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Comparative Antimicrobial Activity, In Vitro and In Vivo, of Soft *N*-Chloramine Systems and Chlorhexidine

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Antimicrobial activity of the following four new *N*-chloramine compounds was evaluated: two chlorinated simple amino acids, a chlorinated half-ester of succinic acid, and a chlorinated half-ester of glutaric acid. For comparison, the known bactericidal agents 3-chloro-4,4-dimethyl-2-oxazolidinone and chlorhexidine were evaluated by the same procedure. The contact germicidal efficiency screen was used to examine the in vitro bactericidal activity of all six compounds in the absence and presence of 5% horse serum or 5% Triton X-100. The four new compounds were found to have greater germicidal activity than the other compounds tested and to exhibit low toxicity and skin irritation values. The in vivo bactericidal activity was evaluated in two studies. In the occlusion test, three of the four new compounds plus chlorhexidine diacetate were tested. The *N*-chloramines were significantly superior to chlorhexidine in preventing the expansion of the normal flora under occlusion. In the scrub test, a gloved-hand wash method was used to compare the antimicrobial effect of a 1% solution of the chlorinated half-ester of succinic acid in triacetin with that of a commercial germicidal hand wash containing 4% chlorhexidine gluconate. The two preparations exhibited essentially the same hand-degerming activity.

New germicidal agents are constantly being sought. Compounds with excellent germicidal activity, low toxicity, and nonirritating properties are needed as disinfectants when low levels of bacteria on the skin are desired.

Several germicide-containing products available today are specifically designed to disinfect the skin. Some of the more widely used products contain chlorhexidine as the active ingredient. The germicidal activity of chlorhexidine and the activity of the products containing chlorhexidine have been studied extensively (1, 6–8).

A series of *N*-chloramine compounds was studied in this laboratory and screened for germicidal activity. Four of the compounds recently synthesized show great promise as germicidal agents and as topical antiseptics in particular. Compounds IIa through IIIb were synthesized by one of us (S.P.) and by Chung Shih, The University of Kansas, Lawrence. Analyses of the compounds are as follows. Calculated for $C_5H_9Cl_2NO_2$: C, 32.29; H, 4.84; N, 7.53. Found: C, 32.58; H, 4.70; N, 7.50. Calculated for $C_6H_{11}Cl_2NO_2$: C, 36.03; H, 5.50; N, 7.00. Found: C, 36.61; H, 5.60; N, 6.85. Calculated for $C_8H_{13}Cl_2NO_4$: C, 37.23; H, 5.08; N, 5.43. Found: C, 37.18; H, 5.20; N, 5.10. Calculated for $C_9H_{15}Cl_2NO_4$: C, 39.72; H, 5.56; N, 5.15. Found: C, 39.90; H, 5.60; N, 4.98.

Figure 1 shows structures of the compounds we discuss. Compound I is the previously described 3-chloro-4,4-dimethyl-2-oxazolidinone (5). Representing the new series of *N*-chloramines are IIa and IIb, simple chlorinated amino acids, and IIIa and IIIb, chlorinated half-esters of succinic and glutaric acid, respectively. Compound IV is the reference compound, chlorhexidine [bis(*p*-chlorophenyl-diguanido)hexane].

MATERIALS AND METHODS

Contact germicidal efficiency screen (CGE). The organisms used were: *Staphylococcus aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6051-1, *Salmonella typhimurium* ATCC 13311, and *Pseudomonas aeruginosa* ATCC 14502. These organisms were maintained as stock cultures at 4°C on slants of nutrient agar obtained from BBL Microbiology Systems (Cockeysville, Md.). They were transferred and biochemically diagnosed monthly.

Overnight cultures of the test organisms were grown at 37°C in tryptose phosphate broth obtained from Difco Laboratories (Detroit, Mich.). After 15 h, cultures were centrifuged at 2,000 rpm for 15 min, and the supernatant was replaced with fresh warm tryptose phosphate broth. After 2 h of incubation, absorbance of the culture at 650 nm was determined, and the culture was then diluted with nutrient broth (Difco) to 10^7 colony-forming units (CFU)/ml, using a previously determined standard curve. Resulting bacterial suspensions were maintained at 4 to 8°C throughout the screen.

