REVIEW ARTICLE

DISINFECTION

A Critical Review of Methods and Mechanisms

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A recent correspondent to the *Pharmaceutical Journal*[1] asked the pertinent question why neither the British Pharmacopœia nor the British Pharmaceutical Codex specifies any biological tests in monographs for disinfectants and antiseptics. There are good reasons in favour of such specifications, for we know that variations in the constituents of some disinfectant preparations can affect significantly their germicidal properties. A good example is the influence of fatty acids on the phenol coefficient of lysol. There are equally good reasons why at present a range of tests should not be included. First, because the various techniques available require a certain skill acquired only by constant practice before reproducible results can be obtained. Secondly, having obtained the results, there is still a good deal of uncertainty about how to translate the findings into practical usage. When the wide variety of disinfectant preparations which are available is considered and the diversity of application is appreciated, the complexity of the problem will be realised.

With any disinfectant, the primary questions to be answered are (1) is it active against all types of organisms, (2) is its activity influenced by organic matter, (3) is its action rapid or slow, (4) does temperature and concentration affect it, and (5) has it undesirable corrosive or irritant properties? Finally, there is the economic aspect to be considered. Some of these questions will be discussed in the following pages.

The Process of Disinfection

The Role of Bacterial Enzymes

The only criterion by which we can judge whether a microbial cell is alive or dead is by its ability to reproduce and proliferate. Reproduction is a natural outcome of the metabolic life cycle of the cell which is constituted, in effect, of a complex chain of enzyme-catalysed reactions. These reactions involve assimilating nutrient substances from the surrounding medium and building it up into protein and similar cellular material; they determine, therefore, the growth characteristics and other properties of the cell. Karström[2] has classed the bacterial enzymes into two groups: (*a*) the constitutive essential enzymes, fundamental to the life of the cell and formed independently of growth conditions, and (*b*) the adaptive enzymes, produced only as required according to prevailing cultural conditions. Clearly the most important in terms of disinfection are those comprising the first group, although adaptations resulting in mutant forms of the original culture can be significant; thus, Berger and Wyss[3] believe

the high resistances to phenol of some cells in a bacterial culture to be due to mutants with certain adaptive abilities.

The nature and properties of bacterial enzymes have been discussed at length by Gale[4]. Like other enzymes, they have complex protein structures and they are specific in catalysing one particular reaction or, in some cases, a particular type of reaction. Because of this specificity it follows that the bacterial cell must carry a multiplicity of enzymes to complete the chain of reactions necessary to fulfil its growth and reproductive cycle. If the chain is broken the cell becomes moribund and dies. Some disinfectants act on a whole series of the enzymes, whilst others are specific against one enzyme only. The disinfecting action of phenols, alcohols, acids and the salts of the heavy metals can be attributed to their denaturing action on the protein moiety of the enzymes generally, and examples of specific interferences are found with the acridines, which are thought to combine with the bacterial coenzymes[4]; with the sulphonamides, which inhibit the enzyme responsible for metabolising *p*-aminobenzoic acid, an essential growth factor[5], and with the esters of *p*-hydroxybenzoic acid which are also said to block an essential enzyme system[6].

Clearly, there must be considerable differences in the enzyme make-up of the various types of bacteria, for through these their individual characteristics are determined. Changes must also take place in the enzyme balance of the cell during its life cycle. Moreover, it is known that the enzymes are sited in different parts of the organism, some in the protoplasm, others in the surface membrane. From these facts may be deduced some explanation for the differences in resistance under adverse conditions which occur not only between types and species of organisms, but also between individual cells of a single population.

Although enzyme interference appears to be the effective mode of action of disinfectants in the majority of cases—interference arising from coagulation, denaturation or other breakdown of the protein moiety of the particular enzymes concerned—it must not be assumed that it represents the action of all forms of disinfection. Several investigators (see Rahn[7]) have claimed that disinfectant action is not due to enzyme inactivation but to reactions within the cell mechanisms concerned with reproduction, but whether these two opinions are in fact different is a matter of conjecture. However, in certain instances, disinfection must be dissociated with enzyme interference. Thus, it has been shown by electron microscopic studies that quaternary ammonium compounds cause release of cell constituents[8] and produce lysis[9] at least at lower concentrations, the action being attributed to lipoprotein complexes being split[10]; penicillin prevents diffusion of the essential metabolite, glutamic acid, into the cells of *Staphylococcus aureus*[11], and cell disruption can be brought about by various physical means.

