Disinfection of Water with Quaternary Ammonium Salts Insolubilized on a Porous Glass Surface

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Insoluble quaternary ammonium salts bound to porous glass showed antibacterial activity. An agent designated as G_{12}, which had a dodecyl alkyl chain, was selected for some antibacterial tests on comparison of it with the agent reported previously. The antibacterial activity of G_{12} toward *Escherichia coli* was mainly due to the adsorption of cells and therefore gradually decreased during continuous treatment of a cell suspension. The lost G_{12} activity was completely recovered by washing with ethanol, and the activity of refreshed G_{12} decreased in the same manner as that of fresh G_{12}. The lost activity was, however, always recovered only by ethanol treatment. This indicated that G_{12} might interact with cells more strongly by means of a hydrophobic force than an electrostatic one. The antimicrobial spectrum showed that G_{12} was effective against not only bacteria but also yeasts.

If an insolubilized agent has an antimicrobial activity, such an agent will be a more suitable disinfectant for liquids than a soluble one because there is no problem of residual toxicity of the agent. Antimicrobial agents which act on surfaces of microbial cells, e.g., the outer membrane, may display their antimicrobial activity only on contact with microbial cells without permeation into the cells. If so, such agents may not lose their activity on insolubilization. From this viewpoint, various workers have already tested some agents and found insolubilized agents with antimicrobial activities (1, 3–11, 18–21, 23). We also attempted to insolubilize some quaternary ammonium (QA) salts by ionic (12, 14) or covalent (15) binding. But with the former, a small amount of QA salts was released from the carrier during the disinfection process (12, 14). Moreover, with the latter, the durability of their activity was poor because of their adsorption of dissolved air (15), and also cell adsorption by them caused their apparent antimicrobial activity and the loss of their activity (13). Walters et al. also found that the algicidal activity of an insoluble QA salt diminished with time (23). However, on covalent binding our insoluble QA salt had no bactericidal activity but only a bacteriostatic one (13), although Isquith et al. (4, 5) and Walters et al. (23) hypothesized that insoluble QA salts had microbicidal activity. As for our insoluble QA salts, on covalent binding QA salts were one of the components of insoluble copolymers (15), but Isquith et al. insolubilized a QA salt on various materials.

Porous glass (PG) is sold commercially as a column packing for permeation chromatography and is also used as a support for immobilization of enzymes (24) or biochemical substances (22). PG is very reactive and may be used as a support for insolubilization of QA salts. In this work, we aimed to synthesize QA salts covalently bound to PG and investigate the antimicrobial activity of such agents.

MATERIALS AND METHODS

Synthesis of insoluble QA salts. CPG-10 (Electro-Nucleonics, Inc.) or FPG-250 (Wako Pure Chemical Co.) was used as

a commercial PG (Table 1). PG was soaked in fuming nitric acid for 20 h to clean its surface and to enhance its reactivity, washed with double distilled deionized water until the pH of washing water was above 5.0, and dried at 100°C for 3 h, and subsequently at 200°C for 15 h. The dried PG was kept under vacuum until use.

PG was bound with 3-chloropropyltrimethoxysilane (Tokyo Chemical Industry Co., Ltd.) by reaction at an equal weight ratio for 10 days under toluene refluxing at 110°C with agitation. The silylized PG (G-Si) was washed thoroughly with toluene, then washed with ethanol, and then dried at 80°C for 5 h. The silylized ratio of G-Si was calculated from the chlorine content of G-Si, 1.0 g of PG bound with about 200 μmol of silane.

G-Si was quaternized with various tertiary amines by the following method. The tertiary amines, the N,N-dimethyl-n-alkylamines shown in Table 2, were purchased from Tokyo Chemical Industry Co., Ltd., ICN Pharmaceuticals, Inc., and Ventron Corp., Chemical Division. Each amine with a long alkyl chain (C_8 to C_{18}) was reacted with G-Si in the same way as for the silylization for 1 or 2 weeks. Amines with ethyl or n-butyl radicals were also reacted with G-Si in toluene in a pressure bottle at 100°C for 3 days because of the low boiling points of these amines. After the reaction, the quaternized G-Si (G-Q) was washed, dried as in the silylization, and used for antimicrobial experiments.

