COMPARATIVE EFFICACY OF BACTERICIDAL COMPOUNDS IN BUFFER SOLUTIONS*

PART I

BY H. HESS† AND P. SPEISER

From the Institute of Pharmacy, University of Basle, Switzerland

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A standardised membrane filter technique, adapted to the counting of organisms previously exposed to disinfectants, has been compared statistically with the pour-plate method. The membrane filters were found to be as suitable as pour-plates for counting viable organisms. The filter method is superior in disinfection experiments, since any quantity of test-mixture can be filtered and the danger of bacteriostasis is excluded. In disinfection experiments with high mortality levels, a great increse in variation occurred in replicate tests. Enriched media were not found to be superior to ordinary nutrient agar for the incubation of filter discs. The Gram-positive organisms tested showed a marked decrease in viable cells after storage at room temperature in slightly acid phosphate solutions for 24 hours, whereas in neutral and slightly alkaline solutions the viable count remained constant. The Gram-negative test organisms were not significantly affected by the phosphate buffer solutions.

THE object of this study was to compare the bactericidal activity of a number of disinfectants suitable for pharmaceutical solutions. A review of the literature has shown that there is little information available about the effect of the pH on the bactericidal activity of these compounds; for this reason, tests were made at four pH values from 4 to 8·5. The cell density chosen for the tests was such that the disinfectants were able, in concentrations used in pharmaceutical solutions, to sterilise the suspensions within periods varying between 30 minutes and 24 hours.

CHEMICAL

With three exceptions, the compounds were obtained commercially and complied with pharmacopoeial or analytical requirements or both: thus, no specifications are given for them. The following substances were tested.

Phenols. Phenol; *o*-, *m*-, and *p*-cresol; chlorocresol; *o*- and *p*-chlorophenol; *p*-nitrophenol; guaiacol (*o*-methoxyphenol); *p*-methoxyphenol; methyl, ethyl-, and propyl *p*-hydroxybenzoates; also *p*-ethoxyphenol prepared from *p*-phenetidin; the product, recrystallised from water, having a melting point of 65 to 66° (uncorr.) and a solubility of about 0·9 per cent w/v in water at 20°.

Aromatic alcohols. On different occasions commercial samples did not dissolve in water to give clear solutions even when saturation had not been reached. The impurities, presumably traces of hydrocarbons, could not

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† Present address: c/o CIBA Limited, Basle.

be removed by distillation. The process of purification was carried out as indicated below for 4-chloro-β-phenylethyl alcohol.

The alcohols used were, benzyl alcohol; β-phenylethyl alcohol; γ-phenylpropyl alcohol; phenoxetol (β-phenoxyethyl alcohol); Gecophen (4-chlorophenyl α-glycerol ether). Also included were 4-chlorobenzyl alcohol and 4-chloro-β-phenylethyl alcohol. The chlorobenzyl alcohol was prepared from 4-chlorobenzyl chloride; crystallisation from water gave the required alcohol in colourless needles of a m.p. of 66 to 68° (uncorr.) and b.p. of 116 to 120°/15 mm.; light absorption in ethanol: λ max 266 mμ (log ϵ 2·42); solubility: 0·35 per cent w/v of saline at 20° and 0·30 per cent at 3°. The chlorophenylethyl alcohol was prepared according to Harper and others[1]; purification of the product from traces of water-insoluble compounds was achieved by preparing the monoester of succinic acid and redistilling the saponified product, giving a colourless, virtually odourless liquid; b.p. 80–83°/0·07 mm.; light absorption in ethanol: λ max = 267 mμ (log ϵ 2·55); solubility: 0·48 per cent w/v in water at 20° and 0·46 per cent w/v in saline at 3°.

Organic mercury compounds. Phenylmercuric borate; its solubility is much better in slightly alkaline phosphate solutions; the solution of 0·022 per cent w/v in M/15 phosphate buffer of pH 4 and 5·5 is nearly saturated. Thiomersal.

Quaternary ammonium compounds. Domiphen bromide. Cetyl pyridinium chloride.

