Electrokinetic Studies of Bacteria II: Effect of Cetyltrimethylammonium Bromide on *Streptococcus faecalis*

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Abstract □ Bacteria were treated with different concentrations of cetyltrimethylammonium bromide in solutions of constant ionic strength equal to 0.01 *M*, buffered at pH values of 3.4 and 7.0. The initial negative ζ-potential of the bacterial cell wall decreased in absolute value as the concentration of cetyltrimethylammonium bromide was increased, reached zero in the vicinity of the CMC, and then became increasingly positive. This is interpreted as chemisorption of the surface-active cation by ion exchange to form cetyltrimethylammonium carboxylate, followed by physical adsorption of the entire cetyltrimethylammonium bromide molecule. Lysis of the bacteria in the presence of relatively high concentrations of cetyltrimethylammonium bromide increased the positive value of the ζ-potential of the cell wall. Cetyltrimethylammonium bromide completely inhibited the growth of *Streptococcus faecalis* at a concentration which produced only a 10–30% decrease in the absolute value of the ζ-potential. This seems to indicate that the primary site of attack of the compound is probably not the cell wall.

Keyphrases □ Cetyltrimethylammonium bromide—growth inhibition of *Streptococcus faecalis* □ *Streptococcus faecalis*—growth inhibition by cetyltrimethylammonium bromide □ Electrokinetics—cetyltrimethylammonium bromide effect on ζ-potential of *S. faecalis* □ Zeta-potential of *S. faecalis*—effect of cetyltrimethylammonium bromide

At physiological pH, bacteria are negatively charged (1) owing to carboxylate groups in the cell wall (2, 3). Therefore, the cell wall interacts strongly with cationic surfactants. Although the antibacterial activity of surface-active quaternary ammonium halides does not necessarily result from their interaction with the cell wall, it may be possible to assess their uptake by bacteria through their effect on the surface charge of the cell wall.

The interaction of quaternary surface-active ammonium salts with bacteria has been studied extensively (4–6), including by electrophoresis (7). Cetyltrimethylammonium bromide has been studied most extensively (8–10). It was chosen for the present study because: (*a*) it is commercially used as an antiseptic[1], (*b*) it can be obtained with the *n*-hexadecyl chain free of higher and lower homologs, and (*c*) its physicochemical properties are well known (11–14).

EXPERIMENTAL

Instrumentation and the treatment of data were described in Part I (3). The bacterium, *Streptococcus faecalis*, and the growth medium used were the same as in Part I. The cells were washed and centrifuged three times in buffer solutions without cetyltrimethylammonium bromide. Cetyltrimethylammonium bromide was added only prior to the electrophoretic measurements.

The cetyltrimethylammonium bromide used[2] was recrystallized twice from acetone containing 5% water, treated once with ethyl acetate, and dried. The inorganic salts were ACS reagent grade.

RESULTS AND DISCUSSION

Lysis Caused by Cetyltrimethylammonium Bromide—Cetyltrimethylammonium bromide is known to cause lysis of bacteria, releasing nucleic acids. The absorbance of released material absorbing at 260 nm. had been found to increase with the time that the bacteria were in contact with cetyltrimethylammonium bromide (8). This opened the possibility that lysed nucleic acids, free or combined with cetyltrimethylammonium bromide, might be adsorbed at the cell wall and thereby affect the surface charge of the bacteria. Shorter times of contact of the bacteria with cetyltrimethylammonium bromide were expected to lead to less extensive lysis and less adsorption of extracellular nucleic acids. On the other hand, it was necessary to allow enough time for cetyltrimethylammonium bromide to reach equilibrium adsorption on the bacteria.

