Electrokinetic Studies of Bacteria III: Effect of Polyvalent Metal Ions on Electrophoretic Mobility and Growth of *Streptococcus faecalis*

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Abstract □ The effect of the polyvalent cations calcium, lead, copper, chromium, iron III, and thorium on *Streptococcus faecalis* was investigated by microelectrophoresis and by growth inhibition measurements. Thorium and the (hydrolyzed) ferric ion inverted the charge of the bacteria from negative to positive. The other cations merely reduced the absolute value of the negative ζ-potential at the concentrations investigated, which were limited because all measurements were performed at an ionic strength of 0.01 *M*. The order of effectiveness in changing the ζ-potential was thorium $\cong$ ferric $\gg$ chromium $\gg$ copper > lead > calcium, in accordance with the Schulze–Hardy rule. The effect of the salts on the growth of the bacteria was studied by the pour plate, growth curve, and turbidity methods. The order of effectiveness in inhibiting the bacterial growth was thorium > ferric > copper. The other cations produced no detectable growth inhibition at concentrations up to about 0.003 *M*. With the exception of chromium, there is a rank-order correlation between the ability of the polyvalent metal salts to reduce the ζ-potential of the bacteria and to inhibit their growth.

Keyphrases □ Electrokinetics—effect of polyvalent metal ions on electrophoretic mobility and growth of *Streptococcus faecalis* □ Electrophoretic mobility, *Streptococcus faecalis*—effect of calcium, lead, copper, chromium, ferric, and thorium ions, ζ-potential □ Bacterial growth, *Streptococcus faecalis*—effect of calcium, lead, copper, chromium, ferric, and thorium ions, pour plate, growth curve, and turbidity methods □ *Streptococcus faecalis*—effect of polyvalent metal ions on electrophoretic mobility and growth □ Metal ions, polyvalent—effect on electrophoretic mobility and growth of *Streptococcus faecalis* □ Zeta-potential—calculated for *Streptococcus faecalis*, effect of polyvalent metal ions.

The effect of salts of heavy metals on bacteria has been the subject of many studies. However, the mechanism of their antibacterial action, the "oligodynamic effect," is not understood (1). The effect of heavy metal cations on the charge of the cell wall of bacteria and spores has been assessed by electrophoresis (2–7). A possible correlation between antibacterial action and surface charge modification was never investigated; it is the topic of the present study.

EXPERIMENTAL

Electrophoresis Procedures—The methods were as described previously (8, 9), except as noted. The bacteria, *Streptococcus faecalis*, were incubated in the liquid growth medium at 37° for 18–24 hr., being in the stationary phase when harvested. Their suspensions were centrifuged for 10 min. at 9000 r.p.m. The bacteria were freed of adhering nutrients by resuspending the sediment three times in 0.010 *M* $NaNO_3$ or KCl and centrifuging for 10 min. at 9000 r.p.m. each time. When the polyvalent metals were in the form of nitrates, sodium nitrate was used for washing; it was also added to polyvalent metal salt solutions to adjust the ionic strength. When necessary, potassium chloride was used for the chlorides and sulfates. Electrophoretic measurements and viability assessments were made on bacterial suspensions of ionic strength equal to 0.010 *M*. It was shown (8) that the electrophoretic mobility of *S. faecalis* was not affected by changing buffers and other electrolytes from K^+ to Na^+ salts. Electrophoretic mobilities were measured after the bacteria had been in contact with the polyvalent cations for 30 min. at room temperature. Whenever tested, increases or decreases of 15 min. in the time of contact produced no significant change in mobility. The glass cell used had a cell constant of 62.4 $cm.^{-1}$, compared to 67.6 $cm.^{-1}$ for the plastic cell (8). The cell constants are used to calculate the specific conductance of the suspensions.

The solutions were not buffered in order to prevent precipitation or complexation of the polyvalent cations by the buffers used previously, namely, phosphate, acetate, or phthalate. Fortunately, the electrophoretic mobility of *S. faecalis* had been found (8) to be independent of pH between 4 and 7. Salts of polyvalent cations were freshly dissolved to avoid extensive hydrolysis.

