Influence of Sodium Hexametaphosphate on Selected Bacteria

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ABSTRACT

POST, F. J. (University of California, Los Angeles), G. B. KRISHNAMURTY, AND M. D. FLANAGAN. Influence of sodium hexametaphosphate on selected bacteria. Applied Microbiol. **11**:430–435. 1963.—Sodium hexametaphosphate (HEX), the solvent of calcium alginate wool used in swabbing inanimate surfaces was studied relative to its effect on various bacterial populations, both pure cultures and wild. It was found that bacteria in wild populations were greatly inhibited, and that a percentage reduction of count was directly related to concentration of HEX. Most gram-positive bacteria were prevented from growing on a medium containing 0.1% HEX. This, or a higher concentration, occurred in the final medium when the method recommended in *Standard Methods for the Examination of Dairy Products* was followed. Growth of *Sarcina lutea* occurred on media with higher concentrations than that of inhibition (0.05%), if $MgSO_4 \cdot 7H_2O$ was incorporated in the medium. Gram-negative bacteria were capable of growing in higher concentrations, even up to 10% HEX. A large percentage of the cells of some strains (represented by *Pseudomonas fluorescens*) were lysed on contact with HEX. Lysis could be prevented by the addition of NaCl or $MgSO_4 \cdot 7H_2O$. The evidence presented suggests that HEX, a phosphate-glass water-softening sequestrant, interferes with divalent cation metabolism, notably magnesium ion, and possibly others, producing cell division inhibition and loss of cell-wall integrity. The mechanism of action was not elucidated.

Two techniques for swabbing surfaces to obtain an estimate of bacterial contamination are currently approved (American Public Health Association, 1960). The more recent method involves the use of calcium alginate, a polysaccharide derived from seaweed, which dissolves readily in solutions of a sodium phosphate glass commonly called sodium hexametaphosphate (HEX). Theoretically, this technique should provide a more accurate estimate of bacterial contamination than the older cotton swab technique, since all bacteria removed from the surface are released from the fibers and are available for plating, whereas many bacteria are believed to remain entrapped in the cotton swab fibers even after vigorous shaking.

Ever since the introduction of the calcium alginate technique by Higgins (1950), its relative merits have been debated. Higgins (1950) reported considerably improved bacterial recovery relative to the cotton swab. Later investigators have been divided. Some feel it is superior to the cotton swab (Cain and Steele, 1953; Trednick and Tucker, 1951; O'Neill and Reed, 1956), some consider it no different (Barnes, 1952; O'Keefe, 1958; Barton, Gorfien, and Carlo, 1954), and at least one rates it slightly inferior (Angelotti et al., 1958). The results of these authors varied considerably, even among those who felt alginate recovery was superior. This variability and the report of Strong, Woodburn, and Mancini (1961), suggesting that a related food additive, sodium alginate, was slightly toxic to bacteria, led to an examination of possible toxicity of the various ingredients used in the technique

MATERIALS AND METHODS

Chemicals and media. HEX was manufactured by the Fisher Scientific Co., New York, N.Y. Calgon, a commercial form of HEX containing an unreported amount of "soda ash and other carbonates of sodium" was made by Calgon Co., Pittsburgh, Pa. Calcium alginate wool (Calgitex) was obtained from Splain and Lloyd, Inc., Milford, Ohio. Nutrient media were dehydrated products of Difco.

Organisms. Activated sludge samples from a sewage treatment plant served as wild populations. Pure cultures were obtained from the laboratory collection. Cultures were subcultured for at least 2 days prior to use, and cultures (18 to 24 hr) were used to prepare inocula or cell suspensions.

Screening tests. In the American Public Health Association (1960) technique, the alginate swab is placed in 5 ml of 0.0044% phosphate buffer and dissolved by adding 1 ml of a 10% Calgon or HEX solution. A 1-ml amount of this, if added to 17 ml of agar during pour plating, resulted in a 0.1% HEX concentration in the medium. Nutrient agar with HEX in concentrations of 0.01 to 2% was prepared and autoclaved. A second set of media with a quantity of alginate equal to the weight of a normal swab (25 to 50 mg) was also used. Activated sludge was serially diluted, and the bacterial population was determined by the pour plate technique. Nutrient broth plus HEX prepared in a similar manner was inoculated with 1 loopful of a 24-hr pure broth culture. Controls were plain nutrient agar or broth.

