4H), 3.8 (s, 2H), 3.2–3.7 (m, 4H), 3.0 (s, 6H), 1.4–2.9 (m, 4H), 2.3 (s, 3H), and 1.2 (s, 6H) ppm.

Anal.—Calc. for $C_{18}H_{29}ClN_2O_4S \cdot H_2O$: C, 51.11; H, 7.39; N, 6.62. Found: C, 50.70; H, 7.37; N, 6.59.

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N-Halo Derivatives V: Comparative Antimicrobial Activity of Soft *N*-Chloramine Systems

JAMES J. KAMINSKI, M. MAUREEN HUYCKE, SALLY H. SELK, NICOLAE BODOR [x], and TAKERU HIGUCHI

Abstract □ Comparative antimicrobial activity studies for certain new classes of soft *N*-chloramines derived from α-aminoisobutyric acid and 2-amino-2-methyl-1-propanol were examined using the minimum inhibitory concentration (MIC) and/or the contact germicidal efficiency (CGE) procedures. Several factors significantly influence the antimicrobial activity of the soft *N*-chloramines: (*a*) the aliphatic chain length in a homologous series, (*b*) the degree of chlorination of the nitrogen atom, (*c*) the solution pH, (*d*) the presence of a denaturant, and (*e*) the nature of a positive charge.

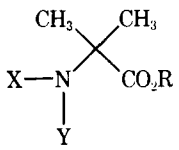
Keyphrases □ *N*-Halo derivatives—low chlorine potential, soft *N*-chloramines, antimicrobial activity evaluated and compared □ *N*-Chloramines, soft—series with low chlorine potential, antimicrobial activity evaluated and compared □ Antimicrobial activity—*N*-chloramine derivatives of α-aminoisobutyric acid and 2-amino-2-methyl-1-propanol evaluated □ Structure–activity relationships—*N*-chloramine derivatives of α-aminoisobutyric acid and 2-amino-2-methyl-1-propanol evaluated for antimicrobial activity

Recently, the *N*-chlorinated derivatives of α-amino acids and their related derivatives were synthesized and their various reactions were studied (1). Based on the stability and reactivity of the nitrogen–chlorine bond in these *N*-chloramines, new types of soft *N*-chloramines were developed which are derivatives of 2-amino-2-methyl-1-propanol (2).

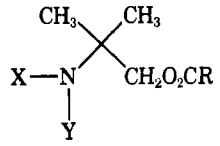
RESULTS AND DISCUSSION

A comprehensive and systematic investigation of the relationship between the antimicrobial activity of the *N*-chloramines and the various factors influencing the polarity, reactivity, and stability of the nitrogen–chlorine bond was examined using the following compounds. Class I represents the esters of the monochloro and dichloro derivatives of α-aminoisobutyric acid; Class II represents the corresponding diketopiperazines. Compounds in Class III have an even lower chlorine potential than those in Class I, while a number of the compounds are structural isomers, such as the III*h*–I*e*, III*i*–I*d*, III*k*–I*f*, and III*l*–I*g* isomeric pairs, which permit a direct comparison of the structure–activity relationship. Compound IV is a stable derivative of 2-amino-2-methyl-1-propanol, representing a whole series of *N*-chloro-2-oxazolidinones. Class V represents the corresponding 1,3-oxazolidine derivatives characterized by a lower chlorine potential and an enhanced soft character.

Minimum Inhibitory Concentration (MIC)—The antimicrobial activity of the various soft *N*-chloramine systems was examined first



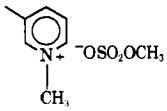
I*a*: X = Cl, Y = Cl, R = CH_3
I*b*: X = H, Y = H, R = $C_6H_{13} \cdot HCl$
I*c*: X = H, Y = Cl, R = C_6H_{13}
I*d*: X = Cl, Y = Cl, R = C_6H_{13}
I*e*: X = H, Y = H, R = $C_8H_{17} \cdot HCl$
I*f*: X = H, Y = Cl, R = C_8H_{17}
I*g*: X = Cl, Y = Cl, R = C_8H_{17}
I*h*: X = H, Y = H, R = $C_{12}H_{25} \cdot HCl$
I*i*: X = H, Y = Cl, R = $C_{12}H_{25}$
I*j*: X = Cl, Y = Cl, R = $C_{12}H_{25}$
I*k*: X = H, Y = H, R = $C_{14}H_{29} \cdot HCl$
I*l*: X = H, Y = Cl, R = $C_{14}H_{29}$
I*m*: X = Cl, Y = Cl, R = $C_{14}H_{29}$
I*n*: X = H, Y = H, R = $C_{18}H_{37} \cdot HCl$
I*o*: X = H, Y = Cl, R = $C_{18}H_{37}$
I*p*: X = Cl, Y = Cl, R = $C_{18}H_{37}$
I*q*: X = H, Y = H, R = $(CH_2)_2O(CH_2)_2O(CH_2)_3CH_3 \cdot HCl$
I*r*: X = H, Y = Cl, R = $(CH_2)_2O(CH_2)_2O(CH_2)_3CH_3$
I*s*: X = Cl, Y = Cl, R = $(CH_2)_2O(CH_2)_2O(CH_2)_3CH_3$