The Dynamics of Disinfection

The death rate of a bacterial population under the influence of any disinfecting agent, chemical or physical, is dependent on the temperature of treatment and the concentration of the disinfectant as well as on the

resistance characteristics of the particular organism concerned. The former are fixed characteristics but the latter is a biological variable. Investigations into the rate of death of bacteria during disinfection have been made by several workers, amongst whom one of the earliest was Chick[12]. She originally postulated that under a given set of conditions, the course of disinfection follows that of a unimolecular reaction and so yields a straight line response when the log. survivors are plotted against time. However, Chick, and others, recorded numerous exceptions in which a sigmoid curve was generally obtained. Henderson Smith[13] was able to demonstrate a change from the sigmoid to an exponential form by increasing the rate of disinfection, and thus was able to reconcile the discrepancies recorded earlier. He believed the change to be due to the initial lag phase being completed so rapidly that it cannot be detected experimentally, and so concluded that a false impression is given of the initial mode of response which makes the emergence of the exponential form more apparent than real. This opinion was supported by Jordan and Jacobs[14]. Withell[15] investigating the action of a number of germicidal substances, obtained three types of response: (*a*) those which give sigmoid time-survivor curves, (*b*) those which give exponential curves, and (*c*) those which give a lag phase followed by an exponential curve. Not infrequently the same organism under ostensibly the same conditions gave varying types of curve when the experiments were repeated. These observations led him to the main conclusion that "the different rates of destruction of bacteria under the influence of a bactericide is determined essentially by differences in the manner in which the resistances of the organisms are distributed." In phenol coefficient tests, where the end-point is that of a complete kill, these observations are significant.

Because of the variations in death rates, Withell was unable to accept the suggestion made much earlier by Phelps[16] that bactericides could be evaluated by comparing the values of the constant k in the expression:—

$$k = \frac{1}{t_1 - t_2} \log \frac{n_1}{n_2}$$

where n_1 = number of viable organisms at time t_1, and n_2 = number of viable organisms at time t_2. Phelps himself did not find k to be constant for the whole disinfection process, and suggested that a mean of several estimations might be used for calculating a "coefficient." Withell rejected this suggestion in favour of using a probit–log. survivor-time curve, which gives a straight line when there is a normal distribution of resistance of the cells.

In an extensive study of the effect of phenol on *Bacterium coli*, Jordan and Jacobs[14] did not agree that this was universally applicable, and quoted several examples where there was a change of slope in the probit-log survival-time line. Berry and Michaels[17] supported this view and concluded the probit-log time relationship over the whole range of mortalities is sigmoid, but over the range of probits 4 to 6 believed from their own experiments that linearity might reasonably be assumed. Since, however,

they agreed that this could not be taken as a generalisation, and in their own experiments with ethylene glycol and its mono-alkyl ethers they were unable to obtain parallel regressions, they considered that bactericidal efficiencies could not be compared by this means.

Jordan and Jacobs[14] realised that many of the difficulties in assessing the factors influencing rate of kill arise from variables such as fluctuation in resistance of the culture and errors due to sampling, and they devised an elaborate cultural and testing technique designed to eliminate these as far as possible. Under these conditions they obtained responses indicating an initial lag phase followed by a slow but increasing death rate which merged into a second phase of constant death rate. This continued until towards the end of the disinfection period when there was a slight decline in the death rate. The decline was thought to be partly due to difficulties in obtaining reliable survivor counts when the mortality exceeded 95 per cent. Because of this and of other cogent reasons, Jordan and Jacobs were of the opinion that, after the initial lag, death rate could be considered to remain constant to the virtual end of the disinfection.

This approach may be satisfactory for the purpose of determining factors such as the concentration and temperature coefficients of germicides, but, as Jordan and Jacobs inferred, in disinfectant testing it serves only to expose the fallacies of the present phenol coefficient methods which use virtual sterilisation as the end-point. Such an end-point is manifestly unsuitable because from the foregoing observations it is most susceptible to variations. Moreover, it is well known that routine laboratory cultures do not have a fixed normal distribution of resistance; also the hazards of picking up the odd surviving cell in a small sample increases as the disinfection approaches completion. These points will be discussed later.

The Effect of Temperature and Concentration on Disinfectant Activity

Clearly it is of considerable practical importance to know the effects of temperature and of concentration on the rate of disinfection. Many attempts have been made in the past to calculate by formulæ the temperature coefficient and the concentration exponent, or dilution coefficient, but according to McCulloch[18] "such formulæ have not proved entirely reliable, probably because of the complicated nature of the phenomenon of disinfection, including as it does the result of many diverse influences not readily expressed by simple equations." Nevertheless, some knowledge of these performance characteristics is essential in order to assess the practical value of a germicide. A single assessment at one temperature and with one end-point is not adequate. Ideally, the rate of kill at two concentrations and at two temperatures should be ascertained, from which it is possible to assess the two coefficients; these should then be confirmed by experimental data at other points. Rahn[19] has suggested that the death times at two or three concentrations at least should be determined.

In general, the activity of a germicide increases as the temperature rises, but exceptions have been noted[7,19,20]. According to Cooper and Haines[21] high coefficients are associated with oxidizing reactions and low coefficients

with reducing reactions. The temperature coefficient is an exponential function, and it appears to be related to the concentration exponent. Each substance has its own temperature coefficient, which is subject to variation according to the range of temperatures being considered and often with the test organism. These points were well demonstrated by Tilley[22] with a series of phenols and alcohols.

Although several different formulæ have been proposed for calculating the temperature coefficient, Jordan and Jacobs[23], like McCulloch[18], were of the opinion that none was really adequate. They found the graph of log. (killing time -10) against temperature to fit the Pearl-Verlhaust equation[24], and therefore suggested this as the most suitable means of calculating the coefficient. In practice, the value seems to be used only occasionally, probably because most germicides are required for use at one or two temperatures only, usually at 37° C. or at about 20° C., to which interest is confined.

