Relationship between the Effects on Bactericidal Activity of Selected Disinfectants and the Hydrophobic Characters of Dibasic Acid Diesters

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We prepared test solutions which contained 80% (v/v) ethanol and 0.2% (w/v) chlorhexidine (CH) or benzalkonium chloride (BC) with or without a dibasic acid diester. After complete evaporation of the ethanol from the solution on filter paper, an overnight broth culture (*Staphylococcus aureus*) was repeatedly inoculated onto the filter paper, and viable bacterial counts were measured at 5 min after the last inoculation. By comparison with viable counts for CH or BC alone, we estimated the potentiating effects of dibasic acid diester on the bactericidal activity of CH or BC, and confirmed that this activity of the two disinfectants was potentiated in the presence of certain compounds in the homologs of di-*n*-butyl esters of aliphatic dibasic acid, and di-alkyl esters of adipic and phthalic acid. Diisobutyl adipate, one of the most effective diesters, substantially enhanced the bactericidal activities of benzethonium chloride, cetyl pyridinium chloride and didecyl dimethyl ammonium chloride, as well as CH and BC, but not those of polyhexamethylene biguanide or alkyldiaminoethyl glycinate. The potentiating effects of dibasic acid diesters observed for both CH and BC seemed to be affected by the hydrophobic character of these diesters themselves and are also expressed well by a particular quadratic equation as a function of these characters: namely, capacity factors, as determined by high-performance liquid chromatography.

Keywords bactericidal activity; disinfectant; dibasic acid diester; hydrophobicity; quantitative structure–activity relationship; *Staphylococcus aureus*; chlorhexidine; quaternary ammonium compound

Introduction

In previous studies,[1] we found that the bactericidal activity of chlorhexidine digluconate (CH) on filter paper was substantially enhanced in the presence of certain emollients such as diisopropyl adipate (**11**), diisobutyl adipate (**12**), polyglycerol and polyoxyethylene glyceryl monococoate (HE). It was further demonstrated that an alcoholic solution containing both CH and emollients (**12** and HE) exhibited effective antimicrobial activity against normal flora as well as artificial contaminants applied to a hand surface and was comparable to an alcoholic preparation containing CH alone.

As the activity of CH was enhanced by dibasic acid diesters such as **11** and **12**, it was of interest to learn whether other such diesters caused similar effects. In the study at hand, we investigaged the relationship between the effects of dibasic acid diesters on the bactericidal activity of several disinfectants, including CH and the hydrophobic characters of the diester groups tested.

Materials and Methods

Disinfectants Table I lists the commercial sources and brand names of the tested disinfectants, the active ingredients and their abbreviations.

TABLE I. Type and Source of Disinfectants Tested

Disinfectant and source	Active ingredient	Abbr.
Hibitane (Sumitomo Seiyaku, Ltd.)	Chlorhexidine digluconate, 20%	CH
Cation F2-50 (Nippon Yushi, Ltd.)	Benzalkonium chloride, 50%	BC
Hyamine 1622 (Rohm and Haas, Ltd.)	Benzethonium chloride, 99%	HA
Cation DDC-50 (Sanyo Kasei Kogyo, Ltd.)	Didecyl dimethyl ammonium chloride, 50%	DDC
Vantocil IB (ICI, Ltd.)	Hydrochloride of polyhexa-methylene biguanide, 20%	IB
Levon 15 (Sanyo Kasei Kogyo, Ltd.)	Sodium alkyl diaminoethyl glycinate, 30%	AG
Cetyl pyridinium chloride (Tokyo Kasei Kogyo, Ltd.)	Cetyl pyridinium chloride, >99%	CPC

Dibasic Acid Diesters Table II lists the various dibasic acid diesters tested whose purities were ascertained by gas chromatography (GC) or high performance liquid chromatography (HPLC) to be more than 98%.

Test Organism and Medium We used *Staphylococcus aureus* ATCC 25923 as the test organism and nutrient broth and agar (Nissui Seiyaku, Ltd.) for reconstituting and subculturing the organism. Overnight broth cultures (37°C, 16 h) were used for all tests.

