Biocontrol Science, 1997, Vol. 2, No. 2, 61−66

Original

Existence of an Optimal Concentration for Bactericidal Activity of Quaternary Ammonium Compounds

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Received 10 November 1996/Accepted 20 February 1997

The bactericidal activities of different types of quaternary ammonium compounds (QAC) were measured in distilled water at 0, 20 or 25℃ for concentrations between 0.001% (w/v) and 25% (w/v). Four different gram-negative bacteria were used as test organisms. In a qualitative suspension test, the existence of a concentration for maximum activity was demonstrated, depending on the temperature, and the type of QAC used and the organism tested. In a quantitative test, *Pseudomonas aeruginosa* and *Alcaligenes xylosoxidans* 9 isolated from a benzalkonium chloride concentrate, were used as test organisms with different resistances to QACs. The bactericidal activities of QACs showed a parabolic curve when plotted against concentration. In the case of dodecyl-, tetradecyl- and hexadecyltrimethylammonium bromide, the optimal concentration for the bactericidal activity of the homologous salts shifted toward lower concentrations as the chain length increased, and was found to be in the vicinity of critical micelle concentration.

Key words : Bactericidal activity/Cationic surfactant/Quaternary ammonium compounds/Colloidal association/Critical micelle concentration.

INTRODUCTION

During a study to measure the viability of the bacterial strains isolated from contaminated 10% (w/w) benzalkonium chloride (BAC) preparations and other disinfectant solutions, Furuta et al. (1992) observed that the organisms could survive or grow not only at lower BAC concentrations but also at higher.

Such findings have led us to the question whether a similar concentration-activity relationship exists between other quaternary ammonium compounds (QAC) and organisms. We are interested in the phenomenon of bacterial survival in the presence of a QAC, especially in the range of higher concentrations, since it may be necessary to consider the nature of disinfectants themselves as well as the characteristics of bacterial cells involved in the mechanism of resistance to disinfectants. Existence of an optimal concentration range is also important from the practical point of view.

Many workers have related various colloidal and/or hydrophobic properties of QACs to their antimicrobial action and discussed the intrinsic activity of QACs in the lower concentration range but not in the higher range (Cella et al., 1952; Daoud et al., 1983; Deluca and Kostenbauder, 1960; Ecanow and Siegel, 1963; Laycock and Mulley, 1970; Pernak et al., 1995; Tomlinson et al., 1977; Weiner et al., 1965).

In the present study, we investigated relationships between the concentrations and the bactericidal activities of QACs, using different types of QACs against gram-negative bacteria, and will discuss the concentration-dependence of the activities of QACs in connection with their physicochemical properties.

MATERIALS AND METHODS

QACs

Reagent-grade compounds were obtained from the

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following sources: Alkyl (hexyl, octyl, decyl, dodecyl, tetradecyl and hexadecyl) trimethylammonium bromides (C_nTAB, where n represents carbon number of alkyl chain), didecyldimethylammonium bromide (DDAB) and dodecylpyridinium chloride (C_{12}PC), Tokyo Chemical Industry, Tokyo; hexadecylpyridinium chloride (C_{16}PC), Wako Pure Chemical Industries, Osaka. Technical-grade compounds were obtained from the following sources: benzalkonium chloride (BAC) [Cation F2-50, 50 %(w/w) active], Nippon Oil & Fats, Tokyo ; benzethonium chloride (BEC) [Hyamine 1622, >99% (w/w) active], Rhom & Haas, Tokyo ; didecyldimethylammonium chloride (DDAC) [Cation DDC-50, 50% (w/w) active, containing 10% (w/w) ethanol], Sanyo Chemical Industry, Kyoto.

Bacteria and growth conditions

The organisms used in this study were *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028 and *Alcaligenes xylosoxidans* 9 (Furuta et al., 1992) [isolated from a contaminated 10% (w/w) benzalkonium chloride preparation].

Bacteria were grown in 1/20 strength nutrient broth (1.5 g/*l*; Nissui Pharmaceutical, Tokyo) at 25℃ for about 48 h on a reciprocating shaker and this suspension was used without washing as the inoculum for all experiments. It is known that growth conditions using weaker nutrient broths and lower temperatures than those commonly used decrease the sensitivity of bacteria toward germicides (Brown and Williams, 1985; Jono and Higashide,1986).

