Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. VII. Synthesis and Antibacterial Activity of Polymeric Phosphonium Salts and Their Model Compounds Containing Long Alkyl Chains

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SYNOPSIS

Various polymeric phosphonium salts containing long alkyl chains (C_{10}—C_{18}) and their corresponding model compounds were prepared, which possess the same hydrophobic structure as that of the common disinfectants (quaternary ammonium salts), and their antibacterial activities were evaluated by means of the viable cell counting method against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative). The polymer with the decyl group exhibited a higher activity than that of the corresponding model compound, particularly against the Gram-positive strain. Furthermore, antibacterial activity of the polymers was found to decrease as the chain length increased. In contrast with the polymers, the antibacterial activity of the corresponding model compounds increased as hydrophobicity of the substituents increased. The antibacterial activity was strongly dependent on the structure, particularly on the length of the alkyl chain. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Use of quaternary ammonium salts as disinfectants started early in the 1930s. Domagk found that benzalkonium chlorides were outstandingly effective for disinfection of skins and superior to phenols in the activity of killing bacteria.[1] Currently, the derivatives of benzalkonium and quaternary ammonium salts have been widely used in disinfection. The cationic disinfectants have a variety of structures: They are provided with the common structural features, positive charge, and a fairly hydrophobic part in a single molecule. In most of the quaternary ammonium salts, one long alkyl chain is covalently bonded to the positively charged nitrogen atom. The antibacterial activity is strongly dependent on the structure, and the length of the alkyl chain has been found to affect significantly the antibacterial activity.[2,3] For example, in a series of benzalkonium salts, those of 12–14 carbon atoms have been shown to possess the maximum activity. Thus, in the cationic disinfectants, the hydrophobic parts in the molecule play a significant role. On the other hand, very little study has been performed so far on the antibacterial activity of phosphonium salts with a long alkyl chain, because dimethylalkylphosphines as starting materials may not be readily available or are difficult to handle.

In a previous article, we reported that polymeric phosphonium salts with trialkyl groups (C_2—C_8) can exhibit a higher antibacterial activity than that of the corresponding low molecular weight model compounds.[4] In the present study, we describe the preparation of various polymeric phosphonium salts with a single long alkyl chain and the corresponding model compounds that possess the same hydrophobic structure as that of the common disinfectants (quaternary ammonium salts) and the evaluation of their antibacterial activity by means of the viable cell counting method.

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EXPERIMENTAL

Preparation

4-Chloromethylstyrene and 4-ethylbenzyl chloride were used without further purification. Dimethyldecylphosphine, dimethyltetradecylphosphine, and dimethyloctadecylphosphine were synthesized by the reported method.[5] The structures of phosphonium monomers and the corresponding model compounds used in this study are shown in Figure 1. ^{1}H-NMR measured on a Hitachi R-600 spectrometer showed the presence of water molecules in all compounds as expected from the highly hygroscopic nature of phosphonium chlorides. Poor agreement between calculated and found values of elemental analysis is most probably due to the absorption of water. Therefore, in the estimation of the calculated values of the elemental analysis, water molecules were included as described in the following section. Melting points of **1**, **2**, and **3** could not be determined because of polymerization during heating. Mass spectra (FAB) were measured on a JEOL JMS-AX 505W mass spectrometer.

Dimethyldecyl(4-vinylbenzyl)phosphonium Chloride (1)

4-Chloromethylstyrene (2.23 g, 14.6 mmol) was added dropwise to a vigorously stirred solution of dimethyldecylphosphine (1.97 g, 9.74 mmol) in methanol (2 mL) at room temperature under an atmosphere of nitrogen. The reaction mixture was left with stirring at room temperature for 40 h. The reaction mixture was then poured into an excess of diethyl ether. The precipitated crystal was collected by centrifugation, washed with diethyl ether, and dried under vacuum. The product was purified by reprecipitation of the dichloromethane solution into a large excess of diethyl ether. Yield 68%. NMR ($CDCl_3$, δ): 0.8–1.0 [3H, broad, $P-CH_2-(CH_2)_8-CH_3$], 1.0–1.6 [16H, m, $P-CH_2-(CH_2)_8-CH_3$], 2.03 [6H, d, $P-(CH_3)_2$], 2.3–2.7 [2H, m, $P-CH_2-(CH_2)_8-CH_3$], 4.28 (2H, d, $-CH_2-P$), 5.29 (1H, d, vinyl proton), 5.72 (1H, d, vinyl proton), 6.71 (1H, dd, vinyl proton), 7.38 (4H, s, aromatic protons), MS (FAB+) *m/e* 319 ($C_{21}H_{36}P^+$).