The quaternized ratio of G-Q was measured by using eosin Y (Wako Pure Chemical Co.), one molecule of which combines with one molecule of QA salt. Fifty milligrams of G-Q and 0.5 ml of 10 mM eosin Y-ethanol solution were mixed together in a 10-ml capped tube and shaken at 30°C. After 24 h, the eosin Y solution was diluted with ethanol, and the optical density at 525 nm of the solution was measured. The amount of QA salt on G-Q was calculated from the decrease in eosin Y concentration. G-Si could not combine with eosin Y at all. QA salt contents of G-Qs and their designations are shown in Table 2, and their structures are shown in Fig. 1.

PG (CPG-10 II) was also reacted with n-octadecyltriethoxysilane (Tokyo Chemical Industry Co., Ltd.) at an triethoxysilane (Tokyo Chemical Industry Co., Ltd.) at an

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TABLE 1. Description of PGs used for insolubilization of QA salts

PG	Mesh size	Mean pore diam (nm)	Pore distribution (%)	Pore vol (ml/g)	Specific surface area (m²/g)	Bed vol (cm³/g)
CPG-10 I	80-120	25.9	±8.2	1.31	118	3.4
CPG-10 II	80-120	22.6	±6.2	0.85	94	2.5
FPG-250L	80-120	23.5	±20	0.81	90	2.0
FPG-240D	50-80	25.6	+50~−17	0.72	ND[a]	2.6

[a] ND, Not determined.

equal weight ratio for 7 days by the same method as for the quaternization in a pressure bottle. After the reaction, the silylized PG (G-S$_{18}$) was washed and dried as described above. The silylized ratio of G-S$_{18}$ was presumed to be ca. 300 μmol/1.0 g of PG. G-S$_{18}$ differed from G-Si in structure (Fig. 1).

Preparation of cell suspensions. *Escherichia coli* K-12 OUT 8401 was used mainly, and *Micrococcus luteus* ATCC 4698, *Staphylococcus aureus* 209P, *Sarcina lutea*, *Salmonella typhimurium* DB21, *Klebsiella pneumoniae* OUT 8017, *Pseudomonas aeruginosa* ATCC 17653, *Serratia marcescens*, *Candida utilis* OUT 6020, *Hansenula anomala* OUT 6079, and *Saccharomyces cerevisiae* OUT 7020 were used for the antimicrobial spectrum. All of these strains are maintained in our laboratories.

These microbes were cultivated, collected, and washed according to the previous method (15). Collected cells were suspended at a prescribed cell concentration in sterilized distilled deionized water. Each cell suspension was kept at 30°C for 30 min before use.

If necessary, *E. coli* was labeled with [6-³H]thymidine (2 Ci/mmol, Amersham International PLC) by the method given in a previous paper (13).

Treatment of a cell suspension with G-Q. A prescribed amount of G-Q, PG, G-Si, or G-S$_{18}$ was packed in a column and treated with ethanol for 5 h to make it hydrophilic. The agents were then continuously washed with 1,500 ml of sterilized distilled deionized water. A cell suspension was passed through the column at a constant flow rate at 30°C, and the effluent was collected in an aseptic test tube after 10 min from the initiation of flow of a cell suspension. Subsequently, the flow rate was changed, and the effluent was also collected as above. In another experiment, a cell suspension was continuously treated at a constant flow rate for a long period, and the effluent was collected at prescribed times. A ³H-labeled cell suspension was also treated by the same method as above.

The number of surviving cells in each collected sample

was determined by the same method as described in a previous paper (15), and radioactivity in a sample was also measured by the same method as in a previous paper (13). In all experiments, the number of control cells did not vary with the experimental period.

RESULTS

Antimicrobial activity in each G-Q. A cell suspension of *E. coli* (10⁶ cells per ml) was treated with 1.2 g of each G-Q with various residence times at 30°C, and G$_{12}$I and G$_{12}$II were used as G$_{12}$. G-Qs with long alkyl chains (C$_8$ to C$_{18}$) showed high antibacterial activity, and, in particular, no surviving cells were detected in the effluent treated with G$_{10}$ (Fig. 2). G$_2$ and G$_4$ showed less activity than other G-Qs and also showed higher activity with a shorter residence time (a higher flow rate), different from other G-Qs (Fig. 2). When a cell suspension was treated with PG, G-Si, or G-S$_{18}$ with a residence time of 60 s, the percentage of survivors in each effluent was 55, 52, and 100%, respectively.