Survival studies. Cultures were washed from nutrient agar slants with 0.05% peptone-water. The resulting

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suspension was adjusted to an optical density (OD) of 0.3, using a Spectronic-20 colorimeter at the 525-mμ wavelength. Dilutions were made so that, upon adding 1 ml to the appropriate flask of test substrate, a final density of 10^6 cells per ml was approximated. Test substrates were 10% HEX or Calgon, 10% HEX or Calgon plus 25 to 50 mg of alginate and phosphate buffer. Samples were removed at intervals of 3, 6, 9, and 24 hr, serially diluted, and the population was determined by placing four or more 0.01-ml drops on the surface of nutrient agar plates. Preliminary work showed that this technique gave the same results as the pour plate technique with a considerable saving of time and equipment. Plates were incubated at the optimal growth temperature of the organism and counted at 24 hr.

OD studies. OD changes in suspensions of cells were determined in glass tubes with a Spectronic-20 colorimeter. Distilled water, peptone-water, and nutrient broth substrate tubes were prepared and autoclaved. Pure cultures were washed from 24-hr nutrient agar slants with distilled water. A suspension was prepared so that, when 0.2 ml was added to 9 ml of the test substrate, an OD reading of approximately 0.1 for growth studies or 0.3 for lysis studies resulted. HEX or other inorganic salts were prepared in 2% solutions and autoclaved separately if required. A sufficient quantity of these solutions was added to the tubes of substrate plus cells to give the desired concentration. Changes in OD were followed at various time intervals.

Inhibition studies. Solid or liquid media were prepared as in the screening studies, except variable amounts of inorganic salts were added. The quantitative reactions of various concentrations of cells were observed by placing quadruplicate 0.01-ml drops on the agar surface or making duplicate pour plates of serial dilutions of cells so that there were 30 to 3×10^6 cells per drop or ml. Counts were made at 24 hr and compared with the control plates. When broth was used, growth inhibition or reversal was reported as present or absent. Controls without HEX were used in all cases.

Miscellaneous. Routine observations of cells were made under the oil-immersion objective of a microscope by use of hanging-drop or slide preparations stained lightly with 0.5% safranine.

RESULTS

Screening tests. Results of the population determination of activated sludge in various HEX concentrations will be found in Fig. 1. It was clear immediately that HEX caused considerable inhibition and that calcium alginate had little or no influence, although the alginate may have offered some protection. The inclusion of alginate fibers in plain nutrient agar had no demonstrable effect on the bacterial numbers. Only when HEX was present did inhibition occur. Apparently, a direct relationship exists between concentration and percentage of kill based on the control count.

A group of organisms commonly found in sewage and on surfaces was studied in HEX-nutrient broth. As clearly seen in Table 1, species response to HEX corresponds closely to Gram reaction. With two notable exceptions, the gram-negative bacteria as a group tolerated considerably higher concentrations of HEX than did the gram-positive bacteria. However, a considerable variation existed within these two groups and, where more than one strain was used, within species as well.

Survival study. To answer the question whether absence of growth in HEX-broth meant death of the cells, a group of species exhibiting growth, no growth, and representing both Gram reactions was chosen for survival studies in the highest concentration of HEX previously used. An inoculum of 10^6 cells per ml was used for ease of determining survivors and so the dilutions required would minimize any residual HEX carry-over (Fig. 2). The pattern of survival or death when compared with growth occurrence in HEX-broth (Table 1) indicates that one can not always predict the results. For example *Pseudomonas aeruginosa* grew in 10% HEX-broth but showed a decline in numbers in 10% HEX alone. Thus, growth inhibition of some species and strains occurred, but death was not always the result of the inhibition. Where death did occur in the presence of HEX alone, most cells died within the first 3 hr. To eliminate any possibility of HEX carry-over,