Experimental Methods

Buffer solutions. An isotonic phosphate buffer was used since Myers[2] pointed out that different buffer systems would exert different actions on *Escherichia coli.* It consisted of H_3PO_4, plus sodium phosphate with the appropriate amount of KCl added. Although the buffering capacity of this system is small at pH values of 4 and 8·5, it was sufficient for the purpose of the present tests. The phosphates and the phosphorus acid were dissolved under aseptic conditions in freshly distilled water from an all-glass still (Table I).

TABLE I

Preparation of isotonic phosphate buffer solutions

pH	M/15 Phosphorus acid + 0·645 per cent potassium chloride	M/15 Sodium dihydrogen phosphate $NaH_2PO_4 \cdot 2H_2O$ + 0·51 per cent potassium chloride	M/15 Disodium hydrogen phosphate Na_2HPO_4 + 0·38 per cent potassium chloride
4	8 ml.	+ 992 ml.	
5·5		940 ml.	+ 60 ml.
7		350 ml.	+ 650 ml.
8·5*		15 ml.	+ 985 ml.

* This solution has a pH of 8·55, after 2 days it falls to about 8·5.

Test solutions. The test solutions were prepared under aseptic conditions by dissolving the disinfectants in the buffer solutions, if necessary on a water bath. The solutions were filled, in amounts of 9 ml., into

aluminium-capped test-tubes, 15 × 160 mm., and inoculated with 1 ml. of bacterial suspension. Stock solutions in distilled water were prepared only from the quaternaries and from thiomersal. Phenols were dissolved in isotonic disodium hydrogen phosphate solution and the pH was adjusted as required. All pH readings were made with a glass: calomel electrode system.

Test organisms. Seven freshly isolated strains of *Escherichia coli* (type I, 44° C.-positive), three freshly isolated and one laboratory strain of *Pseudomonas pyocyanea*, and five freshly isolated strains of *Staphylococcus aureus*, were tested for their resistance to phenol. The most resistant strains were chosen, but results obtained with these were frequently checked with other strains. A culture of the *Staph. aureus* F.D.A. strain (No. 209) at our disposal was not sufficiently resistant and was used only in certain preliminary experiments; other strains also included one gravis and one atypical strain of *Corynebacterium diphtheriae*, one strain of *Serratia marcescens*, and one of *Streptococcus faecalis*.

Nutrient media. Cultures were maintained on agar slopes and subcultured every month; from these, new slopes were inoculated every week for test suspensions. The nutrient agar contained 0·5 per cent of peptone (Difco), 0·3 per cent of beef extract (Difco), 0·3 per cent of sodium chloride, 0·2 per cent of desiccated di-potassium hydrogen phosphate, and 2 per cent of agar; the pH was 7·4 to 7·5 before autoclaving at 120° for 20 minutes. The slopes for *C. diphtheriae* were enriched with 0·5 per cent of yeast extract (Difco) and an additional 0·5 per cent of peptone. With *Staph. aureus*, modifications of the nutrient agar did not induce a higher resistance to phenol. The agar was reduced to 1 per cent for the pour-plates and the media used in the disinfection experiments. In these, the nutrient agar was enriched with 0·5 to 1 per cent of yeast extract, an additional 0·5 per cent of peptone, 1 per cent of dextrose, and—when mercurials were tested—0·05 per cent of sodium thioglycollate.

Preparation of the test suspensions. The cultures were grown on slopes for 24 hours at 37° after which they were washed off with 5 ml. of water and the suspensions centrifuged at 3000 r.p.m. for 15 minutes. The centrifuged cells were resuspended in 5 ml. of phosphate buffer, pH 7, and shaken with small glass beads for 5 minutes to break up clumps. This washed suspension was diluted further with buffer so that the cell density in the final test-mixture was about 3×10^5. Plate counts made from each suspension revealed that the variation in the number of viable cells was not more than ± 50,000, and did not affect the results of the disinfection experiments.

