

New Polymeric Biocides: Synthesis and Antibacterial Activities of Polycations with Pendant Biguanide Groups

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Acrylate monomers with pendant biguanide groups were successfully synthesized, and their homopolymers and copolymers were prepared with acrylamide. These cationic disinfectants of polymeric forms exhibited high antibacterial activity against gram-positive bacteria, whereas they were less active against gram-negative bacteria. It was found that the activity of the polymeric disinfectants was much higher than that of the monomeric species, and the difference in activity between the polymers and the monomers was discussed on the basis of their contributions to each elementary process of the lethal action.

Quarternary ammonium salts and biguanides, both of which are positively charged at physiological pH, have been used widely as effective antibacterial agents. Their common features are the presence of a positively charged part and a fairly lipophilic part in the same molecule (12). Since the early work of Rose and co-workers (4, 13), biguanides have been employed widely as antimicrobial agents. Currently, chlorhexidine is one of the most popular disinfectants because of its broad spectrum of antibacterial activity, high kill rate, and nontoxicity toward mammalian cells (7).

At the present stage of study, the sequence of elementary events in the lethal action of the cationic disinfectants may be summarized as follows (7): (i) adsorption onto the bacterial cell surface; (ii) diffusion through the cell wall; (iii) binding to the cytoplasmic membrane; (iv) disruption of the cytoplasmic membrane; (v) release of K^+ ions and other cytoplasmic constituents; and (vi) precipitation of cell contents and the death of the cell. Electrophoretic measurements clearly demonstrate that the bacterial cell surface is usually negatively charged. The adsorption of polycations onto the negatively charged cell surface is expected to take place to a greater extent than that of monomeric cations because of the much higher charge density carried by the polycations. Furthermore, binding to the cytoplasmic membrane is also expected to be facilitated by the polycations, compared with that by the monomeric cations, because of the presence of a large number of negatively charged species (such as acidic phospholipids and some membrane proteins) in the membrane (7). Thus, the disruption of the membrane and the subsequent leakage of K^+ ions and other cytoplasmic constituents would be enhanced by the polycations. These considerations would lead to the expectation that cationic disinfectants of polymeric forms exhibit higher antibacterial activity than those of monomeric or dimeric forms.

To examine the advantage of the cationic disinfectants of polymeric forms in antibacterial activity, we prepared ethylenic biguanide derivatives which were either homopolymerizable or copolymerizable to high-molecular-weight polymers. We chose biguanide compounds because of their broad spectrum of activity and nontoxicity as mentioned above. These polymers were found to exhibit higher antibacterial activity than the relevant monomeric species, which would enable us to look into polymer effects in bactericidal action with reference to polymer structure and molecular weight.

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MATERIALS AND METHODS

Preparation. The synthetic route for monomers with pendant biguanide groups is shown in Fig. 1.

4-(2-Hydroxyethyl)aniline hydrochloride(II). p-Aminophenethylalcohol (Tokyo Kasei) (100 g; 0.73 mol) was dissolved in dioxane (1,000 ml), and after filtering, dry hydrochloric acid gas was passed through the solution with vigorous stirring at room temperature for 1 h. The precipitated salt was collected and dried under vacuum (crude product, 120 g; yield, 95%). It was then recrystallized from 2-propanol. Melting point (mp), 170 to 172°C; nuclear magnetic resonance (NMR) (CD_3OD , δ), 2.83 (2H, t, $-CH_2-$), 3.80 (2H, t, $-CH_2OH$), 5.30 (s, broad, NH_3-), 7.40 (4H, s, aromatic).