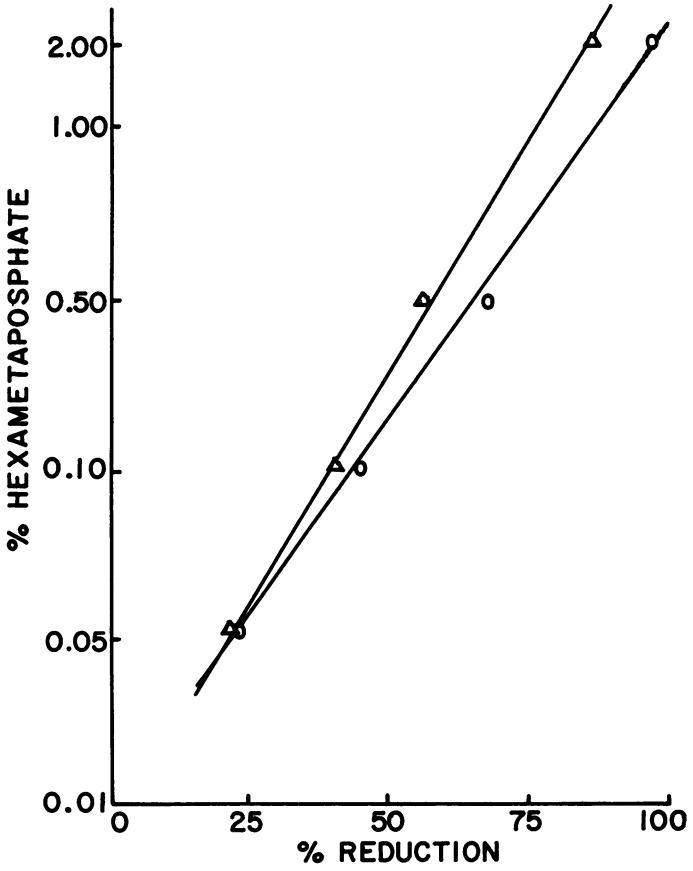

FIG. 1. *Effect of HEX concentration on determination of the bacterial population of activated sludge. Symbols: ○, activated sludge; △, activated sludge plus calcium alginate.*

TABLE 1. *Growth response of selected bacteria to various concentrations of HEX in nutrient broth*

Organism	HEX (%)				
	0.05	0.1	0.5	2	10
Gram-negative					
Aerobacter aerogenes	+	+	+	+	+
Escherichia freundii	+	+	+	+	+
E. coli (2 strains)	+	+	+	+	+
Salmonella typhimurium	+	+	+	+	+
S. typhosa	+	−	−	−	−
Serratia marcescens	+	+	+	+	+
Shigella flexneri	+	+	+	+	+
Pseudomonas aeruginosa (11 strains)	+	+	+	+	+
P. aeruginosa (1 strain)	+	+	+	+	−
P. fluorescens	+	+	+	−	−
P. geniculata	+	+	+	+	−
Pseudomonas sp.	+	+	+	−	−
Aeromonas punctata	+	+	+	+	−
Proteus vulgaris	+	+	+	+	−
Alcaligenes faecalis	+	+	+	−	−
Gram-positive					
Streptococcus faecalis (2 strains)	+	+	+	+	−
Bacillus brevis	+	+	−	−	−
B. subtilus (2 strains)	±	±	−	−	−
Staphylococcus aureus (1 strain)	+	+	−	−	−
S. aureus (5 strains)	+	−	−	−	−
S. aureus (2 strains)	−	−	−	−	−
Staphylococcus sp. (1 strain)	+	+	−	−	−
Staphylococcus spp. (7 strains)	+	−	−	−	−
Staphylococcus sp. (1 strain)	−	−	−	−	−
Sarcina lutea (2 strains)	−	−	−	−	−