Test Solutions Test solutions were prepared using 80% (v/v) ethanol

TABLE II. Dibasic Acid Diesters Tested, Their Capacity Factors and Their Potentiating Effects on CH and BC

Dibasic acid diester	Source[a]	Capacity[b] factor (k')	log (N_0/N)[c]	
			CH	BC
1 Di-*n*-bytyl oxalate	R	0.478	−0.100	0.203
2 Di-*n*-butyl malonate	R	0.371	0.087	0.753
3 Di-*n*-butyl succinate	R	0.476	0.170	0.677
4 Di-*n*-butyl glutarate	S	0.627	0.403	1.303
5 Di-*n*-butyl adipate	R	0.908	0.490	1.602
6 Di-*n*-butyl suberate	S	1.337	0.393	1.227
7 Di-*n*-butyl sebacate	R	2.621	0.347	0.747
8 Dimethyl adipate	R	0.047	0.053	−0.009
9 Diethyl adipate	R	0.171	0.097	0.007
10 Di-*n*-propyl adipate	R	0.356	0.093	0.222
11 Diisopropyl adipate	R	0.290	−0.043	0.433
12 Diisobutyl adipate	R	0.844	0.507	1.643
13 Di-*tert*-butyl adipate	S	0.600	0.330	1.337
14 Di-isoamyl adipate	S	1.294	0.383	1.707
15 Di-*n*-hexyl adipate	S	2.609	0.407	1.043
16 Di-(2-ethylbutyl) adipate	S	3.509	0.433	1.180
17 Di-(2-ethylhexyl) adipate	R	7.009	0.060	0.160
18 Diisodecyl adipate	R	16.198	0.003	0.097
19 Dimethyl phthalate	R	0.053	0.103	−0.097
20 Diethyl phthalate	R	0.175	0.147	0.113
21 Di-*n*-butyl phthalate	R	0.821	0.577	1.377
22 Diisobutyl phthalate	R	0.747	0.603	1.417
23 Di-(2-ethylhexyl) phthalate	R	6.982	0.053	−0.160
24 Diisodecyl phthalte	R	17.493	0.077	−0.247

a) R, reagent (Tokyo Kasei Kogyo, Ltd.); S, the gift of and synthesized by the late Prof. M. Okahara of Osaka University. *b*) Determined by HPLC (Table III). *c*) Potentiating effect: N_0=average counts after exposure to the disinfectant alone; N=average counts after exposure to the disinfectant and the dibasic acid diesters.

solutions containing each disinfectant and dibasic acid diester (0.2% (w/v) respectively). As a control, 80% (v/v) ethanol solutions containing each disinfectant alone were prepared.

Bactericidal Test The apparatus to evaluate the bactericidal activity of the residue of the test solutions on filter paper was as shown in Fig. 1. A filter paper (Toyo Roshi No. 50, 20 × 20 mm), was placed on the top of a glass cylinder (outside diameter = 25 mm, length = 65 mm) which was fixed in the center of a glass beaker (inside diameter = 50 mm, depth = 70 mm). A 50 μl volume of the test solution was poured onto the filter paper and allowed to dry completely for 2 h within the apparatus covered by a petri dish; then, a 10 μl volume of the overnight broth culture was inoculated on the center of the paper and similarly dried for 1 min. The inoculating and drying procedures were repeated five times. Five min after the last inoculation, we inserted the filter paper into a screw-capped bottle (inside diameter = 55 mm, depth = 100 mm) containing 50 ml of the sampling solution (sterile distilled water containing 0.3% cocoyl amide ether sulfate (Sanamide CF-3, 30% active, Nippon Yushi, Ltd.) as an inactivating surfactant) and 20 g of glass beads (diameter = approx. 3 mm). Viable bacteria were recovered from the filter paper by manually shaking it up and down for 45 s (approx. 3 strokes/s). The surfactant added for inactivation had no inhibitory effect on colony counts and quenched the action of disinfectants (data not shown). A 0.5 ml sample was withdrawn from the sampling solution and serially diluted 10-fold in the same solution. Each of the 0.5 ml of the serial dilutions was placed on a plate, mixed with nutrient agar, and incubated aerobically at 37 °C for 48 h. Colony forming unit (CFU) were counted and described as counts per 0.5 ml of the sampling solution in log CFU. This procedure was repeated for each test solution three times.

Analysis of variance was used to test the significance of differences in log CFU between test solutions.

The potentiating effects of dibasic acid diesters on the bactericidal activity of each disinfectant were reported as $\log N_0/N$, where N_0 = average counts after treatment by a test solution containing a disinfectant alone, and where N = average counts after treatment by a test solution containing a disinfectant and one of the dibasic acid diesters.