Experiments designed to determine optimum contact times are summarized in Table I. The buffer solutions used contained potassium acetate plus cetyltrimethylammonium bromide of combined ionic strength equal to 0.01 *M*; their pH was 7.0–7.1. The bacteria were incubated for 24 hr. at 37°. This corresponds to the stationary phase of their growth cycle (3). After harvesting, the bacteria were suspended three times in 0.020 *M* potassium acetate and centrifuged. They were finally suspended in the cetyltrimethylammonium bromide–potassium acetate buffers, and the electrophoretic mobilities were measured after the times of contact specified in Table I. It is seen from the table that the 30-min. contact time of the bacteria with cetyltrimethylammonium bromide was long enough for the cell wall to reach equilibrium uptake of cetyltrimethylammonium bromide yet short enough to prevent noticeable interaction between any nucleic acids, which might have been freed by lysis, and the cell wall, with or without the participation of cetyltrimethylammonium bromide.

The increased conductance of the bacterial suspension in 0.00200 *M* cetyltrimethylammonium bromide after 120 min. contact time and the increased positive mobility of the bacteria indicate that lysis had become measurable at that time. Leached cell constituents were probably adsorbed by the cell wall and may have promoted the adsorption of additional cetyltrimethylammonium bromide.

The bottom row of Table I refers to an experiment where soluble cell constituents were added to a bacterial suspension containing cetyltrimethylammonium bromide in order to simulate the effects of extensive lysis. Suspended bacteria were lysed by 2 min. of intense ultrasonic irradiation. The suspension was centrifuged, and 3 cm.^{3} of the supernate, with an absorbance at 260 nm. of 0.35, was added to 50 cm.^{3} of the bacterial suspension to which cetyltrimethylammonium bromide had been added 10 min. previously. The addition of extracellular nucleic acids caused a 30% increase in the electrophoretic mobility of the bacteria toward the negative pole in the presence of 0.0020 *M* cetyltrimethylammonium bromide. The same phenomenon of increased positive charge density due to nucleic acids in the presence of cetyltrimethylammonium bromide had also been observed in the case of the bacterial suspension in 0.0020 *M* cetyltrimethylammonium bromide after a contact time of 120 min., but to a lesser extent (third row of Table I). In this case, the nucleic acids originated from lysis of the cells induced by cetyltrimethylammonium bromide.

The increase in the positive charge density of the bacteria can be ascribed to the purine and pyrimidine bases of nucleic acids ad-

[1] Under the brand names Cetrimide BP and Cetavlon.
[2] Matheson, Coleman and Bell, technical grade.

Table I—Effect of Time of Contact and of Lysis on Electrophoretic Mobility of *S. faecalis* in Presence of Cetyltrimethylammonium Bromide (I)

Time of Contact, min.	Electrophoretic Mobility[a] in —Solution of I of Concentration— 0.00010 *M*	0.00200 *M*	Specific Conductance in 0.00200 *M* Solution of I, μmhos/cm.
5	−1.64 ± 0.04	1.72 ± 0.06	1220
30	−1.69 ± 0.04	1.73 ± 0.03	1220
120	−1.65 ± 0.04	1.90 ± 0.04	1280
10		1.73 ± 0.03	1220
10[b]		2.25 ± 0.03	1400

[a] In (microns/sec.)/(volts/cm.); average of 10 measurements ± standard deviation of the average. [b] Plus nucleic acids (see text).

sorbed on the cell wall. Attachment of these nucleic acid molecules probably occurred through binding of their phosphate groups to cetyltrimethylammonium bromide molecules which had become physically adsorbed on the cell wall prior to the addition of nucleic acids. This increase in the positive charge density of the bacteria by adsorbed nucleic acids can occur only if most or all of the free phosphate groups of these nucleic acid molecules are neutralized by cetyltrimethylammonium bromide. There was a sufficiently large excess of cetyltrimethylammonium bromide in solution to neutralize all phosphate groups of the added extracellular nucleic acids.

An alternative explanation for the increase in the positive charge density of the bacteria treated with cetyltrimethylammonium bromide upon subsequent addition of extracellular nucleic acids is also based on the complex coacervate formed between the nucleic acids and the molecules of cetyltrimethylammonium bromide physically adsorbed on the cell wall. Because of the excess cetyltrimethylammonium bromide in solution, this coacervate in turn physically adsorbed a larger amount of cetyltrimethylammonium bromide molecules than had been adsorbed on the cell wall originally, *i.e.*, prior to the addition of nucleic acids to the suspension. The greater density of cetyltrimethylammonium bromide molecules physically adsorbed on the cell wall in the presence of the adsorbed nucleic acids resulted in a greater density of positive charges.

