Hemolysis of Erythrocytes by Antibacterial Preservatives II

Quaternary Ammonium Salts

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The hemolytic activity of various alkyldimethylbenzyl ammonium chlorides on rabbit blood was determined. Hemolytic activity increased with increasing alkyl chain length from C_8 to C_{18}. With the exception of the tetradecyl homolog, hemolytic activity of quaternary ammonium salts having the same alkyl group increased with chlorine substitution on the benzyl group. Correlations with reported microbiological activities and critical micelle concentrations have been made.

IN A PREVIOUS report (1) the hemolytic activities of benzalkonium chloride and benzethonium chloride were reported. These cationic surfactants hemolyzed rabbit and human erythrocytes at very low concentrations, and, on a weight basis, were about 200 times as effective as phenol in causing total hemolysis. These high hemolytic activities corresponded to high phenol coefficients reported for the above-mentioned quaternary ammonium salts.

The purpose of this investigation was to study the relationship of the chemical structure of a series of cationic surfactants to their hemolytic activity. Compounds chosen for study were alkyldimethylbenzylammonium chlorides and alkyldimethyl chlorine-substituted benzylammonium chlorides, where the alkyl group varied from an 8-carbon-atom straight chain to an 18-carbon-atom straight chain.

EXPERIMENTAL

Materials.—The quaternary ammonium salts (quats) used in this study are shown in Table I and were supplied by the Sterling Winthrop Research Institute, Rensselaer, N. Y. Reagent grade sodium chloride was used.

Preparation of Solutions.—Stock solutions (0.025 or 0.10%) of each quaternary ammonium salt were prepared in 0.9% sodium chloride solution. Aliquots of these were diluted with 0.9% sodium chloride solution to prepare the proper working concentrations. Several of the salts were not completely soluble and the insoluble materials settled out in stock solutions as immiscible liquids. These stock solutions were shaken vigorously before withdrawing aliquots. All solutions used in this study were assayed for cationic surfactant content.

Method of Assay for Cationic Surfactant.—The amount of quaternary ammonium salt in solution was determined by the titrimetric assay method of Barr *et al.* (2) as modified by Auerbach (3). The sample was placed in a 50-ml. glass-stoppered cylinder and 5 ml. of pH 9.5 phosphate buffer, 15 ml. of 0.0001 *N* dioctyl sodium sulfosuccinate, 1 ml. of bromophenol blue indicator, and 5 ml. of chloroform were added. The excess anionic agent was titrated with a 0.0001 *N* cetyldimethylbenzylammonium chloride solution to the end point as indicated by the appearance of a blue color in the chloroform layer.

Collection of Blood.—Rabbit blood was collected by heart puncture, defibrinated, and aerated in the same manner as described by Grosicki and Husa (4). A fresh blood sample was used in each experiment.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis of erythrocytes in various quaternary ammonium salt solutions was described in the first paper of this series (1).

Complete hemolysis of rabbit erythrocytes in quat solutions gave absorbance readings indicating greater than 100% hemolysis. This increase in absorbance readings was attributed to a darkening of the red color by the quaternary ammonium compounds. Experiments were carried out to quantitate this higher absorbance using a procedure described in an earlier paper (5). Readings for complete hemolysis of rabbit blood in quat solutions were 3 to 5% higher than hemolysis readings in distilled water.

To determine the effect of different blood concentrations on hemolysis, experiments were carried out in which various amounts of blood were added to a fixed volume of test solution. A standard for 100% hemolysis was prepared for each of these experiments by hemolyzing, in distilled water, the same concentration of blood that was used in the various quat solutions.

The pH of each test solution was determined before blood was added and at the end of hemolysis experiments using a Beckman model G pH meter.

