Electrokinetic Studies of Bacteria IV: Effect of Acridines on *Streptococcus faecalis*

HANS SCHOTT

Abstract □ The effect of the following acridines on the electrophoretic mobility of *Streptococcus faecalis* was studied at constant values of pH and ionic strength: acridine hydrochloride, 9-hydrazinoacridine acetate, 9-aminoacridine hydrochloride, acriflavine hydrochloride, and proflavine dihydrochloride. Increasing times of contact of the bacteria with acridines rendered the electrophoretic mobility less negative or more positive due to the interaction with nucleic acids released from the bacteria through the action of the acridines. Stirring of the bacterial suspensions minimized this effect. Hence, electrophoretic mobilities were measured on samples withdrawn from stirred bacterial suspensions at successive time intervals after the addition of the acridines, and the mobilities were extrapolated to zero time. The mobility–concentration curves had similar shapes for all acridines. With increasing concentration of the acridinium salts, the negative mobilities or ζ-potentials of the bacteria decreased slowly at first but at a much faster rate as they approached zero. When the neutralization of the carboxylate groups in the bacterial cell wall by chemisorption of acridinium ions was complete, the ζ-potential was reduced to zero because the resultant acridinium carboxylates were only slightly dissociated. Further concentration increases inverted the electric charge of the bacteria to positive by physical adsorption of the entire acridinium salts. The positive mobilities increased with the acridine concentrations, rapidly at first but at slower rates for still higher concentrations. The acridine concentrations required to reduce the mobility or ζ-potential of the bacteria to zero were lower in more acid media. For four of the five acridines, there was an inverse relationship between these concentrations and the base strength of the acridines. The acridines of highest pKa were most strongly chemisorbed by the carboxylate groups in the cell wall, saturating them at the lowest concentrations. Relatively high concentrations of acridines caused the bacteria to agglomerate. This agglutination was not due to a reduction in the absolute value of the ζ-potential; it was probably caused by nucleic acids leaked from the bacteria or by their complex coacervates with the acridines, possibly through bridging. Aminoacridine hydrochloride and proflavine dihydrochloride did not inhibit the growth of *S. faecalis* even at concentrations 10 times greater than those at which they reduced the ζ-potential to zero. This suggests that the primary site of attack of these acridines is not the cell wall.

Keyphrases □ Electrokinetics—effect of acridines on electrophoretic mobility of *Streptococcus faecalis* □ Electrophoretic mobility, *Streptococcus faecalis*—effect of acridines, ζ-potential □ *Streptococcus faecalis*—effect of acridines on electrophoretic mobility □ Acridines—effect on electrophoretic mobility of *Streptococcus faecalis* □ Zeta-potential—calculated for *Streptococcus faecalis*, effect of acridines

Previous work had shown that *Streptococcus faecalis* is negatively charged at pH values above 2.3 owing to carboxylate groups in the cell wall (1–3). The interaction with a surface-active quaternary ammonium halide antiseptic consisted of chemisorption which progressively neutralized the charge of the bacteria with increasing concentration, followed by physical adsorption which reversed their charge to positive (2).

Acridine derivatives were selected to examine the effect of the base strength of a series of compounds containing basic nitrogen atoms, or the extent of ionization of their salts, on their adsorption by the bacterial cell wall. Acridines have been investigated extensively by Albert and coworkers (4–6). They range from weak to strong bases and are often available as hydrochlorides. While the site of the antibacterial action of the acridines is not the cell wall (6), it is interesting to ascertain if there is any relationship between their adsorption at the cell wall as studied by electrophoresis and their bacteriostatic effectiveness. Because of the porosity of the cell wall, acridines also come into contact with the cytoplasmic membrane. Cetyltrimethylammonium bromide modified the permeability of the membrane of *S. faecalis*, promoting the leakage of nucleic acids (2). This may be the mechanism of its antibacterial action.

EXPERIMENTAL

Materials—The following acridines were used as received: acridine hydrochloride[1], 9-aminoacridine hydrochloride[1], acriflavine hydrochloride (3,6-diamino-10-methylacridinium chloride hydrochloride)[1], proflavine dihydrochloride (3,6-diaminoacridine dihydrochloride)[1], and 9-hydrazinoacridine[2] (5). (The ring numbering is that of the International Union of Chemistry.) Cetrimonium (cetyltrimethylammonium) bromide was purified as described previously (2), and the salts used were ACS reagent grade. Water was redistilled in an all-quartz still and boiled out just prior to use.

Procedures—The bacteria, *S. faecalis* (ATCC 9790), the low-phosphate liquid growth medium, and the electrokinetic techniques were described previously (1). Electrophoretic mobilities were measured with the glass cell (3).