Bactericidal studies

Two methods were used to assess the bactericidal activity of QACs, designated as the qualitative and quantitative suspension test (Reybrouck, 1992).

In the qualitative suspension test, 0.5 ml of the bacterial suspension was added to 4.5 ml of QAC solutions and diluted stepwise with sterilized distilled water, and the mixtures were kept at 0, 20 or 25℃ in a water bath. At various preestablished times, 0.025 ml aliquots were withdrawn from each reaction mixture and each aliquot was immediately diluted to 5 ml with nutrient broth (30 g/*l*) supplemented with 4% (w/w) polysorbate 80 and 0.3% (w/w) lecithin as an inactivator. Bacterial survival was determined by the presence of turbidity in the cultures after incubation for 48 h at 30℃.

In the quantitative suspension test, the reaction mixtures were kept at 25℃ for 10 min for *A. xylosoxidans* or at 0℃ for 30 s for other organisms, and 0.5 ml aliquots of the mixtures were transferred to 4.5 ml of a neutralizer consisting of 6% (w/w) polysorbate 80 and 2% (w/w) lecithin. Immediately thereafter, serial dilutions were made in another neutralizer consisting of 4% (w/w) polysorbate 80 and 0.3% (w/w) lecithin. 0.5 ml of each dilution were poured onto nutrient agar plates supplemented with 4% (w/w) polysorbate 80 and 0.3% (w/w) lecithin and the plates were incubated for 48 h at 25℃ before counting.

The bactericidal activity of QACs was defined as log reduction [log (N_0/N)], where N_0 and N are survival bacterial counts after exposure to distilled water and a QAC solution, respectively.

RESULTS

To demonstrate the existence of an optimal condition in the concentration-activity relationship of QACs, test organisms were exposed to various concentrations of QACs. Exposure time and temperature were altered, depending on the organism and its particular sensitivity to QACs.

Results of the qualitative suspension test are indicated in Tables 1, 2 and 3. Table 1 shows the bacte-

TABLE 1. Effect of concentration and exposure time on the bactericidal activity of didecyldimethylammonium chloride against *A. xylosoxidans* at 20° and 25℃.

Temperature (℃)	Exposure time (min)	Concn [%(w/v)]								
		0.001	0.005	0.01	0.05	0.1	0.5	1	5	10
20	1	+[a]	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	+
25	1	+	+	+	−[a]	+	+	+	+	+
	5	+	+	+	−	−	+	+	+	+
	10	+	+	+	−	−	+	+	+	+
	20	+	+	+	−	−	+	−	+	−

[a] +, growth ; −, no growth.

TABLE 2. Effect of concentration and exposure time on the bactericidal activity of quaternary ammonium compounds against *E. coli* at 0℃.

QAC	Exposure time (s)	Concn [% (w/v)]								
		0.001	0.005	0.01	0.05	0.1	0.5	1	5	10
DDAC	15	+[a]	+	−[a]	−	−	−	−	−	−
	30	+	+	−	−	−	−	−	−	−
	60	+	−	−	−	−	−	−	−	−
	300	+	−	−	−	−	ND	ND	ND	ND[b]
BAC	15	+	+	+	+	−	−	−	−	−
	30	+	+	+	+	−	−	−	−	−
	60	+	+	+	+	−	−	−	−	−
	300	+	+	+	−	−	ND	ND	ND	ND
BEC	15	+	+	+	+	+	−	−	+	+
	30	+	+	+	+	−	−	−	−	+
	60	+	+	+	+	−	−	−	−	−
	300	+	+	+	−	−	ND	ND	ND	ND

[a] +, growth ; −, no growth.
[b] Not done.

TABLE 3. Effect of concentration and exposure time on the bactericidal activity of quaternary ammonium compounds against *S. typhimurium* at 0℃.

