[CONTRIBUTION FROM THE RESEARCH DIVISION OF ARMOUR AND COMPANY]

The Relationship of Charge Density, Antibacterial Activity and Micelle Formation of Quaternary Ammonium Salts

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An earlier hypothesis that the antibacterial activity of quaternary ammonium salts is influenced by the charge density on the nitrogen atom has not been confirmed. An inverse relationship between antibacterial activity and critical micelle concentration has been shown in the series of compounds studied. It is suggested that steric effects are a major factor in determining the tendency toward micelle formation and the biological activity of cationic surface active agents.

An earlier publication from this Laboratory reported observations on the relation of certain structural characteristics to the antibacterial activity of higher aliphatic quaternary ammonium salts[2]; the hypothesis was presented that this activity is influenced by the charge density on the nitrogen atom. In order to evaluate this hypothesis the present investigation was undertaken.

A series of four phenyl-containing quaternary ammonium salts is compared with the series of cyclohexyl analogs. In both series the cyclic structure is separated from the nitrogen atom by from 0 to 3 methylene groups. Thus with the phenyl-containing series interaction of the high electron density ring with the nitrogen atom is progressively reduced. If this interaction is an important factor in determining the antibacterial activity of the compounds the cyclohexyl series should show approximately equal activities for all members. Also a very marked difference should be observed between the first members of each series in which the ring is attached directly to the nitrogen atom such that the cyclohexyl compound should be much more active than the phenyl.

The narrow range of biological activity observed among the compounds under investigation necessitated the development of a more critical bacteriological test.

Biological activity of the present series of compounds has also been related to the tendency for colloidal association as determined by critical concentration for micelle formation.

Experimental

The phenyl-containing quaternary ammonium salts were identical with those used in the earlier study.[2] In order to obtain the cyclohexyl analogs several preparative routes were employed. Attempts to purify the cyclohexyl-containing quaternary ammonium salts by crystallization from a variety of solvents were unsuccessful. Although these salts are precipitated by cooling to very low temperatures the compounds do not crystallize nicely and are obtained as viscous hygroscopic liquids at room temperature. Elemental analysis yielded unsatisfactory values. On the basis of the preparative procedures employed and spectrophotometric measurements the products were considered to be sufficiently pure for biological testing.

(2-Cyclohexylethyl)-dodecyldimethylammonium Chloride.—A solution of 9 g. of dodecyldimethylphenethylammonium chloride in 100 cc. of ethanol was treated with palladium black in order to remove catalyst poisons. The solution was filtered and subjected to catalytic hydrogenation over platinum at 90° and 600 p.s.i. for 16 hours. Attempts to purify the product by crystallization from a variety of solvents were unsuccessful. Infrared spectrophotometric examination indicated the absence of substituted phenyl groups (14.3 μ absorption). Ultraviolet spectrophotometric measurements (at 260 mμ) indicated the presence of 0.9% of the original phenyl-containing compound.

(3-Cyclohexylpropyl)-dodecyldimethylammonium Chloride.—A solution of 9 g. of (3-phenylpropyl)-dodecyldimethylammonium chloride in 100 cc. of ethanol was treated with palladium black, filtered and then hydrogenated over platinum at 100° and 500 p.s.i. for four hours. Infrared spectrophotometry showed no substituted phenyl group but ultraviolet indicated 12.7% of the original salt.

Cyclohexylmethyldodecyldimethylammonium Chloride.—A solution of 15 g. of benzylamine hydrochloride in 100 cc. of ethanol was hydrogenated over platinum at 80° and 1,000 p.s.i. for 18 hours. The resulting solution showed no substituted phenyl groups by infrared examination. The cyclohexylmethylamine was obtained by treatment of the solution with base, separating and drying over sodium sulfate. The yield was 12 g. A mixture of 11.3 g. of cyclohexylmethylamine and 12.5 g. of dodecyl bromide was heated on the steam-bath for six hours, then allowed to stand overnight. The mixture was treated with aqueous base and the organic layer washed four times with 25-cc. portions of water. The base was added to 50 cc. of 10% hydrochloric acid. The insoluble amine hydrochloride was filtered and triturated with cold water to remove cyclohexylmethylamine hydrochloride. The N-cyclohexylmethyldodecylamine was crystallized from ethyl acetate yielding 8.0 g. of product. This amine salt together with 8.4 g. of sodium bicarbonate, 50 cc. of methanol and 25 cc. of methyl chloride was heated in an autoclave at 100° for two hours. After cooling, the contents of the autoclave were filtered, evaporated to dryness and crystallized from ethyl acetate at about −70°. Infrared spectrophotometry showed no substituted phenyl group; ultraviolet showed 1.2% of the phenyl-containing quaternary ammonium salt.