The bacteria were harvested after 20–25 hr incubation at 37°, which brought them into the stationary phase of their growth cycle and produced an uncorrected absorbance of 0.70 ± 0.02 at 640 nm. The suspensions were centrifuged for 15 min at 3000 rpm. The bacteria were freed of adhering nutrients by resuspending the sediment twice in 0.020 *M* potassium acetate and centrifuging for 15 min at 3000 rpm. They were suspended in potassium acetate or potassium chloride solutions, and acridine solutions were added to bring the suspensions to the desired concentration and ionic strength. The suspensions containing acridines were usually kept in the dark prior to measuring electrophoretic mobility because of the light sensitivity of many acridines. However, exposure to light produced no significant effect on mobility in those instances where comparison measurements were made with suspensions exposed to light and kept in the dark.

The inhibitory effect of two of the acridines was assessed by the pour plate method as described previously (2, 3). The presence of nucleic acids in solution was estimated using a visible–UV recording spectrophotometer[3] equipped with 1-cm quartz cells. The single change in procedure, namely, stirring of bacterial suspensions after drug addition, will be discussed.

The *Streptococcus pyogenes* culture[4] was incubated in the same liquid growth medium as *S. faecalis* in a shaking bath at 37° for 26 hr, at which time the uncorrected absorbance at 640 nm was 0.38–0.43. Purification methods were the same as for *S. fae-*

[1] K & K Laboratories, Plainview, N.Y.

[2] Supplied by Professor Adrien Albert, John Curtin School of Medical Research, Australian National University, Canberra City, Australia. The author thanks him for suggesting the use of 9-hydrazinoacridine and for supplying a sample.

[3] Perkin-Elmer double-beam spectrophotometer, Coleman 124.

[4] From the culture collection of the Department of Microbiology of Temple University Schools of Pharmacy and Dentistry.

Table I—Influence of Stirring on the Effect of 9-Aminoacridine Hydrochloride and of Proflavine Dihydrochloride on the Electrophoretic Mobility of *S. faecalis* at Constant Ionic Strength

Concentration of 9-Aminoacridine Hydrochloride[a], *M*	pH	Stirring Level	Electrophoretic Mobility[b] (Specific Conductance[c]) after Contact Times of: 5 min	15 min	35 min	45 min	60 min
0.0060	7.1	Occasional slow	−0.78 ± 0.03 (2750)	−0.58 ± 0.03 (2750)	0 (2750)	+0.56 ± 0.01 (2750)	+0.40 ± 0.01 (2710)
0.0060	7.1	Continuous slow	−0.78 ± 0.04 (2750)	−0.63 ± 0.02 (2750)	0 (2750)	+0.53 ± 0.02 (2750)	+0.49 ± 0.03 (2680)
0.0060	7.1	Continuous fast	−0.86 ± 0.04 (2750)	−0.46 ± 0.02 (2620)	−0.40 ± 0.02 (2600)	−0.43 ± 0.03 (2560)	−0.53 ± 0.03 (2540)
			10 min	30 min	40 min	60 min	
0.0082	6.9	None	−0.69 ± 0.03 (2710)	+0.69 ± 0.02 (2710)	+0.64 ± 0.03 (2710)	+0.55 ± 0.02 (2680)	
0.0082	7.1	Occasional slow	−0.65 ± 0.03 (2710)	+0.59 ± 0.03 (2680)	+0.56 ± 0.04 (2650)	+0.56 ± 0.03 (2620)	
0.0082	7.1	Continuous slow	−0.58 ± 0.02 (2710)	+0.23 ± 0.01 (2680)	+0.48 ± 0.03 (2650)	+0.40 ± 0.02 (2620)	
0.0082	7.0	Continuous fast	−0.61 ± 0.03 (2710)	−0.53 ± 0.01 (2600)	−0.45 ± 0.02 (2580)	−0.42 ± 0.02 (2580)	
Concentration of Proflavine Dihydrochloride[d], *M*			5 min	15 min	25 min	40 min	
0.0015	5.2	None	−1.20 ± 0.05 (690)	−1.08 ± 0.04 (690)	−0.82 ± 0.03 (690)	−0.80 ± 0.05 (690)	
0.0015	5.2	Continuous fast	−1.02 ± 0.02 (690)	−0.91 ± 0.03 (690)	−0.88 ± 0.03 (690)	−0.84 ± 0.04 (690)	

[a] Ionic strength adjusted to 0.03 *M* with potassium acetate. [b] Units of (micrometers per second)/(volts per centimeter); average of 10 or more measurements ± standard deviation of the average. [c] Units of μmhos per centimeter. [d] Ionic strength adjusted to 0.0065 *M* with potassium acetate.