4-(2-Hydroxyethyl)phenyldicyandiamide(III). The procedure reported by Curd et al. was followed (3). A mixture of II (80 g; 0.46 mol), sodium dicyanamide (Kanto Chemical; 90% pure; 45.6 g), and distilled water (920 ml) was stirred at 90°C for 2.5 h. After cooling to room temperature, the precipitate was collected and dissolved in 2 N sodium hydroxide (100 ml) at 60°C. The insoluble part was removed by filtration, and the filtrate was made acidic (pH 2) with hydrochloric acid. The pale cream precipitate was collected, washed with water, dried under vacuum (64 g; yield, 68%) and then recrystallized from 2-propanol: mp, 172 to 174°C; NMR ($DMSO-d_6$, δ), 2.70 (2H, t, $-CH_2-$), 3.67 (2H, t, $-CH_2OH$), 6.92 (2H, s, dicyandiamide), 7.25 (4H, s, aromatic), 9.02 (1H, s, dicyandiamide). Elemental analysis: Calculated: C, 58.80; H, 5.92; N, 27.44. Found: C, 58.78; H, 5.98; N, 27.45.

4-(2-Acryloyloxyethyl)phenyldicyandiamide(IV). A solution of III (15 g; 0.074 mol) dissolved in tetrahydrofuran-water (10:1 [vol/vol]; 99 ml) was cooled in an ice bath; to this solution acryloyl chloride (Tokyo Kasei; 45 ml; 0.55 mol) was added dropwise with stirring over a period of 2 h, and the reaction mixture was left overnight at room temperature. It was then poured into a large excess of water, and the precipitate was collected and dried under vacuum (18 g; yield, 95%). It was recrystallized from an acetone-benzene (1:2 [vol/vol]) mixture. mp, 157 to 159°C; NMR ($DMSO-d_6$, δ), 2.90 (2H, t, $-CH_2-$), 4.30 (2H, t, $-CH_2O-$), 5.9-6.4 (3H, m, vinyl), 6.95 (2H, s, dicyandiamide), 7.30 (4H, s, aromatic), 9.05 (1H, s, dicyandiamide). Elemental analysis: Calculated: C, 60.45; H, 5.46; N, 21.69. Found: C, 60.10; H, 5.21; N, 21.52.

N^1 -4-(2-Acryloyloxyethyl)phenyl- N^5 -4-chlorophenylbiguanide hydrochloride(VI). 4-Chloroaniline hydrochloride (5.5 g;

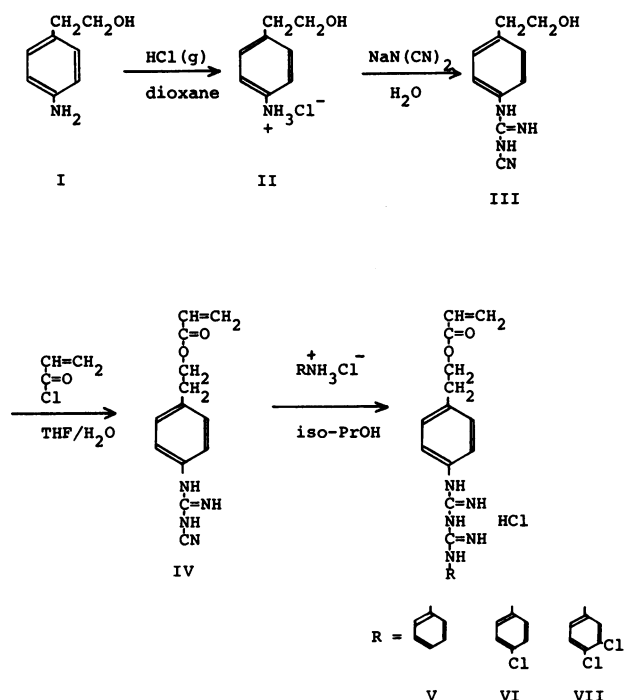


FIG. 1. Synthetic route for monomers with pendant biguanide groups.