Determination of the Hydrophobic Parameters of Dibasic Acid Diesters Concerning the hydrophobic parameters of dibasic acid diesters, we used capacity factors (k'), which were directly determined by HPLC. Operating conditions of HPLC are shown in Table III. Capacity factors were calculated from the retention time of the test materials (t_R) and that of a marker material (potassium iodide) (t_0) as follows: $k' = (t_R - t_0)/t_R$.

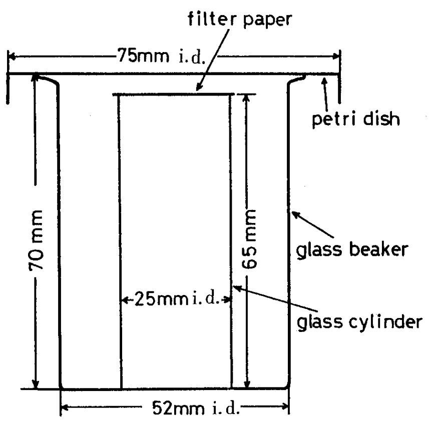

Fig. 1. Apparatus for Bactericidal Test

TABLE III. Experimental Conditions of HPLC

Instrument	LC-6A (Shimadzu Seisakusho, Ltd.)
Column	Nucleosil 100-5C_{18} (Macherey-Nagel/D-5160 Duren)
Column temperature	40 °C (CTO-6A, Shimadzu Seisakusho, Ltd.)
Detector	UV 215 (SPD 6A, Shimadzu Seisakusho, Ltd.)
Mobile phase	0.05 M $HClO_4$: CH_3OH = 25 : 75
Flow rate	1.0 ml/min

Results

Effect of Dibasic Acid Diesters on the Bactericidal Activity of Chlorhexidine Digluconate and Benzalkonium Chloride The effects of dibasic acid diesters on the bactericidal of CH and BC are summarized in Table II as $\log N_0/N$. The activity of CH and BC was influenced by the type of diester; the most effective compounds existed in each homolog of di-*n*-butyl esters of dibasic acids, and dialkyl esters of adipic and phthalic acid. The diesters which significantly potentiated the activity of CH and BC were common with the exception of **1** and **2**, which potentiated for BC but not for CH.

Under our experimental conditions, these dibasic acid diesters themselves had no bactericidal activity in the absence of a certain disinfectant such as CH or BC, although some of them were slightly bacteriostatic on agar plate (data not shown).

Effect of Diisobutyl Adipate on the Bactericidal Activities of Disinfectants **12**, one of the most effective diesters for both CH and BC was investigated to determine if it showed similar effects on the bactericidal activities of other disinfectants. In the presence of **12**, the activities of HA, CPC and DDC were potentiated, but those of IB and AG were not affected (Fig. 2). Of the disinfectants tested, DDC had the greatest activity against *S. aureus* in both the absence and presence of **12**.

Relationship between the Hydrophobic Character of Dibasic Acid Diesters and Their Effects on the Bactericidal Activity of CH and BC As described above, several effective diesters were recognized among the homologs of di-*n*-butyl esters of dibasic acids and dialkyl esters of adipic and phthalic acid. It may thus be speculated that the potentiating effects of diesters on the bactericidal activities of disinfectants are closely related to the hydrophobic character of the diesters. Therefore, we determined capacity factors (k') (Table II) for the hydrophobic parameters and studied the relationships between $\log k'$ and the potentiating effects ($\log N_0/N$) for CH and BC. Plottings of $\log N_0/N$ *versus* $\log k'$ demonstrated that no potentiating effect of diesters appeared in the range of $\log k'$ less than -0.55 or

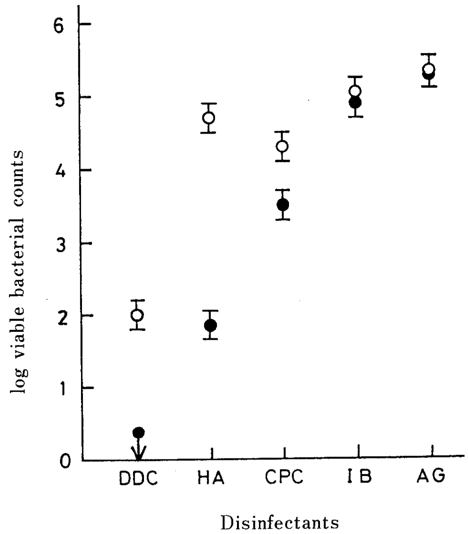

Fig. 2. Effect of Diisobutyl Adipate (**12**) on the Bactericidal Activities of Five Disinfectants against *S. aureus* on Filter Paper
○, disinfectant alone; ●, disinfectant and **12**.

more than 0.85. $\log k'$ of 19 types of diesters were in the -0.55 to 0.85 range.