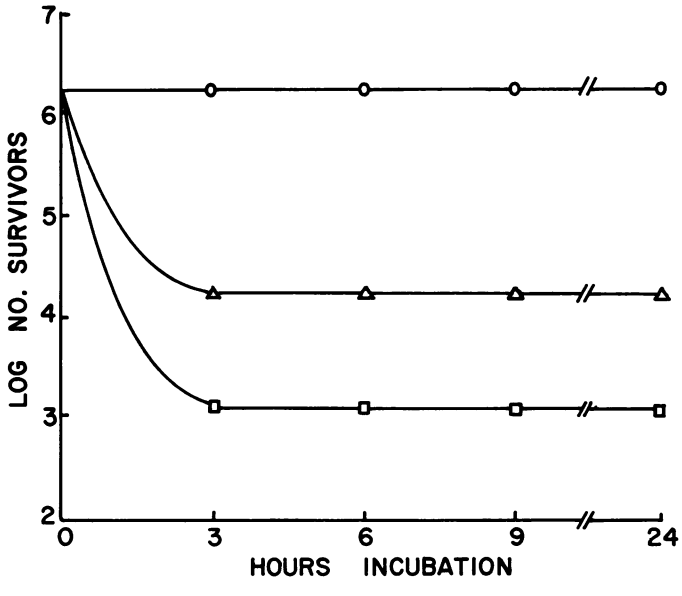

FIG. 2. *Survival of selected bacteria in the presence of 10% HEX in distilled water. Symbols: ○, Shigella flexneri, Escherichia coli, Aerobacter aerogenes, Salmonella typhimurium, S. typhosa, Sarcina lutea, Streptococcus faecalis, Staphylococcus aureus, and all controls; △, Pseudomonas aeruginosa; □, P. fluorescens and Staphylococcus sp.*

several organisms were studied by the membrane filter technique instead of by drop plating with identical results.

On the basis of the survival study, the following two organisms were chosen for further study: *P. fluorescens*, because it showed growth in intermediate HEX-broth concentrations yet showed death in the 10% solution, and *Sarcina lutea*, because it showed no growth in any HEX-broth concentration yet showed no death in the 10% solution.

OD studies. When *P. fluorescens* was (i) grown in nutrient broth or (ii) cells were added to nutrient broth, distilled water, or 0.05% peptone-water to give a final OD of 0.3 and then HEX was added to the tube, a sharp continuous decline in OD occurred. Results of a typical experiment are presented in Fig. 3. That this decline represented death was demonstrated by passing a diluted portion of the tube contents through a membrane filter and incubating it on pads of double-strength nutrient broth. Reductions of 60 to 90% occurred. Microscopic observations of the suspensions indicated considerable cellular debris. Tubes with nutrient broth in which a decline in OD had been noted were incubated at 30 C, and, after 24 hr or more, growth had resumed.

OD decline followed by growth suggested the possibility that salt imbalance may have caused lysis and that tolerant surviving cells were able to grow after a period of adjust-

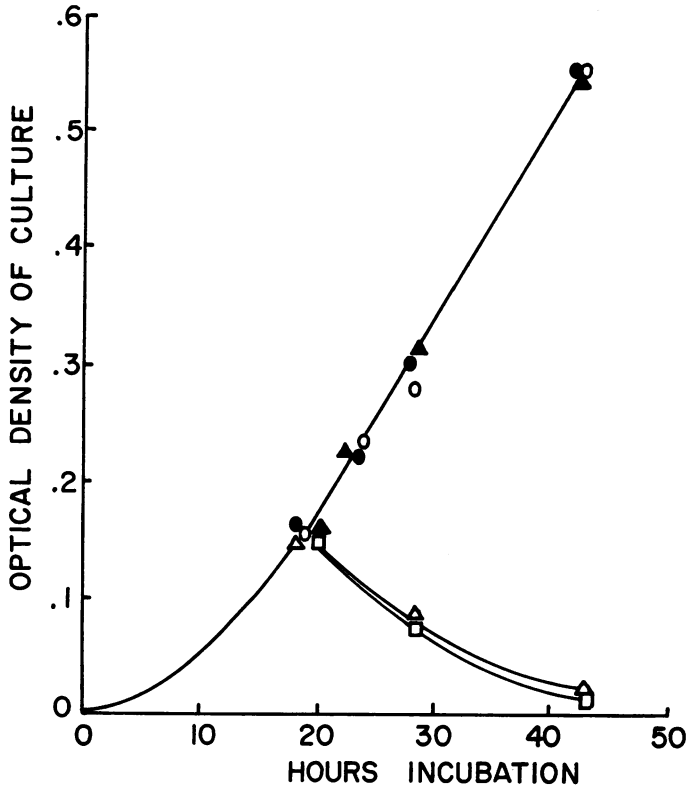

FIG. 3. *Optical density response of Pseudomonas fluorescens cells growing in nutrient broth to the addition of HEX and the protective effect of NaCl and $MgSO_4 \cdot 7H_2O$. Concentrations are those in the substrate tube. ○, control; △, 0.1% HEX; □, 0.06% HEX; ●, 0.1% HEX plus 0.1% NaCl; ▲, 0.1% HEX plus 0.05% $MgSO_4 \cdot 7H_2O$.*