G$_{12}$ was used in the following experiments because it had antibacterial activity and the same alkyl chain as the insoluble QA salt reported in previous papers (13, 15).

Durability of antibacterial activity of G$_{12}$. A cell suspension of *E. coli* (5 × 10⁵ cells per ml) was continuously treated with various amounts of G$_{12}$III with a residence time of 3 min at 30°C. The antibacterial activity of G$_{12}$ decreased with in-

TABLE 2. QA salt content of G-Qs

Alkyl chain	PG	QA salt content (μmol/g)	Designation
C_2H_5-	FPG-250L	83.4	G$_2$
C_4H_9-	FPG-250L	73.4	G$_4$
$C_8H_{17}-$	FPG-250L	23.1	G$_8$
$C_{10}H_{21}-$	FPG-250L	21.4	G$_{10}$
$C_{12}H_{25}-$	FPG-250L	19.2	G$_{12}$I
	FPG-250L	61.2	G$_{12}$II
	CPG-10 I	72.1	G$_{12}$III
	FPG-250D	55.1	G$_{12}$IV
$C_{14}H_{29}-$	FPG-250L	48.8	G$_{14}$
$C_{16}H_{33}-$	FPG-250L	23.6	G$_{16}$
$C_{18}H_{37}-$	FPG-250L	15.6	G$_{18}$

(a)

$$\text{(G)-O-Si-(CH}_2\text{)}_3\text{-Cl}$$

(b)

$$\text{(G)-O-Si-(CH}_2\text{)}_3\text{-N}^+\text{-C}_n\text{H}_{2n+1}\cdot\text{Cl}^-$$

n: 2~18

(c)

$$\text{(G)-O-Si-C}_{18}\text{H}_{37}$$
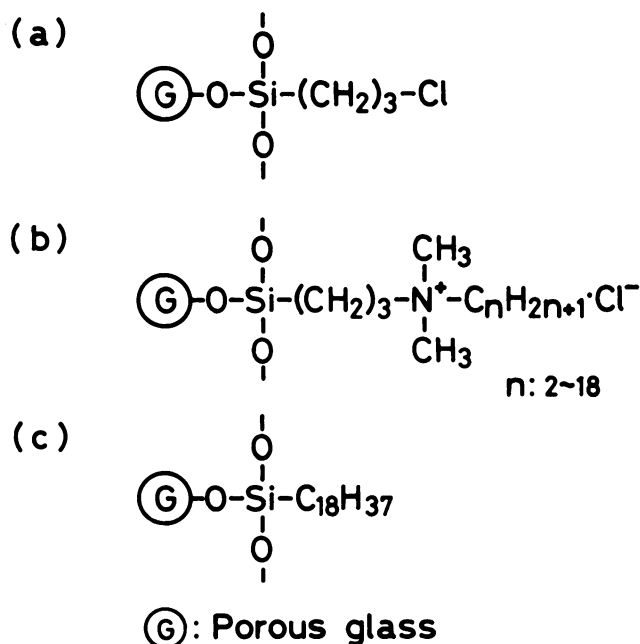
(G): Porous glass

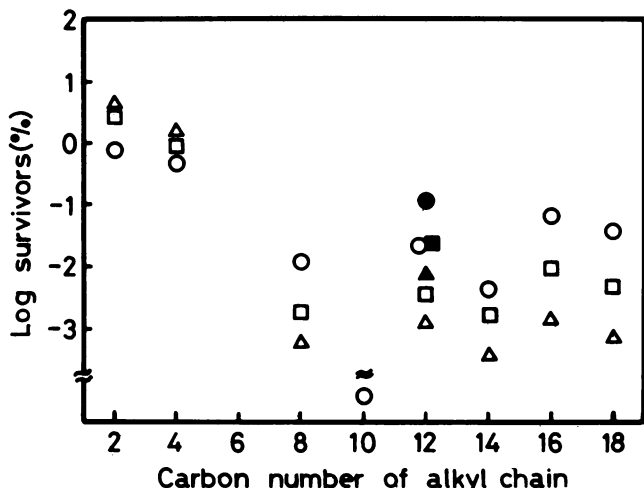FIG. 1. Proposed structures for G-Si (a), G-Q (b), and G-S$_{18}$ (c).