With one exception, the values of k' obtained in each homolog of dibasic acid diester followed the expected hydrophobic series of the compounds. k' value of **1** was higher than predicted: it was higher than that of **2**, which has more methylene groups than **1**; hence, a certain factor other than hydrophobic character may be involved in determining the k' value of **1**. **1** was therefore excluded from analysis.

The potentiating effects for CH and BC, obtained by linear multiple regression analysis, are seen in Eq. 1 and 2, respectively:

$$\text{CH}: \log(N_0/N) = -1.063 \pm 0.267 (\log k')^2 + 0.365 \pm 0.141 (\log k') + 0.483 \pm 0.060 \quad (1)$$
$$(n=18,\ r=0.910,\ s=0.088,\ F=35.93,\ -0.55 < \log k' < 0.85)$$

and,

$$\text{BC}: \log(N_0/N) = -2.907 \pm 0.753 (\log k')^2 + 0.630 \pm 0.399 (\log k') + 1.472 \pm 0.170 \quad (2)$$
$$(n=18,\ r=0.911,\ s=0.249,\ F=36.76,\ -0.55 < \log k' < 0.85)$$

where n, r, s, F, and $\pm$ represent number of compounds,

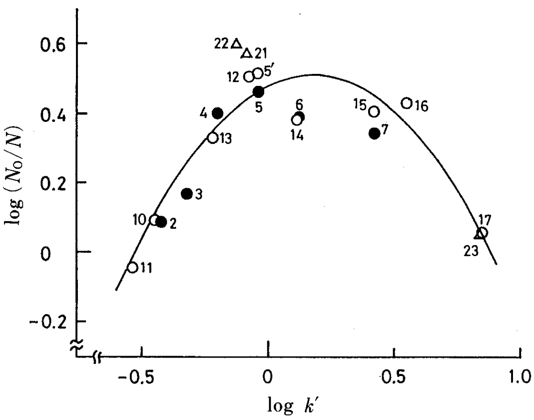

Fig. 3. Relationship between $\log N_0/N$ for CH and $\log k'$ of Dibasic Acid Diesters

●, di-n-butyl esters of aliphatic dibasic acids; ○, di-alkyl esters of adipic acid, △, di-alkyl esters of phthalic acid.

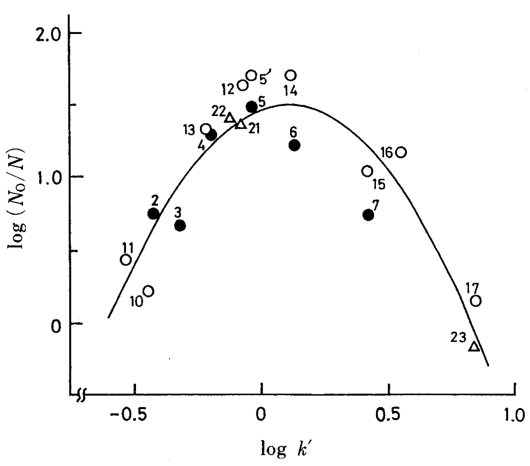

Fig. 4. Relationship between $\log N_0/N$ for BC and $\log k'$ of Dibasic Acid Diesters

●, di-n-butyl esters of aliphatic dibasic acids; ○, di-alkyl esters of adipic acid; △, di-alkyl esters of phthalic acid.

correlation coefficient, standard deviation, ratio of variance, and 95% confidence limit of each constant of equations, respectively.

Equations 1 and 2 for CH and BC are illustrated in Figs. 3 and 4, respectively, which represent the potentiating effects of each diester for CH or BC.

Discussion

We were interested in whether dibasic acid diesters other than **11** and **12**, which were earlier found to enhance the bactericidal activity of CH,[1a] exhibit similar effects on CH, and, if so, whether all these diesters enhance the activities of other disinfectants.

