COMPARATIVE EFFICACY OF BACTERICIDAL COMPOUNDS IN BUFFER SOLUTIONS

PART II

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A number of phenols, aromatic alcohols, organic mercury compounds and quaternary ammonium compounds have been examined, by the membrane filter method, to assess their antibacterial action at four different pH values against various Gram-positive and Gram-negative organisms. The killing rate of the compounds tested varied with the pH. The phenols, in the undissociated state, and the aromatic alcohols were most effective against Gram-negative cells at a slightly alkaline pH, and least effective at a pH of 5·5 to 7, whereas against Gram-positive cells, the compounds were least bactericidal at a pH of 7 to 8·5. Upon Gram-positive as well as Gram-negative cells, phenylmercuric borate was more potent in alkaline solution, thiomersal, in acid solution. This difference is connected with the opposite charges of the active ions. A reduction in bactericidal activity was noted in acid solutions of domiphen bromide and cetyl pyridinium chloride although the activity of the latter was less influenced by pH. The importance of the partition ratio between water and a lipid phase for the bactericidal efficacy of phenols and aromatic alcohols is stressed, and a new approach to the testing of these compounds is proposed. 4-Chloro-β-phenylethyl alcohol yielded promising results as a new bactericidal agent. *Pseudomonas pyocyanea* was more rapidly killed by the compounds tested than *Escherichia coli*.

IN Part 1 of this work[1], a technique for the counting of bacteria by means of membrane filters was described and analysed statistically. This technique has been used in a large number of experiments, the results of which are discussed in the following pages.

EXPERIMENTAL METHODS

The details of the technique employed as well as the compounds and organisms tested have been described in Part I[1]. The cell density was 3×10^5/ml.; 10 ml. of the test-mixture was filtered through Co 5 membrane filters which were incubated on enriched nutrient agar with 0·05 per cent of thioglycollate for mercurials; three filter pads were used for each pH. Figures based on a logarithmic scale are used to present the counts, each column representing the "mean" of the three replicates, and the highest as well as the lowest count is indicated.

RESULTS AND DISCUSSION†

Bactericidal Action of Phenols and Aromatic Alcohols

Influence of the pH. Figures 1 and 2 give the results obtained with *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas pyocyanea*, *Streptococcus faecalis*, *Serratia marcescens* and *Corynebacterium diphtheriae*

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† Only a small number of the counts obtained can be reproduced here. Values of n (concentration exponents) and Q_{10} (temperature coefficients), calculated on the basis of mortalities exceeding 99·9 per cent, may be summarised as follows: Phenols and aromatic alcohols: $n = 6$, $Q_{10} = 4{\cdot}3$ (calculated on the basis of the difference between 20° and 37°); mercurials: $n = 2$; quaternaries: $n < 2$.

tested against phenol and β-phenylethyl alcohol. For the Gram-negative species, the minimal action (that with most survivors) was found to be on the acid side, at about pH 5·5 for *E. coli*, and at pH 5·5 to 7 for *Ps. pyocyanea* and *Serr. marcescens*. For the Gram-positive cells, on the other hand, minimal action was at the slightly alkaline pH values of 7 to 8·5 whereas between pH 7 and 5·5, a large increase in bactericidal action was recorded. Similar results were obtained with the other phenols and aromatic alcohols tested; dissociating phenols, however, were found to behave differently.