ment. Inclusion of inorganic salts, such as $MgSO_4$ or NaCl, in HEX-broth eliminated any OD decline and permitted *P. fluorescens* to grow normally in nutrient broth or peptone-water (Table 2 and Fig. 3). Studies with 0.1% HEX in distilled water indicated $MgSO_4 \cdot 7H_2O$ at 0.05% or higher was effective in reversal as was NaCl at 0.1% or higher. Other salts also offered some protection from lysis. The presence of calcium alginate did not reverse lysis nor did $CaCl_2$ as determined by the membrane-filter technique. A single experiment with sodium ethylenediaminetetraacetic acid (EDTA) in place of HEX did not produce lysis. As also indicated in Table 2, various medium ingredients offered no protection.

Microscopic examination of *P. fluorescens* cells, which ultimately grew in the presence of 1% HEX-broth. showed gross morphological changes, such as elongation of cells from a normal length of 3 to 4 μ to one of 20 to 30 μ, Club and curved forms were also present. Phenomena of this sort have been reported where there has been interference with cell division (Grula, 1960; Brock, 1962). No such forms were noted in those organisms which grew well in 10% HEX, e.g., *Escherichia coli*.

S. lutea showed no lysis under any of the conditions tested nor did 10% HEX kill any cells (Fig. 2). However, *S. lutea* would not grow in broth until the HEX concentration was reduced below 0.05%. Inclusion of 0.02% or more $MgSO_4 \cdot 7H_2O$ permitted normal growth in the presence of 0.1% HEX-broth, and NaCl had no effect (Table 3).

Inhibition studies. S. lutea was completely inhibited by 0.05% HEX when 2.6×10^5 cells per drop were placed on a nutrient agar medium. Some reversal of this inhibition was noted when $MgSO_4 \cdot 7H_2O$ was included in the same medium (Table 4). Although reversal was not complete, 0.1% $MgSO_4 \cdot 7H_2O$ seemed to be the most effective. A consideration of the results presented in Tables 3 and 4 suggests that only a few of the original cells were able to reproduce when magnesium sulfate was added. Use of Tryptone-glucose-yeast extract agar (TGE) plus HEX instead of nutrient agar as the enrichment substrate was somewhat less inhibitory (Table 5), permitting growth of *S. lutea* in the presence of 0.05%, but not 0.1%, HEX.

Table 6 shows that *P. fluorescens* was greatly inhibited above 1.0% HEX, although the inhibition was completely reversed by the addition of 0.1% $MgSO_4 \cdot 7H_2O$. It is rather interesting to note that the combination of HEX and

TABLE 2. *Change in optical density (OD) of Pseudomonas fluorescens cells suspended in various substrates*

Substrate	Maximal change in OD in 24 hr		
	With 0.1% HEX	Without HEX	Difference*
Distilled water	−.14	−.03	.11
NaCl (0.9%)	−.11	−.10	.01
NaCl (0.1%)	−.07	−.08	.01
NaCl (0.05%)	−.11	−.05	.06
$MgSO_4 \cdot 7H_2O$ (0.1%)	0	0	0
$MgSO_4 \cdot 7H_2O$ (0.05%)	−.02	−.01	.01
$MgSO_4 \cdot 7H_2O$ (0.01%)	−.07	−.03	.04
Na_2SO_4 (0.1%)	−.12	−.05	.07
KH_2PO_4 (0.1%) at pH 7.0 (NaOH)	−.07	−.04	.03
K_2SO_4 (0.1%)	−.10	−.04	.06
$NaHCO_3$ (0.1%) at pH 7.0 (NaOH)	−.08	−.04	.04
NaH_2PO_4 (0.1%) at pH 7.0 (NaOH)	−.10	−.04	.06
KCl (0.1%)	−.09	−.04	.05
Peptone-water (0.05%)	−.16	+.01	.17
Nutrient broth	−.14	.26†	.40
Yeast extract (0.1%)	−.07	.14†	.21
Proteose peptone (0.1%)	−.07	.12†	.19
Tryptone (0.1%)	−.07	.17†	.24
Calcium alginate (0.05%)	−.11	−.05	.06

* A difference of .03 OD units was considered significant.
† Growth occurred in these substrates.

