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Effect of Alkyl Chain Length of Benzalkonium Chloride on the Bactericidal Activity and Binding to Organic Materials

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We investigated the effect of chain length on the bactericidal activity of benzalkonium chloride during a short contact time by counting survivors. The C_8 and C_{10} homologues had very weak bactericidal activity. The bactericidal concentrations of the C_{12} and C_{14} homologues were 6—400 μg/ml at 10 min of contact at 25 °C against 15 strains of test bacteria. The C_{16} and C_{18} homologues showed variable bactericidal activity towards the 15 strains tested.

As the carbon chain was lengthened, the killing rate decreased and inhibition of the bactericidal activity by organic materials increased. The bactericidal concentrations of the C_{12} homologue at 1 min of contact at 25 °C were 5000 μg/ml in a suspension of 2.5% dried yeast, and 2500 μg/ml in a 10% solution of human serum. Those of the C_{16} homologue were more than 10000 μg/ml in both cases.

We assayed unbound benzalkonium chloride in solutions of bovine serum albumin by high-performance liquid chromatography and found that the bactericidal activity in a solution of bovine serum albumin arose from it.

These results show that from a practical point of view, C_{12}-benzalkonium chloride is the most effective component of the homologues of benzalkonium chloride.

Keywords——benzalkonium chloride; alkyl chain length; bactericidal activity; short contact; dried yeast inhibition; human serum inhibition; bovine serum albumin binding; HPLC; unbound benzalkonium chloride

Introduction

Benzalkonium chloride (abbreviated as BAC) as listed in the pharmacopoeia of Japan (10th Ed.)[1] is a mixture of alkyldimethylbenzylammonium chlorides of the general formula $(C_6H_5CH_2N(CH_3)_2R)Cl$, in which R represents an alkyl group no shorter than n-C_8H_{17} and no longer than n-$C_{18}H_{37}$. The chain length of the alkyl group affects the bactericidal activity. The activity of BAC against *Salmonella typhi* was found by Fujita[2] to be maximum with the $C_{14}H_{29}$ homologue. Shelton *et al.*,[3] working with alkyltrimethylammonium bromides, showed that the $C_{16}H_{33}$ homologue had the strongest activity towards *Staphylococcus aureus* and *Eberthella typhosa* (*Salmonella typhi*) , but that the activity of homologues shorter than $C_{14}H_{29}$ was stronger towards *E. typhosa* than towards *S. aureus*. This finding suggested that the effect of the chain length of the quaternary ammonium moiety depends on the strains tested.

The bactericidal activity of BAC is broad and its killing rate is fast against many clinical isolates.[4] This paper deals with the effect of chain length on the bactericidal activity of this compound in a short contact time in the presence of organic materials against clinical isolates, including opportunistic pathogens. We also assayed the binding of homologues of the alkyl group of BAC to bovine serum albumin by high-performance liquid chromatography (HPLC) to study the relation between bactericidal activity and the amount of unbound benzalkonium chloride.

Materials and Methods

Organisms——The clinical isolates were obtained from several hospitals in 1982—1983. The environmental isolates were collected from our laboratories in 1983. *Achromobacter guttatis* A-39 and *Alcaligenes faecalis* 572, which had both been isolated from 10% benzalkonium chloride,[5] were provided by the National Institute of Health, Japan. *Staphylococcus aureus* FDA 209P, *Pseudomonas aeruginosa* IFO 3080, *Pseudomonas cepacia* ATCC 17774, *Proteus mirabilis* ATCC 21100, *Serratia marcescens* IFO 12648, and *Escherichia coli* NIHJ-JC2 were used as standard strains.

Compounds——Octyl, decyl, dodecyl, tetradecyl, hexadecyl, and octadecyl dimethylbenzylammonium chlorides (abbreviated as C_8-, C_{10}-, C_{12}-, C_{14}-, C_{16}-, and C_{18}-BAC) were obtained from Sanyo Chemicals, Ltd., Japan, as 47.2%, 48.8%, 49.3% and 40.7% solutions and 97.5% and 95.7% solids, with purities of 97.9%, 98.0%, 97.8%, 94.9%, 96.4%, and 94.5%, respectively. Alkyl (59—63% $C_{12}H_{25}$–, 29—34% $C_{14}H_{29}$–, 6.8—7.2% $C_{16}H_{33}$–) dimethylbenzylammonium chloride (abbreviated as OSN) used was OSVAN® (10% solution, Daigo Nutritive Chemicals, Ltd., Japan).

Assay of Bactericidal Concentration——Disinfection experiments were done in wells of microtitration plates[4] at 25 °C (abbreviated as the MTP method). First, 250 μl of a test solution of BAC was poured into each well of the microtitration plate using a sterilized disposable syringe attached to a STEPPER™ (TRIDAK Division, Indicon Inc., U.S.A.). Test bacteria growing in SCD medium (soybean-casein digest broth) (Daigo) at 35 °C for 24 h were diluted to about 10^7 cfu/ml with sterilized deionized water, and 10 μl of this suspension was added to the test solution and stirred using a sterilized toothpick. After 5 and 10 min of contact, 10 μl of the reaction mixture was transferred to 3 ml of SCDLP medium (soybean-casein digest broth with lecithin & polysorbate 80) (Daigo) to neutralize the BAC, and the mixture was incubated at 35 °C for 72 h.