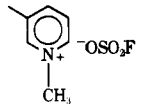
IIIa: X = Cl, Y = Cl, R = CH_3
IIIb: X = H, Y = H, R = $(CH_2)_2CH_3 \cdot HCl$
IIIc: X = H, Y = Cl, R = $(CH_2)_2CH_3$
IIId: X = Cl, Y = Cl, R = $(CH_2)_2CH_3$
IIIe: X = H, Y = Cl, R = $C(CH_3)_3$
IIIf: X = Cl, Y = Cl, R = $C(CH_3)_3$
IIIg: X = H, Y = H, R = $(CH_2)_4CH_3 \cdot HCl$
IIIh: X = H, Y = Cl, R = $(CH_2)_4CH_3$
IIIi: X = Cl, Y = Cl, R = $(CH_2)_4CH_3$
IIIj: X = H, Y = H, R = $(CH_2)_6CH_3 \cdot HCl$
IIIk: X = H, Y = Cl, R = $(CH_2)_6CH_3$
IIIl: X = Cl, Y = Cl, R = $(CH_2)_6CH_3$

IIIm: X = Cl, Y = Cl, R =

IIIn: X = Cl, Y = Cl, R =



IIIo: X = Cl, Y = Cl, R =

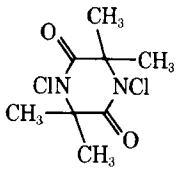


using the MIC procedure. This method is based on incubation of the microorganism tested with solutions containing different concentrations of the soft *N*-chloramine. The MIC of the soft *N*-chloramine is the lowest concentration of the *N*-chloramine for which growth of the microorganism tested is not observed. The MIC is indicative of the *N*-chloramine's bacteriostatic activity but may also represent the *N*-chloramine's bactericidal activity.

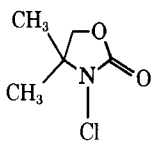
Differentiation between the bacteriostatic or bactericidal activity of the *N*-chloramine can be deduced by subculturing the test organism from the MIC and observing the growth or expiration of the organism. However, regardless of the inhibitory mode of bacterial growth, it is important to consider that the activity of the *N*-chloramine observed using this procedure is determined essentially under equilibrium conditions. The microorganism tested and the different concentrations of the *N*-chloramine can attain an equilibrium state. The antimicrobial activity of the soft *N*-chloramine observed under these conditions can be considered its equilibrium thermodynamic activity.

The MIC's of three representative soft *N*-chloramines containing nitrogen–chlorine bonds of different polarity were determined (Table I). Comparison between the MIC's determined for these agents (II, IV, and V*a*) suggests that as the polarity of the nitrogen–chlorine bond is decreased, the MIC against both Gram-positive and Gram-negative microorganisms is increased. The MIC's for the soft *N*-chloramines of α-aminoisobutyrate esters and of 2-amino-2-methyl-1-propyl carboxylates also were determined (Tables II and III).

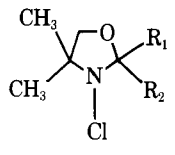
Comparison between the MIC's determined for the α-*N*-chloroamino and α-*N,N*-dichloroaminoisobutyrate homologous esters (Table II) suggests that the dodecyl homologs (I*i* and I*j*) exhibit the optimum antimicrobial activity against a broad spectrum of Gram-positive, Gram-negative, and fungal organisms. However, compared



II



IV



V*a*: $R_1 = R_2 = CH_3$

V*b*: R_1R_2 =

V*c*: R_1R_2 =

Table I—MIC[a] of *N*-Chloramines Containing Nitrogen–Chlorine Bonds of Different Polarity

Compound	*Staph. aureus*	*Strep. pyogenes*	*E. coli*	*S. typhimurium*	*B. subtilis*
II	237	>473[b]	118	237	118
IV	1052	>1052	1052	1052	1052
V*a*	3260	3260	3260	3260	3260

[a] Expressed in parts per million of compound. [b] Greater than indicates that the MIC for the test organism is greater than the highest concentration of the compound that could be tested.

to the MIC determined for dodecyl α-aminoisobutyrate hydrochloride (I*h*), it appears that the activity observed for I*i* and I*j* is primarily an effect of the inherent activity of the parent molecular structure. Unlike the MIC's of the *N*-chlorinated α-aminoisobutyrates, comparison between the MIC's determined for the 2-*N*-chloroamino- and the 2-*N,N*-dichloroamino-2-methyl-1-propyl carboxylates (Table III) suggests that these *N*-chlorinated products are no more efficacious antimicrobial agents than their unchlorinated parent precursors.