RESULTS AND DISCUSSION

All quaternary ammonium compounds studied were highly active in causing hemolysis of rabbit erythrocytes. Figure 1 illustrates typical hemolysis curves obtained when rabbit blood was added to various concentrations of quat solutions. The minimum concentrations of quaternary ammonium salts causing complete hemolysis are shown in Table I along with their reported microbiological activities (6). Hemolytic activity of these compounds increased with an increase in alkyl chain length from C_8 to C_{18}. Microbiological activity increased with an increase in alkyl chain length to an optimum

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TABLE I.—CONCENTRATIONS OF QUATERNARY AMMONIUM COMPOUNDS CAUSING COMPLETE HEMOLYSIS OF RABBIT ERYTHROCYTES AND COMPLETE KILLING OF *S. aureus*

	RR′(CH₃)₂NCl		Min. Concn. in moles/L. Causing 100% Hemolysis[a]	Complete Kill of *S. aureus*[b]
Compd.	R	R′		
1	C_8H_{17}	Benzyl	9.5×10^{-3}	...
2	$C_{10}H_{21}$	Benzyl	7.7×10^{-4}	1.6×10^{-3}
3	$C_{10}H_{21}$	2-Chlorobenzyl	3.4×10^{-4}	3.8×10^{-4}
4	$C_{10}H_{21}$	4-Chlorobenzyl	2.0×10^{-4}	2.5×10^{-4}
5	$C_{10}H_{21}$	2,4-Dichlorobenzyl	1.4×10^{-4}	2.2×10^{-4}
6	$C_{12}H_{25}$	Benzyl	9.0×10^{-5}	1.8×10^{-4}
7	$C_{12}H_{25}$	2,4-Dichlorobenzyl	3.7×10^{-5}	5.4×10^{-5}
8	C_8H_{17}–$C_{18}H_{37}$ (mixed)	Benzyl	7.4×10^{-5}	...
9	$C_{12}H_{25}$–$C_{14}H_{29}$ (mixed)	2-Chlorobenzyl	4.8×10^{-5}	...
10	C_8H_{17}–$C_{18}H_{37}$ (mixed)	3,4-Dichlorobenzyl	3.3×10^{-6}	...
11	$C_{14}H_{29}$	Benzyl	3.0×10^{-5}	6.3×10^{-5}
12	$C_{14}H_{29}$	2,4-Dichlorobenzyl	4.1×10^{-5}	5.1×10^{-5}
13	$C_{16}H_{33}$	Benzyl	2.9×10^{-5}	1.2×10^{-4}
14	$C_{16}H_{33}$	2-Chlorobenzyl	2.2×10^{-5}	1.8×10^{-4}
15	$C_{18}H_{37}$	Benzyl	2.4×10^{-5}	4.4×10^{-4}
16	$C_{18}H_{37}$	2-Chlorobenzyl	1.8×10^{-5}	6.5×10^{-4}

[a] All values are for a 1:101 concentration of blood in quaternary solution and represent an average of at least two blood samples. All solutions contain 0.9% NaCl. [b] Data from Ross *et al.* (6). That concentration killing the organism in 10 min., but not in 5 min., was considered the minimum killing concentration.

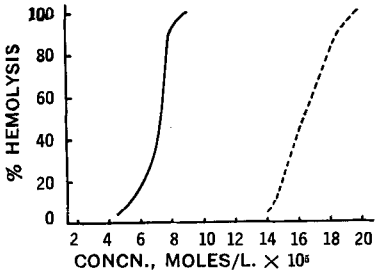

Fig. 1.—Hemolysis of rabbit erythrocytes in quat solutions at 37°. Key: ---, dodecyldimethylbenzylammonium chloride; ——, decyldimethyl-4-chlorobenzylammonium chloride.