The new series of *N*-chloramines along with com-

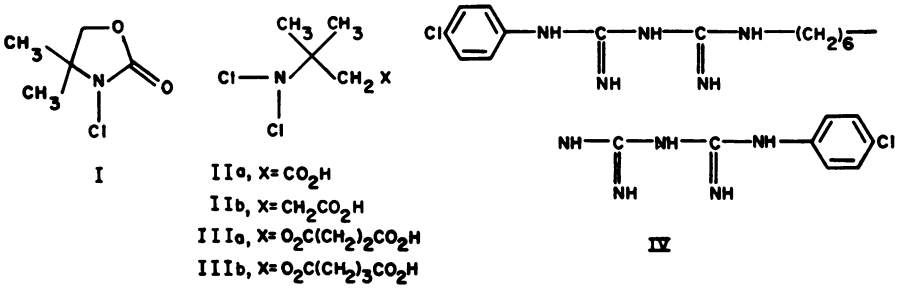

FIG. 1. Chemical structures of 3-chloro-4,4-dimethyl-2-oxazolidinone (I), four new *N*-chloramine compounds (IIa, IIb, IIIa, and IIIb), and chlorhexidine (IV).

pound I and chlorhexidine diacetate (Sigma Chemical Co., St. Louis, Mo.) were weighed into sterile flasks and, immediately before screening, dissolved in 0.1 M NaOAc buffer, pH 4.6. The denaturing agents used were a 10% solution of tissue culture-desiccated horse serum (serum; Difco) in sterile distilled water and a 10% solution of alkyl phenoxy polyethoxy ethanol (Triton X-100) obtained from Rohm-Haas (Philadelphia, Pa.). When used, 2.5 ml of the denaturing agent was mixed with 2.5 ml of double-strength test solution exactly 30 min before the bacterial suspension was added.

The CGE screen was initiated when 0.2 ml of bacterial suspension was added to 5 ml of test solution contained in a sterile glass-stoppered flask. At intervals of 0.5, 2.5, 5, 10, and 15 min, a loopful (3-mm-diameter Nichrome loop) of inoculated test solution was subcultured into 5 ml of sterile nutrient broth and blended, the high dilution serving to stop the action of the compound. All of the subculture tubes were incubated at 37°C for 7 days and observed for growth (turbidity). The first subculture time at which no growth was present was considered the sterilization time.

Controls were run with a 5-ml tube of the buffer inoculated with 0.2 ml of bacterial suspension. A loopful of inoculated buffer was streaked on a nutrient agar plate to ensure purity and viability of the test organisms. Loopfuls of inoculated buffer and of test solution were transferred to a 5-ml tube of nutrient broth. Turbidity after 24 h at 37°C indicated the dilution was great enough to stop the action of the compound.

Skin irritation study. The test animals were 8-week-old New Zealand male rabbits weighing an average of 1.5 kg. Hair was clipped from the back and flanks of the animals.

Compounds IIa, IIIa, and IIIb and chlorhexidine diacetate were dissolved or suspended in 0.1 M NaOAc buffer, pH 4.6, at concentrations of 0.1, 1.0, and 5.0%. The 12 test solutions were applied once on each of three rabbits. The occlusion and scoring procedure described previously (2) was followed with one major change. Occlusion of test preparations on rabbit skin was achieved with "adhesive plasters for patch test" obtained from Torrii Yakuhin Corp. (Tokyo, Japan). The adhesive plasters (test strips) consisted of six gauze circles (1.5-cm diameter) mounted on the sticky side of a strip of plastic adhesive tape (17 by 3.5 cm). Each gauze circle (patch) was inoculated with 100 μl of a test solution immediately before a test strip was placed on a rabbit. The tape adhered to the skin extremely well, thus sealing and isolating each patch. One test strip was placed on either side of and parallel to the backbone of the rabbit.

After the test strips were removed, the primary irritation index (PII) was recorded for each treated spot. The maximum possible PII was 8. A PII $\leq$ 2 indicated mild irritant, 2 < PII $\leq$ 6 indicated moderate irritant, and PII > 6 indicated severe irritant.

Occlusion test. A total of nine subjects were tested. After the control study described below, a test panel of five remained. Each subject used only Ivory bar soap (Proctor and Gamble, Cincinnati, Ohio) 2 weeks before and during the study.

The topical evaluation procedure previously described (7) was followed except that the 24-h occlusion was achieved with the test strips described in the irritation study. Each patch was inoculated with 100 μl of sterile water or a test solution immediately before a test strip was placed on the volar forearm of a subject.