Cyclohexyldodecyldimethylammonium Chloride.—A mixture of 20 g. of cyclohexylamine and 25 g. of dodecyl bromide was heated on the steam-bath for ten hours. After treatment with aqueous base the organic layer was separated, dried over sodium sulfate and distilled through a Vigreux column. A yield of 7 g. of N-cyclohexyldodecylamine was obtained; b.p. 200° (15 mm.). The secondary amine together with 4.2 g. of sodium bicarbonate, 15 g. of methyl chloride and 50 cc. of methanol was heated in an autoclave at 100° for three hours. After removal of the solvent the quaternary ammonium salt was crystallized from methyl acetate at a low temperature. Ultraviolet spectrophotometry indicated the complete absence of phenyl groups.

Critical concentrations for micelle formation were determined by the method and in the equipment described in earlier publications from this Laboratory.[3] Critical concentrations are listed in Table I.

TABLE I

QUATERNARY AMMONIUM CHLORIDES, $[R(CH_3)_2C_{12}H_{25}N]Cl$

	R	C.M.C., M		R	C.M.C., M
1	C_6H_5	0.00765	5	C_6H_{11}	0.0090
2	$C_6H_5CH_2$	.0081	6	$C_6H_{11}CH_2$	.0072
3	$C_6H_5CH_2CH_2$	.0041	7	$C_6H_{11}CH_2CH_2$	.0030
4	$C_6H_5CH_2CH_2CH_2$	.0031	8	$C_6H_{11}CH_2CH_2CH_2$	.0014

(1) To whom communications regarding this work should be addressed.

(2) J. A. Cella, D. N. Eggenberger, D. R. Noel, L. A. Harriman and H. J. Harwood, THIS JOURNAL, **74**, 2064 (1952).

(3) D. N. Eggenberger, F. K. Broome, R. A. Reck and H. J. Harwood, *ibid.*, **72**, 4135 (1950); D. N. Eggenberger and H. J. Harwood, *ibid.*, **73**, 3353 (1951).