0.034 mol), IV (8.7 g; 0.034 mol), and 2-propanol (30 ml) were refluxed in the presence of a small amount of hydroquinone for 15 min. The precipitate which formed on cooling was collected, washed with 2-propanol, dried under vacuum (9.7 g; yield, 69%), and recrystallized from 2-propanol: mp, 204 to 206°C; NMR (CD_3OD , δ), 2.93 (2H, t, $-\text{CH}_2-$), 4.33 (2H, t, $-\text{CH}_2\text{O}-$), 4.77 (6H, s, biguanide), 5.8-6.4 (3H, m, vinyl), 7.3 (4H, s, aromatic), 7.35 (4H, s, aromatic); (DMSO- d_6 , δ), 7.0-7.6 (broad, biguanide). Elemental analysis: Calculated: C, 54.04; H, 5.01; N, 16.58; Cl, 16.79. Found: C, 54.14; H, 4.81; N, 16.15; Cl, 16.13. N^1 -4-(2-Acryloyloxyethyl)phenyl- N^5 -phenylbiguanide hydrochloride (V) and N^1 -4-(2-acryloyloxyethyl)phenyl- N^5 -3,4-dichlorophenylbiguanide hydrochloride (VII) were prepared similarly (yield, 49% for V and 45% for VII; their structures were confirmed by NMR).

Polymerization. Homopolymers of V, VI, and VII. Polymerization was carried out at 60°C in dimethylformamide (DMF) with 2,2'-azobis(2-amidinopropane)dihydrochloride (Wako Chemical) as an initiator. Each polymerization tube was charged with desired amounts of the monomer, the initiator, and DMF (30 ml) (see Table 1). It was then degassed by three freeze-pump-thaw cycles under high vacu-

TABLE 2. Copolymerization of VI with acrylamide^a

Copolymer	Mol fraction of VI in:		Conversion (%)	M_w^b
	Monomers	Polymers		
VIII	0.200	0.152	92	— ^c
IX	0.400	0.263	90	18,900
X	0.598	0.426	83	34,500
XI	0.793	0.506	83	31,500

^a Solvent, DMF-H₂O (1:1 [vol/vol]); initiator, 2,2'-azobis-2-amidinopropane · 2 HCl; temperature, 60°C; time, 9 h.

^b Determined with a low-angle, laser light-scattering photometer (KMX-6) in methanol.

^c —, Not soluble in methanol.

um, sealed off, and placed in a constant temperature bath at 60°C. After the period indicated in Table 1, the polymerization tube was opened, and the content was poured into an excess of acetone (300 ml). The precipitated polymer was filtered off, washed with acetone, 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 methanol solution into a large excess of acetone. M_w was determined in methanol and listed in the last column of the table. It is evident that the values of M_w are not so different among the three polymers.

Copolymers of VI with acrylamide. Copolymerization was conducted at 60°C in mixed solvent of DMF-water (1:1 [vol/vol]) with the same initiator as that used in the homopolymerization. Each polymerization vessel was charged with the predetermined amounts of VI and acrylamide, whereas the total weight of the monomers was kept constant (100 g/liter). The concentration of the initiator was 4.0 g/liter. The polymerization procedure was the same as that of homopolymerization. The copolymers obtained were highly hygroscopic, so that care was taken not to leave them in air for a long period, particularly when filtered. The conversion for each copolymer is listed in Table 2. The copolymer composition was determined on the basis of the absorption at 257 nm due to the aromatic biguanide hydrochloride. To obtain the value of ϵ for N^1 -4-alkylphenyl- N^5 -4-chlorophenylbiguanide hydrochloride, N^1 -4-(2-hydroxyethyl)- N^5 -4-chlorophenylbiguanide hydrochloride was used as model compound: $\lambda_{\text{max}} = 230 \text{ nm}$ ($\epsilon = 1.83 \times 10^4$); 257 nm ($\epsilon = 1.74 \times 10^4$) in water. The molecular weight is listed in the last column of Table 2.

