THE EVALUATION OF BACTERICIDES

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(With 3 Figures in the text)

CONTENTS

INTRODUCTION

The Rideal-Walker (1903) and Chick-Martin (1908) methods for the evaluation of bactericides both depend on a technique of comparison with a standard bactericide. In this respect they agree with other methods of biological assay; but they depart from the rules of biological assay in one fundamental characteristic. When two samples are compared by their action on living organisms, they should always be samples which contain an *identical* active principle. It has been found in pharmacological work that if two different active principles are compared on the same organism or organ, the method is unsatisfactory because the sensitivity of the test organism to one principle often varies in a different way from the sensitivity of the test organism to the other principle (Gaddum, 1940). From the nature of the problem of evaluating bactericides it is inevitable that two *different* active principles should be compared. It follows that the Rideal-Walker and Chick-Martin methods will not give satisfactory results unless the sensitivity of the test organism to each bactericide varies in the same way. Fig. 1 illustrates this point. It is impossible to measure this sensitivity accurately by the present tests. By comparing the coefficients obtained when different standard times are chosen, a measure of the variation in sensitivity is, however, obtained. The only way in which a variation in sensitivity can be accurately estimated is to follow the course of the reaction between bactericide and organism by a series of viable counts. If this is done, the standard deviation of the logarithm of the survivor times (λ) and its reciprocal (b), the slope of the characteristic curve, can be calculated. If the response of the organism to the two bactericides results in different values of λ, no end-point method of assay with that standard bactericide and organism will yield satisfactory results; counting methods will show why this is so. The remedy would be to use another standard chemical. As far as I am aware there is no information on values of λ and b in comparative bacteriological work. In the absence of such information it would be wise to restrict the present standard tests to substances of analogous chemical nature, i.e. to use phenol as a standard for phenolic bactericides only. This is illustrated by Chick & Martin (1908) where phenol was compared

with Disinfectant 'A' by the Rideal-Walker test, choosing three standard times—2·5, 12·5 and 40 min. The phenol coefficient of Disinfectant A (a mixture of cresols) varied only in a small degree—being 15 and 17 and 16 respectively. It is probable that end-point methods do not give arbitrary results when substances of similar nature are compared. At the same time end-point methods do not indicate from one experiment whether the result is arbitrary or not. Counting methods would demonstrate a difference in response of the organism.

The comparative rates of death of the test organism in two bactericidal solutions permit the calculation of a value, the phenol coefficient, which is given to the bactericide under test. The comparative efficiency of the two bactericidal solutions is estimated approximately by end-point methods. I am not concerned in this part of the paper with

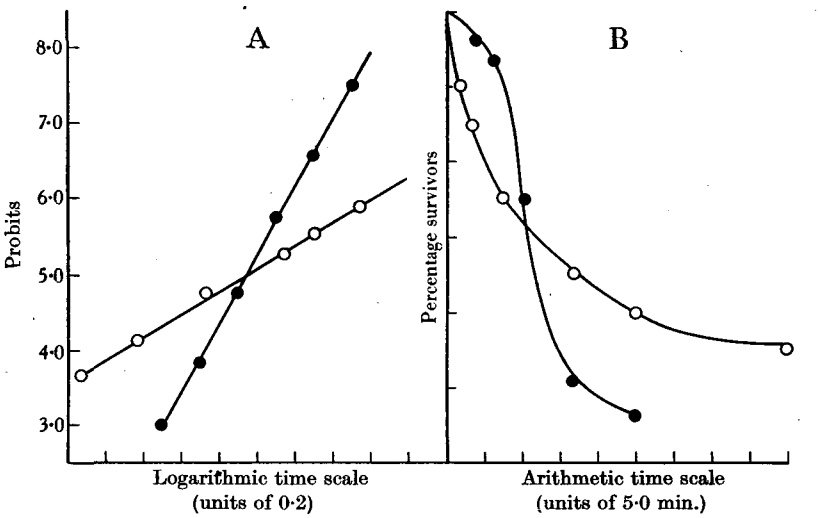

Fig. 1. (A) The two lines represent probit-logarithm of time lines, derived from time-survivor curves of organisms in two lethal solutions. The lines are of different slope, i.e. the standard deviation of the logarithms of the survivor times (λ) is different.

(B) These curves are derived from the same data as 'A'. It represents the approximate form of time-survivor curves corresponding to the curves in 'A'.

Any attempt to compare the activities of two lethal solutions, which yield time-survivor curves similar to those of 'A' and 'B', by calculating relative death-rates, will give results which will vary with the extent of the reaction, over which the death-rate is followed (compare Fig. 3).

the inconsistencies of transferring this value, obtained at one particular pair of concentrations, to a quotient which is apparently intended to apply generally to the test bactericide at any concentration. This question will be referred to in the discussion. The experimental work summarized in this paper is concerned with a method of comparing the bactericidal efficiency of two *solutions* by following the death-rate of the organism in the solutions by viable counts. Two methods of analysing the counts are reported, and later in the paper the theoretical aspects of end-point methods are discussed.