Table II—Effect of Ionic Strength, Time of Contact, Stirring, and pH on Electrophoretic Mobility of *S. faecalis* in Potassium Acetate Buffers

Medium, Potassium Acetate[a], *M*	pH	Specific Conductance, μmhos/cm	Mobility ± *s*[b], (μm/sec)/(v/cm)	Contact Time[c], min
0.010	6.8	1060	−2.30 ± 0.07	30
0.010	7.0	1060	−2.56 ± 0.13	5
		1060	−2.61 ± 0.10	15
		1000	−2.57 ± 0.07	25
		1000	−2.40 ± 0.06	40
0.010	7.0	1060	−2.56 ± 0.13	5
		1060	−2.61 ± 0.10	15
		1000	−2.57 ± 0.07	25
		1000	−2.40 ± 0.06	40
0.030	7.0	2870	−1.86 ± 0.08	10
		2870	−1.73 ± 0.08	20
		2930	−1.82 ± 0.10	30
		2930	−1.70 ± 0.11	45
		2930	−1.70 ± 0.08	60
0.030	7.0	3080	−1.83 ± 0.11	5
		3080	−1.75 ± 0.06	15
		3080	−1.72 ± 0.04	5[d]
		3080	−1.78 ± 0.06	15[d]
0.030	7.0	3050	−1.82 ± 0.06	5
		3050	−1.72 ± 0.04	15
		3050	−1.74 ± 0.08	25
		3050	−1.73 ± 0.07	40
0.030	7.1	3050	−1.73 ± 0.06	5
		3020	−1.73 ± 0.05	15
		3020	−1.75 ± 0.06	25
		3050	−1.69 ± 0.04	40
0.030	7.0	2930	−1.78 ± 0.05	5[e]
		2930	−1.72 ± 0.10	20[e]
		2930	−1.75 ± 0.04	30[e]
0.030 + 0.044 *M* acetic acid	4.7	3000	−1.70 ± 0.10	5
		3000	−1.72 ± 0.08	30

[a] Each entry refers to a different batch of *S. faecalis*. [b] Average of 10 measurements ± the standard deviation of the average. [c] No stirring unless otherwise indicated. [d] With constant fast stirring. [e] With constant slow stirring.

Table III—Electrophoretic Mobility of *S. faecalis* as a Function of Proflavine Dihydrochloride Concentration at Constant Ionic Strength

Concentration of Proflavine Dihydrochloride, *M*	pH	Specific Conductance, μmhos/cm	Mobility[a], (μm/sec)/(v/cm)
Ionic Strength 0.01 *M*[b]			
0	6.8	1060	−2.40[c]
0.0005	6.0	1040	−1.94[c]
0.0010	5.7	1160	−1.31
0.0015	5.5	1080	−0.76
0.0025	5.0	1060	+0.45
0.0050	4.8	1080	+0.87
Ionic Strength 0.03 *M*[b]			
0	7.0	3000	−1.78[c]
0.000025	6.9	2930	−1.71[c]
0.000050	6.8	3010	−1.77[c]
0.00010	6.8	2880	−1.55
0.00020	6.7	2810	−1.12[c]
0.00025	6.7	2940	−1.27[c]
0.00040	6.4	2990	−1.35[c]
0.00050	6.4	2930	−1.07
0.00100	6.2	2870	−0.78
0.0012	6.0	2940	−0.42
0.0014	6.0	2890	−0.48
0.0015	6.0	2930	+0.37[d]
0.0016	6.0	2930	+0.63[d]
0.0018	5.8	2990	+0.43
0.0020	5.8	2950	+0.56
0.0030	5.6	2710	+0.54
0.0040	5.4	2990	+0.80[c]
0.0045	5.4	3020	+0.92
0.0050	5.2	3000	+1.55

[a] Extrapolated to zero time from four sets of 10 measurements performed after 5, 15, 25, and 40 min, respectively. [b] Ionic strength adjusted with potassium acetate. [c] No significant trend with time. [d] Sign of electric charge changed with time.

calis. The bacteria were suspended in the acridine solutions and stirred for the specified time, after which the suspensions were diluted with distilled water. Aliquots were treated by the pour plate method as described (3).

RESULTS AND DISCUSSION

Electrophoresis—*Effect of Stirring*—Preliminary experiments designed to determine optimum contact times of bacterial suspensions with acridines prior to measuring the electrophoretic mobility gave poorly reproducible mobility values; they were found to undergo systematic changes with time in the presence of acridines. This led to the evaluation of the effect of stirring, as summarized in Table I. Fast stirring refers to the use of a magnetic stirrer at the highest speed which did not produce a vortex,

Table IV—Electrophoretic Mobility of *S. faecalis* as a Function of Acriflavine Hydrochloride Concentration at Constant Ionic Strength[a]

Concentration of Acriflavine Hydrochloride, *M*	pH	Specific Conductance, μmhos/cm	Mobility[b], (μm/sec)/(v/cm)
0.0010	4.7	3530	−1.69
0.0020	4.7	3460	−1.02
0.0025	4.7	3430	−0.53
0.0030	4.7	3240	+0.36
0.0030	5.6	3150	−0.48
0.0070	4.7	3060	+0.70
0.0090	4.8	3000	+0.80
0.0150	4.5	2950	+1.11

[a] Ionic strength adjusted to 0.03 *M* and pH adjusted to values listed with potassium acetate and potassium chloride. [b] Extrapolated to zero time from four sets of 10 measurements performed after 5, 15, 30, and 45 min, respectively.