Counting of Viable Cells in Test Solutions——We mixed 5 ml of test solution with 20 μl of a cell suspension in a test tube at 25 °C. Samples were withdrawn 10 and 30 s, and 1 and 10 min intervals. Then 500 μl of reaction mixture was transferred to 9.5 ml of SCDLP medium (Daigo), and the number of viable cells was counted using a Spiral System (Spiral System Instrument Inc., U.S.A.) on SCDLP agar medium after incubation at 35 °C for 24—48 h. Moreover, all SCDLP liquid media containing samples were incubated at 35 °C for 72 h to check for viable cells fewer than 10^2 cfu/ml.

To study the effect of organic materials, 2.5% dried yeast[6] (Asahi Breweries, Ltd., Japan) or 10% fresh human serum was added to the test solution.

Assay of BAC——BAC was assayed by reversed-phase HPLC by the method of Meyer.[7] The HPLC system consisted of a pump (LC-5A, Shimadzu Corporation, Japan), a reversed-phase column (ZORBAX CN, 5 μm, 4.5 mm × 25 cm, DuPont Instruments, U.S.A.), a loop injection system (20 μl, SIL-1A, Shimadzu), a UV detector (SPD-2A, Shimadzu) and a chromato-pack data system (C-R3A, Shimadzu). The mobile phase was 600 ml of acetonitrile (HPLC reagent grade, Wako Pure Chemical Industries, Ltd., Japan) and 400 ml of 0.1 M sodium acetate (special grade, Wako) adjusted to pH 5.0 with acetic acid (analytical reagent grade, Wako). After being filtered through a 1 μm filter and degassed by ultrasonication (Eiko-Seiki, Japan, No. 3, 15 min), the mobile solvent was started through the chromatographic system. The chromatographic parameters were a flow rate of 2.0 ml/min giving a pressure of 1.2 kg/cm^2, a 20-μl loop injector with an analysis time of 13 min, 254 nm detection at 0.02 a.u.f.s. and a chart speed of 0.4 cm/min. The software statistical package supplied by the manufacturer was utilized with the data system.

C_{12}-, C_{14}- and C_{16}-BAC each showed a single peak at a retention time of 8.8, 10.7 and 13.2, respectively. Each calibration graph was linear and passed through the origin (Fig. 1).

Separation of Unbound BAC in a Solution of Bovine Serum Albumin——A 1 ml portion of C_{12}-, C_{14}-BAC or OSN (200—4000 μl/ml) solution was mixed with the same volume of a solution of bovine serum albumin (powder fraction V-Cohn, Wako) (5—160 mg/ml) and incubated at room temperature for 1 h. Then 1 ml of the reaction mixture was put in an ultrafiltration cell[8] (Micropartition system, MPS-1; YMT membrane, Amicon Corp., U.S.A), and centrifuged at 3000 rpm in a centrifuge (Sorvall RC-5B, DuPont). The filtrate up to a centrifugation time of 1 min was discarded and that at more than 2 min was collected and analyzed for unbound BAC using HPLC.

To confirm non-absorption on the membrane, standard solutions of C_{12}-, C_{14}- or C_{16}-BAC (20—2000 μg/ml) were filtered. Recovery of C_{12}-BAC was 97—100% for 80—2000 μg/ml and that of C_{14}-BAC was 100% for 80—640 μg/ml, but that of C_{16}-BAC was only 73—76% for 80—160 μg/ml, so we did not assay unbound C_{16}-BAC.

Results

Effect of Alkyl Chain Length of BAC on Bactericidal Activity in Deionized Water

The bectericidal concentrations of homologues of the long chain alkyl group of BAC assayed by the MTP method at 10 min of contact are shown in Table I. C_8 - and C_{10}-BAC had

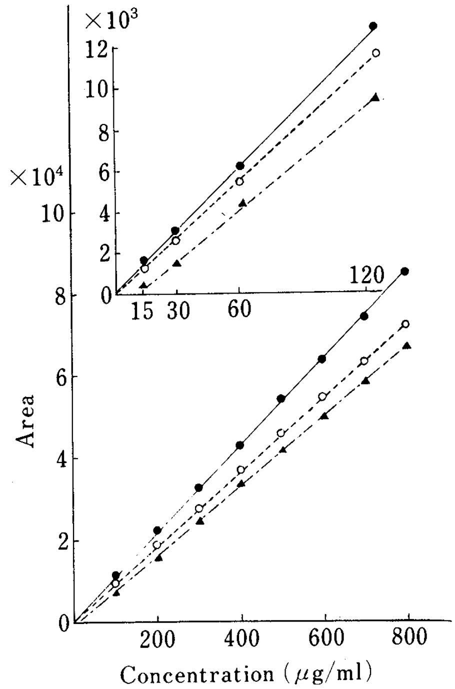

Fig. 1. Standard Calibration Curves for C_{12}, C_{14}, and C_{16}Homologues of BAC

●—●, C_{12}; ○---○, C_{14}; ▲---▲, C_{16}.