QAC	Exposure time (s)	Concn [% (w/v)]								
		0.001	0.005	0.01	0.05	0.1	0.5	1	5	10
DDAC	15	+[a]	+	−[a]	−	−	−	−	−	−
	30	+	+	−	−	−	−	−	−	−
	60	+	+	−	−	−	−	−	−	−
	300	+	−	−	−	−	ND	ND	ND	ND[b]
BAC	15	+	+	+	+	−	−	−	−	−
	30	+	+	+	+	−	−	−	−	−
	60	+	+	+	−	−	−	−	−	−
	300	+	+	+	−	−	ND	ND	ND	ND
BEC	15	+	+	+	+	+	−	−	+	+
	30	+	+	+	+	−	−	−	−	+
	60	+	+	+	−	−	−	−	+	+
	300	+	+	+	−	−	ND	ND	ND	ND

[a] +, growth ; −, no growth.
[b] Not done.

ricidal activities of DDAC against *A. xylosoxidans*, a strain highly resistant to BAC, at 20 and 25℃. At 20 ℃, the cells could survive in the whole concentration range from 0.001 to 10% (w/v), but at 25℃, not in the range from 0.05 to 0.1% (w/v) due to the temperature effect. Tables 2 and 3 show the activities of three kinds of QACs, DDAC, BAC and BEC, against *E. coli* and *S. typhimurium* at a low temperature, 0℃. These organisms were generally sensitive to QACs and were killed rapidly at ambient temperatures, but at 0℃, were observed to survive at concentration levels above 5% (w/v) of BEC although not at those of BAC and DDAC.

The concentration-dependence of QAC activities was investigated in detail by the quantitative suspension test method using *P. aeruginosa* and *A. xylosoxidans* as test organisms. Figure 1 shows the results of the bactericidal activities of DDAC, BAC and BEC against *P. aeruginosa*, which is relatively resistant to QACs, at 0℃ for 30 s. An optimal concentration for the bactericidal activity of each QAC was observed in the concentration-activity relationship. The concentration dependence patterns of four other QACs against *A. xylosoxidans* at 25℃ are shown in

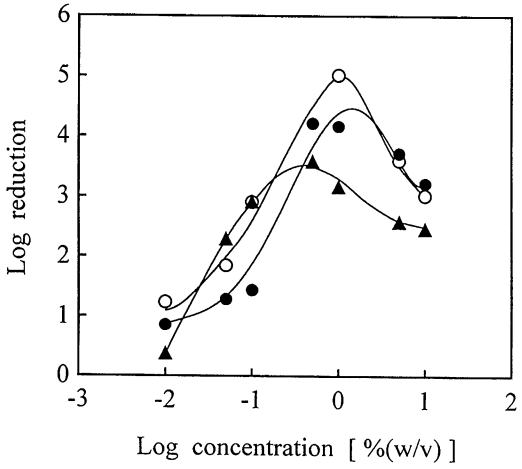

FIG. 1. Concentration dependency of the bactericidal activity of quaternary ammonium compounds against *P. aeruginosa* at 0℃ after 30-s exposure. Symbols : ○, DDAC; ●, BAC ; ▲, BEC.

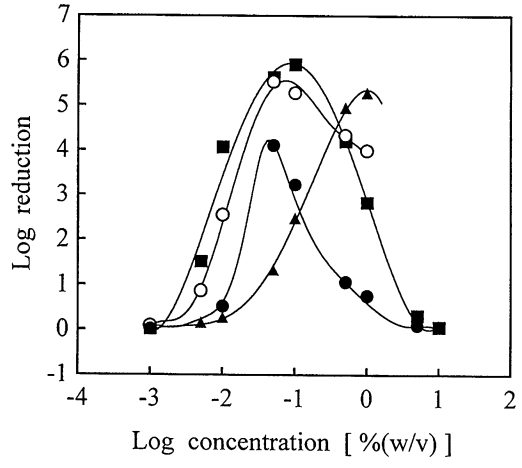

FIG. 2. Concentration dependency of the bactericidal activity of quaternary ammonium compounds against *A. xylosoxidans* at 25℃ after 10-min exposure. Symbols : ▲, $C_{12}PC$; ■, $C_{16}PC$; ○, DDAB ; ●, $C_{16}TAB$.