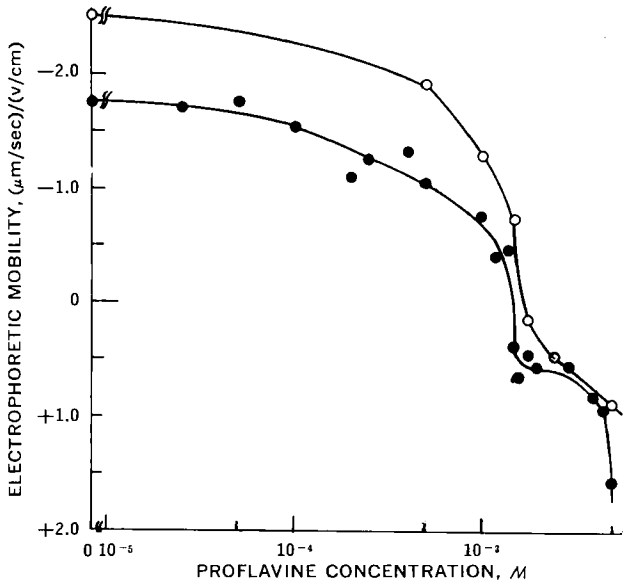

Figure 1—*Electrophoretic mobility of* S. faecalis *as a function of proflavine dihydrochloride concentration. Key:* ○, μ = *0.01* M; *and* ●, μ = *0.03* M.

approximately 10 rps of the magnetic bar. Slow stirring refers to approximately 2 rps.

Negative mobility indicates a negatively charged cell wall, which caused the bacterium to migrate to the positive pole. With or without stirring, mobilities and, hence, ζ-potentials usually became increasingly less negative or more positive with increasing times of contact of the suspended bacteria with acridines. All five acridines showed this effect, which was due to leakage of nucleic acids from the bacteria caused by the acridines, similar to what had been observed with cetyltrimethylammonium bromide (2). The increase in absorbance at 260 nm with time of contact with acridines paralleled the increase in mobility of the bacteria, indicating that nucleic acids released by the acridines were the cause of the change in mobility. In the absence of acridines, no extracellular nucleic acids could be detected spectroscopically, nor did the mobilities change with time in stirred and unstirred suspensions (Table II).

Continuous fast stirring of bacterial suspensions to which acridines had been added produced the smallest increase in mobility with time. Evidently, stirring dispersed the nucleic acids released by a given bacterium, removing them from its vicinity fast enough to prevent substantial adsorption of these nucleic acids or of their complex coacervates with acridines at the cell wall. According to previous work (2), adsorption at the cell wall of nucleic acids released by the action of cetyltrimethylammonium bromide on *S. faecalis* in unstirred suspensions, probably in the form of complex coacervates with the cationic surfactant, changed the charge density of the cell wall to more positive values. In unstirred suspensions with added acridines, nucleic acids just released and/or their coacervates with acridines were slow to diffuse away from the bacteria, increasing the opportunity for their adsorption by the cell wall, with resultant changes in mobility.

To correct this effect, mobilities were measured on samples withdrawn at regular intervals after the addition of the acridines, while the bulk of the bacterial suspension was continuously stirred. Reported mobilities are the values extrapolated to zero time. Even in rapidly stirred suspensions, the charge of the bacterial cell wall occasionally underwent a change of sign with increasing time of contact, usually from negative to positive. This was observed especially for 9-aminoacridine hydrochloride, proflavine dihydrochloride, and acridine hydrochloride; it occurred near dye concentrations producing zero mobility.