Contact Germicidal Efficiency (CGE)—Since the proposed mechanism of bactericidal action for the *N*-chloramines is a result of the direct transfer of positive chlorine from the *N*-chloramine to an appropriate receptor in the cell, the determination of the MIC, which is based on an equilibrium condition, may be an inappropriate means to observe the kinetic effect of the *N*-chloro function in determining the bactericidal activity of the *N*-chloramine. To investigate the kinetic effect of different positive chlorine transfer rates from *N*-chloramines containing nitrogen–chlorine bonds of different polarity on the bactericidal activity, the various soft *N*-chloramine systems were examined using the CGE procedure.

The CGE method is based on treating the microorganism tested with a solution of a given concentration of the soft *N*-chloramine. At various times, aliquots of this solution are subcultured into sterile nutrient broth; the dilution of the aliquot subculture must be sufficient to ensure no further bacteriostatic action of the *N*-chloramine. The subcultured time interval samples are incubated, and the growth behavior of the microorganism tested is observed. The shortest time interval for which growth of the microorganism is not observed is the sterilization time for the soft *N*-chloramine against the particular microorganism tested.

Effect of Homologous Chain Length and Degree of N-*Chlorination*—The sterilization times for the soft *N*-chloramines of α-aminoisobutyrate esters and of the 2-amino-2-methyl-1-propyl carboxylates were determined (Tables IV and V). Comparison between the sterilization times determined for the homologous *N*-chlorinated α-aminoisobutyrates (Table IV) suggests that III*a* is the most active soft *N*-chloramine of the series from a kinetic standpoint. However, an accurate comparison between the sterilization times for the homologs in the series is difficult because of the marked differences in the equilibrium solubilities of the *N*-chloramines.

Nevertheless, the inherent activity of the parent molecular structure for dodecyl α-aminoisobutyrate hydrochloride (I*h*), which is observed in the MIC procedure, is also observed in the CGE procedure. The inherent activity of the parent molecule effectively masks the bactericidal effect of the *N*-chloro function at the relatively low concentrations of the *N*-chloramines. Comparison between the sterilization times determined for 2-(2-*n*-butoxyethoxy)ethyl α-aminoisobutyrate hydrochloride (I*q*) and its *N*-chlorinated products (I*r* and I*s*) supports this hypothesis. The parent molecular structure, I*q*, does not exhibit any antimicrobial activity against any of the microorganisms examined using the CGE procedure. However, comparison of the sterilization times determined for I*r* and I*s* relative to the parent precursor demonstrates the substantial kinetic bactericidal effect of the *N*-chloro function.

Furthermore, comparison between the sterilization times determined for I*r* and I*s* at essentially the same concentration of positive chlorine suggests that the *N,N*-dichloroamino derivative (I*s*) is a more effective antimicrobial agent relative to the *N*-chloroamino derivative (I*r*). Unlike the behavior observed in the determination of the MIC of the *N*-chlorinated products of 2-amino-2-methyl-1-propyl carboxylates, comparison between the sterilization times determined for these derivatives demonstrates that the parent molecular structures are essentially inactive against the microorganisms tested and the kinetic bactericidal effect of the *N*-chloro function is observable for these *N*-chloramines.

Table II—MIC[a] of α-Aminoisobutyrates

Compound	*Staph. aureus*	*Strep. pyogenes*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*A. niger*	*C. albicans*	*T. mentagrophytes*
I*a*	125	>250[b]	125	>250	250	>250	>250	>250
I*b*	>500	>500	>500	>500	>500	>500	>500	>500
I*c*	>121	>61	>121	>121	>61	>121	>121	>121
I*d*	>131	>66	>131	>131	>66	>131	>131	66
I*e*	125	125	250	250	500	250	250	250
I*f*	150	75	150	150	>75	>150	150	140
I*g*	142	71	142	>142	>71	>142	142	142
I*h*	3.9	7.8	15.6	15.6	>500	31.3	15.6	15.6
I*i*	9	9	>131	>131	>66	33	33	17
I*j*	80	10	>159	>159	>80	>159	159	20
I*k*	3.9	7.8	500	>500	>500	62.5	15.6	7.8
I*l*	1.6	6.3	>250	>250	250	250	250	125
I*m*	62.5	31.3	500	500	125	>62.5	125	7.8
I*n*	3.9	7.8	>500	>500	>500	>125	125	62.5
I*o*	139	18	>139	>139	>70	>151	>151	76
I*p*	>118	>59	>118	>118	>59	>118	>118	>118
I*q*	>500	>500	>500	>500	>500	>500	>500	>500
I*r*	>125	>62.5	>125	>125	>125	>125	>250	>125
I*s*	>125	>62.5	>125	>125	>62.5	>125	250	>125

[a] Expressed in parts per million of compound. [b] Greater than indicates that the solvent in the dilution sequence inhibited the test organism at the higher concentrations.