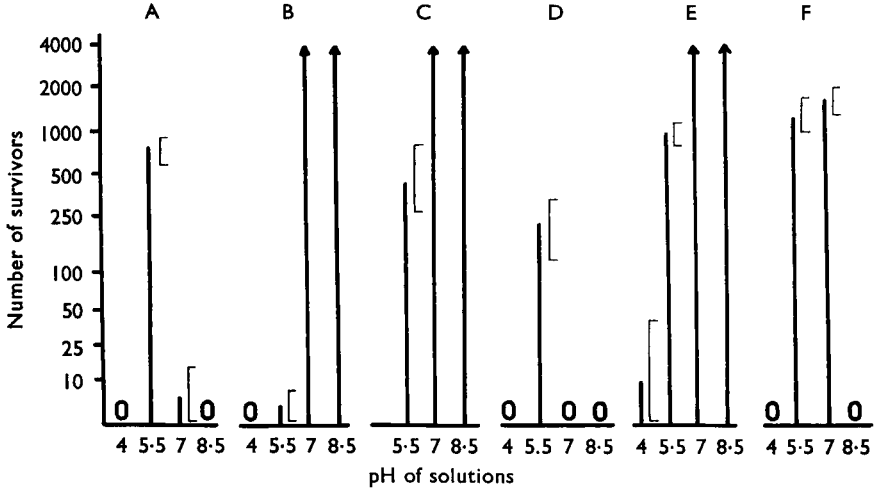


FIG. 1. Membrane filter counts of suspensions of *E. coli* (A, D), *Staph. aureus* (B, E), *Str. faecalis* (C) and *Ps. pyocyanea* (F), exposed to 1 per cent phenol (A, B, C) and 1 per cent (D, E) or 0·5 per cent (F) β-phenylethyl alcohol in isotonic phosphate buffers of four pH values. Each column represents the "mean" calculated on the basis of the logarithms of three parallel counts, brackets indicate lowest and highest counts. An arrow at the top of a column indicates filters too densely grown for a count of single colonies, the limiting count being 2000 to 3000 colonies for the Gram-negative organisms and about 4000 for the Gram-positive ones. For each count, 10 ml. of test-mixture with an initial count of $3 \cdot 10^5$ cells per ml. were filtered, 0 = sterile.

The results with the Gram-negative organisms are contrary to the axiom that phenols are more bactericidal in acid solutions. Especially surprising was that, in general, the efficacy was even more pronounced at pH 8·5 than at pH 4, despite all the phenols tested showing some dissociation at pH 8·5.

One explanation why this characteristic difference between Gram-positive and Gram-negative organisms has not been detected sooner might be that the methods hitherto used e.g., those of Kuroda[2], were not sufficiently sensitive; thus with the more sensitive membrane technique now available these differences are now seen.

As the efficacy of phenols and alcohols was influenced so characteristically by the pH of the solution, we first sought a clue in the different

isoelectric points of Gram-positive and Gram-negative bacteria discussed by Stearn and Stearn[3]. They measured the different uptakes of dye by the cytoplasmic membrane, but to us a difference in the charges of cytoplasmic membranes is difficult to reconcile with the fact that phenols are bactericidal in the molecular form only. It seems that no direct binding of the molecules to acceptor sites can be held responsible; rather, the permeability barrier may be influenced by the external pH. Thus, owing to the difference in barrier between Gram-positive and Gram-negative cells, the penetration of the disinfectants into these cells may not be the same at pH 5·5. Further studies in this field will be necessary to clarify this point.