Effect of Ionic Strength—The addition of indifferent electrolytes, *i.e.*, electrolytes whose cations and anions are not specifically chemisorbed by ionic groups in the surface of the bacteria, generally reduces the absolute values of the ζ-potential or electrophoretic mobility (1). Increasing ionic strength likewise reduces them. As is shown in Table II, a threefold increase in the ionic

Table V—Electrophoretic Mobility of *S. faecalis* as a Function of 9-Aminoacridine Hydrochloride Concentration at Constant Ionic Strength[a]

Concentration of 9-Aminoacridine Hydrochloride, *M*	pH	Specific Conductance, μmhos/cm	Mobility[b], (μm/sec)/(v/cm)
0	7.1	3020	−1.73[c]
0.0005	7.1	2910	−1.35[c]
0.0010	7.1	2890	−0.98[c]
0.0020	7.0	2870	−1.06[c]
0.0050	7.0	2850	−0.57
0.0070	7.1	2810	−0.32
0.0080	7.1	2750	−0.56
0.0085	7.0	2750	−0.36[d], −0.34[d]
0.0090	7.0	2680	+0.29[c]
0.0100	7.0	2680	+0.46
0.0115	7.0	2680	+0.62
0.0150	6.9	2500	+0.67[c]
0.0200	6.9	2460	+0.71[c]

[a] Ionic strength adjusted to 0.03 *M* with potassium acetate. [b] Extrapolated to zero time from four sets of 10 measurements performed after 5, 20, 35–40, and 55–60 min, respectively. [c] No significant trend with time. [d] Sign of electric charge changed with time.

strength of the buffer solution at constant pH reduced the absolute value of the mobility by 30%.

Table II also shows the excellent reproducibility of the electrophoretic mobility measurements and, in the absence of acridines, their independence of time of contact with the bacteria and of stirring. Five series of measurements, made over 10 months in 0.030 *M* potassium acetate blanks, gave a grand average mobility of −1.751 (μm/sec)/(v/cm), with a standard deviation of less than 1%. Three series of measurements in 0.010 *M* potassium acetate blanks gave a grand average of −2.509, with a standard deviation of 1.5%.

Changing the pH from 7.0 to 4.7 at constant ionic strength produced no significant change in mobility in the absence of acridines. The zero point of charge of *S. faecalis* is at a pH of about 2.3 (1).

Effect of Acridines at Constant Ionic Strength—The data for proflavine dihydrochloride (Table III) show that the presence of acridines did not alter the lowering of the absolute value of the electrophoretic mobility by an increase in ionic strength as long as the bacteria remained negatively charged. After charge inversion, however, an increase in ionic strength unexpectedly caused increases in mobility.

A threefold increase in ionic strength (μ) shifted the concentration–mobility curve toward more positive mobilities and lower concentrations (Fig. 1). This reduced the proflavine dihydrochloride concentration producing zero mobility by 24%. Thus, a drug concentration of 0.0015 *M* changed the mobility to −0.76 (μm/sec)/(v/cm) at $\mu = 0.01$ *M* but to +0.37 at $\mu = 0.03$ *M*.

Clumping or aggregation of bacteria was sometimes observed at high concentrations of acridines, *e.g.*, above 0.001 *M* for proflavine dihydrochloride at $\mu = 0.03$ *M*, in 0.01 *M* hydrazinoacridine acetate, and in 0.03 *M* acridine hydrochloride. It became more severe as the time of contact of the dissolved acridines with the suspended bacteria increased. Time of contact affected agglomeration more than mobility.

This aggregation is not a flocculation caused by reduction in the absolute value of the ζ-potential due to adsorption of acridines at the cell wall. Clumping occurred only at relatively high acridine concentrations, regardless of the magnitude of the mobility. In the case of proflavine dihydrochloride, for instance, aggregation was observed from small negative through zero to high positive mobility values. At $\mu = 0.03$ *M*, the compound produced severe clumping at a concentration of 5×10^{-3} *M*, where the ζ-potential was +21 mv according to Eq. 5 of Ref. 1, but none at 7×10^{-5} *M*, where the interpolated ζ-potential was −21 mv. Aggregation seems to be caused by nucleic acids released from the bacteria through the action of the acridines or by nucleic acid–acridine complex coacervates, possibly *via* a bridging mechanism (7, 8).

Acriflavine hydrochloride is dibasic. Thus, in a 0.015 *M* solution, the maximum amount of potassium acetate consistent with an ionic strength of 0.03 *M* gave a pH of only 4.7. To maintain constant conditions in all suspensions, with acriflavine hydrochloride concentration as the only variable, the pH was adjusted to 4.7 with potassium acetate; potassium chloride was added to maintain the ionic strength at 0.03 *M* where necessary. The results are listed in Table IV. One measurement, at an acriflavine hydrochloride concentration of 0.0030 *M*, was made at pH 5.6 in addition to the usual 4.7 value. The charge of the bacteria in the

Table VI—Electrophoretic Mobility of *S. faecalis* as a Function of Acridine Hydrochloride Concentration at Constant Ionic Strength[a]

Concentration of Acridine Hydrochloride, *M*	pH	Specific Conductance, μmhos/cm	Mobility[b], (μm/sec)/(v/cm)
0	6.7	4220	−1.70
0.0050	4.1	4000	−1.59
0.0100	3.5	3500	−1.22
0.0300	3.1	2600	−0.40[c]

[a] Ionic strength adjusted to 0.03 *M* with potassium chloride. [b] Extrapolated to zero time from four sets of 10 measurements performed after 5, 15, 30, and 45 min, respectively. [c] Sign of electric charge changed with time.