The membrane filter technique employed. Cultivation of bacteria on membrane filters was first used for the bacteriological examination of water[3–5]; and subsequently for the testing of skin disinfectants[6–8], as well as for the evaluation of disinfection rates[9]. For the present study, we employed Co 5 membrane filters (Membranfiltergesellschaft, Göttingen) and the corresponding all-metal apparatus. Co 5 filters are nitrocellulose films of 120 μ thickness, the average "pore-diameter"[10] being 500 mμ and the filtering surface 12·5 sq. cm.

For the tests, the filters were "sterilised" in sterile hot water while the tubes with the disinfectant dilutions were placed in a water bath at either 20° ± 0·1° or 37° ± 0·1° half an hour before inoculation with the cell suspensions. After inoculation, the mouth of each tube was flamed and the tubes carefully shaken; this process was repeated once during the longer tests. Cotton-wool plugs are not suitable for the membrane filter method because exact counts cannot be made with accuracy owing to the presence of fibres on the filters. Before filtration, the filter unit was flamed, mounted while hot, and a quantity of sterile water was filtered to cool it. At intervals between 15 minutes and 24 hours (30 minutes, 2 hours, and 24 hours were most currently used), the whole test-mixture was filtered at 1 to 2 ml. per second, after which the filter was washed with 30 to 50 ml. of sterile water. The membrane was then removed and laid on the surface of a prepared nutrient agar plate. The plates were placed in the incubator within 5 minutes and incubated at 37° for 16 to 30 hours before reading. The concentration of agar in the medium was limited to 1 per cent to keep the surface as wet as possible The colonies so developing remain discrete and may be stained by incubating them on nutrient agar containing 0·05 per cent of potassium tellurite[7] for 2 to 4 hours; depending on the species, the colonies are stained brown or black. Counts were made on the dried filters by means of a magnifying lens.

Suitability of the Technique Employed

Influence of the Subculture Medium on the Number of Survivors

Several authors[11–13] have stressed the importance of enriched culture media for cells having survived lethal agents; this opinion, also advocated by Berry and Michaels[14], has not gone unchallenged[15]. We have tested several media with reference to their influence on the survivor count of *E. coli* and *Staph. aureus* by means of the membrane filter method, using phenols, quaternary ammonium germicides and organic mercury compounds and found no significant differences. These media contained various amounts of peptone, yeast extract, meat extract and glucose. The only difference observed was in the rate of growth. We may therefore infer that enriched media are growth-promoting but cannot "revive weakened cells". For this reason a medium containing nutrient agar, 1 per cent of peptone, 1 per cent of dextrose and 0·5 per cent of yeast extract was used in the tests.

Comparison of the Membrane Filter Technique and the Pour-plate Method

A statistical comparison was made with suspensions of *E. coli* and *Staph. aureus* in phosphate buffer pH 7·0 and suspensions of *Ps. pyocyanea* at pH 4·0, 5·5, 7·0 and 8·5. The cell density of the *E. coli* and the *Staph. aureus* suspensions was about 200/ml. and 140/ml. respectively, and the counts were made immediately. For *Ps. pyocyanea* the cell density was about 3×10^5/ml. and the counts were made after storage at 20° for 24 hours. During this time some growth of the organism had taken place, but this is taken into account in the figures recorded in Table II, which shows the comparative counts obtained by the two methods on suspensions

after suitable dilution. These growths probably account for the differences in counts recorded between the duplicate samples, and of the *Ps. pyocyanea* suspension.

TABLE II

COUNTS OBTAINED FROM PARALLEL EXPERIMENTS WITH MEMBRANE FILTERS AND POUR-PLATES

Experiment	Membrane filters (Co 5)				Pour-plates			
	Number of counts	Mean count (x)	Variance (s^2)	Standard deviation (s)	Number of counts	Mean count (x)	Variance (s^2)	Standard deviation (s)
534 (*Staph. aureus*)	18	142	90·9	9·5	19	155	221	15
535 (*E. coli*)	20	201	270·1	16	20	206	217·5	15
533 (*Ps. pyoc.*) immediate counts 8·5 a*	10	313	563·9	24	10	307	567·8	24
7 a	5	305	59·75	8	5	319	444·5	21
533 counts after 24 hours at 20° 8·5 a	5	206	461·75	21	6	170	583·6	24
8·5 b	5	70	88·5	9	7	61	13·1	4
7 a	5	129	163·7	13	5	111	193·5	14
7 b	5	95	119·5	11	5	79	51	7
5·5 a	5	102	212·25	15	5	79	150	12
5·5 b	5	92	49·25	7	5	72	92	10
4 a	6	100	149	12	5	113	128·5	11
4 b	4	79	74·3	9	5	88	143·5	12

* a and b are parallel tubes.