Table III—MIC[a] of 2-Amino-2-methyl-1-propyl Carboxylates

Compound	*Staph. aureus*	*Strep. pyogenes*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*A. niger*	*C. albicans*	*T. mentagrophytes*
III*a*	>62.5[b]	62.5	>62.5	>62.5	62.5	>62.5	>62.5	62.5
III*b*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
III*c*	>62.5	62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
III*d*	>62.5	62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
III*e*	>250	>125	>250	>250	>250	>250	>250	>250
III*f*	250	250	>125	>125	250	>125	>125	>125
III*g*	>62.5	62.5	>62.5	>62.5	62.5	>62.5	>62.5	>62.5
III*h*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
III*i*	>62.5	62.5	>62.5	>62.5	>62.5	>62.5	>62.5	62.5
III*j*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
III*k*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
III*l*	62.5	62.5	>62.5	>62.5	>62.5	>62.5	>62.5	62.5
III*n*	>250	>250	>250	>250	>250	125	>250	125

[a] Expressed in parts per million of compound. [b] Greater than indicates that the solvent in the dilution sequence inhibited the test organism at the higher concentrations.

Table IV—CGE of α-Aminoisobutyrates

Compound	Concentration[a]		Sterilization Time[b], min				
	ppm	ppm, Cl^+	*Staph. aureus*	*Strep. pyogenes*	*E. coli*	*S. typhimurium*	*B. subtilis*
I*a*	887	334	0.5	2.5	0.5	2.5	2.5
I*b*	806	—	>60	10	30	45	30
I*c*	664	106	2.5	0.5	0.5	2.5	0.5
I*d*	79	22	5	2.5	2.5	15	5
I*e*	327		60	10	0.5	15	15
I*f*	275	39	30	0.5	0.5	2.5	2.5
I*g*	71	18	10	2.5	>60	45	15
I*h*	241		0.5	0.5	45	5	2.5
I*i*	159	18	30	0.5	2.5	60	15
I*j*	71	15	30	30	>60	>60	60
I*k*	114		60	2.5	45	15	45
I*l*	83	9	30	0.5	45	>60	30
I*m*	66	13	45	30	>60	>60	45
I*n*	113		10	10	5	30	15
I*o*	59	5	>60	30	>60	>60	45
I*p*	68	11	60	30	30	>60	45
I*q*	2506		>60	>60	>60	>60	>60
I*r*	2263	285	15	2.5	2.5	15	5
I*s*	1142	256	2.5	0.5	2.5	10	2.5

[a] Solubility in 30% methanol—0.1 *M* sodium dihydrogen phosphate, pH 7.0. [b] Time intervals screened were 0.5, 2.5, 5, 10, 15, 30, 45, and 60 min.

Although differences in the equilibrium solubilities of the *N*-chloramines investigated do exist, comparison of the sterilization times determined for the *N*-chloramines (Table V) suggests that III*f* is the most active in the series. Compound III*f* contains a branched (tertiary) aliphatic carboxyl chain and, analogous to the antimicrobial activity observed for the *N*-chlorinated α-aminoisobutyrates, the most

Table V—CGE of 2-Amino-2-methyl-1-propyl Carboxylates

Compound	Concentration[a] ppm	Concentration[a] ppm, Cl+	Sterilization Time[b], min *Staph. aureus*	*Strep. pyogenes*	*E. coli*	*S. typhimurium*	*B. subtilis*
III*a*	1292	458	5	2.5	0.5	2.5	2.5
III*b*	1070	—	>60	>60	>60	>60	>60
III*c*	2078	389	2.5	0.5	0.5	2.5	2.5
III*d*	979	304	2.5	0.5	0.5	2.5	0.5
III*e*	1886	323	5	0.5	0.5	2.5	2.5
III*f*	409	120	5	2.5	2.5	5	2.5
III*g*	1037	—	>60	>60	>60	>60	>60
III*h*	1319	211	2.5	0.5	2.5	5	2.5
III*i*	95	26	10	2.5	10	10	2.5
III*j*	1040	—	>60	30	15	30	5
III*k*	300	43	10	2.5	5	10	0.5
III*l*	85	21	15	5	10	10	2.5