Bacteriological Procedures—Three methods were used to assess the antibacterial activity of the heavy metal salts, designated as pour plate, growth curve, and turbidity methods. In the first two methods, the bacteria were treated in the same way as for the electrophoresis studies. After 18–24 hr. of incubation in the liquid growth medium, when the bacteria were in the stationary phase, the suspensions were centrifuged, washed by centrifugation three times with 0.010 *M* KCl or $NaNO_3$, and suspended for 30 min. at room temperature in solutions containing different concentrations of polyvalent metal salts. The ionic strength of these solutions was adjusted to 0.010 *M* with potassium chloride or sodium nitrate when necessary. In the blank experiments, the bacteria were given the identical treatment except that potassium chloride replaced the polyvalent metal chlorides and sulfates, and sodium nitrate replaced the polyvalent metal nitrates.

In the *pour plate method*, after the suspensions had been standing for 30 min., 1-ml. aliquots were diluted 10^4–10^6-fold with distilled water, and 0.1-ml. portions of the diluted suspension were mixed in petri dishes with 5% molten brain–heart infusion agar[1]. After 20 hr. of incubation at 37°, the number of colonies was counted. The difference between the plate counts of samples treated with potassium chloride or sodium nitrate and with polyvalent cation salts is due to the antibacterial action of the latter.

In the *growth curve method*, the suspensions already described, containing one of the heavy metal salts and/or potassium chloride or sodium nitrate, were left to stand for 30 min. At that time, 1-ml. aliquots were withdrawn and added to 100-ml. portions of a sterile liquid growth medium contained in 300-ml. culture flasks equipped with side arms. The growth medium contained 1% yeast extract[1], 1% bacto-tryptone[1], 0.1% dextrose, 0.050 *M* K_2HPO_4, and 0.050 *M* KH_2PO_4 and had a pH of 6.8. This medium was given a higher phosphate concentration than the 0.0023 *M* concentration of the medium previously used to increase the growth rate of the bacteria. The higher phosphate content reduced the lag time (defined below) from 3.5 to 2.6 hr. The culture flasks were shaken in a 37° bath. Absorbance measurements at 640 nm. were made at hourly intervals by inserting the side arm into a spectrophotometer[2]. During the logarithmic phase, absorbances were measured every 30 min.

The growth curves (plots of absorbance *versus* incubation time) are based on the "adjusted net absorbance" (*AA*), which is the "net absorbance" (*NA*) corrected for light scattering. Net absorbance is the difference between the absorbance after the specified incubation time and the absorbance immediately after inoculation. The relationship between *NA* and *AA* is given (10) by:

$$NA = AA - 0.1(AA)^2 - 0.002(10AA - 2)^2 \quad \text{(Eq. 1)}$$

This equation can be simplified, for *NA* values between 0.196 and

[1] Supplied by Difco Laboratories.
[2] Spectronic 20, Bausch & Lomb Co.

Table I—Effect of Polyvalent Cations on the Electrophoretic Mobility of *S. faecalis*