Effect of Cetyltrimethylammonium Bromide on Electrophoretic Mobility—The data showing the effect of cetyltrimethylammonium bromide on the electrophoretic mobility of *S. faecalis* are summarized in Table II. Two kinds of buffer solutions were used for the electrophoretic measurements. The acid ones contained 0.01 *M* acetic acid plus cetyltrimethylammonium bromide and enough potassium bromide to bring the ionic strength to 0.01 *M*. Their pH, 3.4, was above the isoelectric point of *S. faecalis* (3). The neutral solutions contained cetyltrimethylammonium bromide plus enough potassium acetate to bring the ionic strength to 0.01 *M*. The acid and the neutral solutions had an average specific conductance of 1620 and 1150 μmhos/cm., respectively. The incubation times ranged from 19 to 26 hr.; *i.e.*, the bacteria were in the stationary phase. Duplicate experiments at the extreme times of that range showed no significant difference in mobility.

In neutral solutions, the high values of the electrophoretic mobility toward the negative pole observed at cetyltrimethylammonium bromide concentrations of 0.00200 *M* and above, namely, $U > 2.0$ (microns/sec.)/(volts/cm.), were sometimes poorly reproducible. Some mobilities were as much as 75% higher than others measured at the same cetyltrimethylammonium bromide concentration. Agglutination of the bacteria was also frequently observed at those conditions. Both phenomena can be attributed to extensive lysis; several bacteria were linked together by nucleic acids or other macromolecules which leaked from the cells and bridged the gap between neighboring cells, possibly through coacervation with cetyltrimethylammonium bromide.

By analogy with the interaction of cetyltrimethylammonium bromide with organic polymers containing carboxyl groups (15, 16) or with glass (17) and bentonite (18) containing silicic acid groups, the adsorption process occurred in two stages: chemisorption by the carboxyl groups of the cell wall followed by physical adsorption. Cetyltrimethylammonium bromide uptake by the cell wall resulted in reduced electrophoretic mobility as the negative carboxylate charges were neutralized by the positive charges of the surface-active cation. This is chemisorption *via* the ion-exchange reactions:

$$B\text{–}COO^-Na^+ + C_{16}H_{33}(CH_3)_3N^+Br^- \rightleftharpoons B\text{–}COO^-\ {}^+N(CH_3)_3C_{16}H_{33} + Na^+Br^- \quad \text{(Reaction 1)}$$

at pH 7.0, and:

$$B\text{–}COOH + C_{16}H_{33}(CH_3)_3N^+Br^- \rightleftharpoons B\text{–}COO^-\ {}^+N(CH_3)_3C_{16}H_{33} + H^+Br^- \quad \text{(Reaction 2)}$$

at pH 3.4, where B represents a portion of the cell wall.

By interpolation in Fig. 1, the electrophoretic mobility was reduced to zero at the cetyltrimethylammonium bromide concentration of 3×10^{-4} *M* in the acid solutions and of 7×10^{-4} *M* in the neutral solutions. These two concentrations, therefore, correspond approximately to the completion of the chemisorption and the beginning of the physical adsorption of cetyltrimethylam-

Table II—Electrophoretic Mobility of *S. faecalis* as a Function of Cetyltrimethylammonium Bromide (I) Concentration at Constant Ionic Strength[a]