ANAL: Calcd for $C_{21}H_{36}PCl \cdot 1/4H_2O$ (359.45): C, 70.17%; H, 10.23%; Cl, 9.86%.
Found: C, 70.34%; H, 10.17%; Cl, 9.92%.

Dimethyltetradecyl(4-vinylbenzyl)phosphonium Chloride (2)

2 was prepared similarly to **1**. Yield 87%. NMR ($CDCl_3$, δ): 0.8–1.1 [3H, broad, $P-CH_2-(CH_2)_{12}-CH_3$], 1.1–1.7 [24H, m, $P-CH_2-(CH_2)_{12}-CH_3$], 2.08 [6H, d, $P-(CH_3)_2$], 2.3–2.7 [2H, m, $P-CH_2-(CH_2)_{12}-CH_3$], 4.30 (2H, d, $-CH_2-P$), 5.30 (1H, d, vinyl proton), 5.76 (1H, d, vinyl proton), 6.73 (1H, dd, vinyl proton), 7.39 (4H, s, aromatic protons), MS (FAB+) *m/e* 375 ($C_{25}H_{44}P^+$).

ANAL: Calcd for $C_{25}H_{44}PCl \cdot 1/2H_2O$ (420.06): C, 71.48%; H, 10.80%; Cl, 8.44%.
Found: C, 71.20%; H, 11.02%; Cl, 8.73%.

Dimethyloctadecyl(4-vinylbenzyl)phosphonium Chloride (3)

3 was prepared similarly to **1**. Yield 19%. NMR ($CDCl_3$, δ): 0.8–1.1 [3H, broad, $P-CH_2-(CH_2)_{16}-CH_3$], 1.1–1.7 [32H, m, $P-CH_2-(CH_2)_{16}-CH_3$], 2.07 [6H, d, $P-(CH_3)_2$], 2.2–2.6 [2H, m, $P-CH_2-(CH_2)_{16}-CH_3$], 4.26 (2H, d, $-CH_2-P$), 5.31 (1H, d, vinyl proton), 5.74 (1H, d, vinyl proton), 6.73 (1H, dd, vinyl proton), 7.39 (4H, s, aromatic protons), MS (FAB+) *m/e* 431 ($C_{29}H_{52}P^+$).

ANAL: Calcd for $C_{29}H_{52}PCl \cdot 1/3H_2O$ (473.16): C, 73.62%; H, 11.22%; Cl, 7.49%.
Found: C, 73.48%; H, 11.59%; Cl, 8.02%.

Dimethyldecyl(4-ethylbenzyl)phosphonium Chloride (4)

Reaction of 4-ethylbenzyl chloride (1.19 g, 7.72 mmol) with dimethyldecylphosphine (1.04 g, 5.14 mmol) was carried out at 140°C in bulk for 24 h under an atmosphere of nitrogen. The product was soluble in methanol and washed several times with *n*-hexane and evaporated to dryness. The product was dried under vacuum and then purified by reprecipitation of the dichloromethane solution into a large excess of diethyl ether. Yield 78%; mp 209–210°C. NMR ($CDCl_3$, δ): 0.7–1.0 [3H, broad, $P-CH_2-(CH_2)_8-CH_3$], 1.0–1.7 [19H, m, CH_3-CH_2-, $P-CH_2-(CH_2)_8-CH_3$], 2.10 [6H, d, $P-(CH_3)_2$], 2.3–2.9 [4H, m, CH_3-CH_2-, $P-CH_2-(CH_2)_8-CH_3$], 4.20 (2H, d, $-CH_2-P$), 7.28 (4H, s, aromatic protons), MS (FAB+) *m/e* 321 ($C_{21}H_{38}P^+$).