Antibacterial assessment. Freeze-dried ampules of *Staphylococcus aureus* (IFO 12732) and *Escherichia coli* (IFO 3806) were opened, and a loopful of each culture was spread to give single colonies on nutrient agar and incubated at 37°C for 24 h. A representative colony was picked off with a wire loop and placed in 10 ml of nutrient broth (peptone [Wako Chemical], 10 g; NaCl, 5.0 g; beef extract [Wako Chemical], 5.0 g in 1,000 ml of sterile distilled water [pH 6.8]), which

TABLE 1. Homopolymerization of the biguanide monomers^a

Monomer	Monomer concentration (g/liter)	Initiator concentration (g/liter)	Polymerization time (h)	Conversion (%)	M_w^b
V	100	1.14	6.5	70	11,700
VI	139	0.83	6.5	81	11,900
VII	97	1.18	6.0	57	12,100

^a Solvent, DMF; initiator, 2,2'-azobis-2-amidinopropane · 2 HCl; temperature, 60°C.

^b Determined with a low-angle, laser light-scattering photometer (KMX-6) in methanol.

TABLE 3. Antibacterial activity of biguanide compounds^a

Compound	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Aerobacter aerogenes</i>	<i>Pseudomonas aeruginosa</i>
VI	10-33	33-66	66-100	100-330	100-330
Poly V	100-330	100-330	>1,000	>1,000	>1,000
Poly VI	100-330	100-330	660-1,000	660-1,000	>1,000
Poly VII	100-330	100-330	660-1,000	660-1,000	660-1,000
XII	100-330	100-330	100-330	660-1,000	660-1,000

^a MIC ($\mu\text{g/ml}$) determined by the spread plate method.

was then incubated overnight at 37°C. At this stage, the culture of *S. aureus* contained $\sim 10^8$ cells per ml, and that of *E. coli* contained $\sim 10^9$ cells per ml. By diluting with sterile distilled water, culture containing $\sim 10^6$ cells per ml was prepared for each strain which was used for antibacterial test. Since the biocides were not completely soluble in water as 1% concentrate, they were dissolved in methanol-water (1:9 [vol/vol]) at first and then diluted with sterile distilled water so as to give the correct final concentration when 18.0 ml of the biocide solution was combined with 2.0 ml of the bacterial culture. It was confirmed that methanol used for the preparation of 1% concentrate did not affect the result of the antibacterial tests. Exposure of bacterial cells to the

biocide was started when 2.0 ml of the bacterial culture containing $\sim 10^6$ cells per ml was added to 18.0 ml of the biocide solution which was preequilibrated at 37°C. At the same time, 2.0 ml of the same culture was added to 18.0 ml of saline, decimal dilutions were prepared, and the starting cell concentration was enumerated by the spread plate method. At various contact times 1.0-ml portions were removed and quickly mixed with 9.0 ml of neutralizer solution (20% Tween 80 plus 3% azolectin in nutrient broth), and then decimal serial dilutions were prepared from this by taking 0.2 ml into 1.8 ml of saline and mixing. From these dilutions the surviving bacteria were counted by the spread plate method. After inoculation, the plates were incubated at 37°C, and the colonies were counted after 48 h. The counting was done in triplicate every time.

Conventional antimicrobial susceptibility testing by the spread plate method was conducted as described previously (8).