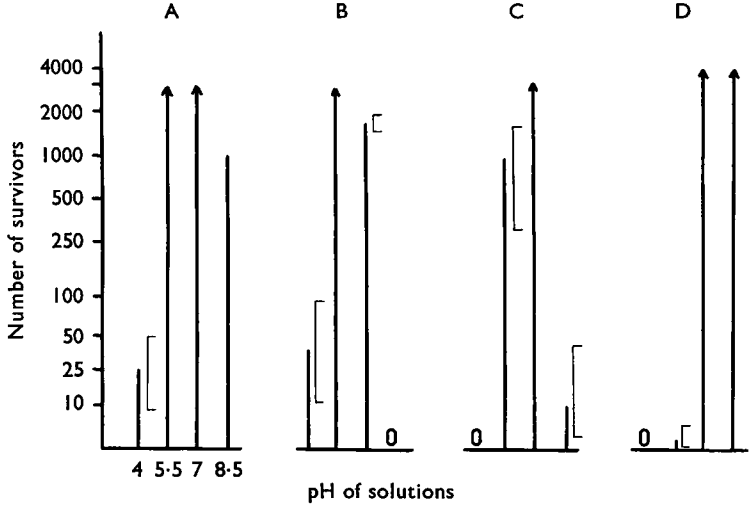


FIG. 2. Membrane filter counts of suspensions of *Serr. marcescens* (A), *Ps. pyocyanea* (B, C, two parallel series), and *Corynebact. diphtheriae* (D), exposed to 0·5 per cent of phenol for 2 hours at 20°. Phosphate buffers, cell density and representation as in Fig. 1.

Influence of the dissociation of phenols. It is well known that th dissociation of phenols increases with increasing pH value, and that this affects their antibacterial properties. This is illustrated in Figure 3 which indicates the bactericidal activities of *p*- and *o*-chlorophenol, propyl-*p*-hydroxybenzoate and *p*-nitrophenol at different pH values against *E. coli.* It shows the rise in dissociation to be proportional to the fall in bactericidal activity. Phenolate ions are therefore not bactericidal, which is in agreement with the statements of other authors. From the Figure, it is possible to estimate the quantitative aspect of the survivor peak at pH 5·5. *o*-Chlorophenol and propyl-*p*-hydroxybenzoate are 3 per cent dissociated at pH 7, which is sufficient to obtain about as many survivors as at pH 5·5. Small differences in concentration seem therefore to be sufficient for a shift in the survivor peak from 5·5 to 7 (or vice versa) with *E. coli.* *p*-Chlorophenol, on the other hand, is

almost 20 per cent dissociated at pH 8·5, with about 20 times as many survivors as at pH 5·5.

The part played by the partition coefficient in the arrangement of the compounds according to their activity. In a preliminary report[4] it was pointed out that our results with phenols and aromatic alcohols are in general agreement with Ferguson's principle[5,6]. This rule, however, stipulating that activity is inversely related to water solubility was not followed by the two chlorinated alcohols, namely, 4-chlorobenzyl alcohol and 4-chloro-β-phenylethyl alcohol. For instance, a 0·021M aqueous solution of 4-chlorobenzyl alcohol (water solubility = 0·021M) exhibited

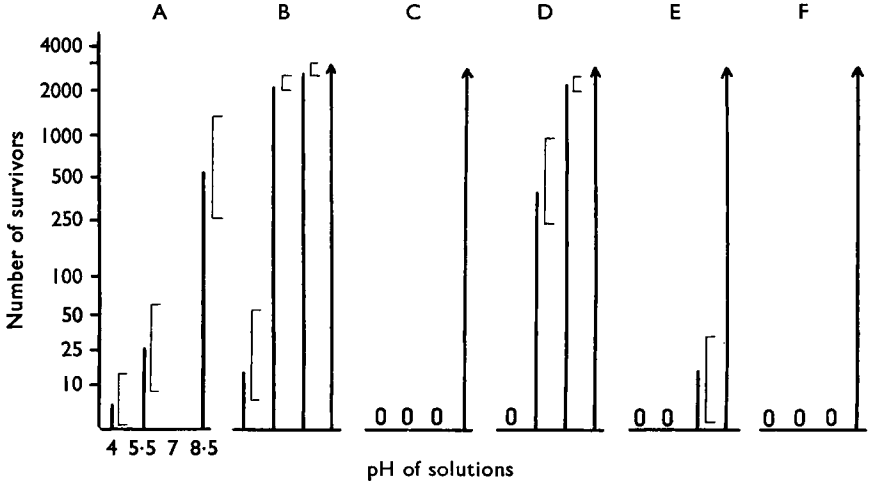


FIG. 3. Membrane filter counts of suspensions of *E. coli* exposed to phenols of varying degrees of dissociation; dissociation rises from left to right. A, *p*-chlorophenol 0·1 per cent for 6 hours at 20°; B, *o*-chlorophenol 0·25 per cent for 30 minutes at 20°, and for 2 hours at 20° in C; D, propyl *p*-hydroxybenzoate 0·03 per cent for 24 hours at 20°; E, *p*-nitrophenol 1 per cent for 30 minutes at 20°; F, *p*-nitrophenol 0·5 per cent for 24 hours at 20°. For buffers, cell density and representation: cf. Fig. 1.