Salt and Concentration[a]	pH	Specific Conductance, μmhos/cm.	Mobility ± *SD*[b], (microns/sec.)/(volts/cm.)
0.010 *M* KCl	7.0, 5.8, 5.3[c]	1370, 1350, 1350[c]	−2.07 ± 0.06, −2.07 ± 0.03, −2.54 ± 0.07[c]
0.010 *M* $NaNO_3$	7.6–5.6, 6.1–5.1	1060, 1060,	−1.94 ± 0.05, −1.96 ± 0.07,
	7.6–8.3, 7.6	1120, 1000	−2.00 ± 0.07, −2.15 ± 0.10
0.010 *M* K acetate	7.2, 7.1, 6.9	1000, 1080, 1060	−2.21 ± 0.09, −2.31 ± 0.07, −2.38 ± 0.09
0.0033 *M* $CaCl_2$	6.2–5.1	810	−1.18 ± 0.02
0.0010 *M* $Ca(NO_3)_2$	7.5–8.7	1000	−1.96 ± 0.07
0.0022 *M* $Ca(NO_3)_2$	6.9–8.5	940	−1.74 ± 0.05
0.0033 *M* $Ca(NO_3)_2$	7.1–8.1, 7.6 5.8	810, 810	−1.52 ± 0.04, −1.57 ± 0.05
0.0010 *M* $Pb(NO_3)_2$	5.2	1090	−1.89 ± 0.06
0.0022 *M* $Pb(NO_3)_2$	5.3	870	−1.69 ± 0.04
0.0033 *M* $Pb(NO_3)_2$	4.7, 5.6	810, 810	−1.21 ± 0.02, −1.27 ± 0.02
0.0010*M* $CuSO_4$ + 0.0060 *M* KCl	5.0, 4.7	1010, 1080	−1.68 ± 0.04[d], −1.69 ± 0.04
0.0025 *M* $CuSO_4$	4.7	540	−1.33 ± 0.03
5×10^{-5} *M* $Cr(NO_3)_3$	5.8	1080	−1.85 ± 0.03
0.00083 *M* $Cr(NO_3)_3$	3.7	950	−1.17 ± 0.02
0.00167 *M* $Cr(NO_3)_3$	3.6	680	−0.82 ± 0.005
0.00042 *M* $Fe(NO_3)_3$ + 0.00748 *M* KCl	3.5	1490	+2.87 ± 0.05
0.00084 *M* $Fe(NO_3)_3$ + 0.00496 *M* KCl	3.1	1350	+3.05 ± 0.06
0.00167 *M* $Fe(NO_3)_3$	2.8	1690	+2.79 ± 0.06
1×10^{-6} *M* $Th(NO_3)_4$	5.2	1150	−2.25 ± 0.03
5×10^{-6} *M* $Th(NO_3)_4$	5.0	1280	−1.04 ± 0.05
1×10^{-5} *M* $Th(NO_3)_4$	4.8	1150	−0.56 ± 0.04
5×10^{-5} *M* $Th(NO_3)_4$	4.2	1280	+1.71 ± 0.04
1×10^{-4} *M* $Th(NO_3)_4$	4.0	1150	+2.22 ± 0.03
2.5×10^{-4} *M* $Th(NO_3)_4$	3.7	1080	+2.37 ± 0.04
5×10^{-4} *M* $Th(NO_3)_4$	3.6	950	+2.52 ± 0.04
1×10^{-3} *M* $Th(NO_3)_4$	3.5	640	+2.96 ± 0.03

[a] Molarity of salt of polyvalent cation when employed. Sodium nitrate was added to nitrates and potassium chloride to chlorides when necessary to bring the ionic strength to 0.010 *M*. When salts of polyvalent and monovalent cations having different anions were mixed, both are listed. [b] Average of 10 or more measurements ± standard deviation of the average. [c] Commas separate results of measurements on different batches of *S. faecalis*, incubated on different days. [d] Washed by centrifugation with 0.0010 *M* $CuSO_4$ + 0.0060 *M* KCl instead of the standard procedure of washing with 0.010 *M* KCl.

about 0.9 (10), to:

$$AA = 1.800 - 0.1\sqrt{321.3 - 330NA} \qquad \text{(Eq. 2)}$$

The adjusted net absorbance of the suspensions is directly proportional to the bacterial concentration, as demonstrated in Fig. 1. *S. faecalis* was grown in the medium described, the suspension was diluted with the growth medium in different proportions, and the absorbance was measured at 640 nm. for each dilution.

Any differences between the growth curves (plots of *AA versus* time) of bacteria treated with polyvalent metal salts and of bacteria treated with sodium nitrate or potassium chloride, such as extended lag phase, reduced growth rate in the exponential phase, or lower bacterial concentration in the stationary phase, are a measure of the antibacterial activity of the former.

The following parameters were investigated in the growth curve method: lag time, generation time, and absorbance in the stationary phase. Lag time and generation time were obtained from semilogarithmic plots of adjusted net absorbance *versus* time.