A series of experiments in which the test organism was inoculated into two bactericides and the death-rate followed has been previously recorded by Withell (1942). The experiments were used in that paper to show that the logarithms of the survival times were approximately normally distributed, and the same set of experiments is used in this paper to estimate the accuracy of a series of determinations of bactericidal activity,

measured by the rate of death of the organisms. All 'experiment numbers' refer to a preceding paper in this series (Withell, 1942), and all counts are given in full in Appendix II to that paper.

THE METHOD

It is not the intention at the moment to describe in detail a new method for comparing bactericides, but to indicate how the fundamental disabilities of the present tests can be remedied in so far as they are concerned with the comparison of two bactericidal solutions. The general conditions for such a test have been carefully set out by Chick & Martin (1908). They mention the following standard conditions which are applicable to the method here described:

(*a*) A standard temperature. The temperature used for all experiments recorded in this work was $20 \pm 0{\cdot}1°$ C.

(*b*) A standard number of organisms. Chick & Martin (1908) stated: 'To obtain a constant result with a particular disinfectant it is essential to work always with the same concentration of organisms, but in comparative experiments this is not necessary as long as they are consistent. Therefore, if the comparison of germicidal value is always made against a standard, the standard being tested at the same time, against the same amount of culture, no inaccuracy occurs even if the number of organisms should vary from time to time.' Such variations are inconvenient, 'as they make it difficult to arrange beforehand the necessary + and − results'. This latter objection does not apply in the same force to experiments involving counting, because many counts are made in each experiment, and as different dilutions are employed, it is only occasionally that a worker cannot follow the course of reaction. I have some evidence, which will be given later, that the concentration of organisms when approximately the same number are added to each bactericide, does not affect the coefficients.

(*c*) The resistance of the culture. The experiments are all comparative, and a variation in resistances will only result in a difficulty in forecasting the necessary + and − results. A change of resistance does not affect counting methods in the same degree as 'end-point' methods for the reason stated in (*b*).

Chick & Martin (1908) showed that if *Bact. typhosum* was kept at 37° C. and successively subcultured, the resistance of suspensions of that organism increased. This is also true for the *Micrococcus* used in most of the comparative work in this paper. Table 1 shows the increase in time for 50% deaths in successive experiments.

TECHNIQUE

The general method of performing these experiments was so arranged that the experiments were as nearly as possible identical. Full details are given in a previous paper (Withell, 1942, Part II), but they are summarized below.

The solutions of bactericides. These solutions were made up immediately before each test in special water that had been doubly distilled on the previous day. The second distillation was from an all-glass Pyrex still, into a sterile Pyrex flask. The water was preserved overnight in plugged flasks, sealed with tin-foil.

The organisms. A *Micrococcus* isolated from the atmosphere, and a strain of *Bact. coli* (type I) were used in a separate series of experiments. These organisms were used as they showed no evidence of clumping in bactericidal solutions. A 24 hr. culture on

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nutrient agar medium was washed off with Ringer solution and approximately the same number of organisms added to each solution under test. The solutions were kept at 20° C. in a thermostatically controlled bath, with variation of ±0·1° C.

The counts. From time to time samples were removed from the bactericide, and the number of viable organisms determined by roll-tube counts (Wilson, 1922; Withell, 1938). Each final dilution was inoculated into five roll tubes, and each mean count is the average of these five separate counts. The medium for a considerable portion of the work was a 6 hr. pancreatic digest of lean bullock buttock beef: later 'C.C.Y.' medium (Gladstone & Fildes, 1940) was used with success. Twelve duplicate experiments were made in which phenol 0·5% and para-chlor-meta-cresol 0·05% were compared using the *Micrococcus*

Table 1

	Phenol 0·5%		Para-chlor-meta-cresol 0·05%	
Exp.	Conc. bacteria per c.c.	Time for 50% deaths min.	Conc. bacteria per c.c.	Time for 50% deaths min.
224	102,600	9·95	257,000	7·08
233	420,400	28·18	375,300	14·13
236	267,000	28·18	262,100	21·38
241	247,000	51·00	287,400	33·88
242	311,600	54·95	297,900	50·12
245	337,800	63·10	364,300	47·86
247	312,400	50·12	303,800	23·99
231	$19{\cdot}6 \times 10^6$	17·78	$17{\cdot}00 \times 10^6$	4·78
238	—	—	$17{\cdot}50 \times 10^6$	22·39
249	$37{\cdot}0 \times 10^6$	63·00	$36{\cdot}67 \times 10^6$	63·00

and four similar experiments with *Bact. coli.* From all these experiments the counts have been analysed in two different ways, each method being designed to estimate the comparative bactericidal activity of these two solutions.

The analysis of the results. An attempt has been made to analyse the results obtained: (*a*) by calculating the comparative reaction rates, (*b*) by estimating the comparative time for 50% response by the organism to the lethal agent.