Before the subjects were selected to participate in the screening of the test solutions, a control study was run. Each subject was screened with a sterile water patch. If the density of aerobic bacteria under that patch did not reach at least 10^5 organisms/cm^2, the subject was rejected. Subjects who passed the control study participated in the screening of test solutions on 2 different test days. A 2-week rest period occurred between test days to permit regrowth of the normal skin microflora.

After the 24-h occlusion, test strips were peeled back carefully. As a patch was lifted, a sterile glass cylinder (area, 1.54 cm^2) was placed on the skin. A 0.5-ml portion of wash fluid consisting of 0.1% Triton X-100 in 0.1 M NaH_2PO_4, pH 7.9, was pipetted into the cylinder. The skin surface was rubbed firmly with a blunt Teflon scrubber for 1 min. The wash fluid was aspirated, and the procedure was repeated. The two washes were pooled, and 10-fold serial dilutions were prepared with half-strength wash fluid.

Portions (0.1 ml) of undiluted wash fluid and of each dilution were spread plated on Trypticase soy agar (BBL) with lecithin and Polysorbate 80 (20 ml of agar per plate). Plates were incubated aerobically at 35°C for 48 h. Colony counts were made under magnification.

Scrub test. The following test preparations were evaluated for degerming activity: Ivory soap; triacetin (Aldrich Chemical Co., Milwaukee, Wis.); 1% (wt/vol)

TABLE 1. CGE of compounds dissolved in 0.1 M NaOAc, pH 4.6, buffer and after a 30-min exposure to denaturant (serum or Triton X-100)

Compound	Concn (μg/ml)	Sterilization time (min) against:				
		S. aureus	*S. epidermidis*	*B. subtilis*	*S. typhimurium*	*P. aeruginosa*
I	1,000	5.0	2.5	2.5	2.5	0.5
	100	>15	10.0	5.0	2.5	2.5
IIa	100	0.5	0.5	0.5	0.5	0.5
	10	2.5	2.5	2.5	0.5	2.5
IIb	1,000	0.5	0.5	0.5	0.5	0.5
	100	2.5	0.5	0.5	0.5	0.5
	10	5.0	10.0	2.5	2.5	2.5
IIIa	1,000	0.5	0.5	0.5	0.5	0.5
	100	2.5	2.5	2.5	0.5	2.5
	10	>15	10.0	2.5	2.5	5.0
IIIb	1,000	0.5	0.5	0.5	0.5	0.5
	100	2.5	2.5	2.5	0.5	2.5
	10	10.0	10.0	2.5	2.5	5.0
IV	200	0.5	0.5	0.5	0.5	0.5
	100	>15	5.0	0.5	5.0	0.5
		After 30-min exposure to serum				
I	300	10.0	5.0	2.5	2.5	2.5
IIa	1,000	0.5	0.5	0.5	0.5	2.5
	300	>15	>15	2.5	2.5	>15
IIb	1,000	0.5	2.5	0.5	0.5	5.0
	300	5.0	10.0	2.5	2.5	>15
IIIa	1,000	2.5	2.5	0.5	2.5	10.0
	300	15.0	>15	5.0	10.0	>15
IIIb	1,000	2.5	2.5	0.5	2.5	10.0
	300	>15	>15	5.0	10.0	>15
IV	300	0.5	0.5	0.5	2.5	>15
		After 30-min exposure to Triton X-100				
I	300	10.0	5.0	2.5	2.5	2.5
IIa	300	0.5	0.5	0.5	0.5	0.5
IIb	300	2.5	2.5	0.5	0.5	2.5
IIIa	300	2.5	2.5	0.5	0.5	2.5
IIIb	300	2.5	2.5	0.5	0.5	2.5
IV	300	0.5	0.5	0.5	2.5	2.5

IIIa in triacetin; Hibiclens (4% [wt/vol] chlorhexidine gluconate with 4% isopropyl alcohol in a nonalkaline base; Stuart Pharmaceuticals, Wilmington, Del.).

Nine subjects volunteered to participate on the test panel. A crossover design was used requiring each subject to wash with the four test preparations on 2 different days. Each subject thus served as his own control. At least 3 days were allowed between treatments to permit regrowth of normal skin microflora.

