10**, **15** and **16**) Furthermore, among the polyionenes with the rigid and alkyl spacers (**11** and **12**), the polyionene with shorter alkyl spacer (**12**) exhibited higher efficiency for phase separation The importance of the polymer conformation in the polymer–membrane interactions has been pointed out recently by several research groups [15–25]

References

1 Rembaum, A , Rile, H and Somoano, R (1970) J Polym Sci B8, 457–466
2 Rembaum, A (1973) Appl Polym Symp 22, 299–317
3 Rajaraman, R , Rounds, D E , Yen, S P S and Rembaum, A (1975) in Polyelectrolytes and Their Applications (Rembaum, A and Selegny, E , eds), pp 163–174, Reidel, Dordrecht
4 Ottenbrite, R M and Myers, G R (1973) J Polym Sci Polym Chem Ed 11, 1443–1446
5 Ideda, T , Yamaguchi, H and Tazuke, S (1990) J Bioact Comp Polym 5, 31–41
6 Ideda, T , Yamaguchi, H and Tazuke, S (1984) Antimicrob Agents Chemother 26, 139–144
7 Ikeda, T , Hirayama, H , Yamaguchi, H and Tazuke, S (1986) Antimicrob Agents Chemother 30, 132–136
8 Broxton, P , Woodcock, P M and Gilbert, P (1983) J Appl Bacteriol 54, 345–353
9 Elferink, J G R and Booij, H L (1974) Biochem Pharmacol 23, 1413–1419
10 White, D A , Lenanrz, W and Schnaitman, C A (1972) J Bacteriol 109, 686–690
11 Costerton, J W , Ingram, J M and Cheng, K -J (1974) Bacteriol Rev 38, 87–110
12 Ikeda, T , Ledwith, A , Bamford, C H and Hann, R A (1984) Biochim Biophys Acta 769, 57–66
13 Kubesch, P , Boggs, J , Luciano, L , Maass, G and Tummber, B (1987) Biochemistry 26, 2139–2149
14 Tanford, C (1961) Physical Chemsitry of Macromolecules, pp 457–525, John Wiley, New York

15 Tamm, L (1987) Biochemistry 25, 7470–7476
16 Skehel, J J, Bayley, P M, Brown, E B, Martin, S R, Waterfield, M D, White, J M, Wilson, I A and Liley, C D (1982) Proc Natl Acad Sci USA 79, 968–972
17 Gething, M J, Doms, R W, York, D and White, J (1986) J Cell Biol 120, 11–23
18 Garcia, L A M, Araujo, S and Chaimovich, H (1984) Biochim Biophys Acta 772, 231–234
19 Subbarao, N K, Parente, R A, Szoka, Jr, F C, Nadasdi, L and Pongracz, K (1987) Biochemistry 26, 2964–2972
20 Myers, M, Mayorga, O L, Emtage, J and Freire, E (1987) Biochemistry 26, 4309–4315
21 Epand, R M, Hui, S W, Argan, C, Gillispie, L L and Shore, G C (1986) J Biol Chem 261, 10017–10020
22 Hancock, R E W (1984) Annu Rev Microbiol 38, 237–264
23 Hartmann, W, Galla, H J and Sackmann, E (1978) Biochim Biophys Acta 510, 124–139
24 Sixl, F and Galla, H J (1981) Biochim Biophys Acta 643, 626–635
25 El Mashak, E M and Tocanna, J F (1980) Biochim Biophys Acta 596, 165–179