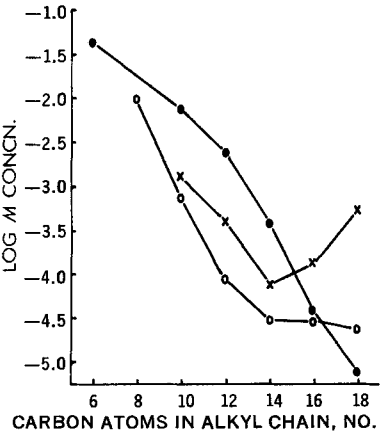

Fig. 2.—Comparison of hemolytic, bactericidal, and critical micelle concentrations for alkyldimethylbenzylammonium chlorides of varying chain length. Key: ●, critical micelle concentrations (dye method) [data from Ross *et al.* (6)]; ×, minimum killing concentrations for *S. aureus* [data from Ross *et al.* (6)]; ○, minimum concentrations causing complete hemolysis of rabbit erythrocytes.

activity at C_{14}, at which point activity decreased with further increase in chain length; hemolytic activity increased slightly with an increase in chain length from C_{14} to C_{18}. Breusch and Hersek (7) studied the hemolytic activities of a series of tertiary *N*-alkylpiperidine hydrochlorides and *N*-alkylpyridinium bromides. They found the activity against pigeon erythrocytes increased with an increase in alkyl chain length and was optimum at C_{14} for the piperidine series and at C_{16} for the pyridine series. However, further increase in chain length decreased the hemolytic activity of these compounds.

The effect of alkyl chain length on activity can be seen in Fig. 2, where comparison of hemolytic and microbiological activities is shown for alkyldimethylbenzylammonium chlorides. Previously reported critical micelle concentrations (6) are also included in Fig. 2 to show the relationship of this physical property to cytolytic activities.

The original pH of each test solution was between 6.4 and 6.8; after adding blood and allowing to stand 45 min. at 37°, the pH increased to approximately 7.5. This change in pH was attributed to the buffering action of rabbit blood.

With the exception of the tetradecyl homolog, hemolytic activity of quaternary ammonium salts having the same alkyl group increased with chlorine substitution on the benzyl group. There was a similar increase in microbiological activity except for the hexa- and octadecyl homologs.

Quantitative comparisons would have varied had other microbiological data been used for comparison and/or had other types (species) of blood been utilized in the hemolytic studies. Ross *et al.* (6) reported slightly different minimum killing concentrations of quaternary ammonium salts for *S. aureus* and *S. typhosa*. Large differences in phenol coefficients of these same quats against *S. aureus* and *E. typhi* have been found (8). In a previous communication (1), the authors reported that higher concentrations of benzalkonium chloride and benzethonium chloride were needed to initiate hemolysis of human erythrocytes than rabbit erythrocytes.

TABLE II.—PER CENT HEMOLYSIS IN QUATERNARY AMMONIUM CHLORIDE SOLUTIONS AFTER ADDITION OF VARIOUS AMOUNTS OF RABBIT BLOOD

Compd.	Concn. Quat in moles/L.[a]	ml. Blood added to 5 ml. Quat Soln. 0.04	0.06	0.08	0.10
7	3.6×10^{-5}	100	78	20	8
11	2.8×10^{-5}	100	53	26	12
11	3.2×10^{-5}	100	86	33	23
12	3.9×10^{-5}	100	70	27	15

[a] In presence of 0.9% NaCl.

Concentrations of quaternary ammonium salts causing complete hemolysis of a 1:101 dilution of blood in test solutions were similar to concentrations causing complete killing of *S. aureus*. However, if another dilution of blood had been used throughout this investigation, the quantitative results would have been different. Table II shows the degree of hemolysis that occurred when different amounts of blood were added to quat solutions of fixed volume and concentration. Per cent hemolysis decreased as the amount of blood added to 5 ml. of quat test solutions was increased. There was also a decrease in actual number of red blood cells hemolyzed at higher blood concentrations which would seem to indicate a decrease in the hemolytic activity of the quaternary ammonium salts in solution. This decreased activity can probably be attributed to partial inactivation of the quaternary ammonium compounds by binding to serum and/or cell membranes. Several experiments were carried out using washed rabbit erythrocytes (washed three times with 0.9% sodium chloride solution), and solutions containing concentrations of quats too low to cause hemolysis of rabbit blood caused complete hemolysis of these washed red cells. Numerous investigators (9, 10) have reported that the activity of quaternary ammonium germicides is decreased by serum as well as other organic substances. Although quantitative results varied with blood concentration, the relative order of activities for the compounds in Table II was not altered.