ANAL: Calcd for $C_{21}H_{38}PCl \cdot 1/2H_2O$ (365.97): C, 68.92%; H, 10.74%; Cl, 9.69%.
Found: C, 68.88%; H, 10.85%; Cl, 9.46%.

Dimethyltetradecyl(4-ethylbenzyl)phosphonium Chloride (5)

5 was prepared similarly to **4**. Yield 82%; mp 215–217°C. NMR ($CDCl_3$, δ): 0.8–1.1 [3H, broad,

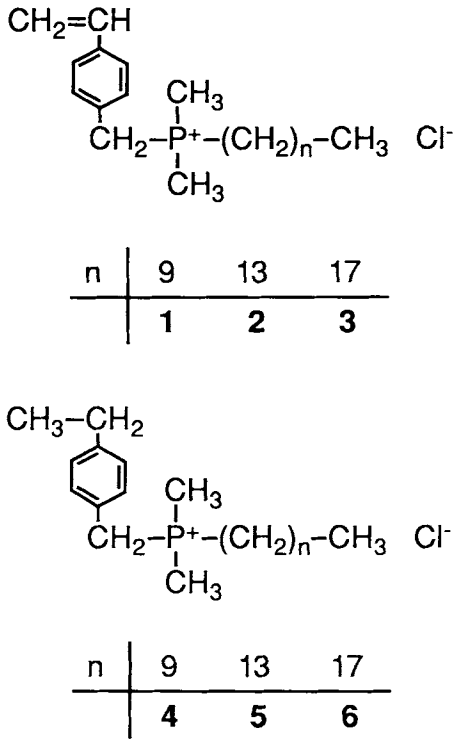

Figure 1 The structure of phosphonium salts used in this study.

P—CH_2—$(CH_2)_{12}$—CH_3], 1.1–1.7 [27H, m, CH_3—CH_2—, P—CH_2—$(\overline{CH_2})_{12}$—CH_3], 2.10 [6H, d, P—$(CH_3)_2$], 2.4–2.9 [4H, m, CH_3—CH_2—, P—CH_2—$(CH_2)_{12}$—CH_3], 4.22 (2H, d, —CH_2—P), 7.29 (4H, s, aromatic protons), MS (FAB+) *m/e* 377 ($C_{25}H_{46}P^+$).

ANAL: Calcd for $C_{25}H_{46}PCl \cdot 1/2H_2O$ (422.07): C, 71.14%; H, 11.22%; Cl, 8.40%.
Found: C, 70.93%; 11.03%; Cl, 8.06%.

Dimethyloctadecyl(4-ethylbenzyl)phosphonium Chloride (6)

6 was synthesized similarly to **4**. Yield 28%; mp 72–75°C. NMR ($CDCl_3$, δ): 0.8–1.1 [3H, broad, P—CH_2—$(CH_2)_{16}$—CH_3], 1.1–1.7 [35H, m, CH_3—CH_2—, P—CH_2—$(\overline{CH_2})_{16}$—CH_3], 2.10 [6H, d, P—$(CH_3)_2$], 2.3–2.9 [4H, m, CH_3—CH_2—, P—CH_2—$(CH_2)_{16}$—CH_3], 4.18 (2H, d, —CH_2—P), 7.29 (4H, s, aromatic protons), MS (FAB+) *m/e* 433 ($C_{29}H_{54}P^+$).

ANAL: Calcd for $C_{29}H_{54}PCl \cdot 1/2H_2O$ (478.18): C, 72.84%; H, 11.59%; Cl, 7.41%.
Found: C, 72.42%; H, 12.17%; Cl, 7.78%.

Polymerization

Polymerizations of **1**, **2**, and **3** were carried out at 60°C for 6 h in methanol/water (vol ratio 1 : 3) or methanol/toluene (vol ratio 1 : 3) with 2,2'-azobis-2-amidinopropane · 2HCl or AIBN as an initiator. Each polymerization tube was charged with the desired amounts of the monomer, the initiator, and solvent (20 mL) (see Table I). It was then degassed by three freeze–pump–thaw cycles under high vacuum, sealed off, and placed in a constant temperature bath at 60°C. After 6 h, the polymerization tube was opened, and the content was poured into an excess of acetone/diethyl ether (vol ratio 1 : 1 or 1 : 2) or acetone/water (vol ratio 2 : 1). The precipitated polymer was filtered off, washed with diethyl ether, and dried under vacuum. The conversion for each polymer is shown in the fifth column of the table. Each polymer was purified by reprecipitation of the chloroform solution into a large excess of diethyl ether. The weight-average molecular weight (M_w) was determined with a KMX-6 low-angle, laser light-scattering photometer in methanol and is listed in the last column of the table.