Lag time, *L*, in hours, is defined (11) by the equation:

$$\log\left(\frac{AA}{0.01}\right) = K(T - L) \qquad \text{(Eq. 3)}$$

where the lag *L* is the time, obtained by extrapolation of the linear portion of the plot corresponding to the exponential growth phase, at which $AA = 0.01$. For time $T = L$, $\log(AA/0.01) = 0$ and $(AA/0.01) = 1$. Instead of specifying the slope *K* of the semilogarithmic plot, the generation time (11) was determined, which is the time required to double the number of cells or the adjusted net absorbance during the logarithmic phase.

In the *turbidity method*, heavy metal salts and/or potassium chloride or sodium nitrate were incorporated into the original, low phosphate growth medium (8, 9) so that the ionic strength of the added electrolytes was 0.010 *M*. These media, contained in culture flasks with side arms, were inoculated with *S. faecalis*, and their absorbance at 640 nm. was measured before and after 24 hr. of incubation at 37° in a shaking bath. After the 24-hr. period, the bacteria were in the stationary phase. Any differences in turbidity increase between suspensions containing polyvalent cations and the blank suspensions containing Na^+ or K^+ are due to the antibacterial action of the polyvalent cations.

RESULTS AND DISCUSSION

Electrokinetic Measurements— The results of the electrophoresis studies are presented in Table I. The pH values were determined just before and just after the mobility measurements. If the difference between the two values was more than 0.5 unit, both values are listed. Single values represent the average pH when the determinations before and after the electrophoretic measurements agreed within 0.5 unit of pH. Negative values for the electrophoretic mobility indicate that the bacteria were negatively charged and migrated to the positive pole. Two samples of the same strain, ATCC 9790, supplied by the American Type Culture Collection about 10 months apart, gave identical mobility values within the precision of the measurements. The earlier measurements were made with the plastic cell (8), while the later measurements were made with a glass cell. The two sets of measurements are combined in Table I.

Polyvalent cations reduced the negative charge of the cell wall progressively as their concentration in the solution was increased while the ionic strength was maintained constant at 0.010 *M*. This change in ζ-potential is due to chemisorption of the cations as exemplified for a divalent cation Me^{+2} by Scheme I, in which W represents the cell wall. Whenever a monovalent Na^+ was replaced by a divalent cation, one fixed negative charge of the cell wall was replaced by one fixed positive charge because polyvalent cations are firmly attached to the carboxylate groups in the wall. Carboxylates of di- and trivalent cations undergo little or no dissociation because the bonds between the carboxylate groups and the cations are chiefly covalent and/or coordinate covalent. Their attachment to

$$W\text{—}COO^- Na^+ + Me^{+2}(NO_3)_2^{-2} \rightleftharpoons W\text{—}COO\cdots Me^+(NO_3)^- + Na^+ NO_3^-$$

Scheme I

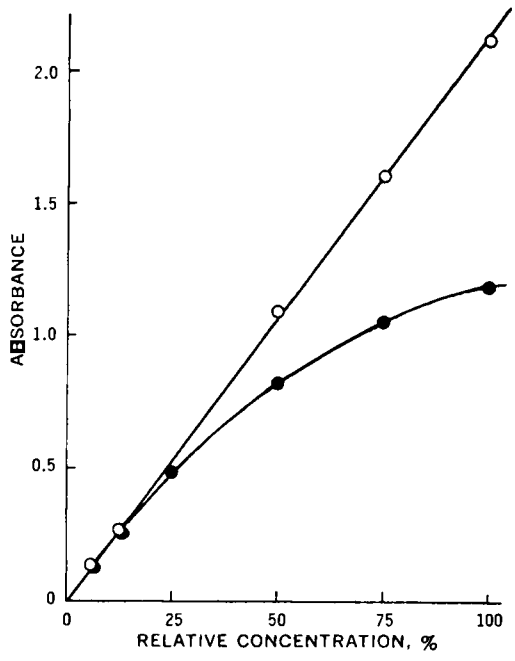

Figure 1—*Growth curve method: effect of dilution on net absorbance of suspensions of* S. faecalis *at 640 nm. Key:* ●, NA; *and* ○, AA.

the carboxylate groups in the cell wall made the di- and trivalent cations an integral part of the bacterium; they were chemisorbed in the Stern layer. Potassium and sodium ions originally bound to the carboxylate groups of the cell wall were extensively dissociated and hence constituted the counterions, being located in the Stern layer and in the diffuse or Gouy double layer (12) surrounding the bacterium. The di- and trivalent cations which replaced them are not dissociated from the carboxylate groups. Therefore, as the reaction (Scheme I) proceeds, the counterions at the cell wall are progressively changed from Na^+ or K^+ to NO_3^-, reducing the negative charge of the cell.