TABLE 3. *Influence of $MgSO_4 \cdot 7H_2O$ and NaCl on the growth of Sarcina lutea in 0.1% HEX-broth*

Substrate (nutrient broth), plus	Increase in OD in 24 hr in	
	HEX	Control
$MgSO_4 \cdot 7H_2O$ (0.2%)	.12	.19
$MgSO_4 \cdot 7H_2O$ (0.1%)	.16	.21
$MgSO_4 \cdot 7H_2O$ (0.02%)	.21	.16
NaCl (0.2%)	.01	.12
NaCl (0.1%)	.02	.17
NaCl (0.02%)	.03	.17
Control	.02	.21

TABLE 4. *Partial $MgSO_4 \cdot 7H_2O$ reversal of Sarcina lutea growth inhibition on HEX-nutrient agar**

HEX (%)	$MgSO_4 \cdot 7H_2O$ (%)			
	0	0.1	1.0	5.0
0.1	0	0	10^1†	0
0.05	0	6.5×10^4	3.6×10^4	1.5×10^3
0.005	2.3×10^5	2.5×10^5	2.2×10^5	6×10^3
Control	2.6×10^5			

* Results are given in numbers of organisms. See text for details.
† A few microscopic colonies at 10^1.

TABLE 5. *Comparison of two media in supporting growth of Sarcina lutea in the presence of HEX**

HEX (%)	Medium†	
	NA	TGE
0.1	0	0
0.05	0	2.7×10^7
0	2.5×10^7	2.5×10^7

* Results are given in numbers of organisms. See text for details.
† NA = nutrient agar; TGE = Tryptone-glucose-yeast extract agar.

1.0% $MgSO_4 \cdot 7H_2O$ was more toxic than the 1.0% salt alone. The reason for this was not determined. The incorporation of 0.025% calcium alginate into the medium also reversed this inhibition but did not prevent lysis in HEX-broth.

Substitution of Calgon for HEX gave essentially the same results in all instances.

DISCUSSION

It seems clear that HEX used to dissolve calcium alginate swabs inhibits the growth of certain bacteria when incorporated into plating media. This particularly affects the gram-positive bacteria. Some gram-negative bacteria may be lysed as a result of their contact with HEX during swab dissolution. This could be a significant factor if the number of cells was initially low. These two factors, growth inhibition and lysis of cells, may account for much of the variation reported by authors who have studied this technique. A comparison of the methods used by the various authors is presented in Table 7. The usual procedure in dissolving the swab is to add 1 ml of a 10% HEX solution to a quantity of diluent in which the alginate swab is placed to be dissolved. The use, by most investigators, of Ringer's solution as diluent, because of its NaCl content, would have a tendency to protect sensitive cells from lysis and thus minimize this as an explanation for differences. Use of 0.0044% phosphate in a buffer (American Public Health Association, 1960) apparently has little protective effect. Upon plating the dissolved alginate solution, a further dilution of the HEX takes place. If 1 ml is added to 17 ml of agar, the range of HEX concentrations in which the organisms used by the authors listed in Table 7 must grow is 0.034 to 0.125%. These figures would be slightly higher if less agar were used. These concentrations (except 0.034% of one study) are within the range of inhibition of many of the gram-positive bacteria as determined in the present study.

TABLE 6. *Effect of HEX and $MgSO_4 \cdot 7H_2O$ on the growth of Pseudomonas fluorescens on solid media**

HEX (%)	$MgSO_4 \cdot 7H_2O$ (%)		
	0	0.1	1.0
2.5	10^1†	1.3×10^7	0
2.0	10^3†	1.5×10^7	0
1.5	10^3†	1.4×10^7	0
1.0	1.3×10^7	1.7×10^7	0
0.75	1.6×10^7	1.3×10^7	0
0.5	1.6×10^7	1.1×10^7	0
0	1.4×10^7	1.3×10^7	1.3×10^7

* Results are given in numbers of organisms. See text for details.

† Poor growth, no individual colonies.

The medium chosen may also play a role in the results. Nutrient agar was used as the base enrichment in the present study. The standard procedure (American Public Health Association, 1960) calls for TGE. TGE agar appears to reduce HEX toxicity to *S. lutea* somewhat, permitting growth in 0.05% HEX, whereas no growth occurred on nutrient agar at this concentration. The standard method, however, would result in a final HEX concentration of 0.1% or higher, an amount not supporting growth of this test organism on either medium.