The dilution coefficient is a somewhat more important factor. It is exponential and again varies with the type of disinfectant. The value is subject to a large experimental error, as shown by Tilley[25], so that numerous replicates are necessary to obtain the correct value. The most generally accepted formula for calculating the concentration exponent is,

$$\frac{\text{log. initial no. organisms}}{\text{log. survivors}} = Ktc^n$$

where K = the reaction velocity constant; t = time of disinfection; c = the concentration of the disinfectant and n = the concentration exponent. With a fixed end-point such as that of a complete kill, this can be simplified to,

$$tc^n = \text{a constant } \textit{or}$$
$$n \log c + \log t = \text{a constant.}$$

According to Jordan and Jacobs[26] this expression fits observed values when t is the *virtual sterilisation time*, but different values of n are obtained when 99·9 per cent. or 90 per cent. mortality times are used.

The significance of the concentration exponent is that when it is high the germicidal activity of a substance, and consequently its disinfecting time, is markedly affected by small changes in concentration. On this basis, therefore, it is essential to measure the death-rate of a disinfectant at not less than two concentrations; information obtained from a single test with an end-point determined at a fixed interval of time is incomplete, and can be misleading.

Bacteriostasis

Because of the differences in virility of the individual cells of a bacterial population, it is not difficult to appreciate that, with certain disinfectant treatments, the less resistant cells are killed easily, others may be partially damaged or inhibited, whilst the most resistant ones may be completely unaffected. The proportions of cells falling into these categories depend on the conditions of disinfection, and cessation of viability in a population

must be a progressive phenomenon. There can be no sharp distinction and no sudden transition between bacteriostatic and bactericidal conditions. In this connection, Dubos[27] wrote "the difference between bacteriostatic and bactericidal effect is often of a quantitative rather than of a qualitative nature," and Price[28] was of the same opinion stating that "sensitivity to inhibition and sensitivity to death cannot be separated. . . . Indeed, it may be questioned whether one often sees inhibition free from death, except in so far as one preceeds the other." These findings were supported on a quantitative basis by Cook[29] as a result of his evaluations of the bacteriostatic activities of phenol against a variety of organisms. On the other hand, Rahn and Van Esseltine[30] believed bactericidal and bacteriostatic actions to be fundamentally different, the latter being determined entirely by the reversibility of enzyme and other reactions. But in a complex and delicately balanced system such as that of the bacterial cell, the necessary reversibility may easily be upset resulting in the ultimate death of the cell.

Under conditions of prolonged bacteriostasis, it is said that certain changes can take place which ultimately render the cell incapable of reproduction. Clearly, the cell must either adapt itself to proliferate in its new surroundings or it becomes moribund and so must be considered dead. This is of importance in disinfectant testing, for, unless due care is taken to eliminate bacteriostasis, misleadingly high results in disinfectant tests can be obtained. In the majority of instances provision of a culture medium in adequate volume for the surviving bacteria to proliferate is sufficient, but in others a more positive approach is necessary because the disinfectants are strongly adsorbed to the bacterial surface. Thus the mercurials, which act on the thiol receptors of bacteria[31], are most effectively neutralised by adding excess of a thiol compound, such as thioglycollic acid, to the medium; the quaternaries, being cationic surface active agents, are neutralised by certain anionic or non-ionic compounds, such as "Lubrol W"[32] and lecithin in "Tween 80"[33] or in "Lissapol N"[34].

Standard Methods of Testing

The methods of estimating the activities of germicides all stem from the original work of Koch[35] and of Kronig and Paul[36] towards the end of the last century. Progress in the development of testing techniques, somewhat slow at first but rapid during the last two decades, has proceeded in two main directions: (*a*) methods applicable to substances used for the disinfection of inanimate objects, and (*b*) methods applicable to substances intended for use on living tissues, including wound surfaces. The procedures followed in the two types of test are fundamentally different. For preparations included in the first group, commonly called "disinfectants," the principal basis of assessment is by phenol coefficient tests, although other tests are also used which take cognisance of the effect of organic matter either in solution or in suspension, of the types of organism to be treated whether they are sporing or non-sporing, and of the physical state of the organisms whether they are in suspension or on surfaces. For germicides intended for disinfecting living tissues, that is those used

clinically in surgery or in personal hygiene and conveniently called "antiseptics," a more diverse series of tests are available depending on whether the preparation is to be used in liquid form or compounded in solids, pastes or ointments for disinfecting the skin, for oral application or for treatment of wounds. The type of infection to be dealt with by this group must be taken into account. Host tissue cell toxicities are also important, but they are outside the scope of this present discussion; so are also the recently discovered chemotherapeutic agents and antibiotics, except in so far as the studies of the latter has revealed much valuable information on the mechanism of disinfection generally[5,11,37].