In this cation-exchange process, the tetravalent cation thorium actually inverted the sign of the charge of the bacteria from negative to positive. The di- and trivalent cations could not be used at high enough concentrations to cause charge reversal because of the upper concentration limit corresponding to the ionic strength of 0.010 *M* adopted in all electrophoresis measurements.

When combining the information from Fig. 2 with Table IV of *Reference 8*, it is seen that cation concentrations required to produce

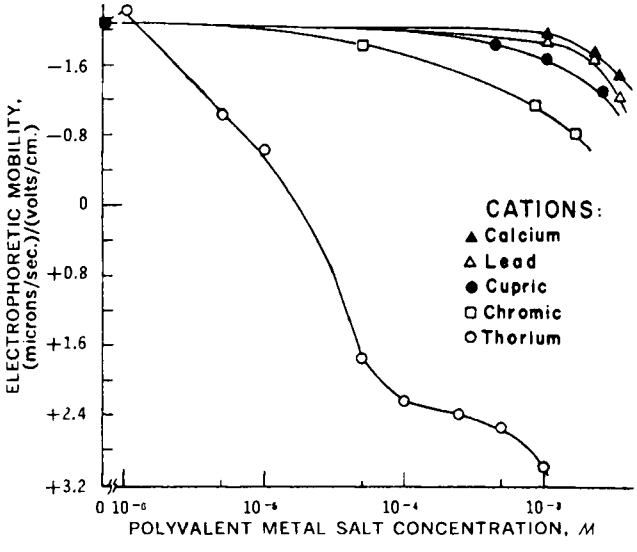

Figure 2—*Effect of the concentrations of various polyvalent cations on the electrophoretic mobility of* S. faecalis *at constant ionic strength.*

Table II—Effect of Polyvalent Metal Salts on the Growth of *S. faecalis* by Pour Plate Method

Salt	*M*[a]	Average Count[b]	*SD*[c]	*n*[d]
Cupric sulfate	0(0.010 *M* KCl)	93	26	11
	0.0001	80	7.1	4
	0.0010	50	8.7	6
	0.0025	21	10	8
Calcium chloride	0.0033	70	—	2
Thorium nitrate	0(0.010 *M* $NaNO_3$)	80	5.2	13
	1×10^{-6}	10	4	4
	1×10^{-5}	0.0063	0.0006	3
	1×10^{-4}	0.0068	0.0004	3
	1×10^{-3}	0.0000	—	2
Chromic nitrate	0.00084	81	—	2
	0.00167	67	—	2
Ferric nitrate	0.00042	60	—	2
	0.00084	0.0	—	2
Lead nitrate	0.00333	62	—	2

[a] Constant ionic strength of 0.01 *M* achieved by addition of potassium chloride or sodium nitrate. [b] Determined at or prorated to a dilution of $1:10^7$. [c] Standard deviation of average count. [d] Number of plates counted.

comparable reductions in electrophoretic mobility and, hence, in ζ-potential are in the ratio of 1:100:900:8000 for Th^{+4}, Cr^{+3}, the average of the three divalent cations, and K^+. Qualitatively, this is in accordance with the Schulze–Hardy rule (12).

The ratio is higher by an order of magnitude than the ratio of the flocculation concentrations for tetra-, tri-, di-, and monovalent counterions predicted by the Derjaguin–Landau–Verwey–Overbeek theory, namely, $1/4^6:1/3^6:1/2^6:1/1^6 = 1:5.6:64:4100$ (12). The most likely explanation for this discrepancy is the occurrence of specific interactions between the polyvalent cations and the cell wall. The theoretical flocculation values are based on lack of specificity, the charge of the counterion being the only factor considered in the reduction of the ζ-potential by added electrolytes. In view of the affinity of the carboxylate group for heavy metal cations, specific interactions must have occurred and probably account for most, if not all, of the low concentrations or high concentration ratios at which the polyvalent cations caused the observed reductions in electrophoretic mobility.