the same bactericidal activity as 0·013M solution of 4-chloro-β-phenylethyl alcohol (water solubility = 0·0294M). However, when these solutions of equal activity were partitioned in hexane:water, 4-chlorobenzyl alcohol achieved a concentration of 0·0106M and 4-chloro-β-phenylethyl alcohol a concentration of 0·0091M in the hexane, the concentrations in the lipid phase thus being similar. Richardson and Reid[7] obtained similar results with α-ω-di-*p*-hydroxyphenylalkanes, as did Shukis and Tallman[8] with aliphatic mercurials. Crisp and Barr[9] confirmed Ferguson's rule giving data on the activities of alcohols and phenols required to immobilise barnacle nauplius larvae. They too stressed that the efficacy of phenols and alcohols is governed by the concentration reached in the biophase. This phase was felt to be a bulk lipid rather polar in character and not an interface between lipid and water.

Our experiments have convinced us that it is possible to use a simple approach to test phenolic and alcoholic disinfectants. Starting from the hypothesis that different concentrations of phenols and aromatic alcohols elicit an identical bactericidal activity when they reach the same molar concentration in a lipid phase after partitioning, reference to physical constants of the compounds in question will allow a comparison of the activities of different compounds to be made. The ratio molar concentration in water: molar concentration in lipid phase after partitioning, provisionally termed "activity index", is a constant for a given compound, provided the same lipid phase is used and that the phenols are undissociated. Once the "activity indices" have been determined experimentally, different compounds can be compared by calculating the concentrations in water which are required to obtain a similar concentration in the lipid phase. The calculated concentrations will probably not be strictly equal in their bactericidal activity since other factors are involved, nevertheless, this approach might be useful for a preliminary comparison.

It has long been known that upon ascending a homologous series, one observes, as a rule, the antibacterial activity to increase and then to decrease again. This fact, already explained in a simple manner by Ferguson[5], may be interpreted from our point of view as follows. On the one hand, the activity of the compounds augments with increasing solubility in lipids. On the other hand, the disinfectant has to be dissolved in water in order to reach the hydrophilic outer layers of the cells. In a homologous series, therefore a certain point will be reached where a further, significant increase in lipid solubility cannot be attained whilst at the same time the solubility in water is lowered, and this means that the activity is necessarily reduced. As Ferguson pointed out, the position of the fall in action depends on the resistance of the organisms; it occurs earlier with *Staph. aureus* than it does with *Salmonella typhi*, for example. One may predict that phenols and aromatic alcohols capable of reaching a high concentration in the cell lipids yet remaining soluble to some extent in water will possess superior killing power. Chlorocresol and 4-chloro-β-phenylethyl alcohol are examples of such compounds.

Our results are in favour of the existence of a lipid barrier at the cell surface. This barrier is very probably represented by the cytoplasmic membrane[10].