Concentration of I, *M*	Electrophoretic Mobility[b] at pH 3.4–3.5	pH 6.9–7.1
0	−2.04 ± 0.04, −2.07 ± 0.06[c]	−2.21 ± 0.05, −2.18 ± 0.07
0.00005	−1.51 ± 0.05	−2.0 ± 0.08
0.00010	−0.92 ± 0.07	−1.75 ± 0.02, −1.64 ± 0.04, −1.68 ± 0.02, −1.65 ± 0.04
0.00025	−0.44 ± 0.07	−1.53 ● 0.03
0.00032	0.35 ± 0.03	−1.08 ± 0.05
0.00039	0.76 ± 0.05	—
0.00050	—	−0.58 ± 0.03, −0.61 ± 0.05
0.00055	0.89 ± 0.02	−0.52 ± 0.02
0.00060	—	−0.28 ± 0.01
0.00075	—	0.26 ± 0.01
0.00085	—	0.24 ± 0.02
0.00100	1.42 ± 0.05, 1.32 ± 0.03	0.63 ± 0.04
0.00150	1.51 ± 0.05, 1.77 ± 0.04	1.50 ± 0.03, 1.48 ± 0.04
0.00200	1.41 ± 0.05, 1.42 ± 0.05, 1.31 ± 0.02	1.45 ± 0.06, 1.71 ± 0.04, 1.83 ± 0.03, 2.97 ± 0.04, 1.79 ± 0.03, 2.10 ± 0.03
0.00250	1.54 ± 0.03	1.58 ± 0.04
0.00300	1.49 ± 0.02, 1.52 ± 0.03	1.67 ± 0.03, 1.73 ± 0.03, 2.92 ± 0.06

[a] Ionic strength = 0.01 *M*. [b] As (microns/sec.)/(volts/cm.); each number represents the average of 10 measurements ± standard deviation of the average. [c] Commas separate repeat measurements.

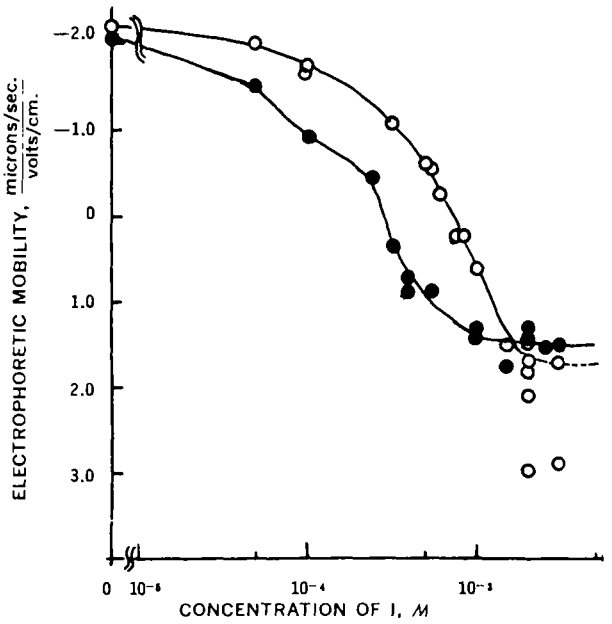

Figure 1—*Electrophoretic mobility of* S. faecalis *as a function of cetyltrimethylammonium bromide (I) concentration at two pH values and the constant ionic strength of 0.01* M. *Key:* ○, *pH 7.0; and* ●, *pH 3.4.*

monium bromide at the respective pH values. They bracket the value of the CMC of cetyltrimethylammonium bromide which, at an ionic strength of 0.01 *M*, is 5.5×10^{-4} *M* (14).

One would expect the chemisorption to be completed at a lower cetyltrimethylammonium bromide concentration in the neutral than in the acid medium, because any hydrobromic acid formed by ion exchange in the former is neutralized by the buffer. The bromide ions of potassium bromide added to the acid buffer to bring its ionic strength to 0.01 *M* should shift the adsorption equilibrium toward still lower cetyltrimethylammonium bromide uptake compared to the neutral medium, to which no potassium bromide had been added. The adsorption of cetyltrimethylammonium bromide by cellulosic fibers, wool, and nylon is reported to increase with increasing pH of the solutions (19).