Phenol Coefficient Tests

The principal methods of determining the germicidal activities of disinfectants are by one or more of the phenol coefficient tests, of which the two official British methods are the Rideal-Walker[38] and the Chick-Martin tests[39]; the Food and Drug Administration (F.D.A.)[40] and more recently the Association of Official Agricultural Chemists (A.O.A.C.)[41] methods are used in the United States. They all use the same basic principles, namely, phenol is the reference standard; the test solutions are simple dilutions in water, with the exception of the Chick-Martin test which employs yeast as added organic matter; the test organisms are selected strains of *Salmonella typhi* or of *Staphylococcus aureus*; the killing time is relatively short, being measured in minutes; the end-point is that of a virtual complete kill, and germicidal activity is expressed as a coefficient related to the lethal effect of phenol.

In this country, the Chick-Martin coefficient serves under the Diseases of Animals Act, 1950, as a basis for calculating dilution awards for phenolic disinfectants; it is also recommended as a means for selecting suitable disinfectants for certain hospital uses[42]. In the United States, a dilution award of 20 times the phenol coefficient is usually given for a disinfectant, provided it is sustained in the recently introduced "Use-Dilution Confirmation" test.

Full details of all of the techniques will not be discussed here but it is desirable to mention some of their salient features and then to draw attention to some of their disadvantages. The Rideal-Walker method was the first phenol coefficient test to be devised. It was originally published in 1903, since when it has undergone several modifications leading to its present form. All other tests are, in effect, modifications and improvements (*sic*) on the original method. The Rideal-Walker test uses a selected strain of *Salm. typhi* (*N.C.T.C.* 786) grown in a medium containing 2 per cent. of peptone (Allen and Hanbury's Eupeptone No. 1), 1 per cent. of Lab-Lemco and 1 per cent. of salt. Serial dilutions of the disinfectant and of phenol at 17 to 18° C. are inoculated with a 24 hour culture, and subcultures are made into the standard broth at $2\frac{1}{2}$ minute intervals. The phenol coefficient is obtained by dividing the lowest concentration of the disinfectant which kills the culture in $7\frac{1}{2}$ but not in 5 minutes by the lowest concentration of phenol which gives the same response. The U.S.F.D.A. method uses a somewhat different medium

containing 1 per cent. of Armour's peptone, 0·5 per cent. of Liebig's beef extract and 0·5 per cent. of salt. The Hopkin's strain of *Salm. typhi* is used and the end-point of the test is the lowest concentration of disinfectant and phenol killing in 10 minutes but not in 5 minutes at 20° C. The culture medium used in the F.D.A. test gives organisms of rather higher resistance than those grown in Rideal-Walker medium, and this is said to result in somewhat lower phenol coefficients. The A.O.A.C. method is a modification of the F.D.A. method, the principal point being that one of a number of subculture media may be used depending on the nature of the disinfectant substance under consideration; thus, a thioglycollate medium is used for dealing with mercurial disinfectants and a lecithin-Tween broth for cationic surface-active substances.

It is of interest to note that quite recently the "Use-Dilution Confirmation" test was introduced[43] as a supplement to the A.O.A.C. phenol coefficient method. The purpose of this test is to confirm that a dilution award on a disinfectant of 20 times the phenol coefficient is, in fact, satisfactory. In this test, small metal "penicillin assay" cylinders, 10 in number, are infected with a test organism, either *Salmonella choleraesuis* or a *Staph. aureus*, dried for a short period and then immersed for 10 minutes in the chosen disinfectant dilution. Each cylinder is then transferred to a nutrient broth and incubated. A satisfactory test requires complete absence of growth. In the event of any growth, the disinfectant dilution must be adjusted appropriately.

Both this and the Chick-Martin technique are more realistic in terms of practical usage. The Use-Dilution test takes into account the effect of organisms dried on a surface, and the Chick-Martin test includes organic matter in the form of yeast cells in the disinfectant dilutions. Both are important, as dried organisms may be more difficult to sterilise, and it is well known that organic matter generally depresses the activity of most disinfectants.

Disadvantages of Phenol Coefficient Tests

Phenol is the chosen standard for most disinfectant tests because (*a*) its disinfecting properties are well established, (*b*) it is a compound of known stability and purity, and (*c*) it is desirable in all tests of this type to have a standard reference material as a control. Phenol is not, however, an ideal standard, for it has a high dilution coefficient and killing rate, and its physical characteristics in solution are often quite unlike those of the disinfectant fluids with which it is being compared. Thus, as pointed out by Berry[44], all phenol coefficient tests contravene a fundamental requirement of all biological tests, namely, that like should be compared with like. There is as yet no satisfactory solution to the problem. *p*-Chloro-*m*-cresol has been tried with no greater success[45].

The result of a phenol coefficient test simply gives the information that under certain conditions a certain dilution of the disinfectant in water will kill a selected strain of *Salm. typhi* or *Staph. aureus* in a given short time. These conditions can give only limited information on the actual value of the disinfectant in practice, and, therefore, the tests can be treated only as

minimum performance tests or as a means of batch standardisation. Moreover, if the germicides are markedly different chemically from phenol, the results may be misleading. They give no information on the effect of time, concentration and temperature, of the influence of organic matter and of the significance of other bacteria. It is only when the A.O.A.C. "Use-Dilution Confirmation" or the Chick-Martin technique is employed that a phenol coefficient test begins to approach practicality.