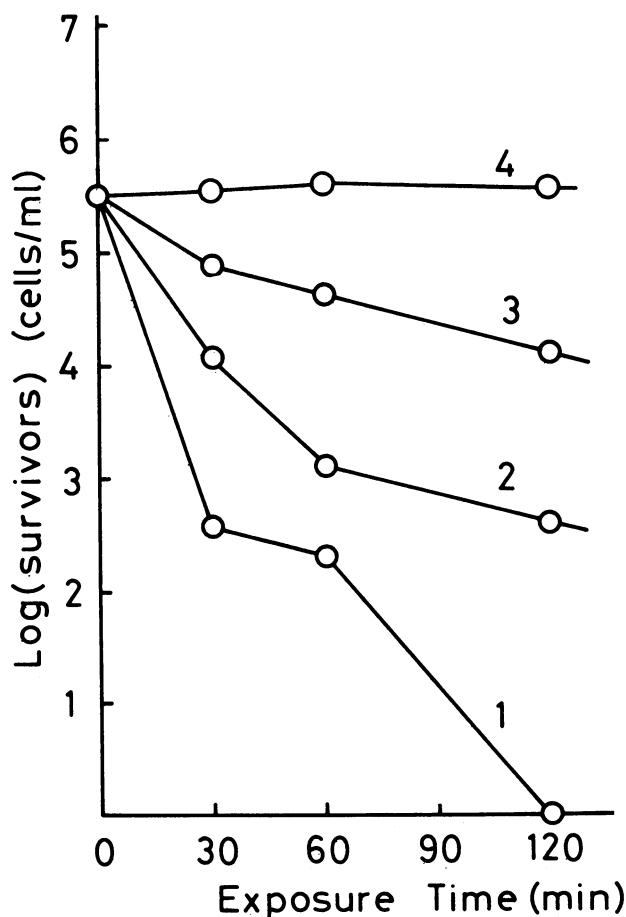


FIG. 2. Log(survivors) versus exposure time plots for the homopolymers against *S. aureus* were as follows: 1, poly V; 2, poly VI; 3, poly VII; and 4, control. Concentration, 1.2 μM , based on the monomer unit (0.5 $\mu\text{g/ml}$ for poly VI).

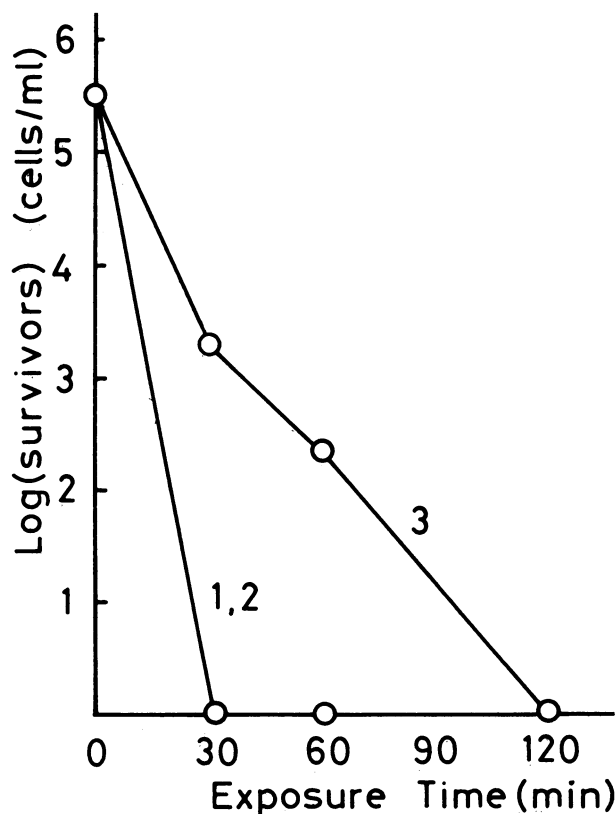


FIG. 3. Log(survivors) versus exposure time plots for the homopolymers against *S. aureus* were as follows: 1, poly V; 2, poly VI; and 3, poly VII. Concentration, 2.4 μM , based on the monomer unit (1.0 $\mu\text{g/ml}$ for poly VI).