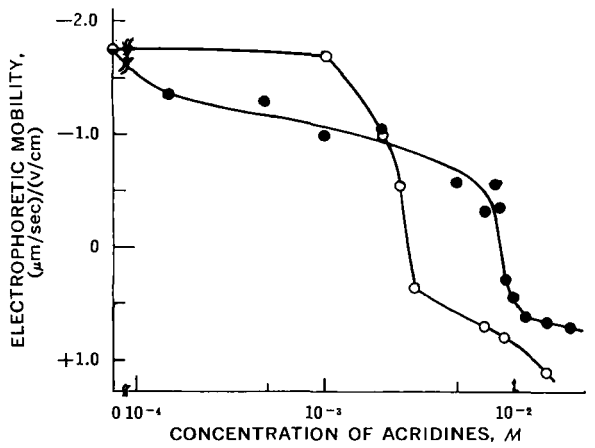

Figure 2—*Electrophoretic mobility of* S. faecalis *as a function of the concentrations of acriflavine and aminoacridine. Key:* ○, *acriflavine hydrochloride; and* ●, *9-aminoacridine hydrochloride.*

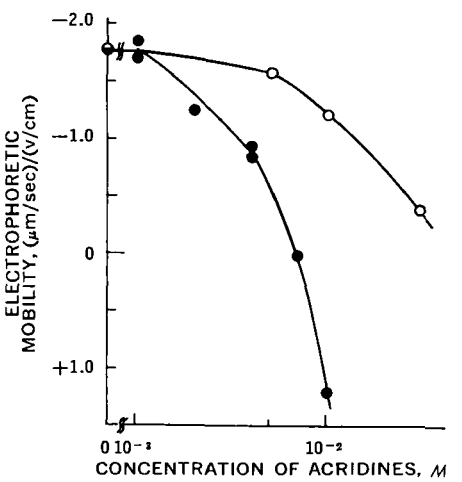

Figure 3—*Electrophoretic mobility of* S. faecalis *as a function of the concentrations of acridine and hydrazinoacridine. Key:* ○, *acridine hydrochloride; and* ●, *9-hydrazinoacridine acetate.*

Table VII—Electrophoretic Mobility of *S. faecalis* as a Function of 9-Hydrazinoacridine Concentration at Constant Ionic Strength[a]

Concentration of 9-Hydrazinoacridine, *M*	pH	Specific Conductance, μmhos/cm	Mobility ± *s*[b], (μm/sec)/(v/cm)
0	4.7	3000	−1.72 ± 0.10[c], −1.68 ± 0.07[d]
0.001	4.5	3060	−1.71 ± 0.08[c], −1.84 ± 0.09[d]
0.002	4.6	2810	−1.25 ± 0.07[c], −1.23 ± 0.04[d]
0.004	4.7	2930	−0.96 ± 0.06[c], −0.84 ± 0.05[d]
0.007	4.4	3000	0[c], 0[d]
0.010	4.4	3120	+1.14 ± 0.06[c], +1.38 ± 0.08[d]

[a] Ionic strength adjusted to 0.03 *M* with potassium acetate; pH adjusted with acetic acid. [b] Each number represents the average of 10 measurements ± the standard deviation of the average. [c] Measured after 7–10-min contact time. [d] Measured after 20–23-min contact time.

medium of higher pH was still negative while it had already been inverted to positive in the more acid medium. The acriflavine concentration required to reduce the ζ-potential of the bacteria to zero is apparently higher in the less acid medium, similar to what had been observed for cetyltrimethylammonium bromide (2).

The results obtained with 9-aminoacridine hydrochloride and acridine hydrochloride are summarized in Tables V and VI, respectively. Acridine is the weakest base of those studied. A solution containing 0.0295 *M* potassium acetate and 0.0005 *M* acridine hydrochloride produced a precipitate, presumably of the free base. Therefore, no attempts were made to buffer acridine hydrochloride solutions with potassium acetate. The hydrochloride was dissolved in water, and potassium chloride was added to maintain the ionic strength at 0.03 *M*. The resultant solutions, although acidic, had pH values above the isoelectric point of *S. faecalis*, which is at 2.3 (1).

Since the sulfate and hydrochloride of 9-hydrazinoacridine are poorly soluble in water (5), the compound was used as the acetate. The ionic strength was adjusted to 0.03 *M* with potassium acetate while enough acetic acid was added to bring the pH value to 4.4–4.7 and to dissolve the base. The data of Table VII indicate that the effect of contact time on mobility was less pronounced for hydrazinoacridine acetate than for aminoacridine hydrochloride and proflavine dihydrochloride.