The washing procedure required the hands to be rubbed together with a test preparation for 2 min. The hands were rinsed with purified water for 1 min and dried with one paper towel before donning sterile, prepowdered, latex surgeon's gloves. The gloves were not rinsed before use.

To recover aerobic skin bacteria, 50 ml of sterile normal saline was added to the gloved hand. A cloth tourniquet was fastened over the glove at wrist level. The subject rapidly opened and closed the hand for 60 s. The glove was carefully removed and shaken to suspend the bacteria in the saline.

After three of the washes (the base-line wash described below, Ivory soap wash, and triacetin wash), 2-ml samples were removed from the glove and added to 2 ml of neutralizer solution (0.035 M KH_2PO_4 with 0.22% [wt/vol] lecithin, 1.55% Polysorbate 80, and enough NaOH to bring the solution to pH 7.2). A 1:5 dilution of this 1:2 dilution was made with the neutralizer solution, and this was followed by serial 10-fold dilutions to 10^{-3}. Portions (0.2 ml) of the initial 1:2 dilutions and 0.1-ml portions of succeeding dilutions were spread plated in duplicate on Letheen agar (20 ml of agar per plate; BBL). After the 1% IIIa-triacetin and Hibiclens washes, 3-ml samples were removed from the gloves and added to 3 ml of neutralizer solution. These samples were then diluted as described above. Portions (2 ml) of the initial 1:2 dilution and 1-ml portions of succeeding dilutions were added to 20-ml Letheen agar pours, previously melted and held at 50°C. The inoculated pours were blended, poured into petri dishes, and allowed to cool. All plates were incubated at 35°C for 48 h. Colonies were counted under magnification.

To determine the base-line level of aerobic skin bacteria, both hands were sampled immediately after a single 2-min wash with Ivory (base-line wash).

To determine the effects of the test preparations on normal flora, an initial 2-min wash with Ivory was

TABLE 2. Rabbit skin irritation

Compound	Concn (%)	PII
IIa	0.1	0
IIIa	0.1	0
IIIb	0.1	0.2
IV	0.1	0
IIa	1.0	0.3
IIIa	1.0	0.2
IIIb	1.0 (suspension)	0
IV	1.0	0
IIa	5.0 (suspension)	5.2
IIIa	5.0 (suspension)	4.7
IIIb	5.0 (suspension)	3.7
IV	5.0 (suspension)	0.7

TABLE 3. Expansion of normal flora: bacterial density[a]

Test date	n	Geometric mean (CFU/cm^2)	Mean log $CFU/cm^2 \pm SE$
1	24	6.01 ± 10^6	6.54 ± 0.10
2	10	5.43 ± 10^6	6.30 ± 0.24
Combined	34	5.76 ± 10^6	6.47 ± 0.10

[a] Differences were not significant (one-tailed Student t test).

followed by another 2-min wash with one of the four test preparations. One hand of each subject was sampled immediately after the gloves were donned. The other hand was sampled after a glove-wearing period of either 30 min or 2 h.

RESULTS

CGE screen. Table 1 shows the in vitro antimicrobial activity of the *N*-chloramines and chlorhexidine diacetate. The chlorinated amino acids were found to be the most potent bactericides. Compound IIa effected sterilization of all five test organisms in 2.5 min or less at 10 μg/ml. The two chlorinated half-esters, IIIa and IIIb, effected sterilization in 2.5 min or less at 100 μg/ml. Compound I, the chlorinated oxazolidinone, was 10-fold less potent than the half-esters. Due to low solubility, chlorhexidine diacetate was screened at 200 and 100 μg/ml. Exhibiting excellent germicidal activity at 200 μg/ml, chlorhexidine diacetate showed much less activity against *S. aureus* at 100 μg/ml and appeared generally less potent than the II or III series *N*-chloramines.

A 30-min exposure to serum had a denaturing effect on the compounds (Table 1). However, by increasing the concentration of the *N*-chloramines from 300 to 1,000 μg/ml, much of the deactivating effect of the serum was overcome.

The new *N*-chloramines and chlorhexidine diacetate showed little or no decrease in germicidal activity after a 30-min exposure to Triton X-100. Compound I, however, did lose potency.

Skin irritation study. After determining the in vitro antibacterial activity of these new *N*-chloramines and before beginning in vivo testing, some irritation and toxicity information was generated. Even at 1%, a concentration well above the level required for germicidal activity, compounds IIa, IIIa, and IIIb caused only mild or no irritation on rabbit skin (Table 2). Suspensions at 5% caused moderate irritation.