FIG. 2. Antibacterial activity of G-Qs. A cell suspension of *E. coli* (10^6 cells per ml) was treated with 1.2 g of each G-Q with residence times of 10 s (O, ●), 30 s (□, ■), and 60 s (△, ▲) at 30°C. $G_{12}I$ (closed symbols) and $G_{12}II$ (open symbols) were used as G_{12}. No surviving cells were detected with G_{10}, and G_6 was not investigated.

FIG. 4. Relationship between the amount of $G_{12}III$ and the volume of cell suspension treated until the percentage of survivors in the effluent reached >0.01%.

creasing volume of a treated cell suspension for every amount of G_{12} (Fig. 3). In Fig. 3 we can see the relationship between the amount of $G_{12}III$ and the treated volume of a cell suspension at which the percentage of survivors became 0.01%. Figure 4 shows that the durability of the antibacterial activity of G_{12} was directly proportional to its amount.

Cell suspensions of various concentrations were continuously treated with 0.8 g of $G_{12}III$ with a residence time of 6 s at 30°C. The lower the cell concentration, the later its activity began to decline (Fig. 5). But the durability of the activity depended on the total treated cell number, regardless of the cell concentration (Fig. 6).

A cell suspension (5×10^5 cells per ml) was continuously treated with 0.8 g of $G_{12}III$ with a residence time of 3 min at 30°C for 5 days, and subsequently the used $G_{12}III$ was washed with 1,500 ml of distilled deionized water. But its

activity could not be recovered. This $G_{12}III$ was treated with 200 ml of ethanol, washed with 1,500 ml of distilled deionized water, and then used for an antibacterial test. This time, its activity was completely recovered, and it decreased in the same manner as that of fresh $G_{12}III$ (Fig. 7). Nevertheless, the above ethanol washing was useful for recovery of the antibacterial activity of 0.7 g of $G_{12}IV$, which was repeatedly used for treatment of 300 ml of a cell suspension (5×10^5 cells per ml) with various residence times at 30°C. This procedure was repeated five times for the same $G_{12}IV$, and we found that every time the washed $G_{12}IV$ showed the same antibacterial activity as the fresh $G_{12}IV$.

Antimicrobial spectrum of G_{12}. The antimicrobial spectrum of G_{12} was examined at 30°C. A cell suspension of each microbe was continuously treated with 0.7 g of $G_{12}IV$ with various residence times, and fresh $G_{12}IV$ was used against each microbe without the above refreshment with ethanol. The antimicrobial test against *E. coli* was carried out five times, and stable antimicrobial activity of $G_{12}IV$ was found. Therefore, the tests against other microbes were done only
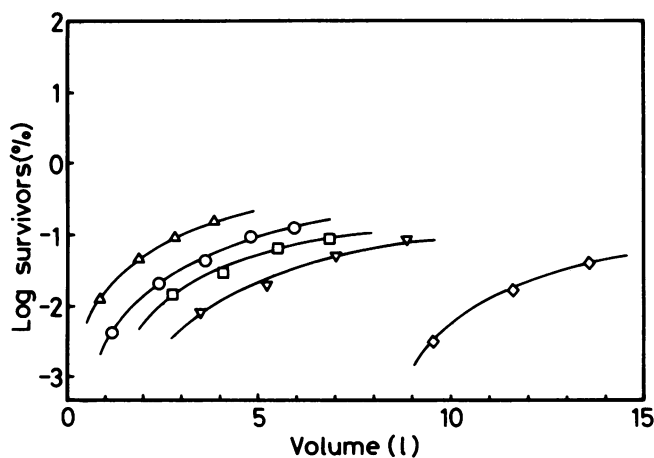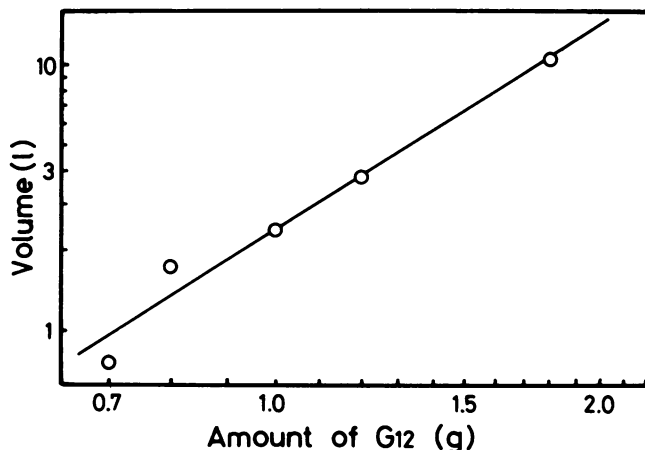