Fig. 2. Three QACs, DDAB, $C_{16}PC$ and $C_{16}TAB$, clearly exhibited maximum activity at approximately 0.1% (w /v). But the concentration for the maximum activity of $C_{12}PC$ might be obscured due to insufficient inactivation at a higher concentration range of this QAC. The difference between the patterns of $C_{12}PC$ and $C_{16}PC$ suggests that optimal concentrations for their maximum activities depend on the alkyl chain length of QACs. Therefore, the homologous compounds of C_n TAB were also examined (Fig. 3). As would be expected, the optimal bactericidal concentration of the homologue was dependent on the alkyl chain length and shifted toward lower concentrations with increase in that length for C_nTAB (n=12, 14 and 16). In

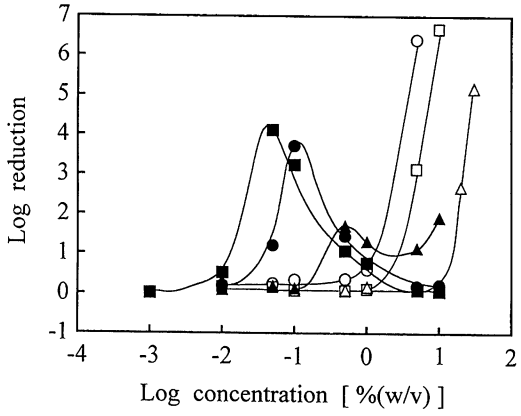

FIG. 3. Concentration dependency of the bactericidal activity of alkyltrimethyl ammonium salts against *A. xylosoxidans* at 25℃ after 10-min exposure. Symbols : △, C_6TAB ; □, C_8TAB ; ○, $C_{10}TAB$; ▲, $C_{12}TAB$; ●, $C_{14}TAB$; ■, $C_{16}TAB$.

addition, the magnitude of the activity at a particular concentration increased in the order of dodecyl < tetradecyl < hexadecyl. In contrast, the optimal concentration for the bactericidal activity of C_nTAB (n=6, 8 and 10) at shorter alkyl chain lengths was not observed.

DISCUSSION

In general, the antimicrobial activity of chemical agents depends on the external environment and on the nature and growth condition of the organisms (Kostenbauder, 1991; Russell, 1982). In our bactericidal test, we lowered the temperature of test solutions and shortened the exposure time in comparison with the usual general conditions for such a test for the determination of the phenol coefficient in order to reduce the sensitivity of the bacteria to the chemicals. As would be expected, an optimal concentration for bactericidal activity against sensitive bacteria such as *E. coli*, *S. typhimurium* and *P. aeruginosa* was also observed in the range of concentrations tested at 0℃ for 30 s (Tables 2 and 3, Fig.1). In this way, one can detect a concentration for the maximum activity under carefully controlled conditions such as temperature and contact period. These findings suggest that the presence of a concentration for a maximum activity may not be related to bacterial species but to the intrinsic nature of QACs themselves.

Therefore, we examined in detail the effect of the molecular structure of QACs, especially the alkyl chain length, on the concentration dependency of the bactericidal activity of the QACs against *A. xylosoxidans*. The relation between the concentration of C_nTAB for a maximum in bactericidal activity (C_{max}) or the magnitude of the bactericidal activity at

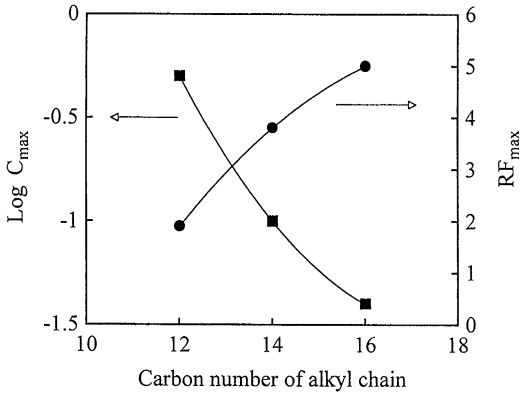

FIG. 4. The relationship between C_{max} or RF_{max} and carbon numbers of alkyl chain of C_nTAB. Symbols: ■, C_{max}; ●, RF_{max}.

C_{max} (RF_{max}) and carbon number of alkyl chain is illustrated in Fig. 4.

C_{max} of C_nTAB clearly shifted toward lower concentration with increase in n. In the case of $C_{12}PC$, it is presumed that if an adequate neutralization could be performed, the optimal concentration for the maximum activity might be similarly found, though this agent could not be sufficiently inactivated in our neutralizing condition above the concentration of 1.0% (w/v). In addition, RF_{max} increased with increasing alkyl chain length. This result is similar to the effect of the alkyl group on the antimicrobial activity in higher dilutions observed by other workers (Daoud et al., 1983; Kourai et al., 1983; Tomlinson et al., 1977). However, RF_{max} values of $C_{12}PC$ and $C_{16}PC$ were almost the same. Although this reason for this is not definite, factors other than alkyl chain length, for example hydrophilic groups, may be primarily involved in RF_{max}.