Bactericidal Action of Organic Mercury Compounds

Figure 4 shows the activities of phenylmercuric borate and thiomersal at four pH values. It is seen that whilst their activities differ greatly, their effects on the Gram-positive and Gram-negative organisms are identical. It is generally agreed that the mechanism of action of mercurials is an interaction with available sulphhydryl compounds since compounds such as thioglycollate can compete with the mercuric ion. The sulphhydryl group has a pKa of 9·1 to 10·8 at 25° in peptides of known structure[11]. According to Cohn and Edsall[11], the pI (isoelectric point) of glutathione is 2·83 and that of cysteine 5·02 to 5·07; because the more alkaline the

solution of these compounds, the greater their negative charges. The difference between the action of phenylmercuric borate (cationic) and thiomersal (anionic) is easily explained by the fact that in more alkaline solutions a greater number of positively charged phenylmercuric ions can be bound by the sulphhydryl groups; for the negatively charged thiomersal, the opposite is true. The interaction must, of course, occur at a site of the cell still influenced by the external pH. The action of mercurials is further elucidated by results like those of Shukis and Tallman[8], who showed that in a series of aliphatic mercurials, the antibacterial action was better when the compounds had a partition coefficient in favour of

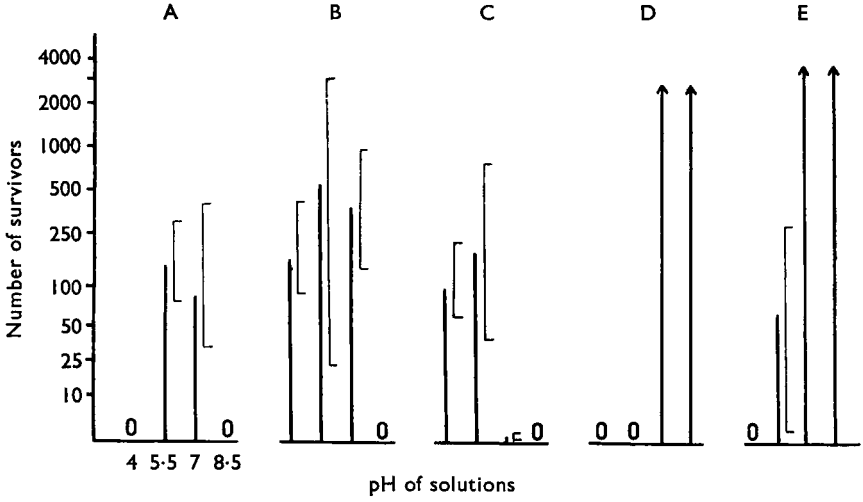


FIG. 4. Membrane filter counts of suspensions of *E. coli* (A, D), *Ps. pyocyanea* (C), and *Staph. aureus* (B, E), exposed to phenylmercuric borate 1:10,000 (A, B, C) and thiomersal 1:10,000 (D, E). Exposure times: 30 minutes at 20° in C, 2 hours at 20° in A and B, 24 hours at 20° in D and E. The phenylmercuric borate solutions were in M/15 phosphate buffers only, the thiomersal solutions were made with the usual isotonic phosphate solutions with potassium chloride. For cell density and representation: cf. Fig. 1.

the lipid phase. We found phenylmercuric borate with its higher solubility in lipids to be much more active than thiomersal which is poorly lipid-soluble.

The action of mercurials seems to proceed in two stages. In the first, the compound is adsorbed on to the cytoplasmic membrane, the adsorption being successful only when the charges of the interacting groups are opposite. The sulphhydryl groups attacked seem to be the same both in Gram-positive and Gram-negative species. The first stage is essentially bacteriostatic and is reversible by thioglycollate. In the second stage, penetration through the lipid barrier, situated most probably in the cytoplasmic membrane, takes place. For this penetration, the water:lipid partition coefficient of the compound is the deciding factor. Once the antiseptic has penetrated, the action is no longer reversible and is therefore bactericidal.