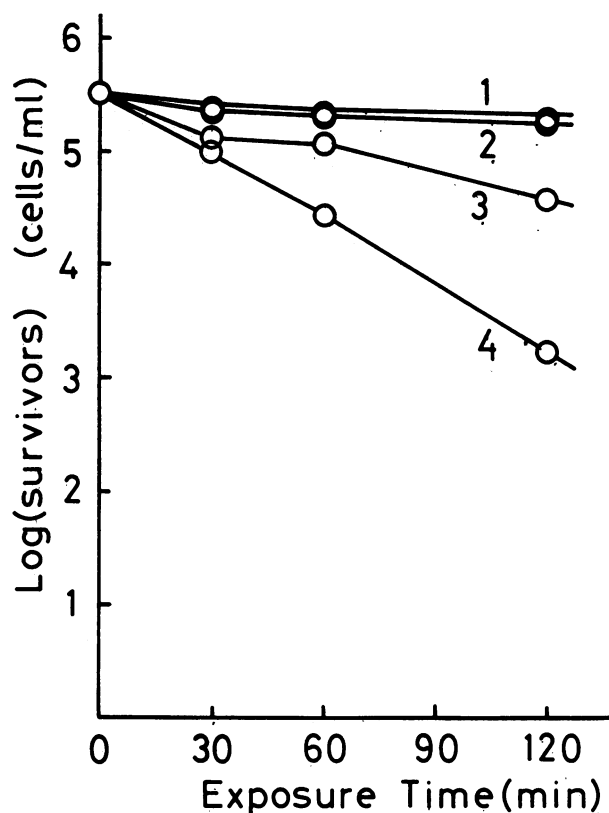


FIG. 4. Log (survivors) versus exposure time plots for the monomer VI against *S. aureus* at the following concentrations: 1, 1.2 μM ; 2, 12 μM ; 3, 120 μM ; and 4, 237 μM .

Measurements. The M_w of polymers was determined with a KMX-6 low-angle, laser light-scattering photometer. The absorption spectra were recorded with a Shimadzu UV-200 spectrometer, and $^1\text{H-NMR}$ spectra were recorded on a JEOL JNM-PM 60.

RESULTS AND DISCUSSION

Many trials have been done to prepare acrylate monomers by esterification of N^1 -4-(2-hydroxyethyl)phenyl- N^5 -4-chlorophenylbiguanide hydrochloride (XII). The latter compound was prepared by refluxing an equimolar mixture of III and 4-chloroaniline hydrochloride in 2-ethoxyethanol for 15 min (yield, 65%). The Schotten-Baumann reaction between XII and acryloyl chloride in pyridine was not successful, giving apparently a triazine derivative with no vinyl group (by NMR), as the conversion of biguanides into guanamines seemed to take place in the presence of acylating agents under basic conditions (11). The same reaction in different solvents (DMF and water) also was not successful, though in DMF a very small amount of the acrylate monomer VI was produced. Esterification of XII by the use of dicyclohexylcarbodiimide and acrylic acid in DMF and pyridine was tried and found not to proceed. Then the strategy in synthetic route was changed: first, dicyandiamide with vinyl group IV was prepared, and then the step for biguanide formation was followed. By this procedure, the acrylate monomers with a pendant biguanide group were successfully prepared despite the fact that biguanides are highly susceptible to condensation with esters to give triazines (11).

Table 3 shows MICs evaluated by the conventional spread plate method. The two figures for each strain indicate the range of MIC: growth of the bacterium could be seen as visual colonies below the lower concentration limit of MIC, whereas no colonies were observed above the higher limit. Consequently, the exact MIC is supposed to lie between these two values. A general trend can be seen from the table that the biguanide monomers and polymers are active against gram-positive bacteria, whereas they are less active against gram-negative bacteria. It is also evident that XII, which has a free hydroxy group, is less active than VI in which the hydroxy group is esterified. Another characteristic seen in the table is that the monomer VI is more active than the polymers.

Here we reached the conclusion that the method of evaluating antibacterial activity of biguanide compounds must be reexamined. The polymeric biguanides are polycations and have a strong tendency to interact with some constituents of media used to cultivate bacteria. They interact strongly with negatively charged species (such as sodium caseinate) and produce insoluble complexes. This complexation may lead to inactivation of the polymeric biguanides when their activity was evaluated on the growth media. To eliminate the interference by the constituents in the growth media, the bactericidal assessment was performed in sterile water.