It is generally accepted that surface-active agents are bactericidal rather than bacteriostatic (11). Quaternary ammonium germicides have been studied extensively with respect to their action against microorganisms, and the lethal action of this group of germicides has been attributed to their surface activity and ability to adsorb to negatively charged sites (12). Gilby and Few (13) reported that the cell membrane's phospholipids are the binding sites for quaternary ammonium compounds. Hotchkiss (14) recognized the direct relationship of surface action and permeability damage to the lethal action of quaternary ammonium germicides, and he demonstrated that when bacterial cells are cytolyzed by surface-active agents, various intracellular substances seep out of the organism.

Hemolysis of human erythrocytes by ionic detergents has been attributed to a lowering of interfacial tension and collapse of the membrane's cholesterol–phospholipid–lipoprotein complex, with the cholesterol monolayer being thought of as the main site of attack (15). Springer (16) reported that lowering of surface tension usually suggests the probability that hemolysis will occur. Love reported that hemolysis by dodecylammonium chloride, like that of sodium dodecyl sulfate, essentially is due to induced cation permeability (17), and it appears to be intimately related to the adsorption of the hemolytic agent (18).

Although quaternary ammonium germicides were not included in the studies of Burnet and Lush (19), they found a very close correlation between the concentrations of sodium desoxycholate, sodium lauryl sulfate, and saponin capable of hemolyzing rabbit cells and the virucidal effect against herpes virus.

In this study the order of hemolytic activities of quaternary ammonium salts was found to be analogous to their microbiological activities, and hemolytic and bacteriolytic concentrations of these compounds were very low. It appears that hemolytic activities of quaternary ammonium salts might be indicative of their bacteriolytic activities.

REFERENCES

(1) Ansel, H. C., and Cadwallader, D. E., *J. Pharm. Sci.*, **53**, 169(1964).
(2) Barr, T., Oliver, J., and Stubbings, W. V., *J. Soc. Chem. Ind.*, **67**, 45(1948).
(3) Auerbach, M. E., private communication, Sterling Winthrop Research Institute, Rensselaer, N. Y.
(4) Grosicki, T. S., and Husa, W. J., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 632(1954).
(5) Cadwallader, D. E., *J. Pharm. Sci.*, **52**, 1175(1963).
(6) Ross, S., Kwartler, C. E., and Bailey, J. H., *J. Colloid Sci.*, **8**, 385(1953).
(7) Breusch, F. L., and Hersek, S., *Hoppe-Seylers Z. Physiol. Chem.*, **291**, 1(1952).
(8) Hinkel, E. T., private communication, Sterling Winthrop Research Institute, Rensselaer, N. Y.
(9) Lawrence, C. A., "Surface-Active Quaternary Ammonium Germicides," Academic Press Inc., New York, N. Y., 1950, pp. 79–82.
(10) Finch, W., "Disinfectants—Their Values and Uses," Chapman and Hall Ltd., London, England, 1958, pp. 84–92.
(11) Newton, B. A., *J. Appl. Bacteriol.*, **23** 345(1960).
(12) Glassman, H., *Bacteriol. Rev.*, **12**, 105(1948).
(13) Gilby, A., and Few, A. J., *J. Gen. Microbiol.*, **23**, 18 (1960).
(14) Hotchkiss, R. D., *Ann. N. Y. Acad. Sci.*, **46**, 479(1946).
(15) Pethica, B. A., and Schulman, J. H., *Biochem. J.*, **53**, 177(1953).
(16) Springer, G., *Bacteriol. Rev.*, **27**, 191(1963).
(17) Love, W. E., *J. Cellular Comp. Physiol.*, **44**, 291 (1954).
(18) *Ibid.*, **47**, 85(1956).
(19) Burnet, F. M., and Lush, D., *Australian J. Exptl. Biol. Med. Sci.*, **18**, 141(1940).