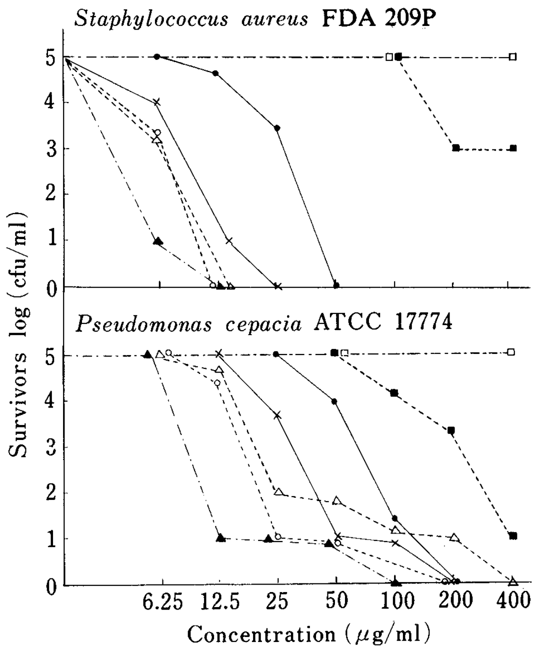

Fig. 2. Number of Survivors after 10 min at 25 °C with Various Concentrations of Homologues of the Long Alkyl Group of BAC and OSN (Alkyl(59—63% $C_{12}H_{25}$-, 29—34% $C_{14}H_{29}$-, 6.8—7.2% $C_{16}H_{33}$-)dimethylbenzylammonium Chloride)

□---□, C_8; ■---■, C_{10}; ●—●, C_{12}; ○---○, C_{14}; ▲---▲, C_{16}; △---△, C_{18}; ×—×, OSN.

very weak bactericidal activity. Their bactericidal concentration range was 400—1000 and that of C_{12}- and C_{14}-BAC was 6.3—400 μg/ml against all test bacteria, including *A. faecalis* 572 and *A. guttatis* A-39. However, C_{16}- and C_{18}-BAC were bactericidal to different extents toward the 15 strains tested. They were effective against gram-positive but not against gram-negative bacteria. Of these homologues, C_{12}-BAC had particularly broad and strong bactericidal activity, and OSN, which is composed mainly of this homologue, had a slightly weaker activity against *A. guttatis* and *A. faecalis* isolated from a 10% solution of BAC than C_{12}-BAC did.

Figure 2 shows the number of survivors in reaction mixtures containing various concentrations of homologues of the long chain alkyl group of BAC after 10 min of contact. Bactericidal activity increased with increasing carbon chain length of the alkyl group from 8 to 16 against *S. aureus* FDA 209P and *P. cepacia* ATCC 17774. C_{18}-BAC had less activity than C_{16}-BAC. *S. aureus* was almost completely killed at 12.5 μg/ml for C_{14}-, C_{16}-, and C_{18}-BAC, 25 μg/ml for OSN, and 50 μg/ml for C_{12}-BAC. *P. cepacia* was killed at 200 μg/ml of C_{12}-BAC and reduced from 10^5 to about 10 cfu/ml over a wide range of concentrations (12.5—200 μg/ml) of C_{14}-, C_{16}-, and C_{18}-BAC and of OSN, but the concentration needed to kill all cells was 100—400 μg/ml, more than that needed in the case of *S. aureus*.

Relation between the Alkyl Chain Length of BAC and Inhibition of Bactericidal Activity by Organic Materials

Bactericidal concentrations of C_{12}-, C_{14}-, and C_{16}-BAC and of OSN at 10 and 30 s and at 1 and 10 min of contact at 25 °C in deionized water, a suspension of 2.5% dried yeast, and a 10% solution of human serum are shown in Table II. As the carbon chain was lengthened, the

TABLE I. Bactericidal Concentrations of Homologues of the Long-Chain Alkyl Group of Benzalkonium Chloride