However, the mobility of the bacteria became zero in neutral solutions at twice the cetyltrimethylammonium bromide concentration as in acid solutions. There was a direct relation between cetyltrimethylammonium bromide concentration and the isoelectric point of *S. faecalis*. As the cetyltrimethylammonium bromide concentration was increased from 0 to 3×10^{-4} to 7×10^{-4} *M*, the isoelectric point increased from a pH value of 2.3 (3) to 3.4 to 7.0. This indicates that amino and other basic groups contributed to the shift in isoelectric point caused by the addition of cetyltrimethylammonium bromide, even though they are present in the bacterial cell wall in much smaller concentrations than carboxyl groups (3). Protonation of basic groups in the acid medium, or a shift in the balance between the carboxyl and amino groups in the cell wall as the former became increasingly neutralized by cetyltrimethylammonium bromide, might account for the effect of cetyltrimethylammonium bromide and of the acidity of the medium on the isoelectric point. The isoelectric point of wool was displaced from a pH value of 3.4 to about 7 by treatment with dodecylpyridinium bromide (20), but keratin has a higher concentration of basic groups than the cell wall of *S. faecalis*. Even for the latter, however, zero electrophoretic mobility appears to mark only approximately the completion of the cetyltrimethylammonium bromide uptake by chemisorption.

The second adsorption step, which conferred a positive charge to the bacteria, was physical adsorption of entire cetyltrimethylammonium bromide molecules (ion-pair adsorption) (15–17). In the chemisorption process, only the cetyltrimethylammonium cations were adsorbed, with the hydrophilic headgroups being oriented toward the cell wall (Reactions 1 and 2). In this process, the cell wall became partly covered with hydrocarbon (cetyl) chains. In the subsequent physical adsorption, the bromide ions of the cetyltrimethylammonium bromide molecules were adsorbed as well. The hydrophilic headgroups were directed toward the aqueous phase, away from the cell wall, thereby conferring a positive charge to the bacteria. The cetyl groups of the physically adsorbed cetyltrimethylammonium bromide molecules probably interacted by hydrophobic bonding with the cetyl groups of the cetyltrimethylammonium ions previously taken up by chemisorption.

Bacteriostatic Effect of Cetyltrimethylammonium Bromide—To determine the effect of cetyltrimethylammonium bromide concentration on its bacteriostatic activity, suspensions of *S. faecalis* in cetyltrimethylammonium bromide solutions were made up at the pH values of 3.2 and 7.3, using acetic acid and potassium acetate, with enough potassium bromide added to the former to bring the ionic strength to 0.01 *M*. After 5- and 30-min. contact times, respectively, aliquots of these suspensions were diluted with water, and 0.1-cm.[3] portions were mixed with 5% molten brain heart infusion agar[3] in plastic petri dishes. After incubation at 37° for 20 hr., the number of colonies was counted. Blank tests were run with bacteria treated for the same length of time with the same buffer solutions, except that the latter contained no cetyltrimethylammonium bromide.

The results are summarized in Table III. The colony or plate counts of suspensions treated with cetyltrimethylammonium bromide are expressed as percentage of the counts of the blank samples at the same pH values. The standard deviation for the plate counts of the blanks was 14%. Therefore, of the intermediate viabilities, only the 69% value is significantly lower than 100%. Values of 6 or 11% are not significantly different from zero.

The most important conclusion is that practically complete inhibition occurred in 5×10^{-5} *M* cetyltrimethylammonium bromide, a concentration at which cetyltrimethylammonium bromide caused only a relatively small reduction in the electrophoretic mobility of *S. faecalis*, namely, about 25% at pH 3.4 and about 10% at pH 7.0 (Table II). This seems to indicate a lack of correlation between the antibacterial activity of cetyltrimethylammonium bromide and its effect on the electrokinetic properties of *S. faecalis*. There is some question whether the antibacterial activity of cetyltrimethylammonium bromide is due to its action on the membrane (5, 8) or on the wall of the bacteria (21). The present results seem to point indirectly to the membrane as the primary site of attack. The electrophoretic mobility, which is a measure of the surface charge density and hence of the cetyltrimethylammonium bromide uptake by the outer portion of the cell wall, showed little correlation with the cetyltrimethylammonium bromide concentration required for complete inhibition.