All phenol coefficient tests suffer the technical disadvantage that their results depend on the assessment of an apparent absolute kill of a given test inoculum. This choice of end-point is unfortunate because it does not necessarily represent sterility but merely "absence of viable organisms in the sample removed and diluted with broth. This sample is small, and a varying percentage of viable organisms may still be alive when the broth yields a negative result."[46] This point had earlier been mentioned by Thaysen[45] who, by actual plate counts, showed that the apparent end-point of total kill is entirely dependent on the amount of sample taken. Vastly different points were obtained with, for example, sub-culture volumes of one loopful and 2·8 ml., the former giving a "kill" in 15 minutes, whereas the latter required 45 minutes. In addition, Thaysen drew attention to the hazards of chance survival of badly damaged cells in the medium selected. The nutritive properties of the culture medium undoubtedly plays an important part in this respect. This is well illustrated by results obtained by Hampil[47] on a series of alkyl resorcinols using media made with two different peptones. For the *iso*hexyl compound she recorded a coefficient of 40·9 in one medium and 75 in the other, and for the heptyl compound coefficients of 49 and 127 respectively. Beef extract can also cause fluctuations in resistance, to such an extent that, according to Goetchius[48], day to day variations in the phenol coefficient can oscillate between 155 and 500. It has also been noted that marked changes in resistance of a culture can be produced by altering the temperature of incubation by as little as 1° C.

The spacings of the dilutions of the phenol and of the disinfectant, and the permitted range of responses of the phenol consequent on the variations in resistance of the cultures, all militate against the precision of results obtained by any phenol coefficient test. Ortenzio *et al.*[49] have recorded variations in resistance of *Salm. typhi* to phenol at dilutions between 1 in 65 and 1 in 100 when grown and tested under standard conditions. The effects of these fluctuations on the values obtained by the F.D.A. method were studied by Rahn[7]. According to his calculations, the death-rate constant for phenol can fluctuate by a factor of 2·4 and still remain within the acceptable lethal limits of the test. Likewise the constant for the disinfectant can fluctuate by a factor of 1·5, giving a total fluctuation for the test of $2{\cdot}4 \times 1{\cdot}5 = 3{\cdot}6$. Rahn stated that a phenol coefficient test cannot be more accurate than the death-rate constants, and so was led to the conclusion that "this [360 per cent.] is the error which the specified conditions present and to which must be added the personal error of the experimenter" to which he added the sweeping statement "They appear to be more accurate because the larger deviations

are not published." The same argument applies to the Rideal-Walker test, although to a rather less extent, because the conditions of the test and the spacings of the dilutions are more closely specified, hence the errors are not likely to be so great. It must also be borne in mind that the deviations quoted are maximal; in practice, the cultural and test conditions in any one laboratory are sufficiently constant to produce lower deviations, but this might not be true of *conditions* between laboratories.

Newer Approaches to Testing

Several new tests, or improvements on existing ones, have been proposed from time to time, all devised with a view to overcoming the difficulties of the present standard methods. The most important points to be dealt with are those concerning the definition of the end-point, namely, the choice of percentage kills up to 100 per cent., and the amount of sample required to assess this. Thaysen[45] was amongst the first to criticise the existing phenol coefficient tests on these grounds, and later Withell[46] proposed determining the time for a 50 per cent. kill and he suggested that this be done with different concentrations of the disinfectant, at different temperatures and with various organisms. Whilst agreeing that an end-point of less than a virtual total kill was desirable, Jordan and Jacobs[26] criticised adversely the 50 per cent. end-point, because the concentration exponent at this mortality level is not constant. In addition, there are obvious technical difficulties in determining a 50 per cent. survival from a large bacterial population. Jordan and Jacobs were of the opinion that the 99 or 99·9 per cent. mortality times would prove more satisfactory, as both give a linear response between log. concentration and log. time. These levels have the further advantage that they can be more easily determined by plate counts.

Needham[50] proposed a nephelometric method in which survivor levels of approximately 3, 2 and 0·75 per cent. are estimated. He chose a sub-culture volume of 0·5 ml. in order to overcome the inherent sampling error of the usual single loopful and he employed a simple peptone medium which he claimed gave more constant and reproducible results than do other more complex media. The end-point is obtained by incubating the sub-culture broth for exactly five hours at 37° C., after which the opacity developed from the surviving proliferating cells is measured nephelometrically and compared with the appropriate dilutions of the standard culture incubated under identical conditions. The mortality curves in relation to concentration for different disinfectants do not always run parallel, but where a comparison of activity between two preparations is required, it is suggested that the means of the concentrations giving the 3, 2 and 0·75 per cent. survivals should be used.