Antibacterial Assessment

Antibacterial activity of the polymers and the corresponding model compounds against *Staphylococcus aureus* (IFO 12732) and *Escherichia coli* (IFO 3806) was explored by the viable cell counting method already reported.[4]

Table I Conditions for Polymerization[a]

Monomer	Monomer Concentration (g/L)	Initiator Concentration (g/L)	Solvent	Conversion (%)	M_w[b]
1	50	0.92	CH_3OH/H_2O	80	89,500
2	40	0.64	CH_3OH/H_2O	60	95,000
3	30	0.20	CH_3OH/toluene	33	107,000

[a] Initiator, 2,2'-azobis-2-amidinopropane · 2HCl (for **1** and **2**); AIBN (for **3**); temperature, 60°C; time, 6 h.
[b] Determined with a low-angle, laser light-scattering photometer (KMX-6) in methanol.

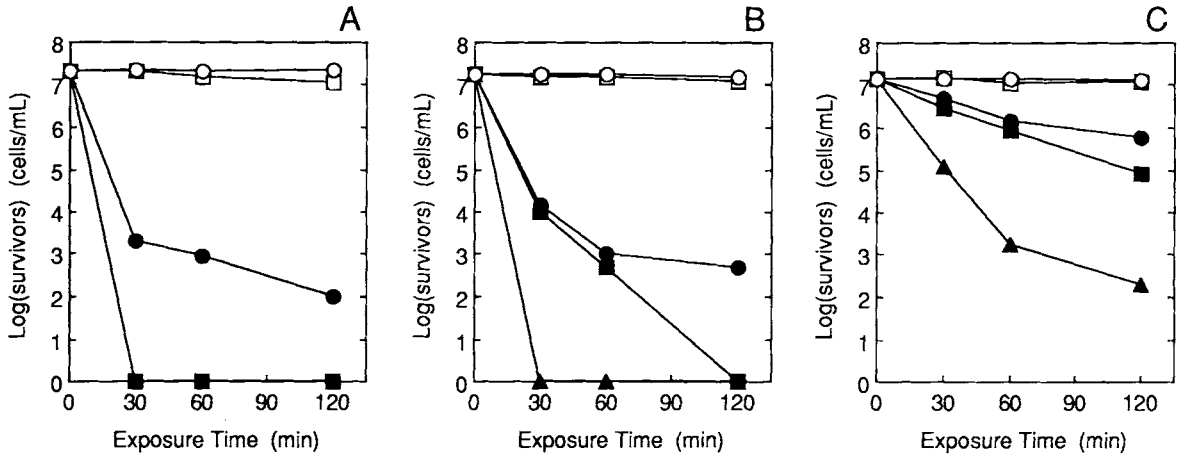

Figure 2 Plots of log(survivors) vs. exposure time for the polymers against *S. aureus*: (A) poly **1**; (B) poly **2**; (C) poly **3**. Concentrations: (○) blank; (▲) 280 μM; (■) 28 μM; (●) 2.8 μM; (□) 0.28 μM, based on the monomer units (100, 10, 1, and 0.1 μg/mL for poly **1**).