Organism	OSN	$[C_6H_5CH_2-N(CH_3)_2-R]$ Cl (R)					
		$-C_8H_{17}$	$-C_{10}H_{21}$	$-C_{12}H_{25}$	$-C_{14}H_{29}$	$-C_{16}H_{33}$	$-C_{18}H_{37}$
S. aureus FDA 209P	25	>1000	500	50	25	12.5	25
Micrococcus sp. H 1689	12.5	800	400	25	6.3	12.5	25
P. aeruginosa IFO 3080	100	1000	500	50	50	100	>400
P. aeruginosa 82-2-32R	200	1000	500	200	100	50	>400
P. cepacia ATCC 17774	200	>1000	500	200	200	100	400
Pseudomonas sp. H 6104	25	800	400	50	12.5	25	50
P. mirabilis 82-1-4	200	>1000	500	200	200	400	400
P. morganii 82-2-11	100	>1000	400	100	50	100	>400
P. mirabilis ATCC 21100	100	1000	500	100	100	100	50
S. marcescens 82-2-52	200	>1000	800	200	200	200	400
S. marcescens IFO 12648	100	1000	500	100	100	100	50
Flavobacterium sp. 82-1-98	200	>1000	500	100	200	>400	>400
E. coli NIHJ-JC2	25	1000	400	25	25	25	100
A. guttatis A-39	500	>1000	>1000	400	400	>400	>400
A. faecalis 572	500	>1000	>1000	200	400	>400	>400

μg/ml. MTP method, in deionized water for 10 min at 25 °C. Inoculum, 10^6 cfu/ml. OSN, alkyl(59—63% $C_{12}H_{25}$-, 29—34% $C_{14}H_{29}$-, 6.8—7.2% $C_{16}H_{33}$-)dimethylbenzylammonium chloride.

bactericidal activity towards *Staphylococcus* sp. increased even at a short contact time, but the activity towards gram-negative bacteria decreased. The bactericidal concentration of C_{16}-BAC against *Staphylococcus* sp. at 10 s of contact was 50—400 μg/ml in deionized water. However, to kill all gram-negative bacteria tested with 1 min of contact, 1000, 5000, >10000, and 1000 μg/ml of C_{12}-, C_{14}-, C_{16}-BAC, and OSN, respectively, were needed. The difference of the bactericidal concentrations after 10 s and after 10 min of contact suggested that the killing rate of BAC decreased with increase in the alkyl chain length.

The bactericidal activity of BAC was inhibited by both dried yeast and human serum. Inhibition by 2.5% dried yeast was stronger than that by 10% human serum. As the carbon chain was lengthened, the inhibition increased. C_{12}-BAC was bactericidal against *P. cepacia, P. aeruginosa, A. guttatis, A. faecalis*, and *S. marcescens* at 5000 μg/ml in a suspension of 2.5% dried yeast and at 2500 μg/ml in a 10% solution of human serum at 1 min of contact, but C_{14}- and C_{16}-BAC could not kill them at 10000 μg/ml for 10 min. The bactericidal concentration of OSN, which consists mainly of C_{12}- and C_{14}-BAC, was >10000 μg/ml in a suspension of 2.5%

TABLE II. Effect of Human Serum and Dried Yeast on Bactericidal Activity of Homologues of the Long-Chain Alkyl Group of Benzalkonium Chloride