A method devised by Bean and Berry[51] particularly for testing disinfectants in soap solutions reverts to a virtual complete kill, but it uses a multiple drop technique to assess accurately the end-point. The scheme of the test is that immediately after the serial dilutions of the disinfectant have been inoculated with the test organism, six uniform drops are delivered into each of a series of sterile tubes at 20° C. After a measured

time, broth is added to each tube which is then incubated and the growths recorded. They later showed[52] that the use of extensive replication and of constant sampling volumes withdrawn immediately after inoculating the bactericide, combined with suitable short sampling intervals, give estimates of mean extinction times which are comparable with those obtained by any other technique. Using a somewhat similar technique, Cook and Wills[53] established a correlation of the extinction method and the percentage survivors at shorter contact times as a means of estimating bactericidal activity. In these opinions and those of Needham, Thaysen, Withell and others we see expressed diametrically opposed ideas about what constitutes the most precise and acceptable end-point.

A number of other proposed testing techniques are worthy of mention, not so much because of their immediate value but to illustrate some of the newer approaches to the problem of assessing germicidal activities. Several investigators have used methods based on the inhibition of specific enzyme activities within the cell. Thus, Roberts and Rahn[54] observed complete enzyme inactivation at bactericidal levels but not with bacteriostatic doses; Sykes[55] suggested that inhibition of succinic acid dehydrogenase activity might be used for this purpose; Sevag and Shelburn[56] correlated closely the retardation of respiration and of growth of streptococci when treated with sulphonamides; Knox *et al.*[57] showed that the death of *Bact. coli* treated with cationic detergents parallels the inhibition of the lactic acid oxidase, and Robertson and Oliver[58] similarly correlated loss of decarboxylase activity with loss of viability in certain organisms after treatment with heat or chemical disinfectants. It is unlikely that any of these methods could become generally applicable mainly because of the extreme divergencies in the modes of action of germicides of different types.

Manometric methods have been suggested[59,60] in which the effect of disinfectants on the oxygen uptake of bacteria is said to parallel their influence on viability. Bronfenbrenner *et al.*[59] found this particularly true for *Staph. aureus* and *Bact. coli* when the respiratory end-point is taken as a reduction of 50 per cent. in oxygen uptake between the fifteenth and twentieth minutes. Hugo[61] reviewed several of the proposed manometric methods and came to the conclusion that such attempts at evaluating disinfectants may lead to false conclusions because their effect on different enzyme systems are not necessarily the same, and the reaction does not necessarily parallel cell viability.

Maurice[62] made the somewhat novel observation that suspensions of bacteria treated with basic dyes increase in turbidity, and the rate of this increase is enhanced by adding various phenols and other compounds. The increase is related to the concentration of antibacterial agent, and so it was found possible to relate the "phenol equivalent activity" obtained from the test with the actual lethal activity of the substance. According to Mandels and Darby[63], microbial cells freshly inoculated into a nutrient medium increase in volume and the increase is related to the viability of the cell population. On this basis, the authors devised a test primarily for testing fungicidal agents but which they claimed could be adapted to

disinfectant testing. Finally, Fischer and Larose[64,65] devised a method for assessing antibacterial activity in terms of affinity for wool. The basis of this test is that there is a common α-keratin structure in wool and in the bacterial cytoplasmic membrane which renders wool and bacteria similar in their responses to disinfectants.

Although each of these tests has its merits and it cannot be denied that the correlations cited must exist under certain circumstances they obviously are of no value for determining the general antibacterial properties of a compound; they neither give any measure of its relative bactericidal and bacteriostatic activities, nor do they give any indication of its selective action against different types or species of bacteria.

Other Testing Techniques

In addition to the standard phenol coefficient tests, a number of other tests have been devised to assess the germicidal activities of certain types of antibacterial preparations intended for specific uses. Because of the interest centring round these tests, they justify some separate consideration. They include the testing of preparations for surface disinfection and for skin and wound disinfection as well as the whole range of creams, ointments and other pharmaceutical preparations used in the treatment of various bacterial and fungal infections.

Tests of this type are all characterised by the facts that they are more realistic than phenol coefficient tests in that the conditions in terms of the menstruum, type of organism, time of exposure, etc., more nearly simulate the actual conditions of usage, and they generally do not use a total kill as the end-point.

Surface Disinfection Tests

Tests for assessing the activities of disinfectants on inanimate surfaces were first devised by Koch[35] and by Kronig and Paul[36], but they have only come into prominence in the last twenty years because of the increasing importance of disinfection in the food and canning industries. Weber and Black[66] were not convinced of the necessity of including deliberately infected surfaces in such assessments, having obtained identical results by a suspension method, but this opinion does not seem to be shared by the majority. The reasons for this are probably twofold: first, most preparations intended for surface disinfection contain a detergent, which obviously plays a significant part in any washing process, and, secondly, organic matter adhering to a surface may exert a marked protective effect on the bacteria.

Jensen and Jensen[67] used a technique in which a test culture is dried on cover slips for a short period after which they are immersed for two minutes in the disinfectant dilutions and then cultured in broth. Mallman and Haines' modification[68] uses infected glass cylinders (this technique was subsequently adopted as the basis for the United States "Use Dilution Confirmation Test"), and Stedman, Kravitz and Bell's modification[69,70] employs small squares of a selected test material—metal, glass, linoleum, etc.—and allows a disinfecting period of ten minutes; a kill of

99·9 to 99·99 per cent. is considered a satisfactory end-point. In later publications Stedman, Kravitz and Bell[71] discuss the significance of detergency and the influence of porous surfaces on the activities of disinfectants.