RESULTS

Figure 2 shows plots of log(survivors) vs. exposure time for poly **1**, poly **2**, and poly **3** against *S. aureus* (Gram-positive). About 10^7 cells/mL of *S. aureus* were exposed to 280, 28, 2.8, and 0.28 μM, based on the monomer units (100, 10, 1, and 0.1 μg/mL for poly **1**), of each polymer in saline. At the concentrations of 280 and 28 μM, poly **1** was capable of killing all the bacterial cells within 30 min of contact. At 2.8 μM, most of *S. aureus* (> 99.999%) were killed within 120 min of contact. At the lowest concentration (0.28 μM), poly **1** showed the antibacterial activity to some extent. These results indicated that poly **1** with the decyl group exhibited a particularly high activity [Fig. 2(A)]. Antibacterial activity of poly **2** with the tetradecyl group is shown in Figure 2(B). At the concentrations of 280 and 28 μM, all the bacterial cells were killed within 30 and 120 min, respectively. At 2.8 μM, > 99.99% of *S. aureus* were killed within 120 min of contact, and poly **2** was inactive at 0.28 μM. With the longest alkyl chain (octadecyl; poly **3**), ca. 99.999, 99, and 90% of the bacterial cells were killed within 120 min at the concentration of 280, 28, and 2.8 μM, respectively. At 0.28 μM, poly **3** was also inactive [Fig. 2(C)]. These results clearly demonstrate that the alkyl chain length strongly affects the antibacterial activity of the polymeric phosphonium salts and the activity decreased as the chain length increased. Furthermore, the concentration dependence in the antibacterial activity could be observed for all the polymers.

Figure 3 indicates the same plots for the model compounds (**4–6**) with different alkyl chains. About 10^7 cells/mL of *S. aureus* were exposed to 280, 28, 2.8, and 0.28 μM (100, 10, 1, and 0.1 μg/mL for **4**) of the model compounds (**4–6**) in saline. At the concentration of 280 μM, **4–6** were capable of killing all the bacterial cells within 30 min of contact. At 28 μM of **4**, ca. 99.99% of *S. aureus* were killed within 120 min of contact [Fig. 3(A)], whereas **5** and **6** killed all the bacterial cells within 30 min of contact [Fig. 3(B) and (C)]. Even at 2.8 μM of **6** with the longest alkyl chain, all the bacterial cells were killed within 30 min of contact [Fig. 3(C)]. On the other hand, **4** and **5** killed only < 90 and ca. 99.99% of *S. aureus* within 120 min of contact, respectively [Fig. 3(A) and (B)]. At the lowest concentration (0.28 μM), all the model compounds were inactive. These results indicate that the model compounds exhibited a high antibacterial activity against *S. aureus* and the activity was in the order of **4** < **5** < **6**. In contrast with the polymer, the antibacterial activity of the model compounds increased as hydrophobicity of the substituents increased. Only poly **1** with the decyl group exhibited a higher activity than that of the corresponding monomeric model compound.

With the antibacterial activity against *E. coli* (Gram-negative), a similar trend to that against *S. aureus* was observed. The antibacterial activity of the polymers against *E. coli* was lower than that against *S. aureus* (see Fig. 4). At the highest concentration of poly **1** and poly **2** (280 μM), ca. 99.9% of *E. coli* were killed within 120 min of contact, whereas only ca. 99% of the strain were killed within

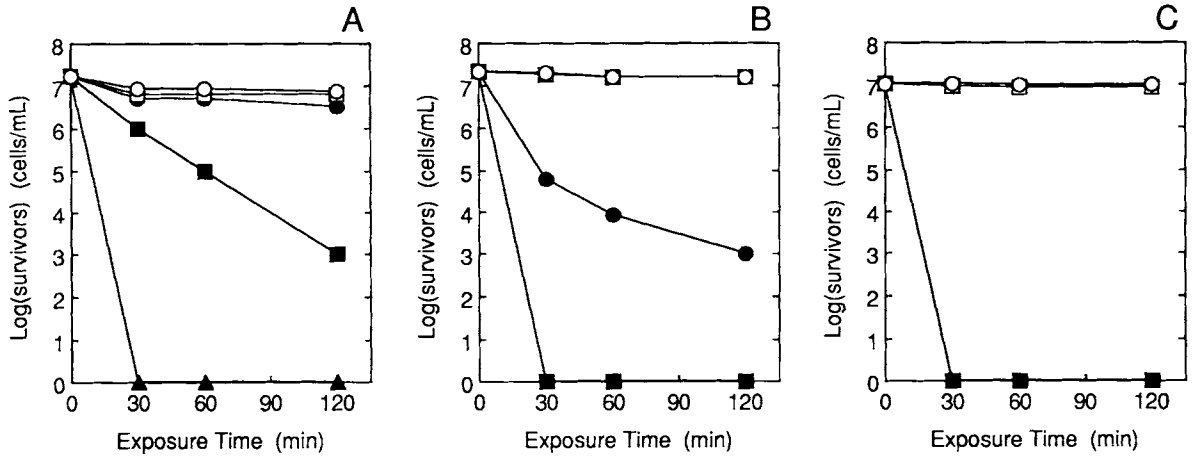

Figure 3 Plots of log(survivors) vs. exposure time for the model compounds against *S. aureus:* (A) **4**; (B) **5**; (C) **6**. Concentrations: (○) blank; (▲) 280 μ*M*; (■) 28 μ*M*; (●) 2.8 μ*M*; (□) 0.28 μ*M*.