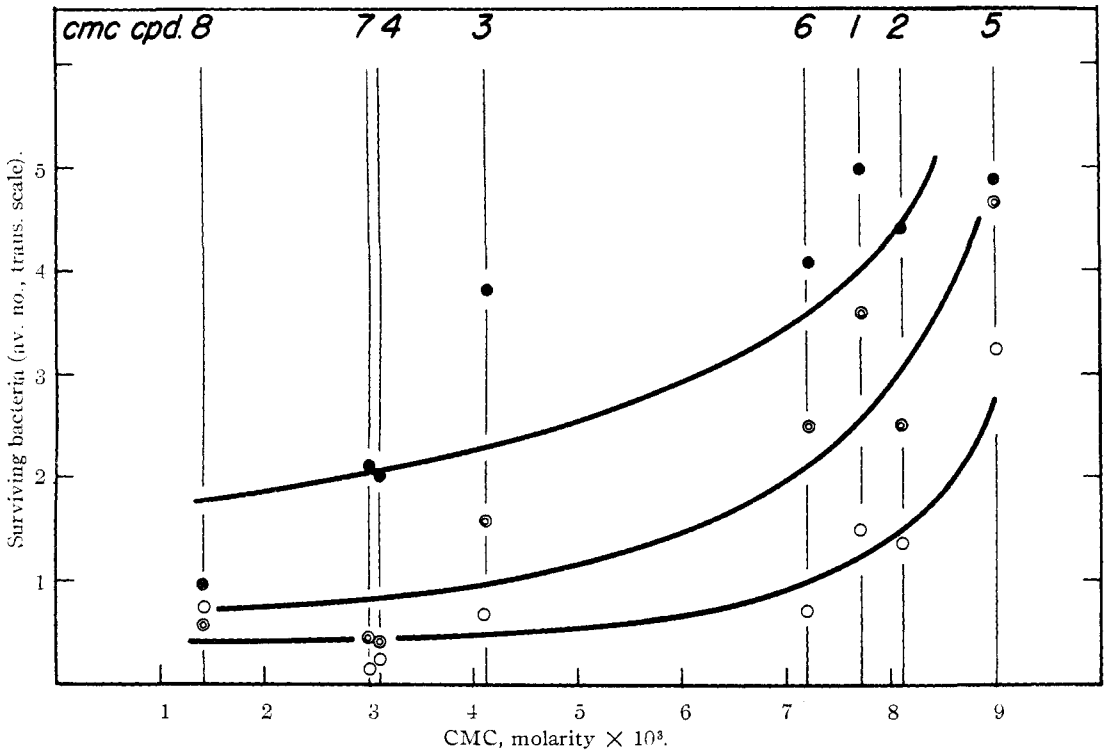

Fig. 1.—Relation of critical micelle concentration to number of surviving bacteria; compound concentration: ○, 0.00003 *M*; ◎, 0.00002 *M*; ●, 0.00001 *M*.

Bacteriological Methods.—A plate count method which gave the numbers of surviving organisms was employed to evaluate the antibacterial activity of the quaternary ammonium compounds.

In the plating procedure used on the medication mixture, a neutralizing agent, lecithin, was added to tryptone glucose extract agar. This neutralizing material did not affect the numbers of countable colonies of the test organisms, *Escherichia coli* and *Micrococcus pyogenes* var *aureus*. For the test suspensions, nutrient broth cultures were incubated 23 to 25 hours, filtered and diluted 1:15 in distilled water. Five tenths cubic centimeter of such suspension was exposed to 5.0 cc. of germicide solution in a test-tube at 37°. About 10 seconds prior to the lapse of the 10-minute interval, 0.5-cc. quantities of the medication mixture were placed in duplicate petri plates then poured with the neutralizing agar at the ten minute interval ± five seconds. The germicidal solutions were of such molarity as to give approximately 99.99% kill during this exposure period and to give countable numbers of colonies when 0.5 cc. of medication mixture was plated directly. For each compound at each of three concentrations (0.00003 *M*, 0.00002 *M*, 0.00001 *M*), the number of tests ranged from 3–9, each test representing two samples (plates) of medication mixture. Results shown in Table II are expressed in terms of a transformed scale. Controls consisted of dilutions of the original bacterial suspensions containing 0.01% of the original number of organisms (3 on the transformed scale).

TABLE II

AVERAGE NUMBERS OF SURVIVING BACTERIA

(Transformed scale.[a])

Compd.	Concn., molarity × 10⁵ 1	2	3
1	5	3.33	1.5
2	4.42	2.5	1.37
3	3.83	1.58	0.68
4	2.04	0.42	0.25
5	4.89	4.67	3.25
6	4.08	2.5	0.71
7	2.12	0.45	0.17
8	0.97	0.58	0.75

[a] Original no. — Trans. scale

0 — 0
1–9 — 1
10–99 — 2
100–999 — 3
1000–9999 — 4
Thousands — 5

Results and Discussion

As with most investigations of antibacterial activity, wide variation among replicates was observed. For purposes of statistical analyses of the data, bacterial counts were treated in terms of a transformed scale.

Table II presents the number of surviving bacteria expressed as average values in terms of the transformed scale for each compound at each of the three concentrations employed. These values are plotted in Fig. 1 against the critical micelle concentrations (CMC) of the eight compounds under investigation. Scattering of points is observed especially among those obtained at the lower concentrations and is possibly the result of errors introduced through adsorption of quaternary salt on the inner surface of the test tube.

It is evident that in general higher biological activity is found in compounds of lower critical micelle concentration. Compounds of lower molecular weight (numbers 1, 2, 5 and 6) possess lower activity. Conversely those of somewhat higher molecular weight (3, 4, 7 and 8) possess higher activity. There appears to be no relation between activity and saturation of the phenyl ring. The earlier hypothesis that proximity of the phenyl ring reduces

activity predicted higher activity for compound 5 than for compound 1 and for compound 6 than for compound 2, but no substantial differences are observed. It must be concluded therefore that either the hypothesis is incorrect and that inductive effects are insignificant or that other equally important compensating factors are in effect.

On the basis of polarity of ring substituted benzyldimethylalkylammonium chlorides as predicted from dipole moments of the corresponding substituted toluene a direct relationship of polarity of substituents with CMC and biological activity has been suggested.[4] In a series of substituted benzyldimethyldodecylammonium chlorides in which the corresponding toluene derivatives possessed dipole moments ranging from 0.4 to 4.40 the CMC range is from 0.28 to 3.6 $\times 10^{-3}$ M. Comparing these observations with those of the present study a wider CMC range (1.4 to 9.0 $\times 10^{-3}$ M) is noted in a series of compounds in which the cyclic substituents possess very low polarity as predicted from dipole moments of the corresponding hydrocarbons. Considering the facts which are available it is suggested that simple steric effects are a major factor in determining the tendency toward micelle formation and the biological activity of cationic surface active agents. These phenomena are perhaps a function of the ability of the molecules to undergo close packing which in turn influences the size of the micelle or the extent of interaction with the bacterial surface.