FIG. 3. Durability of the antibacterial activity of G_{12}. A cell suspension of *E. coli* (5×10^5 cells per ml) was continuously treated with 0.7 g (△), 0.8 g (O), 1.0 g (□), 1.2 g (▽), or 1.8 g (◇) of $G_{12}III$ with a residence time of 3 min at 30°C.
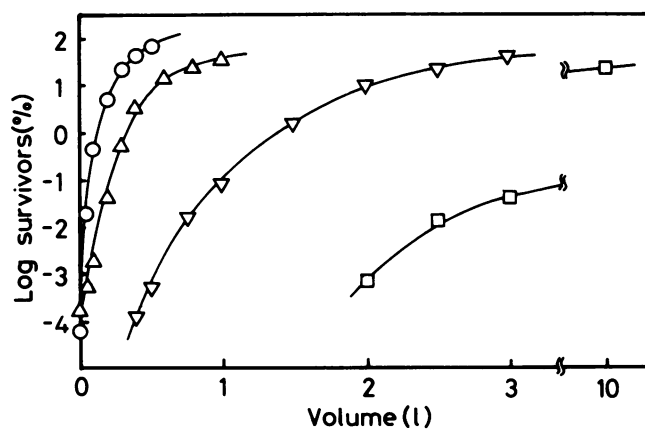

FIG. 5. Effect of cell concentration on the durability of the antibacterial activity of G_{12}. Cell suspensions of *E. coli* were continuously treated with 0.8 g of $G_{12}III$ with a residence time of 6 s at 30°C. The cell concentrations were 3×10^7 (O), 10^7 (△), 3×10^6 (▽), and 5×10^5 (□) cells per ml.
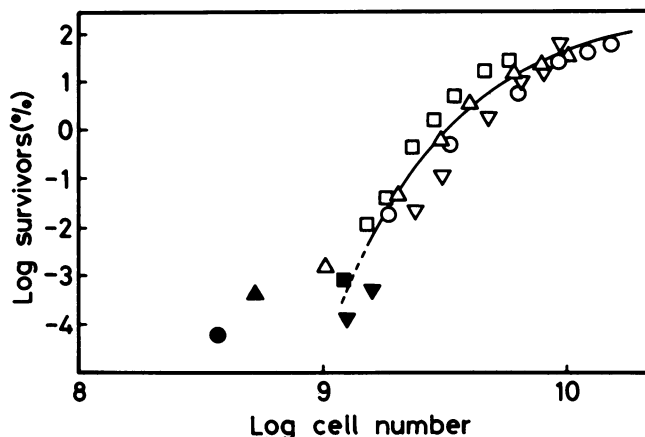
FIG. 6. Effect of the number of treated cells on the durability of the antibacterial activity of G_{12}. Data and symbols are the same as those described in the legend to Fig. 5. Closed symbols indicate data which have large errors because the data were calculated with samples of fewer than 10 colonies per plate.

once. G_{12} showed high antimicrobial activity against gram-positive bacteria, *P. aeruginosa*, *C. utilis*, and *H. anomala*, and surviving cells could not be detected in the effluent in the case of a residence time of 120 s (Table 3). Furthermore, G_{12} also showed antimicrobial activity against enterobacteria and *Saccharomyces cerevisiae* (Table 3).