Bactericidal Action of Quaternary Ammonium Compounds

A comparison of the two compounds tested (Figs. 5 and 6) showed that cetyl pyridinium chloride was more active than domiphen bromide against *Staph. aureus*, 1 : 100,000 of the former compound being more effective in 2 hours than the same concentration of domiphen bromide in 24 hours. This difference was never noted with *E. coli*. While a more pronounced

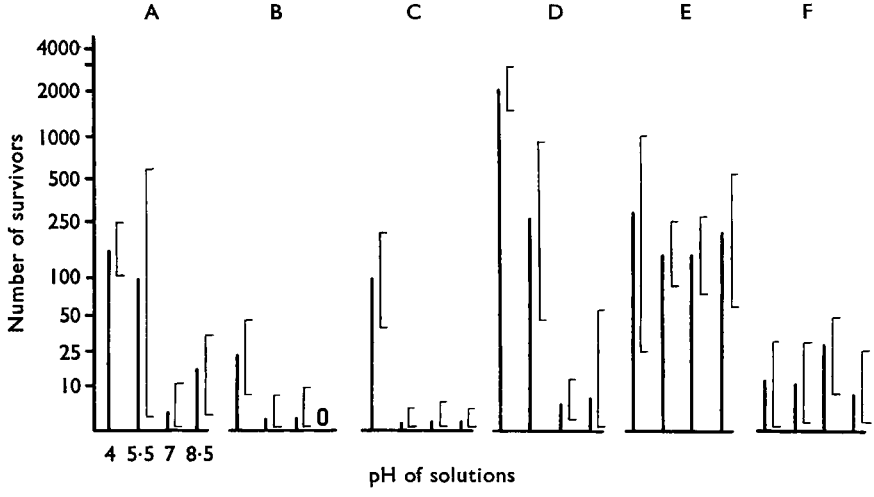


FIG. 5. Membrane filter counts of suspensions of *E. coli* (A, C, E) and *Staph aureus* (B, D, F) exposed to domiphen bromide 1 : 20,000 (A, B), 1 : 50,000 (C, D), and 1 : 100,000 (E, F), Exposure times: 30 minutes at 20° (B), 2 hours at 20° (A, C, D), and 24 hours at 20° (E, F). Experiments B and C were conducted with suspensions of *E. coli* of lesser resistance than those used in A. In experiments B and D, four parallel filtrations were made. For buffers, cell density and representation: cf. Fig. 1.

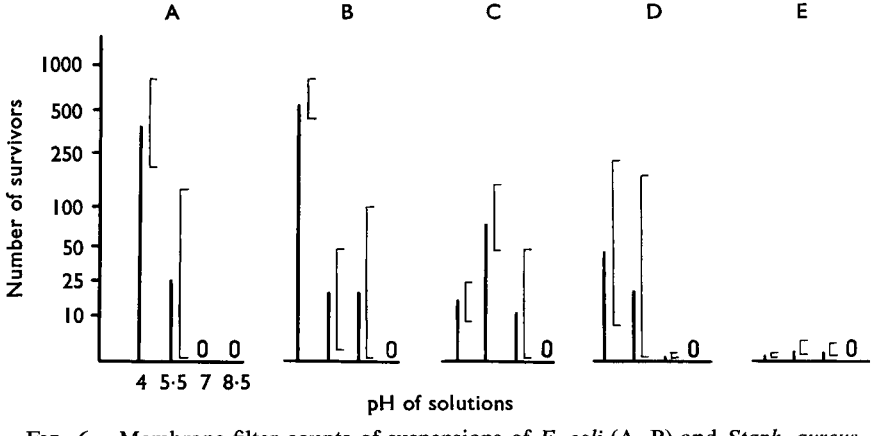
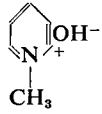
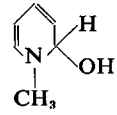


FIG. 6. Membrane filter counts of suspensions of *E. coli* (A, B) and *Staph. aureus* (C, D, E) exposed to cetyl pyridinium chloride 1 : 10,000 for 30 minutes at 20° in A, 1 : 20,000 for 2 hours at 20° in B, 1 : 100,000 for 2 hours at 20° in C, D and E (three parallel series). Buffers, cell density and representation same as in Fig. 1.