Acridine Concentrations Producing Zero Mobility—The uptake of acridines by the cell wall probably occurs in two stages analogous to those described for cetyltrimethylammonium bromide (2). The first stage is chemisorption by ion exchange between the acridinium hydrochlorides and the carboxylic acid or potassium carboxylate groups in the cell wall (1). It reduces the electrophoretic mobility of the bacteria to near zero, since the resultant acridinium carboxylates are only slightly dissociated. The second stage, physical adsorption of the acridinium hydrochlorides by the cell wall, inverts the sign of the ζ-potential to positive. The transition between these two processes corresponds to the steepest portions of the acridine concentration *versus* electrophoretic mobility plots in Figs. 1–3.

The bacterial suspensions used in the electrophoresis measurements contained very small amounts of bacteria, not nearly enough to lower significantly the concentration of the dissolved acridines by adsorption. Therefore, the initial tabulated concentrations of acridines are substantially the same as their equilibrium concentrations.

The concentrations of the different acridine derivatives required to reduce the electrophoretic mobility of the bacteria and, hence, the ζ-potential to zero were obtained by interpolation in Figs. 1–3. These concentrations are inversely related to the affinity of the compounds for the cell wall: lower concentrations correspond to stronger adsorption. The concentrations producing zero mobility are listed in Table VIII together with the pKa values of the acridine derivatives. By applying the equation (4):

$$\text{percentage ionized} = \frac{100}{1 + \text{antilog (pH} - \text{pKa)}} \quad \text{(Eq. 1)}$$

the acridine derivatives are seen to be fully ionized at the pH values prevailing in the bacterial suspensions. For dibasic acridine derivatives, this refers only to the more basic of the two cationic groups.

Table VIII—Acridine Derivative Concentrations Required to Reduce Electrophoretic Mobility (*U*) of *S. faecalis* to Zero[a]

Compound	pKa in Water at 20°[b]	Concentration for *U* = 0	
		M	at pH
Acridine hydrochloride	4.8	0.046	About 3.1
9-Hydrazinoacridine acetate	7.15	0.0070	4.4
9-Aminoacridine hydrochloride	9.99	0.0086	7.0
Acriflavine hydrochloride	12	0.0028	4.7
Proflavine dihydrochloride	9.65	0.00145	6.0

[a] At an ionic strength of 0.03 *M*. [b] From Refs. 4 and 5.

For acridine, hydrazinoacridine, and acriflavine, the concentration required for producing zero mobility increases with decreasing base strength (*cf.*, Table VIII). Aminoacridine does not follow this inverse relationship, probably because the effect of its concentration on electrophoretic mobility was studied in a neutral medium whereas the other compounds were studied in acid media. As shown previously, the concentration for producing zero mobility is lowered by a decrease in pH. In the case of cetyltrimethylammonium bromide, this concentration was lowered from 7×10^{-4} to 3×10^{-4} *M* when the pH was decreased from 7.0 to 3.4 at constant ionic strength (2). If a drop in pH from 7.0 to 4.4–4.7 caused a proportional decrease in the aminoacridine concentration for producing zero mobility, the inverse relationship between this concentration and the pKa would most likely be extended to include aminoacridine. This inverse relationship is expected because the more basic compounds, of higher pKa, are chemisorbed more avidly by the carboxylate groups in the cell wall and, therefore, tie them up or neutralize them at lower concentrations. At the point of complete saturation or neutralization of the carboxylate groups, the ζ-potential of the cell wall is reduced to zero.

Of the five acridine compounds listed in Table VIII, proflavine is the only one whose pKa and concentration for producing zero mobility do not follow the inverse rank order. A decrease in pH from 6.0 to 4.4–4.7 would reduce its concentration for producing zero mobility even more, thereby further accentuating the discrepancy with the other four acridine derivatives.

Antibacterial Action of Acridines—As can be seen from Table IX, *S. faecalis* was remarkably resistant to 9-aminoacridine hydrochloride and to proflavine dihydrochloride, even though these compounds were reported (4) to possess marked antibacterial activity. Therefore, the susceptibility to cetyltrimethylammonium bromide of the lot of *S. faecalis* used in the present experiments was measured and compared with that of another lot of the same strain used 2 years earlier (2). The data of Table IX and those of Table III of Ref. 2 show that the minimum inhibitory concentration of cetyltrimethylammonium bromide was low and of comparable magnitude for the two lots. Thus, the lack of susceptibility to antibacterial agents of the present lot of *S. faecalis* was not general, and it is unlikely that it was due to mutation.