Organism		Bactericidal concentration ($\times 10^3$ μg/ml)$^{a)}$															
		OSN				C_{12}-BAC				C_{14}-BAC				C_{16}-BAC			
		10 s	30 s	1 min	10 min	10 s	30 s	1 min	10 min	10 s	30 s	1 min	10 min	10 s	30 s	1 min	10 min
S. aureus FDA 209P	DW	0.5	0.5	0.2	0.05	5	1	0.5	0.1	1	0.4	0.1	0.025	0.4	0.2	0.025	0.01
	HS	1	0.5	0.5	0.5	1	1	0.5	0.5	5	1	0.5	0.5	0.5	0.5	0.5	0.5
	DY	5	5	2.5	2.5	5	5	2.5	2.5	5	5	2.5	2.5	>10	2.5	2.5	2.5
S. epidermidis IFO 3762	DW	0.2	0.1	0.0125	0.006	1	0.4	0.4	0.025	0.8	0.025	0.025	0.006	0.05	0.006	<0.006	<0.006
	HS	0.5	0.5	0.5	0.5	2.5	1	0.5	0.5	0.5	0.5	0.5	0.5	2.5	0.5	0.5	0.5
	DY	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1
P. cepacia ATCC 17774	DW	5	1	1	0.4	1	0.8	0.5	0.5	>10	0.5	0.2	0.1	>10	>10	0.2	0.05
	HS	2.5	0.5	0.5	0.5	2.5	1	0.5	0.5	>10	5	2.5	0.5	>10	>10	>10	2.5
	DY	10	5	5	2.5	5	5	5	2.5	>10	>10	10	5	>10	>10	>10	>10
P. cepacia H130	DW	5	5	0.2	0.1	5	5	1	0.5	10	5	5	0.2	>10	>10	>10	2.5
	HS	5	1	0.5	0.5	5	1	1	0.5	5	5	2.5	1	>10	>10	>10	5
	DY	>10	5	5	2.5	10	5	5	5	>10	>10	>10	10	>10	>10	>10	>10
P. aeruginosa IFO 13736	DW	0.05	0.05	0.025	0.01	0.1	0.1	0.05	0.025	0.05	0.05	0.025	0.001	0.1	0.025	0.025	0.01
	HS	2.5	1	0.5	0.5	10	5	0.5	0.5	10	5	2.5	1	>10	>10	5	1
	DY	10	10	5	5	10	5	5	5	>10	>10	10	5	>10	>10	10	5
P. aeruginosa 82-2-32	DW	0.1	0.1	0.025	0.01	0.2	0.1	0.1	0.025	0.2	0.2	0.025	0.01	10	0.1	0.05	0.01
	HS	2.5	2.5	0.5	0.5	5	5	2.5	1	10	5	2.5	1	5	5	0.5	0.5
	DY	>10	10	10	10	10	5	5	5	>10	>10	10	10	>10	>10	>10	>10
P. mirabilis ATCC 21100	DW	0.2	0.2	0.05	0.05	0.4	0.2	0.01	0.01	0.2	0.05	0.05	0.025	0.1	0.05	0.01	0.01
	HS	5	2.5	0.5	0.5	5	1	1	0.5	10	2.5	2.5	1	>10	5	5	1
	DY	>10	5	5	2.5	10	5	5	2.5	>10	10	5	5	>10	>10	>10	10
P. mirabilis 82-1-4	DW	5	0.2	0.1	0.01	1	0.4	0.2	0.05	1	0.1	0.05	0.05	>10	0.2	0.05	0.025
	HS	5	1	0.5	0.5	5	2.5	1	1	10	2.5	2.5	0.1	>10	>10	2.5	0.5
	DY	>10	5	5	5	10	10	5	5	>10	>10	10	5	>10	>10	>10	10
P. morganii 82-2-11	DW	0.5	0.2	0.1	0.05	0.2	0.2	0.05	0.05	1	0.05	0.01	0.01	1	0.05	0.05	0.025
	HS	2.5	1	0.5	0.5	10	5	0.5	0.5	10	5	1	1	>10	>10	5	1
	DY	5	5	5	5	10	5	5	5	10	10	5	5	>10	>10	>10	10
S. marcescens 82-2-52	DW	5	0.4	0.2	0.05	>10	0.4	0.4	0.025	>10	0.4	0.2	0.025	>10	>10	0.025	0.025
	HS	5	2.5	1	0.5	10	5	1	0.5	>10	>10	5	2.5	>10	>10	>10	2.5
	DY	>10	>10	5	5	>10	5	5	5	>10	>10	10	5	>10	>10	>10	10
Flavobacterium sp. 82-1-98	DW	0.1	0.05	0.05	0.025	0.2	0.05	0.05	0.25	0.2	0.05	0.025	0.025	0.2	0.1	0.025	0.01
	HS	2.5	0.5	0.5	0.5	5	1	0.5	0.5	5	5	1	0.5	10	5	2.5	0.1
	DY	2.5	2.5	2.5	2.5	5	2.5	2.5	1	10	10	5	1	10	5	5	2.5
A. guttatis A-39	DW	5	5	1	0.2	>10	>10	1	1	>10	>10	10	1	>10	>10	>10	0.5
	HS	5	5	1	0.5	5	5	1	1	>10	>10	>10	>10	>10	>10	>10	>10
	DY	>10	>10	>10	2.5	>10	>10	5	5	>10	>10	>10	>10	>10	>10	>10	>10
A. faecalis 572	DW	1	1	0.2	0.1	1	0.4	0.2	0.2	>10	0.2	0.2	0.2	>10	>10	>10	0.2
	HS	10	2.5	1	0.5	10	5	2.5	0.5	>10	10	10	1	>10	>10	>10	10
	DY	>10	>10	10	5	>10	10	5	5	>10	>10	>10	5	>10	>10	>10	>10

a) Killed from 10^6 to <10 cfu/ml at 25 C. DW, deionized water; HS, 10% human serum; DY, 2.5% dried yeast. OSN, alkyl (59—63% $C_{12}H_{25}$-, 29—34% $C_{14}H_{29}$-, 6.8—7.2% $C_{16}H_{33}$-)dimethylbenzylammonium chloride.