[a] Solubility in 30% methanol—0.1 *M* sodium dihydrogen phosphate, pH 7.0. [b] Time intervals screened were 0.5, 2.5, 5, 10, 15, 30, 45, and 60 min.

active *N*-chlorinated 2-amino-2-methyl-1-propyl carboxylate, III*f*, also is an *N,N*-dichloroamino derivative. With the kinetic bactericidal effect of the *N*-chloro function in the *N*-chloramines established using the CGE procedure, the effects of (*a*) the solution pH, (*b*) the presence of a denaturant, and (*c*) the nature of a positive charge in the *N*-chloramine on the antimicrobial activity of *N*-chloramines containing nitrogen–chlorine bonds of different polarity were examined.

Effect of Solution pH—The sterilization time for the soft *N*-chloramine system as a function of the solution pH was investigated (Table VI). Comparison between the sterilization times determined for these *N*-chloramines suggests that all of the *N*-chloramines investigated appear to be equally effective antimicrobial agents against the less resistant Gram-positive and Gram-negative microorganisms. However, this conclusion is based on an inherent limitation in the time scale of the CGE procedure. Since the shortest time interval that could be examined was 0.5 min, expiration of the organism tested during this interval is not a true indication of the actual contact time required to detoxify the organism.

Despite the inherent limitation in the method, the antimicrobial activity determined for each *N*-chloramine against the less resistant organisms under essentially the same conditions appears to be pH independent. On the other hand, for the more resistant Gram-positive organisms, *Staphylococcus epidermidis* and *Staph. aureus*, only II and IV, which contain the most polar nitrogen–chlorine bonds, exhibit a pH-independent antimicrobial activity. The antimicrobial activity of I*a*, III*e*, III*f*, and V*a*, which contain less polar nitrogen–chlorine bonds, appears to be decreased marginally as the solution pH is increased.

Effect of Denaturant—The sterilization times for the same soft *N*-chloramines under identical conditions but in the presence of a denaturant such as horse serum were determined (Table VII). Comparison between these sterilization times relative to those described in Table VI demonstrates that II, which contains the most polar nitrogen–chlorine bond and should most readily transfer its positive chlorine atom, is also the *N*-chloramine most readily deactivated by the denaturant.

The antimicrobial activity of IV is also decreased by the presence of a denaturant under neutral (pH 7.0) or basic (pH 9.3) conditions. Furthermore, the extent of deactivation appears to increase as the solution pH is increased. The antimicrobial activities of V*a* and III*e*, which contain even less polar nitrogen–chlorine bonds relative to II and IV, are also decreased by the presence of the denaturant. The antimicrobial activity of the *N*-chloroamino derivative (III*e*) is completely removed by the denaturant. On the other hand, the sterilization times determined for the *N,N*-dichloroamino derivative (I*a*) in the presence and the absence of the denaturant are essentially unchanged. The lack of deactivation of I*a* by the denaturant further supports the previous conclusion from the CGE studies (Table IV) that the *N,N*-dichloroamino derivatives are more efficacious antimicrobial agents relative to the *N*-chloroamino derivatives.

Effect of Nature of a Positive Charge—The effect on the antimicrobial activity of the presence and nature of a positive charge in *N*-chloramines containing nitrogen–chlorine bonds of different polarity was examined (Table VIII). Comparison between the sterilization times determined for the *N,N*-dichloroamino derivative (III*m*) and the *N*-chloroamino derivative (V*b*) suggests that both *N*-chloramines are equally effective antimicrobial agents at the concentrations examined.

Comparison between the sterilization times determined for V*b* relative to V*c* demonstrates that, even at the same positive chlorine

Table VI—CGE of *N*-Chloramines as a Function of the Solution pH in the Absence of Horse Serum

Compound	Concentration ppm	Concentration ppm, Cl+	pH	Sterilization Time[a], min *Staph. epidermidis*	*Staph. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*B. bronchiseptica*
	1071	403	4.6	0.5	1	0.5	0.5	0.5	0.5
I*a*	1071	403	7.0	3	3	0.5	1	0.5	0.5
	1071	403	9.3	4	4	0.5	1	0.5	0.5
	404	69	4.6	0.5	1	0.5	0.5	0.5	0.5
III*e*	1886	323	7.0	3	4	0.5	2	1	1
	1704	292	9.3	4	3	1	2	1	1
	489	143	4.6	0.5	2	0.5	0.5	0.5	1
III*f*	409	120	7.0	2	4	1	3	0.5	2
	319	94	9.3	2	2	1	2	0.5	2
	139	41	4.6	2	3	1	2	3	1
II	126	36	7.0	2	4	1	4	5	4
	146	43	9.3	2	4	1	4	4	4
	1332	311	4.6	2	2	0.5	1	0.5	0.5
IV	1146	267	7.0	1	2	0.5	1	0.5	0.5
	1061	247	9.3	2	2	1	1	0.5	1
	1355	289	4.6	0.5	0.5	0.5	0.5	0.5	0.5
V*a*	1463	312	7.0	1	3	0.5	0.5	1	1
	1333	285	9.3	5	5	2	4	2	3

[a] Time intervals screened were 0.5, 1, 2, 3, 4, and 5 min.