bactericidal action of the quaternaries in alkaline solutions is to be expected because of the positive charge of the active molecules, cetyl pyridinium chloride was reported by Quisno and Foter[12] to be equally active between pH 2 to 10. This is partly confirmed by our results (Fig. 6). In spite of its being a weak base (pKb 8·8), pyridine forms quaternary salts which act as strong electrolytes. The quaternary hydroxides, however, exhibit properties[13] that might possibly be able to furnish some explanation for the comparatively milder bactericidal activity at alkaline pH values. Thus, *N*-methylpyridinium hydroxide "a" responds to oxidation with potassium ferricyanide as though it had structure "b".



a



b (Pseudo base)

In alkaline solutions, more of the pseudo base is likely to be formed; this non-ionic compound probably being less bactericidal.

Most workers testing quaternaries have obtained erratic results (see Davies[14]). The explanations generally offered are irregular distribution or agglutination of the cells in the test medium. Combined agglutination and killing have also been advanced to explain the very rapid killing rate in the first few minutes[15,16], followed by a slowing down of the killing process. Davies[14] as well as Du Bois[17] ascribed the persistence of a few survivors to the possibility that cells within agglutinates could no longer be reached by the disinfectant.

To study this further we examined cultures of *E. coli* and *Staph. aureus* in solutions of pH 4 and 7 by phase contrast to see whether agglutination of cells occurred under our experimental conditions. The cell density had to be raised to 15×10^6/ml. for these examinations. *E. coli* was agglutinated by both compounds at 1 : 5000, but not at 1 : 10,000, whereas *Staph. aureus* was agglutinated by dilutions as high as 1 : 200,000. Although agglutination was demonstrated by this method, no differences in survivor counts were found when the cultures were shaken with beads in a lecithin-Tween solution. Our experiments showed the quaternaries to have a very potent bactericidal action which is probably due to the low density of the washed cells employed and to the absence of foreign matter which might act as surface-active adsorbent. The killing of most cells in the first few minutes of exposure is no doubt ascribable to the surface-activity of the quaternaries which are absorbed rapidly by the cytoplasmic membrane, causing disorder and changes in surface charges in this essential part of the cell[18,19]. An important factor seems to be a profound change in permeability. Chaplin[20] described strains of *Serr. marcescens* which were resistant to quaternaries, and he observed that these strains contained more lipids in the cell surface. Similar results were obtained by Dyar[21] with *Staph. aureus*. The persistence of a few survivors might be attributed to differences in the lipid content of the cell surfaces.

Comparison of the Resistance of the Organisms Tested

Our results confirm that *Staph. aureus* is generally more resistant to phenols and aromatic alcohols than *E. coli*. The few experiments with *Str. faecalis* indicate that it is even more resistant than *Staph. aureus*. *Ps. pyocyanea* was more susceptible than *E. coli* to both the phenols and aromatic alcohols as well as to the mercurials and quaternaries tested. However, *Ps. pyocyanea* is still generally regarded as an organism showing marked resistance to disinfectants. Our findings on the efficacy of the quaternaries agree with those of Lawrence[22] and with Ostrolenk and Brewer[23]. Klein and others[24] found the quaternaries to be less active although they used a different method. Berry[25], who tested various organisms against phenoxetol only, suggested that this compound is particularly effective against *Ps. pyocyanea*. However, results of our comparative experiments in which we tested benzyl alcohol, β-phenylethyl alcohol and phenoxetol against *Ps. pyocyanea* show that β-phenylethyl alcohol is the most effective compound. Hence we could not confirm Berry's claim and suggest that the good effect of phenoxetol may be because *Ps. pyocyanea* offers minimal resistance to phenols and aromatic alcohols. After testing a number of typical, freshly isolated strains of the main test organisms we remain convinced that a general high resistance of *Ps. pyocyanea* to disinfectants does not exist although the occurrence of certain highly resistant strains cannot be excluded.

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