Adsorption of E. coli onto G_{12}. A cell suspension of ³H-labeled *E. coli* cells (3 × 10⁷ cells per ml) was continuously passed through a column containing 0.8 g of G_{12}III with a residence time of 30 s at 30°C. After 500 ml of a cell suspension was treated, sterilized distilled deionized water was immediately poured into the column. It was found that the percentage of survivors was less than that of radioactivity in every effluent of a cell suspension. This pattern was also observed in the subsequent washing with distilled deionized water (Fig. 8). This indicated that the effluent contained dead cells and living cells, and furthermore, the decrease of radioactivity in the effluent showed that some
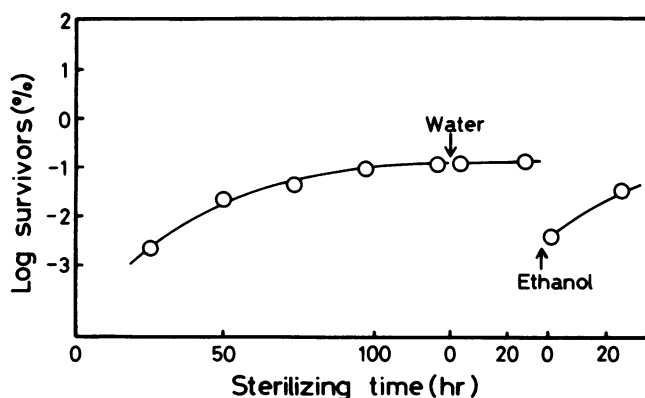
TABLE 3. Antimicrobial spectrum of $G_{12}IV^a$

Microbe	Cell concn[b] (10⁶ cells/ml)	% Survivors at a residence time of:			
		10 s	30 s	60 s	120 s
Staphylococcus aureus	0.5	0.022	0.006	0.003	—[c]
Micrococcus luteus	1.1	0.004	—	—	—
Sarcina lutea	0.6	—	—	—	—
Escherichia coli[d]	0.6	0.202	0.090	0.042	0.010
Salmonella typhimurium	0.6	0.505	0.162	0.041	0.011
Klebsiella pneumoniae	1.0	0.319	0.080	0.023	0.007
Serratia marcescens	1.3	0.026	0.007	0.003	0.002
Pseudomonas aeruginosa	1.0	0.013	0.007	0.001	—
Candida utilis	1.5	0.010	0.002	—	—
Hansenula anomala	1.1	0.028	0.002	0.001	—
Saccharomyces cerevisiae	1.1	0.100	0.019	0.004	0.001

[a] Each microbe was suspended in distilled deionized water and treated with 0.7 g of fresh $G_{12}IV$ at 30°C with each residence time shown.
[b] Initial viable cell count in the influent.
[c] —, No surviving cells were detected.
[d] The average values for five experiments. The data for other microbes were based on one experiment.

cells in the influent were adsorbed by G_{12}. It was deduced from the above results that cell adsorption would be the main mechanism involved in the antimicrobial activity of G_{12}.

DISCUSSION

It is proposed that microbial cells can be adsorbed on solid surfaces by electrostatic or hydrophobic interaction, or both (2, 16, 17, 25). We also reported in a previous work (13) that bacterial cells were firmly adsorbed onto $C_{12}(50)$, the designation for a quaternized divinylbenzene-vinylpyridine copolymer, by an electrostatic force rather than a hydrophobic one. In this work we found that G_{12} adsorbed bacterial cells (Fig. 8) and that the cell adsorption onto it caused a decrease of its apparent antimicrobial activity. The G_{12} completely recovered its activity on being washed with ethanol (Fig. 7), and repeated treatment with ethanol was very effective for G_{12}, although ethanol washing became ineffective after re-
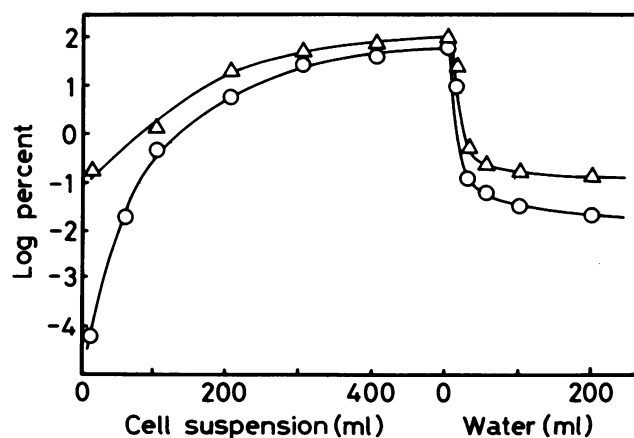

FIG. 7. Decrease and recovery of the antibacterial activity of G_{12}. A cell suspension of *E. coli* (5 × 10⁵ cells per ml) was continuously treated with 0.8 g of G_{12}III with a residence time of 3 min at 30°C for 120 h. Then, after G_{12} was washed with 1,500 ml of distilled deionized water, another cell suspension was treated for 24 h under the same conditions. G_{12} was then washed with 200 ml of ethanol and subsequently with 1,500 ml of water, then used again for the antibacterial test.