(4) S. Ross, C. E. Kwartler and J. H. Bailey, *J. Colloid Sci.*, **8**, 385 (1953).

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[CONTRIBUTION NUMBER 30 FROM THE DEPARTMENT OF BIOPHYSICS, FLORENCE R. SABIN LABORATORIES, UNIVERSITY OF COLORADO MEDICAL CENTER]

Binding of Salt Ions by Bovine γ-Pseudoglobulin[1]

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At *p*H values within its isoelectric zone, bovine γ-pseudoglobulin aggregates at very low salt concentrations, presumably due to association of molecules of opposite charge. In contrast, at *p*H 3.1 the protein aggregates at high but not low NaCl concentrations and at lower concentrations of other supporting electrolytes. The effectiveness of univalent anions in aggregating the protein at *p*H 3.1 increases in the order Cl, Br, NO_3 and ClO_4. In terms of equivalent concentration sulfate appears to fall between bromide and nitrate at high concentration, but is more effective than nitrate at low concentration. Interpretation of these results in terms of binding of anions by the protein is supported by electrophoretic measurements. The mean isoelectric *p*H value of the protein decreases on substitution of Br, NO_3 and ClO_4 for Cl in the buffer solvent, the effectiveness of the substituting anions increasing in the mentioned order. Sulfate also decreases the isoelectric point of the protein when substituted for chloride in the buffer, but the relative effectiveness of this anion as compared with the univalent anions has not been established.

Introduction

Unpublished data obtained in this Laboratory show that whereas the major portion of bovine γ-pseudoglobulin sediments as a single boundary at a rate independent of the *p*H over the range 7.4 to 4.2, the sedimentation constant of the protein decreases by about 10% between *p*H 4.2 and 3.5 and then once again becomes independent of *p*H over the range 3.5 to 2.2. In neutral solutions the sedimentation behavior of γ-globulin is independent of salt concentration over the range 0.02 to 1 M NaCl, whereas at *p*H 3.1 the sedimentation behavior is strongly dependent on salt concentration. The sedimentation behavior of the protein in acidic solutions is also dependent upon the anionic nature of the supporting electrolyte. Experiments designed to help elucidate the nature of the structural changes which occur in acidic solutions and give rise to the observed changes in sedimentation behavior are still in progress. However, the authors wish to report the results of some of these experiments which indicate that the sedimentation behavior of γ-globulin in acidic solutions can be modified appreciably by binding of various anions.

Experimental

Material.—The bovine γ-pseudoglobulin used in these experiments was the water-soluble fraction of Armour Fraction II of Bovine Plasma. A solution containing 3 g. of Fraction II/100 ml. was dialyzed exhaustively against many changes of cold, distilled water; the water-insoluble fraction removed by centrifugation and discarded; and the water-soluble fraction stored in salt-free solution at 2°. The sedimentation behavior of the protein as determined at ionic strength 0.1 and at *p*H 6.8 and 3.1 did not change during several months of storage.

Ultracentrifugal Analysis.—Sedimentation velocity experiments were performed in the Spinco Model E electrically driven ultracentrifuge.[2] A sedimentation cell with a Kel-F centerpiece was used at *p*H 3.1. Runs were made at room temperature on 1.1–1.3% protein solutions at 59,780 r.p.m. The temperature of each run was taken as one degree less than the mean of the rotor temperature at the beginning and end of the run.[3] Distances from the reference line to the meniscus and to the position of the maxima of the schlieren peaks and the areas under the peaks were measured on projected tracings of the photographic records of the sedimenting boundaries. Allowance was made for the stretching of the rotor.[4] Sedimentation constants were

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(2) Specialized Instruments Corporation, Belmont, California.

(3) D. F. Waugh and D. A. Yphantis, *Rev. Sci. Instr.*, **23**, 609 (1952).

(4) G. L. Miller and R. H. Golder, *Arch. Biochem. Biophys.*, **36**, 249 (1952); J. F. Taylor, *ibid.*, **36**, 357 (1952).