Acute oral LD_{50}. To determine the acute oral 50% lethal dose (LD_{50}), 18 to 20-g Swiss white female mice were tested. The compounds tested were dissolved in purified water, and 1.2-fold serial dilutions were made. For each dilution, 10 mice were given 0.2 ml of solution orally. The surviving mice were counted after 6 days, and the LD_{50} was calculated as previously described (4). The acute oral LD_{50} for IIa was 495 mg/kg, and that for IIIb was 785 mg/kg.

Occlusion test. The data generated thus far

TABLE 4. Effect of aqueous solutions on the expansion of normal flora

Compound	Concn (μg/ml)	n	Mean % reduction	Mean log $CFU/cm^2 \pm SE$	P^a	P^b
IIa	1,000	6	99.9	1.95 ± 0.66	***	***
IIIa	1,000	10	99.6	2.48 ± 0.59	***	**
IIIb	1,000	6	99.9	2.68 ± 0.50	***	**
IV	1,000	10	97.5	4.49 ± 0.26	***	
IIa	100	6	85.3	4.97 ± 0.59	***	NS
IIIa	100	10	90.2	4.44 ± 0.45	***	**
IIIb	100	6	98.2	3.92 ± 0.76	***	**
IV	100	10	69.2	5.84 ± 0.24	**	
IIa	10	6	76.7	5.96 ± 0.20	*	
IIIb	10	6	72.2	5.76 ± 0.38	*	

[a] One-tailed Student t test comparing combined log CFU per centimeter squared under control patches with mean log CFU per centimeter squared under the test patch. NS, Not significant; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

[b] One-tailed Student t test comparing mean log CFU per centimeter squared under test compound IV patch with mean log CFU per centimeter squared under *N*-chloramine patches.

TABLE 5. Base-line levels of aerobic skin bacteria

Hand	n	Mean log no. of bacteria/ hand ± SE[a]	Geometric mean of no. of bacteria/ hand
Left	9	6.13 ± 0.12	1.80 ± 10^6
Right	9	5.93 ± 0.13	1.22 ± 10^6
Both	18	6.03 ± 0.09	1.51 ± 10^6

[a] All values were not significantly different ($P < 0.20$).

TABLE 6. Reproducibility of the levels of aerobic skin bacteria recovered immediately after washing on 2 test days[a]

Prepn	Mean log no. of bacteria recovered/hand ± SE			
	n	1st test day	n	2nd test day
Ivory	8	5.81 ± 0.17	8	5.87 ± 0.14
Triacetin	9	5.58 ± 0.09	9	5.52 ± 0.15
IIIa/triacetin	8	5.11 ± 0.15	8	5.06 ± 0.12
Hibiclens	9	5.11 ± 0.15	8	4.84 ± 0.23

[a] Differences were not significant (one-tailed Student t test).

were analyzed, and three of the most promising N-chloramines and chlorhexidine diacetate were evaluated as topical antiseptics for human use.

Due to the change in the occlusion test procedure, controls were run to assure the validity of the new method. Table 3 illustrates the consistency with which the normal flora expanded under the patches of the test strips.

The in vivo germicidal efficiency of the selected N-chloramines was excellent and significantly greater than that of chlorhexidine diacetate (Table 4). A significant reduction in the expansion of the normal flora was observed when IIa and IIIb at 10 μg/ml or chlorhexidine diacetate at 1,000 μg/ml was used.

Scrub test. The chlorinated half-ester of succinic acid was the only N-chloramine evaluated in this study. It exhibited germicidal activity, low toxicity, and long-term stability in triacetin. Chlorhexidine gluconate was evaluated in its marketed form, Hibiclens.

No significant differences were observed in the normal levels of aerobic bacteria recovered from the left and right hands. Thus, base-line geometric and logarithmic mean levels for both hands were calculated (Table 5) and used to evaluate the effect of the test preparations on the normal microflora of the hands.

The crossover design demonstrated a reproducible sampling procedure as well as a sufficient regrowth period between treatments. The mean log number of bacteria recovered immediately after washing did not vary significantly from one day to the next for any of the preparations (Table 6).

Triacetin, 1% IIIa in triacetin, and Hibiclens all caused a significant reduction in the level of bacteria on the hands immediately after washing (Table 7). The 1% IIIa–triacetin solution and Hibiclens caused similar reductions, both of which were significantly greater than reductions effected by triacetin alone or by Ivory soap.