Miscellaneous—An unsuccessful attempt was made to determine the adsorption isotherm of cetyltrimethylammonium bromide on *S. faecalis* by adding small incremental amounts of a 0.1 *M* cetyltrimethylammonium bromide solution to a relatively concentrated bacterial suspension. After each cetyltrimethylammonium bromide addition, the amount of residual or free cetyltrimethylammonium bromide, *i.e.*, of cetyltrimethylammonium bromide not adsorbed by the bacteria, was to be determined by measuring the surface tension of the suspension with a Wilhelmy plate. After each cetyltrimethylammonium bromide addition, the surface tension of the suspension was found to decrease slowly instead of remaining constant. The surface tension of the suspension was increased by cleaning its surface, only to drop again slowly on standing.

This observation is in agreement with slow leaching of macromolecular material from the cells, which is either surface active by

Table III—Antibacterial Effect of Cetyltrimethylammonium Bromide (I)

	Molarity of I					
	5×10^{-5}		5×10^{-6}		5×10^{-7}	
	pH		pH		pH	
	3.2	7.3	3.2	7.3	3.2	7.3
Percent viability[a]:						
After 5 min.[b]	6, 0[c]	0, 0	101, 96	6, 11	96	93
After 30 min.[b]	0, 0	0, 0	103, 95	1, 0	94	69

[a] Plate count in presence of I as percentage of plate count of blank sample. [b] Contact time with I. [c] Commas separate duplicate values.

[3] Difco Laboratories No. 0418.

itself or forms a surface-active coacervate with cetyltrimethylammonium bromide. The equilibration of cetyltrimethylammonium bromide between the bulk solution and the cell wall–solution and solution–air interfaces is fast. The slow drop in surface tension probably resulted from diffusion of nucleic acids with or without bound cetyltrimethylammonium bromide to the surface, which is a slow process.

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Mass Spectrometric Behavior of Cardiac Steroid Aglycones of the Cardenolide Type

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Abstract □ The mass spectra of several cardenolide aglycones are discussed, and the principal modes of fragmentation are outlined. In addition to conventional expulsions of functional groups, the spectra exhibit C_{15}, C_{16}, and C_{17} fragment ions, resulting by elimination of ring D and the side chain as well as ions comprising the latter group with remnants of ring D.

Keyphrases □ Cardenolides—analysis, mass spectroscopy □ Cardiac steroid aglycones, cardenolides—analysis, mass spectroscopy □ Mass spectroscopy—analysis, cardenolides

Constitutionally, the group of cardiac steroid aglycones of the cardenolide type (I) is basically distinguished by an unsaturated γ-lactone side chain (β-oriented) and a β-oriented C-14 hydroxyl group in addition to the ubiquitous C-3 hydroxyl group (mostly β-oriented). The structure is frequently complexed by additional oxygen functions of various types located at various sites of the nucleus. The advent of the technique of mass spectrometry has provided an important tool for structure determination in various classes of natural products (2, 3) but, surprisingly, its application in the group of cardenolides has been extremely limited. Apart from the reported analysis of the spectra for conventional losses of functional groups in several products (4–7), a pattern for fragmentation of the nucleus in digitoxigenin (I) was proposed—without offering a suitable mechanism—by Spiteller (8) and another one was proposed for anhydroafrogenin (II), a C-14 anhydro cardenolide, by Shannon (9).

In the present article, a report is given on the mass spectrometric behavior of certain cardenolide models representing some of the more common types; the proposed fragmentation processes may be found useful in other cases. The molecular ions show up in the spectra, mostly with low intensities, and are always accompanied by abundant ions resulting from the loss of nearly all of the oxygen functional group content: as water from hydroxyl groups, as acetic acid and/or ketene from acetoxyl groups, and as CO (and rarely as CHO) from aldehyde groups. An important feature of frequent appearance is the breakdown of ring A, by a retro-Diels-Alder type of reaction involving loss of butadiene (54 mass units), in the ionized olefin resulting after elimination of the C-3 substituent. This type of breakdown of ring A is possible only with the location of the