120 min of contact with poly **3**. Figure 5 shows plots of log(survivors) vs. exposure time for the model compounds (**4–6**) against *E. coli*. About 10^7 cells/mL of *E. coli* were exposed to 280, 28, 2.8, and 0.28 μ*M* (100, 10, 1, and 0.1 μg/mL for **4**) of the model compounds in saline. At the concentration of 280 μ*M*, **4**, with the shortest alkyl chain, needed a longer time (120 min) to kill the bacterial cells completely, whereas **5** and **6** killed all of *E. coli* within 30 min of contact. The model compounds **5** and **6** showed a high activity even at the concentration of 2.8 μ*M*, and **6** exhibited the highest activity against *E. coli* [Fig. 5(C)]. The antibacterial activity of the phosphonium salts against *E. coli* was evidently different from that against *S. aureus*. In general, the low molecular weight compounds showed a higher activity against the Gram-negative strain than did the corresponding polymeric analogs.

DISCUSSION

Antibacterial Activity of Cationic Biocides with Long Alkyl Chain

It has been reported that the target site of cationic disinfectants is the cytoplasmic membranes of microbes.[2,6,7] The main constituents of the cytoplasmic membrane are membrane proteins and phospholipids. Lipid bilayers and liposomes are frequently used

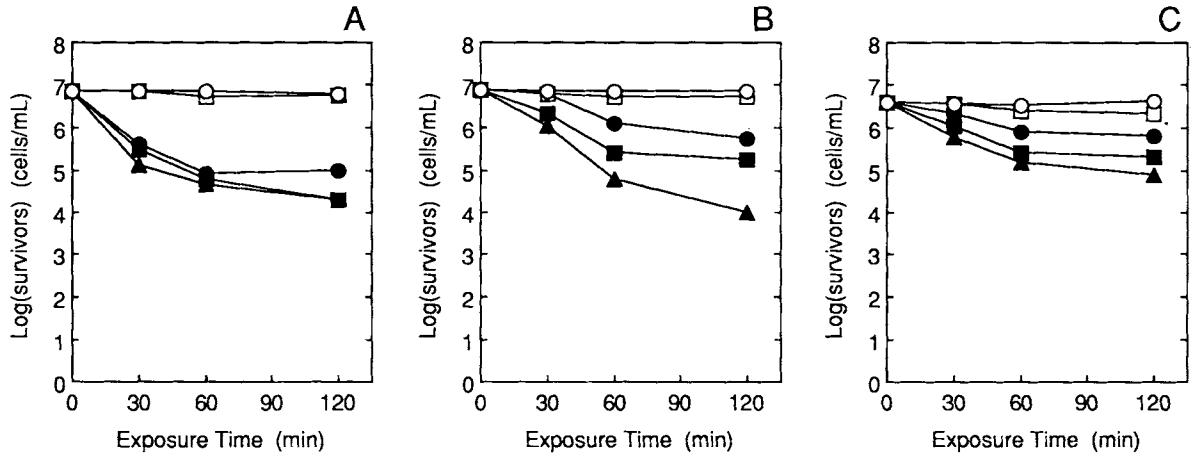

Figure 4 Plots of log(survivors) vs. exposure time for the polymers against *E. coli:* (A) poly **1**; (B) poly **2**; (C) poly **3**. Concentrations: (○) blank; (▲) 280 μ*M*; (■) 28 μ*M*; (●) 2.8 μ*M*; (□) 0.28 μ*M*, based on the monomer units (100, 10, 1, and 0.1 μg/mL for poly **1**).