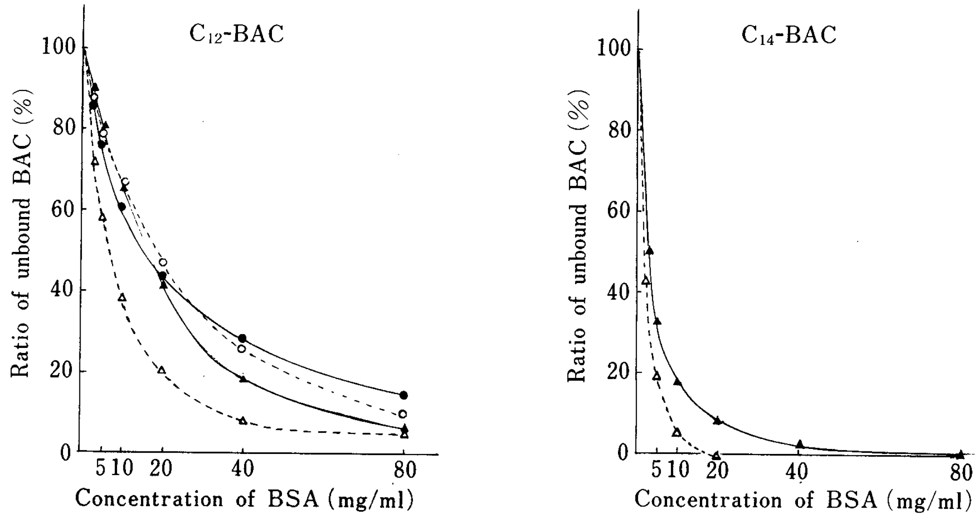

Fig. 3. Ratio of Unbound C_{12} and C_{14} Homologues of BAC in Mixtures with Various Concentrations of Bovine Serum Albumin (BSA)

△---△, 100; ▲—▲, 500; ○---○, 1000; ●—●, 2000 μg/ml.

dried yeast and 1000 μg/ml in a 10% solution of human serum at 1 min of contact. When the contact time was short in the presence of 10% human serum, the bactericidal concentrations against some strains were slightly less than those in water.

Effect of Alkyl Chain Length of BAC on Binding to Bovine Serum Albumin

The unbound BAC in mixtures containing various concentrations of bovine serum albumin was assayed using HPLC after separation from the albumin by ultrafiltration. The percentage of unbound C_{12}- and C_{14}-BAC is shown in Fig. 3. Unbound BAC decreased drastically as the concentration of the albumin was increased to 20—40 mg/ml for C_{12}-BAC and 10 mg/ml for C_{14}-BAC. C_{12}-BAC bound less markedly to the albumin than C_{14}-BAC did. In a mixture of 500 μg/ml of C_{12}- or C_{14}-BAC and 20 mg/ml of the albumin, 40% of the C_{12}-BAC was unbound, but even with only 10 mg/ml of the albumin less than 20% of C_{14}-BAC was unbound. Figure 4 shows that the percentages of unbound C_{12}- and C_{14}-BAC in a solution of OSN, a mixture of C_{12}-, C_{14}- and C_{16}-BAC, are similar to those in separate solutions of each component (Fig. 3). In the presence of bovine serum albumin, the most unbound component in OSN was C_{12}-BAC.

Relation between Binding of BAC to Bovine Serum Albumin and Inhibition of Bactericidal Activity

The bactericidal activity of C_{12}-BAC and OSN was assayed in deionized water and in solutions of 100 and 500 μg/ml of these compounds in the presence of 10—80 mg/ml of bovine serum albumin. The number of survivors of *S. aureus* FDA 209P and *P. cepacia* ATCC 17774 and the concentration of unbound BAC are shown in Table III.

C_{12}-BAC was bactericidal against *S. aureus* at 50—70 μg/ml in deionized water and at 100 μg/ml in a 10 mg/ml solution of the albumin at 10 min of contact. The concentration of unbound C_{12}-BAC in the latter solution, 38 μg/ml, was nearly equal to the bactericidal concentration in deionized water. These results showed that the bactericidal activity of C_{12}-BAC against *S. aureus* in a solution of bovine serum albumin was attributable to the unbound form. Among the components of OSN, the concentration of C_{16}-BAC could not be assayed, since it was absorpted on the ultrafiltration membrane. The bactericidal concentration of OSN was less than that of C_{12}-BAC in deionized water, because C_{16}-BAC had stronger bactericidal activity against *S. aureus* FDA 209P and *P. cepacia* ATCC 17774 than