Table VII—CGE of *N*-Chloramines as a Function of the Solution pH in the Presence of Horse Serum

Compound	Concentration			Sterilization Time[a], min					
	ppm	ppm, Cl^+	pH	*Staph. epidermidis*	*Staph. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*B. bronchiseptica*
	1071	403	4.6	1	2	0.5	0.5	1	0.5
Ia	1071	403	7.0	3	4	1	1	3	1
	1071	403	9.3	4	7	1	3	2	3
IIIe	468	80	4.6	>10	>10	>10	>10	>10	>10
	139	41	4.6[b]	>15	>15	>10	>10	>10	>10
II	126	36	7.0[b]	>15	>15	>15	>15	>15	>15
	146	43	9.3[b]	>15	>15	>15	>15	>15	>15
	1332	311	4.6	3	3	0.5	1	0.5	0.5
IV	1146	267	7.0	6	10	5	4	3	4
	1061	247	9.3	>10	>10	>10	>10	>10	>10
	1355	289	4.6	>10	>10	3	>10	4	6
Va	1463	312	7.0	6	>10	3	5	7	>10
	1333	285	9.3	>10	>10	4	9	4	4

[a]Time intervals screened were 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 min unless otherwise noted. [b]Time intervals screened were 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 15 min.

Table VIII—CGE of *N*-Chloramines Containing a Positive Charge

Compound	Concentration			Sterilization Time[a], min					
	ppm	ppm, Cl^+	pH	*Staph. epidermidis*	*Staph. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*B. bronchiseptica*
IIIm	79	21	4.6	3	5	2	1	2	4
IIIn	3100	565	4.6	4	5	1	2	2	1
IIIo	1112	209	4.6	3	5	1	5	3	5
Vb	1428	231	4.6	1	5	0.5	0.5	0.5	0.5
Vc	2594	267	4.6	>5	>5	>5	>5	>5	>5

[a]Time intervals screened were 0.5, 1, 2, 3, 4, and 5 min.

concentration, the presence of a localized positive charge completely removes the antimicrobial activity of Vc. This observation suggests several points regarding the detoxification mechanism of *N*-chloramines that requires further amplification. The only major difference between Vb and Vc is the presence of a remote, localized, positive charge from the *N*-chloro function. If hydrolytic degradation of each *N*-chloramine to another positive chlorine-containing species, *e.g.*, HOCl, were important, essentially the same antimicrobial activity would be expected for Vb and Vc. The difference observed in their antimicrobial activity suggests that the *N*-chloramine as an entity exerts the bactericidal effect upon the microorganism.

Furthermore, the loss of antimicrobial activity only for the *N*-chloramine containing the localized positive charge suggests that the *N*-chloramine must penetrate the cell wall of the microorganism to exert its bactericidal effect. Since the presence of a remote, localized, positive charge would not be expected to perturb the *N*-chloramine bond polarity significantly and, therefore, the rate at which positive chlorine is transferred, the localized positive charge must inhibit the antimicrobial activity of the *N*-chloramine by preventing effective penetration through the cell wall.

The *N*-chloramines IIIn and IIIo also contain a positive charge. However, in this case, the positive charge of the *N*-chloramine can be effectively delocalized by resonance. Comparison between the sterilization times determined for IIIn and IIIo suggests that the *gegen*-ion does not significantly influence the antimicrobial activity of the *N*-chloramine. Most importantly, the antimicrobial activity of the *N*-chloramine is not altered by the presence of a delocalized positive charge. Although differences in the *N*-chloramine concentrations examined do exist, the sterilization times determined for IIIm relative to IIIn or IIIo demonstrate that the delocalized positive charge in the *N*-chloramine does not significantly affect antimicrobial activity.

Furthermore, the antimicrobial activity of IIIn determined in the presence of a denaturant such as horse serum is identical to that observed in the absence of the denaturant under identical concentration conditions. This observation supports the proposal that delocalization of the positive charge permits sufficient penetration of the *N*-chloramine into or through the cell wall of the microorganism to exert a bactericidal effect. In addition, delocalization of the positive charge apparently prevents a significant protein binding of the *N*-chloramine, which would affect the antimicrobial activity of the *N*-chloramine. The identical antimicrobial activity observed for IIIn in the absence and presence of a denaturant once again demonstrates the superior bactericidal effect of an *N,N*-dichloroamino derivative.