Because of the importance of surface disinfection in dairying a number of tests specific for this purpose have been devised[72–76]. All include milk solids and they differ only in the type of test material used—glass slides, rubber strips, metal strips or metal cans—in the time of disinfection and in the method of assessing the end-point. In general a kill of the order of 99·9 per cent. is accepted.

Testing Quaternary Ammonium Compounds

The most numerous and controversial of these tests are concerned with the surface active cationic group of substances, the quaternary ammonium compounds, and an extensive literature has accumulated around them. The compounds and their properties have been well described by Lawrence[77] and their particular application in the disinfection, or "sanitisation," of food and beverage utensils have been discussed by Resuggan[78]. Difficulties were first encountered in testing these compounds because of their high surface activities and the consequent bacteriostatic carry-over on subculture which led to indeterminate and false-high values. The position was rectified, however, by the discovery of a number of antagonists or inactivating agents, amongst which are phospholipids[79], lecithin with "Tween 80"[33] or with "Lissapol N"[34], suramin sodium[80], agar and milk[81]: nutrient broth alone is not an inactivator[82,83], and this explains the reason for the initial testing difficulties.

The false-negative results frequently obtained were at first thought to be due to the test organisms being massed on the walls of the tube by the action of the quaternary compound, and to overcome this Klarmann and Wright[84] devised a semi-micro test method in which the whole of the test solutions could be cultured. As was to be anticipated, the results obtained were lower, due not so much to the elimination of sampling errors than to the use of a much greater subculture volume. Davies[32] believed the discordant results to be due mainly to the bacteria being clumped and he proposed a testing technique which (*a*) allows a sufficiently large sample to be subcultured to ensure including some bacterial clumps, (*b*) provides means for breaking up the clumps, and (*c*) gives an actual count of surviving bacteria at chosen time intervals. He used a 1 per cent. solution of "Lubrol W" as the inactivating agent. The method is similar to that proposed by Weber and Black[66]. They used two test organisms, *Staph. aureus* and *Bact. coli*, and obtained the end-point by plating the disinfectant mixture, after quenching with lecithin-Tween, at intervals between 15 and 300 seconds contact. They claimed that the only satisfactory end-point is that of a total kill, on the argument that the variable death rates of bacteria under the influence of different disinfectants render other end-points unacceptable. They also claimed that their method gives a reliable practical dilution value if the end-point is assessed after 30 seconds exposure. Cousins[34] employed a somewhat different technique, including

milk solids as organic matter in the disinfectant dilutions. She used only a two minute disinfection period employed lecithin in "Lissapol N" as the inactivator and plated decimal dilutions to obtain the end-point. This method is more realistic in that it takes into account the effect of organic matter, but the choice of only two minutes contact, although simulating the average immersion time of utensils for washing up, is rather short for experimental observations.

Skin Disinfection

In considering skin disinfection, it should be remembered that, owing to its particular structure, it is not possible ever to achieve complete sterilisation of the skin, and so "skin antisepsis" is probably a more appropriate term. Any germicide applied to the skin will only deal with those micro-organisms with which it comes in contact, that is, the transient types in or near the surface; it cannot touch those resident deep in the pores of the skin. For this reason, many workers believe that a type of germicide should be employed which will retain its activity on the skin for some time, and a test on these lines was recently described by Powell and Culbertson[85]. It is similar to one used in the author's laboratory for some years. Briefly the technique consists of applying known dilutions of the germicide to small marked areas of the skin and then, at selected time intervals up to several hours, infecting these areas with a culture of *Staph. aureus* and assessing survivors after ten minute contact by swabbing and plating.

A practical *in vivo* method of assessing the value of skin disinfectants, particularly for those in which soap or other detergents are employed, is that devised by Price[86] or one of the several modifications subsequently suggested[87,88,89]. All of these tests employ some variation of a multiple hand-basin washing technique in which the hands are first washed for a fixed time under controlled conditions with the given germicide dilution and then rinsed in several basins of sterile water which are subsequently plated to count surviving bacteria. Other types of test have been suggested[90–94], but Price[95] has expressed the opinion that "the serial-basin hand-washing test is the only one proposed so far which is able to measure reliably the skin disinfectant action of mechanical cleansing or chemical germicides." The method is, however, cumbersome and requires a large number of test subjects in order to obtain reliable results.

Many skin disinfectants are made with a phenolic germicide in a soap or other detergent base, a typical example being Solution of Chloroxylenol B.P. Varying results on the effects of soaps on the activities of phenolic compounds have been reported; Hampil[96], for example, found that sodium oleate depresses the activities of phenol, cresol and hexylresorcinol, whilst others have recorded enhanced activities. Frobisher[97] and Cade[98] observed variable effects according to the particular phenol used and the concentration of soap in the solution. These differences can be accounted for on the basis that small concentrations of soaps reduce the activities of phenols, but above certain concentrations the soaps give rise to micelle formations and these act as centres for solubilising substances which are

otherwise relatively insoluble. The subject was studied in detail by Bean and Berry[99] using chloroxylenol and benzylchlorophenol in potassium laurate solutions. They showed that the bactericidal properties of these solutions are related to the concentration of the phenols in the micelles and not to the overall concentration in the system as a whole.