TABLE III

SIGNIFICANCE OF THE SUM OF THE PROBABILITIES RESULTING FROM THE *t*-TEST

Values of t obtained from experiments	Degrees of freedom	P
3·146	35	0·006
1·013	38	0·3
0·564	18	0·6
1·394	8	0·2
2·584	9	0·03
3·989	10	0·005
2·129	8	0·07
2·740	8	0·03
2·702	8	0·03
3·763	8	0·008
1·815	9	0·1
1·257	7	0·3

$\chi^2 = 2\Sigma\ (-\log_e P) = 70{\cdot}50$.
For 24 degrees of freedom (number of P multiplied by 2) P $<$ 0·1 per cent.

To obtain the counts, 1 ml. amounts were either distributed into petri dishes and plated in the usual way with nutrient agar or they were filtered on a membrane and the membrane was incubated on nutrient agar of the same composition. Each plate or filter was counted three times and individual counts were within $\pm$ 2 to 3 per cent of the mean. From these counts the variances of the two methods were calculated from the F values and they were found to be not significantly different. The t values were also calculated, but from these calculations it was not possible to decide whether the differences in the counts obtained by the two methods were due to chance. Subsequently, the data were subjected to further analyses by means of the combination of probabilities (Fisher[16]), and the findings from these are recorded in Table III. Using the Table of χ^2, we find a probability of less than 0·1 per cent for 24 degrees of

freedom. The difference between the means must therefore be regarded as highly significant. In the experiments with *Ps. pyocyanea*, all the significant differences appeared when the colony counts obtained with the filter method were higher, and it will likewise be seen that the cell density of the suspensions in question is low. From this statistical analysis it may be inferred that membrane filters are slightly superior when suspensions of low cell density have to be counted. This has been shown for *Ps. pyocyanea* with a probability of error of less than 0·1 per cent, but in our experience, it is also true for other test organisms, in spite of Wolochow's[17] opinions to the contrary. With higher counts, Co 5 filters should be rejected in favour of larger filters.

The Variation in Replicate Tests During the Course of Disinfection

Most authors making a quantitative recording of the number of survivors in disinfection experiments have found an excessive variation in replicate plates or roll-tubes[14]. In our experiments, a mortality level of 99·99 to 99·9999 per cent was currently achieved and so a larger set of replicates were filtered to examine in more detail the variations reported. These experiments were made under conditions as identical as possible, for example, using one test suspension for each series, the same batch of nutrient agar, membranes of the same serial number, and the disinfectant solutions were readjusted to pH 7·0. The initial counts were as usual $3 \cdot 10^5$ cells/ml., and 10 ml. amounts were filtered. Parallel with the larger series, two smaller ones were made also, representing the corresponding routine tests made in triplicate: the findings are summarised in Figures 1–4. Each of the Figures shows a strong divergence from a Poisson series, and this is the type of variation which has been encountered throughout this present study. It has compelled us to represent the results by means of a graphical method in which "mean counts"—represented by dotted lines—were computed from the logarithms of the single counts.