With the comparative antimicrobial activity studies for the various low chlorine potential, soft *N*-chloramine systems accomplished using the MIC and/or CGE procedures, the utility of these agents as effective, soft antimicrobial agents necessitated an evaluation of their hydrolytic stabilities. The hydrolytic stability of three representative soft *N*-chloramines (II, IV, and Va) containing nitrogen–chlorine bonds of different polarity was examined in solutions of pH values corresponding to those in which their antimicrobial activities were determined (Table IX); the rate of change in the concentration of positive chlorine was followed iodometrically. The kinetic data obtained could be interpreted as first-order kinetic processes. The stability of the *N*-chloramines under the experimental conditions was characterized using the observed rate constant and reaction half-life.

Comparison between the hydrolytic stability determined for II and IV as a function of the solution pH is consistent with the degree of polarization of the nitrogen–chlorine bond in the *N*-chloramines. That is, II, which contains the nitrogen–chlorine bond most susceptible to hydrolytic cleavage, is also the *N*-chloramine that contains the most polar nitrogen–chlorine bond. The apparent discrepancy observed between the hydrolytic stability of Va and the degree of polarization of the nitrogen–chlorine bond in the *N*-chloramine is a result of the inherent instability of the 1,3-oxazolidine system. The 1,3-oxazolidine is the mono-*N*-analog of the 1,3-dioxolane system. These systems are susceptible to cleavage under acidic and basic conditions.

Comparison between the hydrolytic stability determined for Va as a function of the solution pH indicates an optimum stability for the *N*-chloramine under neutral conditions and relatively rapid degradation under acidic and basic conditions. This observation supports the hypothesis of cleavage of the 1,3-oxazolidine system and suggests that the loss of positive chlorine from Va is not solely due to hydrolytic cleavage of the nitrogen–chlorine bond.

The degree of polarization of the nitrogen–chlorine bond in II and IV confirmed by the hydrolytic stability studies is also consistent with the explanation of the antimicrobial activity of these *N*-chloramines based on different positive chlorine transfer rates determined by the degree of polarization of the nitrogen–chlorine bond.

Further chemical and microbiological investigations of IV to cor-

Table IX—Hydrolytic Stability of *N*-Chloramines Containing Nitrogen–Chlorine Bonds of Different Polarity

Compound	Solution pH	Rate Constant[a] $\times 10^{-2}$, hr^{-1}	Half-Life, hr
II	4.6	0.06	1260
	7.0	0.21	330
	9.3	1.95	35.5
IV	4.6	0.007	9600
	7.0	0.018	3792
	9.3	0.495	140
Va	4.6	27.7	2.5
	7.0	1.39	50
	9.3	13.9	5.0

[a] Rate constants were determined at 40°.

relate the degree of polarization of the nitrogen–chlorine bond and the rate of positive chlorine transfer to the antimicrobial activity of the *N*-chloramine are forthcoming (3).

EXPERIMENTAL

MIC—Prior to determination of the MIC, all bacteria were cultured for 18–20 hr (37°) in tryptose phosphate broth, except *Streptococcus pyogenes* (ATCC 19615) which was cultured in brain heart infusion broth plus 10% normal horse serum. Immediately prior to testing, each culture was adjusted to an optical density of 0.10 (650 nm) and diluted subsequently into double strength medium to approximately 2×10^5 viable organisms/ml.

Spore suspensions of the fungi, *Aspergillus niger* (ATCC 16404) and *Trichophyton mentagrophytes* (ATCC 9129), were diluted into maltose peptone broth to approximately 2×10^5 spores/ml. *Candida albicans* (ATCC 10231) was adjusted to an optical density of 0.10 and diluted into double strength maltose peptone to approximately 2×10^5 organisms/ml. The other organisms used were: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhimurium* (ATCC 14028), and *Bacillus subtilis* (ATCC 6051).

The diluted cultures were aseptically distributed in 1-ml amounts to five sterile culture tubes for each organism. The test solution of the compound to be investigated was prepared using 0.1 *M* sodium dihydrogen phosphate buffered to pH 7.0 and subjected to twofold dilutions into sterile water. Then 1 ml of each dilution was dispensed to separate tubes containing the organisms. The inoculated tubes were incubated at 37° for 18–20 hr, and the MIC of the compound was determined. Yeast and fungi were tested in the same manner, except that the incubation period was 5 days at 25°.

Controls were run to ensure viability of the organisms, sterility, and lack of bactericidal effect from the buffer.