The organism chosen for testing would also have influenced the results. In pure culture studies, the use of *E. coli* by many authors was fortuitous; unless there are some especially sensitive strains, *E. coli* will grow even in 10% HEX if nutrient is present. Some gram-negative bacteria may, in fact, be inhibited as much as some gram-positive bacteria. *Bacillus subtilus*, used by several investigators, has been shown to grow only poorly in the presence of 0.05 and 0.1% HEX-broth. Of the *Staphylococcus aureus* strains used in the present study, one grew in 0.1% HEX-broth, five grew only in 0.05%, and two did not grow in any. Wild

TABLE 7. *Comparison of techniques employed by investigators for the calcium alginate swab method*

Reference	Medium	HEX (%) to dissolve	HEX (%) in medium	Organism	Diluent	Conclusion (relative to cotton)
Higgins (1950)	Blood agar	1	0.05	*Staphylococcus aureus* *Escherichia coli*	Ringers	Better
Trednick and Tucker (1951)	MacConkey's agar	2.5	0.125	*E. coli*	Ringers	Better
Barnes (1952)	NA*	0.67	0.034	*E. coli, S. albus*	Ringers	Same
Cain and Steele (1953)	TGE†	1	0.05	*S. aureus*	Ringers	Better
Barton et al. (1954)	TGE†	1	0.05	*Bacillus subtilis*	Ringers	Same
O'Neill and Reed (1956)	TGE†	2	0.1	*B. subtilis*, wild	Ringers	Better
Angellotti et al. (1958)	TGE†	1	0.05	*B. subtilis, S. aureus*	Ringers (25%)	Poorer
O'Keefe (1958)	NA*	2	0.1	*Shigella flexneri*	Peptone-water (0.1%)	Same
Robinson (1958)	Selenite F	1.4	0.07	*Salmonella typhosa* *S. paratyphi B*	Selenite F	Better
American Public Health Association (1960)	TGE†	2	0.1	—	Phosphate	—
Walter and Card (1962)	NA*	1.1	0.055	Wild	Phosphate	—

* Equivalent to Difco Nutrient Agar.

† Equivalent to Difco Tryptone Glucose Yeast Extract Agar.

populations used in several studies very likely contain many organisms which cannot grow in the presence of HEX. All of these factors could have affected the results of the various authors, and may offer an explanation of the considerable variability reported.

Although the results of this study do not demonstrate this unequivocally, the cause of the observed inhibition and lysis may lie in a disturbance of magnesium utilization. Webb (1953) has shown that the magnesium requirement of gram-positive bacteria greatly exceeds that of gram-negative bacteria. It must be noted that reversal of gram-positive *S. lutea* is only partial (i.e., recovery of only a few of the original cells), and not all concentrations of HEX can be reversed by $MgSO_4 \cdot 7H_2O$.

Filament formation resulting from interference with cell division has been demonstrated by Grula (1960). Brock (1962) reported filament formation by a strain of *E. coli* when grown in magnesium-deficient media. *P. fluorescens* in the present study exhibited similar filament formation when grown in the presence of 1% HEX-broth. This could be the result of magnesium deficiency, although other explanations are not ruled out.

Further support for magnesium interference comes from consideration of the nature of HEX. HEX is one of the polyphosphate group of cleaners and water softeners; it is soluble in water producing a clear solution of pH 6.8 (Parker and Litchfield, 1962). HEX softens water by chelating ions of magnesium and calcium. Its sequestering ability is much greater for calcium than for magnesium; this may account for the ability of some organisms to grow in the presence of large amounts of HEX, since some may be able to utilize this more weakly bound magnesium. Although magnesium quantitatively is the most important metal in the cell, binding of other metabolically essential metals, such as iron, cannot be overlooked.

The results of this study do not permit much more than speculation on the reason for lysis of some bacteria. According to Brock (1962), magnesium is concerned with cell-wall integrity. A shortage of this element could result in the loss of certain properties, such as maintenance of salt balance. The addition of NaCl may reverse this and permit normal growth. That this is not a complete explanation is evident from the fact that a much stronger chelating agent, EDTA, does not produce lysis. Lysis may or may not be related to magnesium dificiency.

Current work indicates that the shortcomings of the alginate swab technique as presented here can be successfully overcome.

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