The short-term duration of the germicidal activity was assessed by sampling one hand of each subject at 30-min postwash. Triacetin, 1% IIIa in triacetin, and Hibiclens all maintained a significant reduction in the level of bacteria on the hands. Triacetin alone had its strongest effect at 30-min postwash. Washing twice with Ivory soap also caused a significant reduction in bacteria at 30-min postwash.

TABLE 7. Disinfection of hands by various preparations

Prepn	n	Time postwash	Mean % reduction in no. of bacteria/hand	Mean log no. of bacteria/ hand ± SE	P[a]	Statistical comparison of treatments
a. Ivory soap	16	Immediate	23.8	5.84 ± 0.11	NS	
b. Triacetin	18	Immediate	68.1	5.55 ± 0.09	***	a vs b: P = * b vs c: P = ***
c. 1% IIIa/triacetin	16	Immediate	88.7	5.08 ± 0.09	***	a vs c: P = *** b vs d: P = ***
d. Hibiclens	17	Immediate	89.3	4.98 ± 0.13	***	a vs d: P = *** c vs d: P = NS
a. Ivory soap	8	30 min	58.1	5.57 ± 0.17	**	
b. Triacetin	9	30 min	75.3	5.49 ± 0.10	***	a vs b: P = NS b vs c: P = **
c. 1% IIIa/triacetin	8	30 min	89.3	4.87 ± 0.20	***	a vs c: P = ** b vs d: P = *
d. Hibiclens	9	30 min	86.2	4.97 ± 0.21	***	a vs d: P = ** c vs d: P = NS
a. Ivory soap	8	2 h	41.4	5.79 ± 0.14	NS	
b. Triacetin	9	2 h	67.8	5.33 ± 0.23	**	a vs b: P = NS b vs c: P = NS
c. 1% IIIa/triacetin	8	2 h	90.5	4.91 ± 0.22	***	a vs c: P = ** b vs d: P = NS
d. Hibiclens	8	2 h	90.9	4.94 ± 0.17	***	a vs d: P = *** c vs d: P = NS

[a] One-tailed Student t test comparing logarithmic mean of level after test preparation wash with logarithmic mean of base-line level for both hands. Symbols are as in Table 4. NS, Not significant.

The long-term duration of germicidal activity on the microflora of the hands was determined by sampling at 2-h postwash. The 1% IIIa–triacetin solution and Hibiclens both maintained a significant reduction in bacteria. Some regrowth occurred between 30 min and 2 h after the triacetin wash. Regrowth also occurred 2 h after the Ivory soap wash.

DISCUSSION

Chloramines have been regarded as promising germicidal agents. This study demonstrates that a novel series of *N*-chloramines has excellent germicidal activity superior to that of chlorhexidine, a currently marketed germicide.

The four new *N*-chloramines were 10 times more efficient as germicides than chlorhexidine diacetate in the in vitro CGE study. They effected sterilization at a concentration 5- to 50-fold less than the concentration at which skin irritation occurred. Although 5% serum inhibited the activity of *N*-chloramines at 300 μg/ml, germicidal activity was regained by raising the concentration of the *N*-chloramines to 1,000 μg/ml. Chlorhexidine diacetate was also inhibited by serum at 300 μg/ml. The nonionic detergent Triton X-100 had little or no effect on the activity of the new compounds or chlorhexidine diacetate.

The *N*-chloramines were 10 to 100 times more effective than chlorhexidine diacetate in preventing the expansion of the normal skin microflora under occlusion. The *N*-chloramines also caused little skin irritation. In fact, they prevented the expansion of the microflora at concentrations 100- to 1,000-fold less than the concentration at which skin irritation occurred.

Finally, in the scrub test, the 1% nonsudsing triacetin solution of compound IIIa had a handdegerming capability equal to that of the 4% preparation of chlorhexidine gluconate. This was found to be true for all three sampling times.

The data presented indicate that these novel *N*-chloramines are excellent germicidal agents. In both the in vitro and in vivo studies, they were superior to one of the more popular topical disinfectants used today. They have low toxicity and cause skin irritation only at concentrations much higher than that needed for germicidal activity. Combined with their low production cost and long-term stability in triacetin, these new compounds demonstrate the potential to be practical and efficient germicidal agents.

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