Testing Antiseptics

For the large group of preparations popularly known as "antiseptics," a number of tests have been devised according to the purposes for which the preparations are recommended. Thus, with some preparations the nature of the organic matter, serum, pus or saliva, and the types of bacteria to be dealt with may be important, and so tests are devised with strains of staphylococci, streptococci, *Bact. coli*, *Pseudomonas pyocyanea* and *Proteus vulgaris* in the presence of serum or whole blood. Speed of action may also be important, depending on whether the germicide can be left in contact for only a short period of seconds or minutes or for a longer duration of several hours. It is usual to make such assessments at blood heat rather than at normal room temperature, and it may be desirable to have some comparison of the bactericidal and bacteriostatic activities.

With semi-solid pharmaceutical preparations such as ointments and creams, two types of test are generally used, (*a*) one of the many variants of the agar plate diffusion test, such as that quoted by Ruehle and Brewer[40], to assess penetrability, and (*b*) a lethal test to assess the killing properties of the preparation. One of the simplest forms of the latter is to inoculate the surface of a serum agar plate with the test organism, incubate for a few hours to establish growth, smear the semi-solid germicide over the surface and then at selected intervals cut out small discs of the treated agar and culture to determine survivors. Somewhat different methods are given by Foter and Nisonger[100] and by Walters[101].

Several *in vitro-in vivo* type tests have also been devised involving tissue toxicities[59,102], tests for toxicity to leucocytes[103,104] and tests in egg membranes[105] as well as those in which treated infected materials are introduced into experimental animals[106,107,108]. Of the last group of tests, the one most generally accepted is that of Nungester and Kempf[109], a revival of the method first proposed by Christiansen[110], in which the tip of the tail of mice is infected with a selected organism, treated for a short period with the disinfectant and then amputated and inserted in the peritoneal cavity of the animal. The limitation of the test is that a mouse-pathogenic organism must be used, but the method is said to give consistent results.

It has not been possible in this review to consider anti-fungal preparations. Because of their importance both medically and industrially a great deal of attention has been paid to them, but it must suffice here to state that in general fungal spores show considerable variations in resistance between the different genera and species, and they are more resistant than most bacteria. Thus, an effective antibacterial preparation is not necessarily active against moulds. The tests devised for assessing activity

against moulds are many and varied, but they do not appear to be as reliable as those used for bacteria.

Conclusions

The study of disinfection has progressed in two main directions, (*a*) investigations into the mode of action of disinfectants on the bacterial cell, and (*b*) the development of methods of testing their efficiencies under diverse conditions. The first line of investigation has been followed mainly in connection with the antibiotics and other chemotherapeutic agents, as exemplified in the extensive work of Gale[4] and his colleagues[11,111–113], of Albert *et al.*[114,115] and of McIlwain[116], and in several reviews and symposia, e.g.[37,117–120]. It is natural that such investigations should have been concentrated primarily on the groups of substances likely to be of chemotherapeutic value because of their special interest in medicine and the desire to produce even more effective agents. Nevertheless, they also give valuable information on the likely mode of action of disinfectants and germicides generally.

Also embraced in this type of investigation is the fundamental work on the variation in resistance of the cells of a bacterial population consequent on its cultural condition and the development of mutants or variants. Because of the fluctuations observed in routine daily cultures and in cultures grown in different laboratories this aspect is of considerable importance in devising any type of test, phenol coefficient or otherwise, in which the resistance of the test organism may be involved.

Turning to the second group of investigations, that is, those concerned with the development of testing methods, it is clear that there is a strong trend away from the classical phenol coefficient tests to methods which are more directly related to conditions of usage. Whilst it must be agreed that the former have proved of value in the past, and still continue to do so if used in their right context, it cannot be denied that on certain points discussed earlier they are unsound. The methods are acceptable if they are confined to standardising phenolic disinfectants or if the results obtained are considered only to be a means of determining minimum performances.

One of the main questions with any disinfectant test is that of deciding what is the most suitable end-point. Opinions are divided; it sounds better to report in terms of a total kill, but there are cogent arguments against this. This is a matter of fundamental importance and much more work is necessary before an *ex cathedra* opinion can be expressed.

Of the more recent testing techniques proposed, nearly all dispense with any reference standard, and the conditions more nearly represent those encountered in actual use, in the way of the menstruum and test materials employed, the time of contact and temperature of disinfection. This applies particularly to the large and varied groups of germicidal preparations used in surgery and in personal hygiene. In this connection, Reddish[121] has proposed a "panel of methods for testing antiseptics" from which the potentialities of any preparation can be determined. Whilst serving as a useful guide, it should not be assumed that these are

the most suitable and the only tests to apply under all circumstances. It is now accepted that no single test can be devised to give all the essential information.

There seems to be little doubt that, with the expansion in the range of germicidal substances now in use and the many diverse uses to which they are put, the testing of disinfectants and antiseptics, rather lightly dismissed in the past, is assuming a more important role in pharmaceutics and in microbiology generally.

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