Ferric nitrate produced charge reversal and nearly identical positive electrophoretic mobilities at all three concentrations tested, the lowest of which was 4.2×10^{-4} *M*. The tetravalent thorium nitrate produced comparable positive mobilities at a similar concentration, namely, 8×10^{-4} *M*, while the trivalent chromium cation did not reverse the negative charge of the bacteria at concentrations as high as 16.7×10^{-4} *M*. The exceptional behavior of ferric nitrate is ascribed to extensive hydrolysis, which produces a variety of polyvalent hydroxylated cations in solution such as $Fe_3(OH)_4^{+5}$. These are more effective charge-inverting species than Fe^{+3} (13). The occurrence of extensive hydrolysis of ferric nitrate in bacterial suspensions was shown by a deepening of their color to a reddish brown shortly after dissolving the nitrate in water.

Of the divalent cations, Cu^{+2} was the most effective in reducing the electrophoretic mobility and Ca^{+2} the least effective. As shown in Table I, Cu^{+2}, Pb^{+2}, and Ca^{+2} at a concentration of 0.0010 *M*

Table III—Effect of pH on Growth Curve Parameters

Buffer[a]	pH	Lag[b], hr.	Generation Time[b], hr.	Absorbance in Stationary Phase[b]
Phosphate	7.1	2.6	0.37	1.17
Acetate	7.0	2.6	0.38	1.18
Acetic acid + potassium bromide	3.45	4.6	0.46	1.19
Acetic acid + potassium chloride	3.5	2.9	0.40	1.18
Phosphoric acid	2.35	3.0	0.38	1.18

[a] See text for composition. [b] Based on duplicate growth curves.

Table IV—Effect of Heavy Metal Salts on Growth of *S. faecalis* by Growth Curve Method

Salt	Concentration[a], *M*	pH	Lag[b], hr.	Generation Time[b], hr.	Absorbance in Stationary Phase[b,c]
Calcium chloride	0(0.010 *M* KCl)	5.9	2.8	0.38	1.39
	0.0033	6.2	2.4	0.43	1.31
Cupric sulfate	0(0.010 *M* KCl)	6.0	3.2	0.42	1.44
	0.0010 + 0.0060 *M* KCl	4.8	3.6	0.46	1.42
	0.0025	4.7	3.8	0.44	1.42
Lead nitrate	0(0.010 *M* $NaNO_3$)	6.1	2.4	0.43	1.38
	0.0033	5.0	2.8	0.46	1.36
Chromic nitrate	0(0.010 *M* $NaNO_3$)	6.4	2.7	0.41	1.44
	0.000835	3.7	3.0	0.38	1.35
	0.00167	3.6	3.0	0.41	1.42
Thorium nitrate	0(0.010 *M* $NaNO_3$)	6.9	2.7	0.49	1.32
	1×10^{-6}	5.6	3.0	0.51	1.35
	1×10^{-5}	4.8	5.0	0.35	1.31
	1×10^{-4}	4.0	5.4	0.40	1.25

[a] Molarity of salt of polyvalent cation when employed. Sodium nitrate was added to nitrates and potassium chloride to chlorides when necessary to bring the ionic strength to 0.010 *M*. When salts of polyvalent and monovalent cations having different anions were mixed, both salts are listed. [b] Based on duplicate growth curves. [c] Adjusted net absorbance. Average of two readings taken between 21 and 27 hr. of incubation time.

reduced the electrophoretic mobility by 0.41, 0.21, and 0.14 (microns/sec.)/(volts/cm.), respectively. These differences or specific effects are ascribed to increasingly stronger binding of the cations to the carboxylate groups in the cell wall when going from Ca^{+2} to Pb^{+2} to Cu^{+2}, in line with their increasing ability to form stable complexes with carboxylated polymers (14, 15).