CGE—A modified serial dilution analysis was used to determine the time taken for various concentrations of the compounds to sterilize suspensions of the microorganisms. The organisms used and their concentrations (organisms per milliliter) in overnight broth culture were: *Staphylococcus epidermidis* (ATCC 12228), 5×10^6; *Escherichia coli* (ATCC 10536), 10×10^6; *Klebsiella pneumoniae* (ATCC 10031), 12×10^6–13×10^6; *Pseudomonas aeruginosa* (ATCC 9027), 12×10^6–13×10^6; *Staphylococcus aureus* (ATCC 6538), 6×10^6–8×10^6; and *Bordetella bronchiseptica* (ATCC 4617), 3×10^6.

Nutrient Broth—The broth contained 5 g of gelysate peptone and 3 g of beef extract/1000 ml of distilled water, pH 6.9.

Nutrient Agar—The nutrient contained 5 g of gelysate peptone, 3 g of beef extract, and 15 g of agar/1000 ml of distilled water.

Horse Serum T.C.—A 10% horse serum solution in distilled water was freshly prepared and adjusted to pH 7, using carbon dioxide.

Method—A stock solution containing a known concentration of the *N*-chloro compound in an appropriate buffer was prepared. Aliquots of this stock solution were then diluted with equal volumes of distilled water or 10% horse serum to give the solutions to be tested. In some cases, the pH of the test solution was adjusted with 1 *M* HCl. Test solutions were left at room temperature for 30 min prior to their use in the screen.

In the screen, 0.2 ml of an overnight broth culture of an organism was added to 5 ml of the test solution. At time intervals of 0.5, 1, 2, 3, 4, 5, *etc.*, min, a loop of the suspension was subcultured into 5 ml of sterile nutrient broth. The subcultures were incubated at 37° for 7 days and observed daily for evidence of bacterial growth. The time reported for sterilization of a suspension of an organism corresponds to the smallest time interval in which a subculture that gave no growth during 7 days was prepared.

The following controls were conducted:

1. A 0.2-ml aliquot of an overnight culture was added to 5 ml of sterile 0.9% NaCl, and a loop of the suspension was subcultured into 5 ml of sterile nutrient broth. Bacterial growth in the subculture, when it was incubated at 37°, indicated that the overnight culture was viable.
2. A loop of a test solution of an *N*-chloro compound and a loop of a 25-fold dilution of an overnight culture with sterile 0.9% NaCl were added to 5 ml of nutrient broth, and the subculture was incubated at 37°. Bacterial growth indicated that the concentration of chlorinating agent in the subcultures did not inhibit bacterial growth.
3. At the same time intervals that subcultures were made of suspensions of microorganisms in test solutions, a loop of the suspension was subcultured on sterile nutrient agar plates. Following incubation of the plates at 37°, the morphology of the colonies was examined for contamination by foreign organisms.
4. Solutions that were identical to the test solutions but that did not contain the *N*-chloro compounds were subjected to the same screen as the test solutions. The purpose of this control was to ensure that the buffers in the test solutions were not bactericidal.

Replicate experiments demonstrated that there were no significant differences in the results within and between experiments.

The hydrolytic stability of the *N*-chloramines investigated was conducted using the procedure described previously (4).

SUMMARY

The preparation of a number of low chlorine potential, soft *N*-chloramines containing nitrogen–chlorine bonds of different polarity was investigated. These novel *N*-chloramine systems were based on derivatization of 2-amino-2-methyl-1-propanol. Comparative antimicrobial studies for these new classes of soft *N*-chloramines, as well as the soft *N*-chloramines of α-aminoisobutyrate esters and related derivatives (1), were examined using the MIC and/or CGE procedures.

The results of the comparative microbiological investigations demonstrate that the antimicrobial activity of the *N*-chloramine is significantly influenced by a number of factors. Comparison between the MIC's of the *N*-chloramines (Table I) suggests that the bacteriostatic activity is decreased as the nitrogen–chlorine bond polarity is decreased. The kinetic bactericidal effect of the *N*-chloro function in the *N*-chloramines is observable using the CGE procedure. Comparison between the sterilization times determined for the *N*-chloramines (Table VI) suggests that only the *N*-chloramines containing the more polar nitrogen–chlorine bonds give a pH-independent antimicrobial activity against the Gram-positive and Gram-negative microorganisms.

Comparison between the sterilization times determined for the same soft *N*-chloramines, but in the presence of a denaturant (Table VII), suggests that the *N,N*-dichloroamino derivative is the most resistant to deactivation by a denaturant. Furthermore, antimicrobial activity is completely removed by the presence of a localized positive charge in the *N*-chloramine but not significantly influenced by the presence of a delocalized positive charge (Table VIII).

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