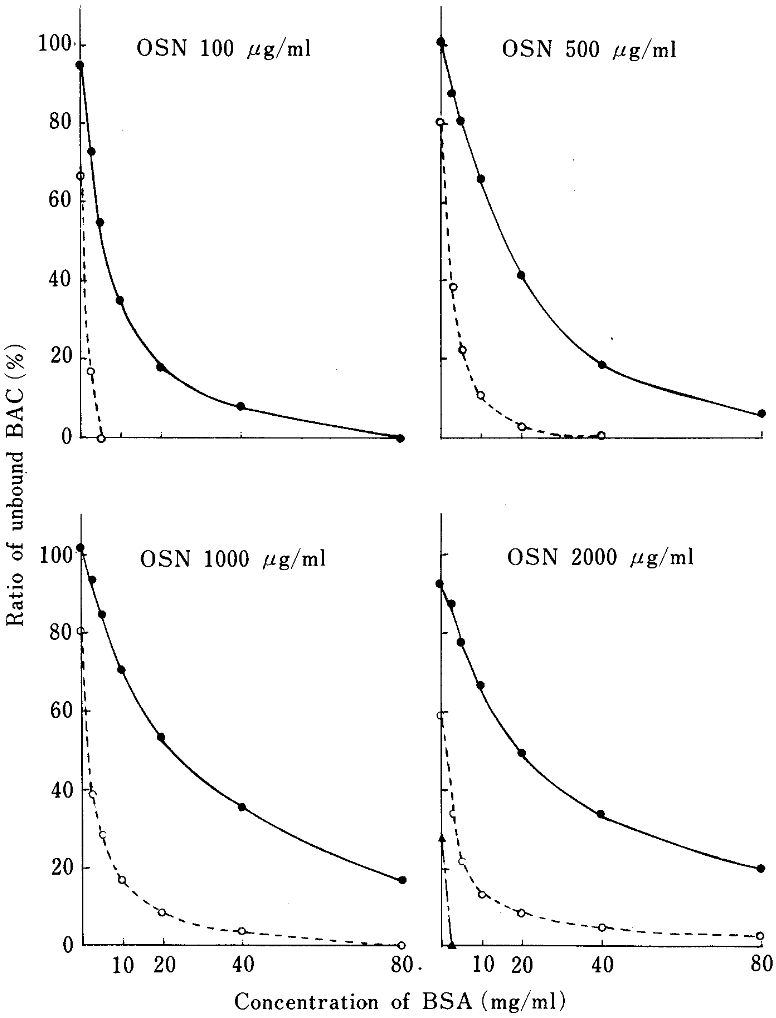

Fig. 4. Binding of the Components of OSN (Alkyl(59—63% $C_{12}H_{25}$-, 29—34% $C_{14}H_{29}$-, 6.8—7.2% $C_{16}H_{33}$-)dimethylbenzylammonium Chloride) to Bovine Serum Albumin (BSA)

●—●, C_{12}-; ○---○, C_{14}-; ▲---▲, C_{16}-homologue of BAC.

C_{12}- and C_{14}-BAC did (Table I). However, in the presence of bovine serum albumin, the bactericidal activity of OSN was equivalent to that of C_{12}-BAC; 20—30 μg/ml for *S.aureus*, and more than 300 μg/ml for *P. cepacia*, in terms of the unbound concentration of C_{12}-BAC.

Discussion

The Pharmacopoeia of Japan (10th Ed.)[1] prescribes that benzalkonium chloride (BAC) is composed mainly of n-$C_{12}H_{25}$ and n-$C_{14}H_{29}$ homologues of the alkyl group, but does not specify the amount of each homologue. In the U.S. National Formulary XVI,[9] the proportion of the n-$C_{12}H_{25}$ homologue is not less than 40% and that of the n-$C_{14}H_{29}$ homologue is not less than 20% of the total.

The mode of the bactericidal action of BAC has been studied by many workers.[10] The working hypothesis is a two-fold action, involving disorganization of the cell membrane first, followed by denaturation of proteins essential to metabolism and growth. Many investigators[11] have studied the effect of the alkyl carbon length on the antibacterial activity of BAC, finding that as the carbon chain of the alkyl group lengthened, the activity became stronger, but that there was a turndown point in activity. The turndown point depended upon

TABLE III. Relationship between Bactericidal Activity and Unbound Concentration of Dodecyldimethylbenzylammonium Chloride (C_{12}-BAC) and OSN in Solutions of Bovine Serum Albumin (BSA)

Organism	BAC μg/ml	BSA mg/ml	C_{12}-BAC Unbound (μg/ml)	C_{12}-BAC No. of survivors (cfu/ml)		OSN Unbound (μg/ml)			OSN No. of survivors (cfu/ml)	
			C_{12}	1 min	10 min	C_{12}	C_{14}	C_{16}	1 min	10 min
S. aureus FDA 209P	200	0	200[a]	<10	0	120	60	14[a]	0	0
	100	0	100[a]	10^2	0	60	30	7[a]	0	0
	70	0	70[a]	10^2	0	42	21	4.9[a]	<10	0
	50	0	50[a]	10^2	<10	30	15	3.5[a]	<10	0
	20	0	20[a]	10^2	<10	12	6	1.4[a]	<10	<10
	10	0	10[a]	10^3	10^2	6	3	0.7[a]	10^2	<10
	100	0	100[a]	0	0	57	20[b]	N.A.	0	0
	100	10	38.4[b]	<10	0	33	0[b]	N.A.	<10	0
	100	20	20.3[b]	<10	<10	21	0[b]	N.A.	<10	0
	100	40	8.3[b]	<10	<10	11	0[b]	N.A.	<10	<10
	100	80	6.7[b]	10^2	<10	5	0[b]	N.A.	10^2	<10
	0	0	0	10^6	10^6	0	0	0	10^6	10^6
P. cepacia ATCC 17774	1000	0	1000[a]	0	0	600	300	70[a]	0	0
	700	0	700[a]	<10	0	420	210	49[a]	0	0
	500	0	500[a]	<10	0	300	150	35[a]	0	0
	400	0	400[a]	10^2	0	240	120	28[a]	<10	0
	300	0	300[a]	10^2	10^2	180	90	21[a]	<10	0
	200	0	200[a]	10^4	10^2	120	60	14[a]	<10	0
	100	0	100[a]	N.A.	N.A.	60	30	7[a]	10^4	10^2
	500	0	500[a]	0	0	304	121[b]	N.A.	0	0
	500	10	328.6[b]	<10	0	198	17[b]	N.A.	<10	<10
	500	20	207.9[b]	10	<10	124	5[b]	N.A.	10^2	<10
	500	40	91.4[b]	10^2	<10	55	1[b]	N.A.	10^2	<10
	500	80	31.5[b]	10^2	10^2	18	0[b]	N.A.	10^2	10^2
	0	0	0	10^6	10^6	0	0	0	10^6	10^6