Bacteriological Results—*Pour Plate Method*—Aqueous solutions of heavy metal salts are frequently acid. To determine how much of their antibacterial activity is due to the heavy metal ions and how much to acidity, it is necessary to determine the effect of acidity on bacterial inhibition in the absence of heavy metals. This was accomplished by exposing the bacteria for 30 min. to the following three buffer solutions of 0.010 *M* ionic strength: 0.010 *M* potassium acetate (pH 7.2), 0.010 *M* acetic acid + 0.0096 *M* KBr (pH 3.3), and 0.00667 *M* phosphoric acid + 0.00580 *M* KCl (pH 2.4). After the 30-min. exposure, aliquots of the suspensions were serially diluted with distilled water in a $1:10^7$ ratio, and the viability of the bacteria was determined by the pour plate method. The average colony counts ($\pm SD$ of the average count) of quadruplicate or quintuplicate experiments were 100 ± 18, 97 ± 6, and 53 ± 12 at the pH values of 7.2, 3.3, and 2.4, respectively. Only the most acid of the treating media produced a significant decrease in viability. None of the heavy metal solutions had pH values below 3.3. Therefore, all inhibitory effects are due to the cations.

Detailed studies with cupric sulfate indicated that the reproducibility of the pour plate method was not very good. Over a 4-week period, eight plates were inoculated with bacteria pretreated with 0.0025 *M* $CuSO_4$, using the same procedures including identical dilutions. The corresponding blanks were inoculated at the same times with bacteria pretreated with 0.010 *M* KCl.

Four of the plates inoculated with bacteria exposed to cupric sulfate had no growth; others had as many as 50 and 76 colonies. However, when the *F* test was applied to the plate counts made at the four levels of cupric sulfate employed (0, 0.0001, 0.0010, and 0.0025 *M*), the trend of increasing inhibition with increasing cupric sulfate concentration was statistically significant: the *F* value obtained exceeded the critical *F* value at the 5% level.

Eleven blank tests, in which the bacteria were pretreated with potassium chloride, were run for the three levels of cupric sulfate. The standard deviation of the average count of the 11 blanks was 28% of that average.

As can be seen from Table II, calcium nitrate, lead nitrate, and chromic nitrate produced no significant inhibition at the concentrations tested; thorium nitrate was the most effective antibacterial agent, followed by ferric nitrate.

Growth Curve Method—The effect of the acidity of the treating solution on the growth of the bacteria was determined by measuring growth curves in the following five buffer solutions: phosphate (pH 7.1), containing 0.00584 *M* KH_2PO_4 + 0.00082 *M* K_2HPO_4; acetate (pH 7.0), containing 0.010 *M* potassium acetate; acetic acid (pH 3.45), containing 0.010 *M* acetic acid + 0.0096 *M* potassium bromide; acetic acid (pH 3.5), containing 0.010 *M* acetic acid + 0.0096 *M* potassium chloride; and phosphoric acid (pH 2.35), containing 0.00667 *M* phosphoric acid + 0.0058 *M* potassium chloride. The ionic strength of the five solutions is approximately 0.01 *M*. After 30 min. contact time, aliquots were incubated and the growth curves determined.

The results (Table III) indicate that the acidity of the treating solution had only a small effect on the three growth curve parameters. The exception is the acetic acid solution containing potassium bromide which caused increases in lag and in generation time. Its pH was a full unit higher than that of the phosphoric acid solution which caused little or no inhibition, indicating that the potassium bromide rather than the pH was responsible for the inhibition caused by the acetic acid potassium bromide buffer. A comparable solution containing potassium chloride instead of bromide caused little inhibition.

Since the lowest pH in the heavy metal solutions was 3.6, any antibacterial activity caused by heavy metal salts is, therefore, due to the cations rather than to a reduction in pH.

Of the results listed in Table IV, the most pronounced inhibition is the increase in lag caused by two thorium nitrate solutions. Lesser inhibitions are the decreased growth in the stationary phase caused by the most concentrated thorium nitrate solution and the increased lag caused by the two copper sulfate solutions.