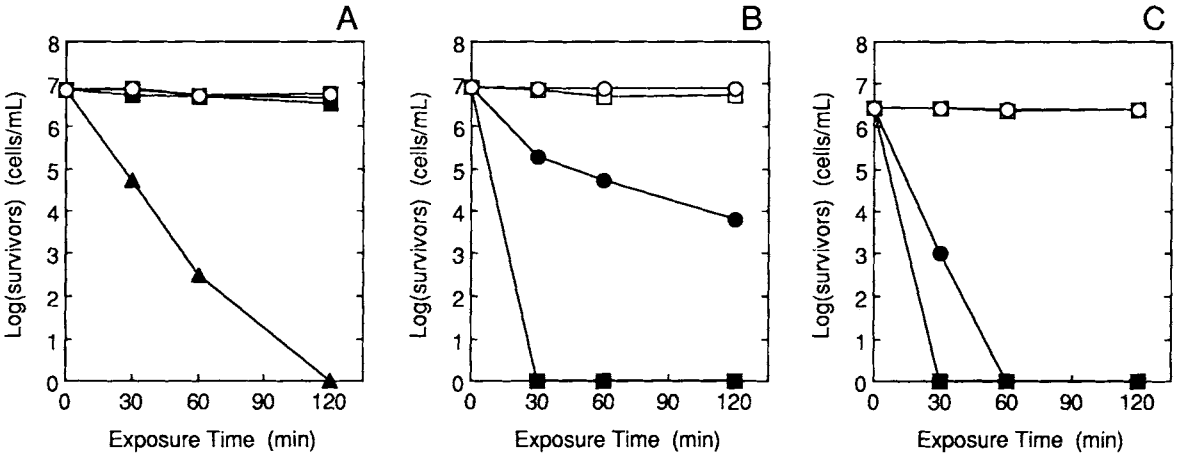

Figure 5 Plots of log(survivors) vs. exposure time for the model compounds against *E. coli:* (A) **4**; (B) **5**; (C) **6**. Concentrations: (○) blank; (▲) 280 μM; (■) 28 μM; (●) 2.8 μM; (□) 0.28 μM.

as experimental models for membranes.[8] The results from these model systems have proved useful for the understanding of phenomena occurring in the more complex biological membranes. Similarly, use can be made of the interactions known to occur between disinfectants and membrane lipids in aqueous solution to facilitate the interpretation of disinfectant effects on membranes. Many studies have been performed so far on the mode of interaction of low molecular weight cationic disinfectants as well as on polymeric forms with lipid bilayer membranes.[9-11] The cations cause aggregation and phase separation in the phospholipids. The phospholipids in the cytoplasmic membranes of bacteria are phosphoglycerides that have both a hydrophilic end (phosphate, often with other polar residues attached to it) and a hydrophobic end (two long-chain fatty acid tails possessing a carbon number of 12–20).[12] The cationic biocides possessing a long alkyl chain are assumed to strongly interact with the cytoplasmic membranes owing to an affinity of the molecular structure, leading to the higher activity. In fact, the model compounds (**4–6**) exhibited a high antibacterial activity against both *S. aureus* and *E. coli,* and their activities outstandingly increased as the chain length of the substituents increased. From these pieces of evidence, it was found that the long alkyl chain was more important to strongly interact with the membranes. Consequently, one of the factors affecting the mode of interaction of cationic disinfectants with membranes (phospholipids) is the hydrophobicity of substrates; in other words, the chain length of the substituents covalently attached to the hydrophilic part.

Effect of Hydrophobic Groups on Antibacterial Activity

Active cationic biocides possess a feature as surfactants since they have hydrophilic and hydrophobic moieties in a single molecule. Many amphiphiles aggregate in aqueous media to form micelles. Surfactants that have proven useful for the solubilization of phospholipids or membrane proteins form micelles themselves in aqueous solution. It has been reported that various types of surfactants including cationic surfactants solubilize the components of biological membranes containing phospholipids, membrane proteins, and cholesterol, which are insoluble amphiphiles.[13-15]

At the present stage, no correlation between membrane solubilization and antibacterial activity has been quantitatively approved; however, solubilization of components in the cytoplasmic membranes would be lethal and result in the death of bacterial cells. The amount of materials being solubilized into the surfactant micelles is affected by such factors as the size of the micelles.[16] The critical micelle concentration (cmc) of surfactants with the same hydrophilic groups decreases with increasing alkyl chain length, namely, micelle size increases as the alkyl chain length increases.[17-19] Another factor affecting the aggregation number of micelles is the free surfactant concentration in aqueous solution. These facts suggest that the surfactants forming large micelles may be favorable to interact with membranes, namely, surfactants with a long alkyl chain form micelles with a large aggregation number; thus, local concentration of surfactants (cationic

biocides) is very high, which is favored in view of interaction with membranes. Validity of this assumption may be evident from the results obtained for the low molecular weight model compounds (**4**–**6**).