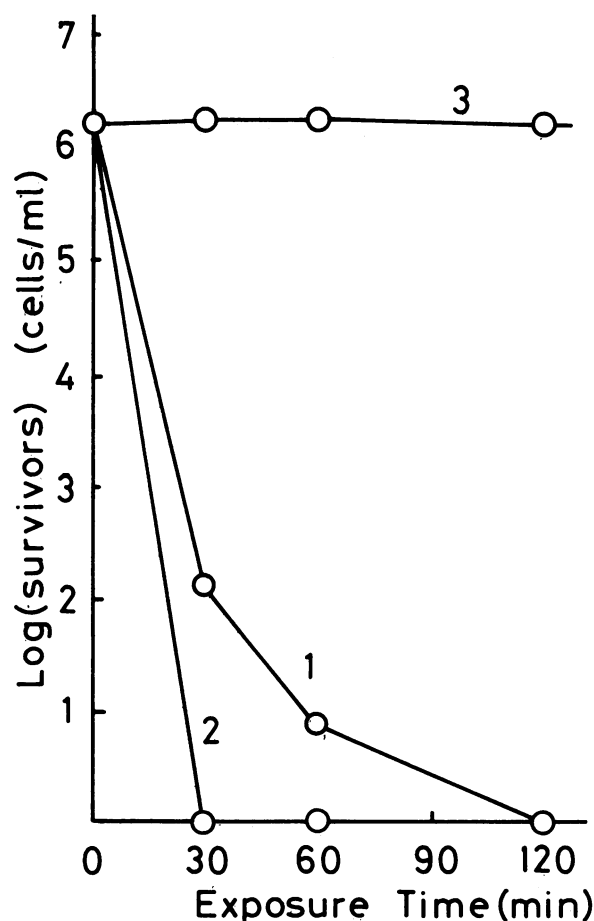


FIG. 5. Log (survivors) versus exposure time plots for VI and poly VI against *E. coli* were as follows: 1, VI; 2, poly VI; and 3, control. Concentration, 95 μM .

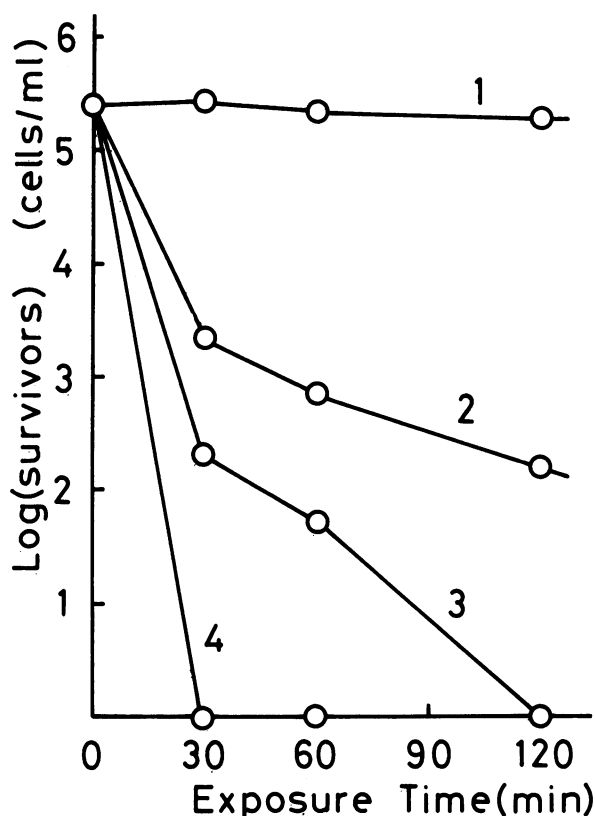


FIG. 6. Log (survivors) versus exposure time plots for the copolymer XI against *S. aureus* at the following concentrations: 1, 0.2 μM ; 2, 1.2 μM ; 3, 2.4 μM ; and 4, 12 μM . The concentrations were calculated on the basis of the biguanide monomer units.