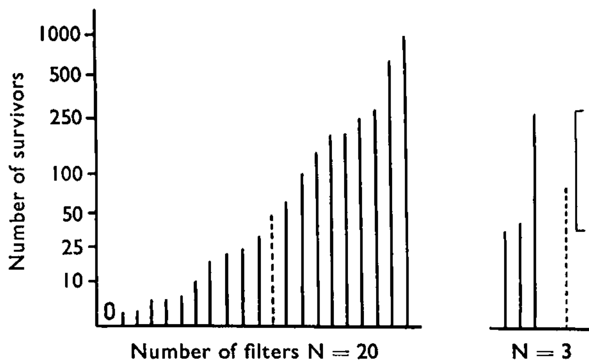

FIG. 1. Membrane filter counts of suspensions of *E. coli* in parallel tubes exposed for 2 hours at 20° to 0·3 per cent of *p*-cresol in isotonic phosphate buffer with a pH of 7·0. Left: Results of a larger series. Right: Results of a routine test. Dotted lines = "means", calculated from the logarithmic values. Each column represents the survivors of 10 ml. of test-mixture with an initial count of $3 \cdot 15^5$ cells/ml., 0 = sterile.

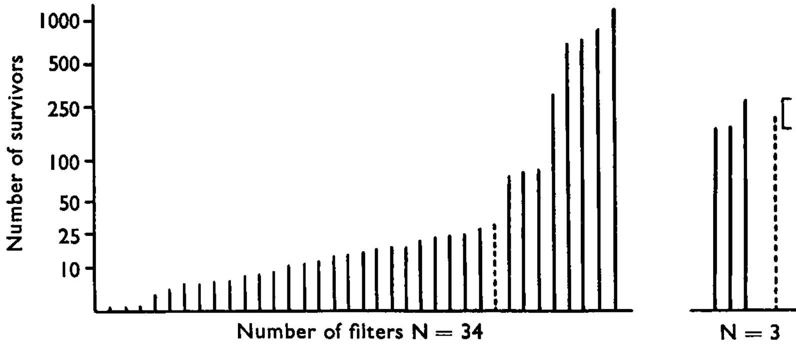

FIG. 2. Membrane filter counts of suspensions of *Staph. aureus* exposed for 24 hours at 20° to 0·2 per cent of 4-chloro-β-phenylethyl alcohol in isotonic phosphate buffer with a pH of 7·0. Left: a larger set of parallel filtrations was made. Right: Records the results of a corresponding routine test. Cell density and representation as in Fig. 1.

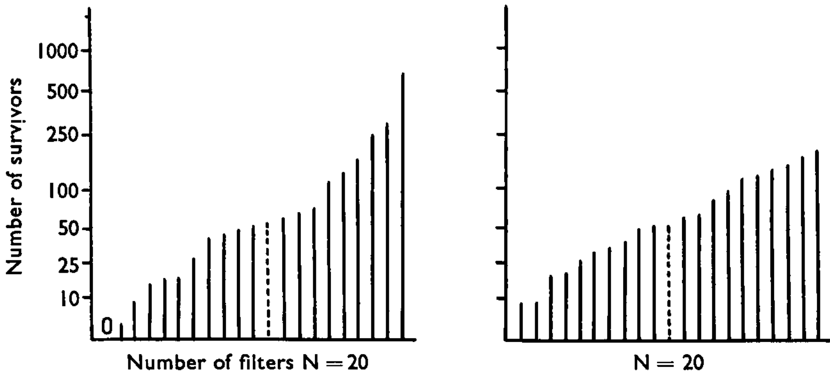

FIG. 3. Membrane filter counts of suspensions of *E. coli* (Left) and *Staph. aureus* (Right) exposed, for 30 minutes at 20°, to solutions of domiphen bromide 1:50,000 in isotonic phosphate buffer with a pH of 7·0. Cell density and representation as in Fig. 1.

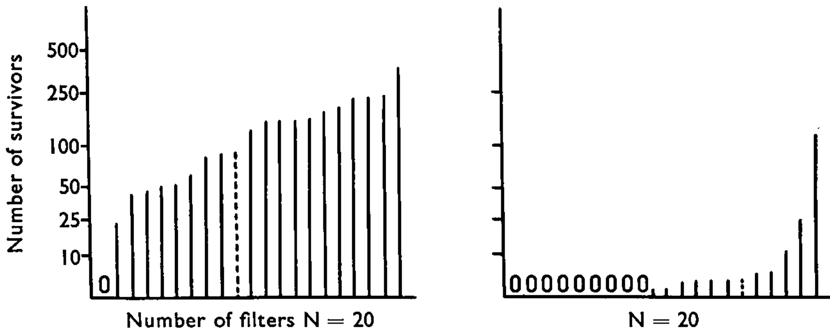

FIG. 4. Membrane filter counts of suspensions of *E. coli* (Left) and *Staph. aureus* (Right) exposed, for 30 minutes at 20°, to cetyl pyridinium chloride 1:100,000 in isotonic phosphate buffer with a pH of 7·0. Cell density and representation as in Fig. 1.