Surface active agents such as QACs having long alkyl chains associate to form micelles at the critical micelle concentration (CMC) and higher. In other words, the monomer (unassociated molecules) concentration remains constant at concentrations above CMC. It also seems that in the action of chemicals in the bactericidal process, the adsorption of monomers onto the bacterial surface may be one of the most important factors in the first step and may be dependent on the monomer concentration.

Tomlinson and coworkers (1977) have investigated the effect of colloidal association on the antimicrobial activity of alkyldimethylbenzyl ammonium chloride homologues and have demonstrated the importance of micelle formation.

At concentrations below the CMC, in the presence of bacteria there exists an equilibrium between the monomer in the solution and that on the cell, i.e.,

$$[\text{monomer}]_{\text{solution}} \rightleftharpoons [\text{monomer}]_{\text{cell}} \quad (1)$$

where $[\text{monomer}]_{\text{solution}}$ and $[\text{monomer}]_{\text{cell}}$ represent the monomer concentration in the solution and on the cell, respectively. It is assumed that this monomeric form is responsible for bactericidal activity. Its concentration in the solution regularly increases with increasing that of QAC, resulting in increased adsorption onto the cell.

Above CMC, the monomer is put in competitive equilibrium with both the cell and micelle as follows;

$$[\text{micelle}] \rightleftharpoons [\text{monomer}]_{\text{solution}} \rightleftharpoons [\text{monomer}]_{\text{cell}} \quad (2)$$

Therefore, when the micelle is present, the monomer is taken out from the solution, thereby altering the equilibrium of (1) and lowering the amount of monomers which contribute to bactericidal activity.

Sexsmith and White (1959a, 1959b) have estimated the concentration of unassociated single long chain ion in an aqueous solution of a QAC by applying the law of mass action to a micelle formation and then have shown that the monomer concentration passes through a maximum as the total concentration of QAC in the solution is increased. This maximum seems to occur in the vicinity of CMC.

Deluca and Kostenbauder (1960) have also shown that the binding of $C_{16}PC$ to nonionic substrates such as methylcellulose and polysorbate 80 is strongly dependent on $C_{16}PC$ concentration, passing through a maximum in the region of the CMC.

The existence of a maximum in the monomer concentration in the aqueous cationic surfactant solution may provide a probable explanation for our experimental results. We thereby examined the relation between CMCs of cationic surfactants and their C_{max} (Table 4), because a maximum in bactericidal activity also occurred in the vicinity of CMC as shown above. The CMCs of QACs tested were similar to their C_{max} values, indicating that the C_{max} is strongly dependent on the CMC. We therefore related the maximum in the bactericidal activity (Figs. 2 and 3) to the ratio of C_{max} and CMC rather than to C_{max}. The variation among values of C_{max}/CMC is very small as compared with the difference in the extremes of C_{max}. Furthermore, the ratio is sufficiently close to a constant in the homologue. However, C_{max}/CMC of alkyl pyridinium salts is about twice as much as that of alkyl trimethylammonium salts. This may be attributed to the difference of polar head group between them or that of the bactericidal mechanism.

In conclusion, we have presumed that the increased bactericidal activity of QAC below C_{max} is due mainly to the increased free QAC ions in the solution, while the depression above C_{max} is mainly due to the micellar formation which prevents the interaction between free QAC ions and bacterial cells. In addition,

TABLE 4. The concentration for the maximum bactericidal activity of quaternary ammonium compounds (C_{max}), CMC and C_{max}/CMC.

QAC	C_{max}[a] (mM)	CMC[b] (mM)	C_{max}/CMC
C_{12}PC	44.3	17.5	2.5
C_{16}PC	2.4	0.9	2.7
C_{12}TAB	15.9	16.0	0.99
C_{14}TAB	3.6	3.6	1.0
C_{16}TAB	1.1	0.92	1.2
DDAB	1.6	1.85	0.83

[a] See Figs. 2 and 3.
[b] The CMC values at 25℃ from Rosen (1989).

the value of C_{max}/CMC may remain constant for a given homologous series of QACs.

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