Figure 2 shows log (survivors) versus exposure time plots for the homopolymers of V, VI, and VII (poly V, poly VI, and poly VII, respectively) against *S. aureus*. Exposure of the polymers to bacterial cells was carried out in sterile water. The concentration of the polymers was 1.2 μM , based on the monomer unit (0.5 $\mu\text{g/ml}$ for poly VI). Poly V was the most active among the three. The difference in activity among the three polymers is not well understood at the moment. Figure 3 shows the log (survivors) versus exposure time plots for the homopolymers against *S. aureus* at 2.4 μM (1.0 $\mu\text{g/ml}$ for poly VI). All of the bacterial cells were killed within 30 min when exposed to poly V and poly VI. At the concentrations higher than 12 μM , all of the bacterial cells were killed within 30 min when exposed to any of the homopolymers. Figure 4 indicates the same plots for the monomer VI. Exposure of the bacterial cells to the monomer VI at the concentration 50 times as high as those of the polymers (120 μM , 50 $\mu\text{g/ml}$) exerted little effect on reducing the number of survivors (Fig. 4, curve 3). From Fig. 2 through 4, it is evident that the polymers (poly V, poly VI, and poly VII) are much more active than the monomer VI when exposed to the bacterial cells without interfering materials.

A similar result was obtained against gram-negative strain *E. coli*. Figure 5 shows a comparison in bactericidal activity between VI and poly VI at the concentration of 95 μM (40 $\mu\text{g/ml}$). It is clear that poly VI is more active than VI in this case as well.

Figure 6 shows the log (survivors) versus exposure time plots for XI, which contains 50.6 mol% of biguanide mono-

mer units in the copolymer against *S. aureus*. The concentrations shown in Fig. 6 were those calculated on the basis of the biguanide monomer units. Copolymers with different biguanide compositions exhibited a similar concentration dependence on bactericidal activity.

The most probable explanation for the higher activity of the polymers may be given by considering their contribution to each elementary process in the lethal action (see above). The bacterial cell surface is negatively charged as evidenced by electrophoretic mobility. Absorption of polycations onto the negatively charged cell surface is supposed to be much more favored than that of monomeric cations on account of the higher charge density of the polyelectrolytes. Thus, process i is expected to be more enhanced for polycations compared with that for monomeric cations.

With respect to process ii, the polycations have a disadvantage. The gram-positive strains have a rather simple cell wall composed of a rigid peptidoglycan layer which allows foreign molecules to come inside without much difficulty (2). Thus, polycations with relatively low molecular weights as used in this study might diffuse easily through the cell walls of gram-positive bacteria. On the other hand, in the case of gram-negative bacteria, there is another bilayer membrane outside the peptidoglycan layer (outer membrane). Because of the outer membrane, foreign molecules are not capable of diffusing easily through the cell wall (2). This could be a disadvantage to the polymeric biocides, since they have larger molecular size than the monomeric ones. However, as is seen in Fig. 5, poly VI with M_w of 11,900 exhibited higher activity against *E. coli* than VI, which might suggest that this type of polycation can reach the cytoplasmic membrane of the gram-negative species after the partial breaking down of the outer membrane.

It is still ambiguous how the biguanides as well as other cationic disinfectants interact with the cytoplasmic membrane with subsequent disruption, although it has been reported that they interact strongly with negatively charged species present in the membrane such as acidic phospholipids and some membrane proteins (1, 6, 9, 10, 14). Binding of the polymeric biguanides to the cytoplasmic membrane (process iv) is supposed to take place more preferably than that of the monomeric ones due to stronger interaction of the former with negatively charged species present in the membrane. This would result in faster disruption of the membrane (process iii) by the polymeric biocides with subsequent release of the cytoplasmic constituents (process v), followed by the death of the cells (process vi). Although the polymeric biocides have a disadvantage in diffusing through the cell walls, overall activity of the polymeric biocides would, after all, be higher than that of the monomers.

In conclusion, the evaluation of antibacterial activity of the polymeric biguanides is complicated by the fact that they interact with some constituents of culture media, and because of this incompatibility evaluation of bacteriostatic activity on growth media will not be precise. In a clean system in which there are no interfering materials such as negatively charged macromolecules, the polymeric biguanides are much more active than the monomeric species. The higher activity of the polymers may be accounted for by their stronger interactions with the cell surface and the cytoplasmic membrane of bacteria as the primary process of the lethal action.

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