Viability of the Test Organisms in the Buffer Solutions

Suspension of the different organisms used were tested for their viability at different pH values at 20° for up to 24 hours; the number of viable cells was determined by plating, and sometimes parallel counts were made by means of membrane filters. Only the essential data are given here; for detailed results, see reference 18.

At a pH of 4, the mortality of *Ps. pyocyanea* was about 70 per cent and that of *E. coli* even less. Moderate growth occurred at the other pH values, with exception of *E. coli* at pH 8·5 where the viable count remained constant. *Staph. aureus* and *C. diphtheriae* were very sensitive to an acid pH; at pH 4, the suspensions were almost non-viable after 24 hours. The freshly isolated strains of *Staph. aureus*, used in the tests because of their higher resistance to phenol, were more easily killed by an acid pH than the F.D.A. strain at our disposal. At pH 7 and 8·5, the number of viable Gram-positive cells remained approximately constant.

Discussion

Statistical analysis has shown that the membrane filter technique adopted here is reliable when used to evaluate comparatively small numbers of viable cells in suspensions. The use of filters in disinfection experiments therefore has several obvious advantages: any quantity of test-mixture may be filtered, the filters may be washed to eliminate the danger of bacteriostat carry-over, except for mercurials, and colony growth occurs under uniform aerobic conditions.

The sensitivity of the membrane filter method is reflected in the results obtained with the three cresols. These are said to have equal bactericidal activity when measured with the phenol coefficient[19]. We have found, however, that *p*-cresol was most effective, closely followed by *o*-cresol, *m*-cresol being much less effective. The difference obtained can be ascribed only partly to the longer period of exposure, namely 30 minutes.

The disadvantages of end point methods in assessing disinfectants have been summarised by Berry[20], Berry and Michaels[21], Withell[22,23], and Wilson and Miles[24]. Nevertheless, in recent papers[25,26], end point methods have still been advocated because quantitative tests are greatly hindered by agglutination of the test suspension, and sterility has always been the desirable state. As for the counting methods, the mortality levels used therein have varied greatly. Jordan and Jacobs[27] preferred a very high mortality level of 99·999999 per cent ("virtual sterilisation time"), starting from an initial count of $3{\cdot}3 \times 10^8$. Other workers have suggested 99·9 or 99 per cent killing levels. Withell[22] introduced an LT50, the advantage being that bacteriostasis is avoided to a large extent in the subculture, and also that excessive variation in replicate tests is said to be reduced thereby. Jordan and Jacobs[27,28] found most discrepancies in their results when the mortality exceeded 95 per cent. Berry and Michaels[14] also observed some excessive variation in their disinfection experiments, even when using intermediate mortality levels, whereas for cells not having been in contact with disinfectants the distribution of

replicate counts followed a Poisson series. They tried to explain the difference by assuming that damaged organisms had more exacting growth requirements, and then went on to quote several authors in support of their thesis. As another possible explanation, Jordan and Jacobs have suggested that a certain percentage of the cells already damaged might be killed by the temperature of melted agar during the preparation of the plates or roll-tubes. Every heterogeneity of this type in colony formation would increase the distribution error already present and thus cause a rise in variation. These authors therefore suggested that a more adequate technique be adopted to handle the larger variation.

The membrane filter technique appears to be the answer to this problem. It yielded satisfactory results when untreated cells were counted; the danger of heat bacteriostasis is excluded since no heat is used, and, finally, growth takes place under equal conditions for all cells. Moreover, by filtering the whole test-mixture, the distribution error can be greatly reduced.

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