As a further check on the reliability of the susceptibility testing, a bacterium reported to be quite susceptible to acridines (4), *S. pyogenes*, was treated with different concentrations of aminoacridine hydrochloride, proflavine dihydrochloride, and cetyltrimethylammonium bromide, using the same pour plate technique. Growth was completely inhibited by low concentrations of all

Table IX—Effect of Antiseptics on Growth of Bacteria by Pour Plate Method[a]

Organism	Compound	Concentration[b], *M*	pH	Viability[c], %
S. faecalis	9-Aminoacridine hydrochloride	0.0001	6.8	106 (106), 92 (93)
		0.005[d]	7.1	93 ((89))
		0.01	6.6	84 (105) ((86)), 98 (84) ((98))
		0.01[d]	7.0	98 ((102))
		0.02[d]	7.0	78 ((65))
		0.05	6.2	106 (106)
		0.06[d]	6.8	65 ((89))
		0.08[d]	6.8	79 ((66))
		0.10	6.2	84 (105)
		0.10[d]	6.7	70 ((81))
	Proflavine dihydrochloride	0.0001	3.9	88 (96), 104 (82)
		0.001	2.8	67 (72), 74 (71)
		0.005	1.9	93
		0.005[d]	5.2	100 (95)
		0.01	1.7	75 (77)
		0.01[d]	4.6	((97))
		0.02[d]	4.0	49, ((79))
		0.04[d]	1.5	((73))
	Cetyltrimethylammonium bromide[e]	1×10^{-5}	7.0	0 (0)
		1×10^{-4}	7.0	0 (0)
S. pyogenes	9-Aminoacridine hydrochloride	1×10^{-6}	7.0	112
		1×10^{-4}	7.0	0 (0) ((0))
		1×10^{-3}	6.9	0 (0)
	Proflavine dihydrochloride	1×10^{-6}	5.1	0 (0)
		1×10^{-4}	4.2	0 (0) ((0))
	Cetyltrimethylammonium bromide[e]	1×10^{-5}	6.9	0 (0)
		1×10^{-4}	6.9	0 (0)

[a] Contact time of bacteria with compounds = 30 min. [b] Solvent for compound and medium for blank was redistilled water unless otherwise indicated. [c] Average colony count of three or more plates expressed as percentage of average count of three or more plates incubated with blank samples, *i.e.*, bacterial suspensions treated under identical conditions except for the absence of antiseptic compounds. Figures without parentheses, between single parentheses, and between double parentheses refer to suspensions stirred continuously and slowly, continuously and fast, and unstirred, respectively. Commas separate duplicate sets of results. [d] Solvent for compound and medium for blank was 0.03 *M* potassium acetate. [e] Solvent for compound and medium for blank was 0.01 *M* potassium acetate.

three antibacterials (*cf.*, Table IX). These two observations corroborate the finding that *S. faecalis* is quite resistant to aminoacridine hydrochloride and proflavine dihydrochloride.

These two acridines, which were strongly chemisorbed at the cell wall of *S. faecalis*, did not inhibit its growth even at concentrations exceeding by more than an order of magnitude the concentrations at which they reduced the ζ-potential of the bacterium to zero, *i.e.*, the concentrations at which their chemisorption to the cell wall was completed. This implies that the primary site of attack of the acridines on the bacteria is not the cell wall, a conclusion corroborated by the extensive literature on the mode of action of acridines (6, 9). They combine with nucleic acids and inhibit the enzymes involved in nucleic acid synthesis, possibly in the cytoplasmic cell membrane.

REFERENCES

(1) H. Schott and C. Y. Young, *J. Pharm. Sci.*, **61**, 182(1972).

(2) *Ibid.*, **61**, 762(1972).

(3) *Ibid.*, **62**, 1797(1973).

(4) A. Albert, S. D. Rubbo, R. J. Goldacre, M. E. Davey, and J. D. Stone, *Brit. J. Exp. Pathol.*, **26**, 160(1945).

(5) A. Albert, *J. Chem. Soc.*, **1965**, 4653.

(6) A. Albert, "Selective Toxicity," 4th ed., Methuen & Co., Ltd., London, England, 1968, pp. 260–276.

(7) T. W. Healy and V. K. LaMer, *J. Colloid Sci.*, **19**, 323(1964).

(8) H. E. Ries and B. L. Meyers, *Science*, **160**, 1449(1968).

(9) E. F. Gale, E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring, "The Molecular Basis of Antibiotic Action," Wiley-Interscience, New York, N.Y., 1972, pp. 188–213.

ACKNOWLEDGMENTS AND ADDRESSES

Received July 17, 1973, from the *School of Pharmacy, Temple University, Philadelphia, PA 19140*

Accepted for publication September 6, 1973.

The advice and assistance of Miss C. Y. Young and the experimental work of Miss G. S. Durelli are gratefully acknowledged.