In the bactericidal test we repeated the inoculation procedure five times on filter paper, since the potentiating effect became stronger with repeated inoculations, though not always significant with only one.[1b]

In the experiment using CH or BC, the most effective compounds existed in each homolog of di-n-butyl of dibasic acids, and dialkyl esters of adipic and phthalic acid.

12 was of interest as one of the most effective compounds and enhanced the activities of CH, BC, HA, CPC, and DDC, but not those of AG or IB. Under our experimental conditions, the bactericidal activities differed greatly depending on the type of disinfectant; DDC was the most effective, exhibiting strong activity in both the absence and presence of **12**. In contrast, IB was not active, and AG only slightly active; in these cases which essentially lack disinfectant activity, the potentiating effects of the ester were not observed. Moreover, it is noteworthy that **12** actually potentiated the activities of all the quaternary ammonium compounds tested.

Because of the existence of optimal compounds in the homologs of each diester, it was anticipated that the potentiating effect would be related to their hydrophobic character. Partition coefficients ($\log P$) in an octanol–water system have often been used to carry out quantitative structure–activity relationship (QSAR) studies.[2] However, the method requires experimental determination of the $\log P$ in many instances, the procedures for which are both troublesome and time-consuming. Therefore, capacity factors (k'), determined by HPLC, were used here as the hydrophobic parameter rather than well-known partition coefficients, since $\log k'$ was shown to be linearly related to $\log P$.[3]

With respect to the potentiating effect obtained by plotting $\log k'$ *versus* $\log N_0/N$, the compounds appearing in ranges of $\log k'$ less than -0.55 and more than 0.85 showed no effect; 19 compounds were in the range of -0.55 to 0.85. **1**, however, was excluded from analysis, since, as stated, a factor other than the hydrophobic character may be involved in the determination of $\log k'$. Eighteen compounds were thus used for linear multiple regression analysis and two quadratic equations were obtained which clearly showed the relationship between the hydrophobic characters and the potentiating effects of dibasic acid diesters. Their correlation coefficients were 0.910 for CH and 0.911 for BC. The values of $\log k'$ giving maximal effects are calculated as 0.175 for CH and 0.108 for BC. This difference seems less significant, since the most effective compounds were common in the two cases.

Such quadratic QSARs have been described in several articles,[2b,3,4] where the compounds which are themselves active have been examined. However, no data have been reported on dibasic acid diesters which *per se* display activity. Furthermore, it is not entirely clear whether the potentiating effects of certain additives on an active compound can be represented as a quadratic QSAR.

It is evident that the potentiating effects of dibasic acid diesters on CH or BC depend on the hydrophobic characters of the diesters, as described above.

Hydrophobic compounds such as *n*-alkanols and anaesthetics are well known to interact with and perturb the biological membrane, and to affect its functions.[5] Furthermore, the biological activities of these hydrophobic compounds are closely related to their hydrophobic characters,[6] for example, partition coefficients (*n*-octanol/water). The dibasic acid diesters tested here are all hydrophobic compounds and expected to interact with the biological membrane in a similar manner, although they have no bactericidal activity. Thus, dibasic acid diesters having optimal hydrophobic characters could to some degree enhance the bactericidal activity of the disinfectants.

An interaction between dibasic acid diesters and the cell membrane could also be explained on the basis of solubility parameters. Vaughan and Wright[7] reviewed the article by Rehn *et al.*[8] on their investigations of 1,3-dihydroxybutane, and demonstrated both that solubility has a major bearing on the activity and that this activity/solubility relationship is unique for each species: that is, each microbial strain has a solubility parameter of maximum activity. The solubility parameter may be derived from the molecular weight, boiling point and specific gravity of the compound, using Hildebrand's equation; for example, that of **5**, one of the most effective compounds, is calculated about 8.5. This value is very close to the solubility parameter (about 8.3) at which the activity against *S. aureus* peaks as illustrated by Vaughan and Wright.[7] As a consequence, **5** may be quite compatible with the organisms, thus being solubilized into the cell membrane, perturbing the membrane and facilitating the penetration of the disinfectant into its target sites.

In summary, we demonstrated that dibasic acid diesters with appropriate hydrophobic characters enhanced the bactericidal activities of disinfectants including CH and quaternary ammonium compounds. The addition of hydrophobic compounds such as **5** and **12** may be useful as a measure to enhance the activities of disinfectants.

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