Turbidity Method—The only cation that showed any inhibition on the growth of *S. faecalis* according to the turbidity method was copper (Table V). Thorium and ferric ions caused no significant inhibition at concentrations at which they completely inhibited the growth of bacteria in the pour plate method. This is evidently due to lowering of the concentration or even disappearance of the free cations due to one of the following three reactions: hydrolysis of the heavy metal salts in the relatively alkaline nutrient broth (initial pH 6.5) causing precipitation of basic salts or hydroxides, particularly of the trivalent and tetravalent cations; precipitation of the phosphates (initial phosphate concentration 0.0023 *M*); and complexation by polypeptides and other organic compounds present in the liquid growth medium. Increased turbidity on addition of salts to the growth medium was noticed for thorium and especially for lead. The marked turbidity that developed when lead nitrate was added to the growth medium was cleared by the addition of nitric acid. It renders the apparent inhibition of bacterial growth by lead nitrate suspect.

Calcium chloride apparently stimulated the growth of the bacteria somewhat. Growth stimulation is well known for salts of magnesium, another alkaline earth metal (16).

The pour plate and the growth curve methods differed from the turbidity method in that the bacteria were treated with unbuffered solutions of the heavy metal salts in the former two. The only possible additives were potassium chloride or sodium nitrate. This precluded the precipitation or complexation of the polyvalent cations by ingredients of the growth medium prior to their coming into contact with the bacteria; the bacteria were treated with the cations at their full nominal concentrations.

Table V—Effect of Heavy Metal Salts Added to Low Phosphate Growth Medium on Growth of *S. faecalis*

Additive	Net Absorbance[a]
None	0.87–0.03 = 0.84
0.010 *M* KCl	0.87–0.04 = 0.83
0.0033 *M* $CaCl_2$	1.00–0.04 = 0.96
None	0.75–0.03 = 0.72
0.010 *M* KCl	0.80–0.02 = 0.78
0.0010 *M* $CuSO_4$ + 0.0060 *M* KCl	0.48–0.08 = 0.40
None	0.80–0.03 = 0.77
0.010 *M* KCl	0.85–0.02 = 0.83
0.0025 *M* $CuSO_4$	0.51–0.08 = 0.43
None	0.75–0.03 = 0.72
0.010 *M* $NaNO_3$	0.75–0.02 = 0.73
0.00333 *M* $Pb(NO_3)_2$	0.80–0.28 = 0.52
None	0.85–0.04 = 0.81
0.010 *M* $NaNO_3$	0.90–0.04 = 0.86
0.00042 *M* $Fe(NO_3)_3$ + 0.00748 *M* $NaNO_3$	0.95–0.05 = 0.90
None	0.80–0.04 = 0.76
0.010 *M* $NaNO_3$	0.85–0.03 = 0.82
0.00167 *M* $Fe(NO_3)_3$	0.85–0.10 = 0.75
None	0.75–0.04 = 0.71
0.010 *M* $NaNO_3$	0.75–0.03 = 0.72
0.00167 *M* $Cr(NO_3)_3$	0.80–0.08 = 0.72
None	0.75–0.04 = 0.71
0.010 *M* $NaNO_3$	0.75–0.03 = 0.72
0.0010 *M* $Th(NO_3)_4$	0.82–0.13 = 0.69

[a] Uncorrected absorbance at 640 nm. after 24 hr. of incubation at 37° minus initial absorbance.

Another shortcoming of the turbidity method is that it measured the effect of the heavy metal salts on the bacteria only in the stationary phase, whereas the growth curve method included the lag and exponential phases as well.

CONCLUSIONS

Combining the results of the methods for measuring growth inhibition gives the following order of decreasing antibacterial activity for the cations employed in this study: thorium > ferric > copper. The remaining cations were ineffective at the concentrations employed. Sprowls and Poe (17) likewise found the antibacterial activity of ferric and copper salts to be greater than that of salts of lead and of other transition metals.

The order of decreasing effectiveness in reducing the electrophoretic mobility was: thorium ≅ ferric ≫ chromium ≫ copper > lead > calcium. With the exception of the chromium ion, there is a correlation in the rank order of growth inhibition and of reduction in the absolute value of the ζ-potential. This indicates that the strength with which polyvalent cations are attached to the outer portion of the bacterial cell wall is similar to the strength with which they are attached to the site where they exert their strongest antibacterial action.

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