The extent of solubilization depends mainly on the amount of surfactants bound to membranes, and surfactants generally cause solubilization of components in membranes at concentrations above cmc. However, it has been reported that the anionic surfactants induce the membrane solubilization and the denaturalization of many membrane proteins at concentrations below the cmc.[20,21] In addition, the adsorption of cationic surfactants onto the cell envelop is expected to be facilitated in comparison with that of anionic and nonionic surfactants since the bacterial cell surfaces are negatively charged. Such high local concentrations of surfactants at the cell envelop may be advantageous to accelerate the aggregation of surfactants. Therefore, the aggregates of cationic surfactants are assumed to enhance the interaction with the cytoplasmic membrane even at concentrations far below the cmc.

In polymeric surfactants, the polysoaps such as polymeric quaternary ammonium salts and pyridinium salts are known to form micelles by their single polymer chain at lower concentrations, and the hydrophobic groups in a molecule influence the size of assembly.[22] The effects of changes in the hydrophobic groups attached to the same polar head of a surfactant ion on micelle size and solubilization efficiency were investigated for the polysoaps. Their solubilization ability was improved with increasing side-chain length and degree of polymerization.[22] However, in the case of the cationic biocides, disinfectants must overcome the bacterial cell wall in order to reach their target sites: cytoplasmic membrane. Since the cell wall possesses a meshlike structure, disinfectant molecules of a small size are expected to diffuse freely through the cell wall. Diffusion through the cell wall is considered to become difficult for molecules with increasing molecular size. Such molecular weight dependence on antibacterial activity has been investigated for the polycationic biocides against intact cells and protoplasts that are freed from the cell wall.[23] The bell-shaped dependence of the antibacterial activity on the molecular weight was observed for the intact cells, whereas the antibacterial activity against protoplasts monotonously increased with increasing molecular weight. These results clearly indicate that the exclusion at the cell walls operates for polymers possessing high molecular weight (large molecular size). Therefore, the alkyl-chain-length dependence observed for the polymers with different alkyl chain lengths (poly **1**, poly **2**, and poly **3**) can be interpreted on the basis of their size of assembly, which is attributed to the long alkyl chain. Since these polymers have similar molecular weights, the only factor affecting the antibacterial activity is the alkyl chain length. Polycationic biocide possessing an optimal size of assembly is expected to show the maximum antibacterial activity, although it is still ambiguous how the polycationic biocides interact with the cytoplasmic membrane with subsequent disruption.

Strain Dependence of Sensitivity

Cationic disinfectants are, in general, more active against Gram-positive bacteria than they are against Gram-negative bacteria. The cell wall of the Gram-positive strain is composed mainly of peptidoglycan and teichoic acid and the overall structure is somewhat meshlike.[24,25] On the other hand, the structure of the cell wall of the Gram-negative bacteria is much more complicated than that of the Gram-positive species. There is another layer outside the peptidoglycan layer called the outer membrane that is composed mainly of lipopolysaccharides and phospholipids.[24] A significant role of the outer membrane is to protect a bacterial cell from attack by foreign compounds such as disinfectants. Thus, the much lower sensitivity of Gram-negative bacteria toward antibacterial agents is due mainly to the presence of the outer membrane. As described in the preceding section, Gram-negative strains were less sensitive toward the polymers than were Gram-positive strains. Because of the large molecular size, diffusion of the polymers through the cell wall is expected to become difficult for *E. coli*. In contrast, the low molecular weight model compounds exhibited a high activity against the Gram-negative strain, which may be interpreted in terms of favorable diffusion through the cell wall since their molecular size is very small. Consequently, the overall activity of phosphonium biocides would be determined by the structure of the substituents and the molecular size.

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