a) Theoretical. *b*) Assayed. N.A.; Not assayed. OSN, alkyl(59—63% $C_{12}H_{25}$-, 29—34% $C_{14}H_{29}$-, 6.8—7.2% $C_{16}H_{33}$-)-dimethylbenzylammonium chloride.

the microorganisms tested. In our results from a homologous series (C_8—C_{18}) of BAC, this point was C_{18} against *S. aureus* and C_{16} towards gram-negative bacteria when the contact time was short (Table I). Blois and Swarbrick[11a] suggested from their study of the interaction of BAC and insoluble monolayers of biological materials that the turndown in activity of BAC is probably related to more than one physical property of the compounds. One is the length of the hydrocarbon chain: the longer the chain, the greater the tendency for the molecules to be adsorbed at the surfaces of bacteria. Another is the reduction in aqueous solubility of the molecules as the carbon number increases. Tomlinson *et al.*[11b] reported that there was a logarithmic relationship up to C_{14} and then a turndown in activity with greater length in the case of the minimum inhibitory concentration of a homologous series (C_8—C_{18}) of BAC, and suggested that the turndown in activity related to colloidal association.

Nakagawa *et al.*[11c] investigated the relationship between the absorption of lauryl pyridinium chloride (LPyC), a quaternary ammonium chloride, and the hydrophobicity of cell surfaces of *E. coli* strains that differed in their susceptibility to LPyC. They showed that the absorption of LPyC on cells was one factor affecting the susceptibility.

When the carbon chain is longer than C_{14}, the solubility and the critical micelle concentration of BAC are extremely low.[12] Therefore, the absorption of BAC with a long-chain alkyl group might be affected by small changes of the cell surface. There are many factors which might be affected by the alkyl carbon length of BAC, producing changes in the bactericidal activity, for example, aqueous solubility, aqueous critical micelle concentration, and lipophilicity, as well as the characteristics of the cell surface of the microorganisms used.

It has not previously been reported that alkyl chain length affects the killing rate of BAC. As the carbon chain was lengthened, we found that the killing rate decreased (Table II). C_{12}- and C_{14}-BAC were effectively bactericidal with all bacteria tested, even at a short contact time (30 s to 1 min).

Moreover, inhibition of bactericidal activity by organic materials was least for C_{12}-BAC (Table II). To clarify the relationship between inhibition of the bactericidal activity of BAC by organic materials and binding of BAC to them, we assayed unbound C_{12}- and C_{14}-BAC in a solution of bovine serum albumin by HPLC. As the carbon chain was lengthened, aqueous solubility decreased.[12] The binding to bovine serum albumin increased with increase in the carbon chain; C_{14}-BAC bound to bovine serum albumin 2.5—3.7 times more than C_{12}-BAC did (Fig. 3). The bactericidal activity of BAC in a solution of bovine serum albumin depended on the amount of unbound BAC (Table III).

The proportion of protein in human serum is about 6.5%.[13] In a 10% solution of human serum, which was estimated to contain 0.65% protein as albumin, we thought that 70—90% of C_{12}-BAC would remain unbound when its concentration was 500—1000 μg/ml (Fig. 4). Judging from the concentration that remained, the bactericidal activity would not be expected to decrease substantially. However, our results (Table II) show that other factors in human serum also inhibited the activity.

Human serum tended to stimulate bactericidal activity against *S. aureus* and *P. cepacia* when the contact time was short. Similar stimulation of the action of BAC, chlorhexidine gluconate, and TEGO-51 by calf serum was reported by Yo *et al.*[14] Fresh serum from vertebrates is lethal to some bacteria,[15] but the control containing only human serum had no such activity in our case. Human serum may make the surfaces of some bacteria more sensitive to BAC.

These results suggest that C_{12}-BAC is the most effective component of the homologous series of BAC in the presence of organic materials. The proportions of n-$C_{12}H_{25}$ and n-$C_{14}H_{29}$ are specified in the prescription of BAC in the U.S. National Formulary XVI. The greater the content of C_{12}-BAC in a homologous series of BAC, the more effective the mixture should be as a sanitizer from a practical point of view.

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