FIG. 8. Relationship between survivors (○) and radioactivity (△) in the effluent after treatment with 0.8 g of G_{12}III. A cell suspension of ³H-labeled *E. coli* (3 × 10⁷ cells per ml) was treated with G_{12}III with a residence time of 30 s at 30°C, and subsequently distilled deionized water was passed through the column at the same flow rate.

peating it three times for $C_{12}(50)$ (15). This difference might be due to their QA salt contents. The QA salt content of G_{12} was only 19 to 72 μmol/g (Table 2), much less than that of $C_{12}(50)$, which was 880 to 1,170 μmol/g (15). It was, therefore, deduced that the electrostatic interaction between cells and G_{12} was weaker than that between cells and $C_{12}(50)$. On the other hand, the effect of hydrophobic interaction might become stronger in G_{12} than $C_{12}(50)$, because an electrostatic force was generally more predominant than a hydrophobic one. G_{12} could be, therefore, completely reactivated with ethanol, which prevents hydrophobic interaction, although the complete reactivation of $C_{12}(50)$ required treatment with alkaline solutions because of the electrostatic interaction (13).

The durability of the antimicrobial activity of G_{12} (Fig. 7) was superior to that of $C_{12}(50)$ (15), and dead cells were detected in the effluent treated with G_{12} (Fig. 8) but not that treated with $C_{12}(50)$ (13). These results would also be due to the low QA salt content of G_{12}. Adsorbed cells might be more readily desorbed from G_{12} than $C_{12}(50)$ by flow shear stress because of the poor electrostatic interaction with G_{12}, and so it was assumed that an active site of G_{12} could repeatedly act for many cells. In other words, a cell would come into contact with G_{12} many times during passage through a column. In the case of $C_{12}(50)$, adsorbed cells would be desorbed from $C_{12}(50)$ with difficulty because of the firm electrostatic interaction, and so cells passing through a column would not be able to come into contact with $C_{12}(50)$ once the $C_{12}(50)$ was covered with adsorbed cells. These assumptions led to the conclusions that only cells which came into contact many times with an insoluble QA salt died, and that insoluble QA salts which could desorb cells easily possessed more durable microbicidal activity. G_{12} was, therefore, superior to $C_{12}(50)$ in antimicrobial action. According to the above conclusions, however, if the QA salt content of $C_{12}(50)$ decreases to the same degree as in G_{12}, $C_{12}(50)$ may also have a microbicidal action. So it is necessary to study the antimicrobial activities of G_{12} and $C_{12}(50)$ with various QA salt contents.

G-Si and G-S_{18}, which are uncharged, hydrophobic agents, were ineffective for treatment of a cell suspension. Even though G-S_{18} had an alkyl chain ($-Si-C_{18}$) as long as that of G_{14} ($-Si-C_3-N^+-C_{14}$), it had no activity at all. Moreover, G_2 and G_4, which are charged and less hydrophobic than other G-Qs, showed antibacterial activity (Fig. 2). These results indicated that an electric charge was also necessary for an insoluble agent to act on microbial cells.

Isquith et al. (4, 5) and Walters et al. (23) have already insolubilized QA salts on various materials and concluded that they had microbicidal activity. Their insoluble QA salts had the same structure as G_{18}, although the supports of QA salts were different. Nevertheless, the antimicrobial activity of G-Qs was mainly due to cell adsorption on them. The insoluble QA salt of Isquith et al., therefore, might decrease the number of surviving cells due to cell adsorption on it, as in the case for our insoluble QA salts. There was, however, a possibility that the insoluble QA salt of Isquith et al. had bactericidal activity because the QA salt content was 1.16 μmol/g (5), much less than that of the G-Qs (Table 2), and because a low QA salt content might give an insoluble QA salt bactericidal activity as discussed above.

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