(*a*) *Calculation of comparative reaction rates*

Phelps in 1911 suggested that bactericides should be estimated by following the death-rates of the test organism. From the counts obtained the death-rate is calculated from the formula

$$k = \frac{1}{t_1 - t_2} \log_{10} \frac{N}{n},$$

where $t_1 - t_2$ is the time interval over which the reaction rate is calculated, N the number of viable organisms at time t_1, and n the number of viable organisms at time t_2. When k is estimated in stages over the reaction from count to count, the mean k is calculated. From the values of k at different concentrations and at different temperatures, the concentration exponent n' (Watson, 1908) and the temperature coefficient θ (Chick, 1908, 1910; Phelps, 1911) can be estimated; and from n the true reaction velocity (constant at all concentrations) can be calculated from the formula

$$KC^{n'}t = \log_{10} \frac{B}{b},$$

where K is the true reaction velocity, C is the concentration of disinfectant, n' the concentration exponent, t the time necessary for disinfection, B the initial number of organisms,

and b the final number of organisms. These characteristics K, n', and θ, Phelps suggests, should be determined for each disinfectant and would form a much more accurate and complete estimation of disinfectant activity than any tests based on end-point methods or the calculation of a coefficient from one estimation at one concentration.

This paper deals with the basis of Phelp's suggestion, i.e. the estimation of comparative bactericidal efficiency by a comparison of reaction rates, for K, n, and θ are all estimated by comparative counting experiments.

In Table 2 will be found the mean reaction rates (i.e. mean k's) from each comparative experiment calculated over the whole period of the reaction. The extent of the reaction which has been followed varies in each case, as it is difficult, if not impossible, to forecast exactly the reaction rates. This will be seen from the mean values of k for each solution.

Table 2. *Death-rates of a* Micrococcus *exposed to phenol* 0·5% *and para-chlor-meta-cresol* 0·05%

	Phenol 0·5%			Para-chlor-meta-cresol 0·05%			
Exp.	Initial conc. of organisms per mil.	Mean k	Extent of reaction followed %	Initial conc. of organisms per mil.	Mean k	Extent of reaction followed %	Ratio of mean k's $\frac{k \text{ P.-c.-m.-c. } 0{\cdot}05}{k \text{ phenol } 0{\cdot}5}$
224	102,600	0·0418	96	257,400	0·0463	98	1·12
229	178,800	0·0309	97	140,600	0·05705	99	1·85
231	19,620,000	0·0164	85	17,020,000	0·0273	98	1·66
233	420,400	0·01029	52	375,300	0·0192	53	1·86
236	267,000	0·01186	74	262,100	0·01252	76	1·05
237	211,500	0·08010	53	189,800	0·01013	65	1·26
238	15,180,000	0·00569	50	17,500,000	0·00843	70	1·48
241	247,400	0·01087	70	287,400	0·01324	95	1·23
242	311,600	0·00927	74	297,900	0·11080	78	1·20
245	337,800	0·00438	72	364,300	0·00776	90	1·76
247	312,400	0·01240	99	303,800	0·02404	99	1·94
249	36,890,000	0·00587	94	36,670,000	0·00633	93	1·08
							Mean = 1·46
			Similar data using *Bact. coli*				
262	17,160,000	0·0525	97	21,080,000	0·0371	67	0·700
267	359,800	0·1017	99	341,300	0·0831	99	0·830
268	251,700	0·1152	99	200,400	0·0948	99	0·830
269	369,700	0·0247	83	291,000	0·0240	84	0·975
							Mean = 0·844

In each case the extent of the reaction which has been followed is noted in Table 2. In the last column is given the ratio of the mean reaction rates of phenol 0·5% and para-chlor-meta-cresol 0·05%, taking the mean k for phenol 0·5% as unity.

As far as I am aware Table 2 summarizes a set of comparative experiments which has not appeared in the literature, and gives an estimate of the error of experiments which compare bactericidal activity by methods involving counting. The mean relative activity of 0·05% para-chlor-meta-cresol solution compared to phenol 0·5% was 1·46 (against the *Micrococcus*) and 0·844 (against *Bact. coli*). The mean percentage deviation from the mean of these observations on the ratio of activity of the two solutions made with the *Micrococcus* was 20·1 (12 experiments). The standard deviation was approximately 25%. The four observations using *Bact. coli* had a mean percentage deviation from the mean of 6·8. This difference in the relative activity when two different organisms are used has been noticed before in a more striking manner—Chick & Martin (1908, Table VI, p. 672) record the 'carbolic acid coefficient' of mercuric chloride as about 80·0

when measured against *Bact. paratyphosum* and less than 0·21 when measured against *Staph. aureus*. Phelps's (1911) original suggestion was made on the assumption that the logarithm of the survivors plotted against time *always* gave a straight line. It is obvious from more recent work that this is not so (Henderson Smith, 1921, 1923; Withell, 1938, 1942). The most usual deviation from the straight line is a lag in the rate at the commencement of the reaction between organism and disinfectant. When a lag phase occurred in the experiments quoted by Withell (1942) it often lasted until 50% of the organisms had succumbed. It is therefore important to ascertain whether the relative reaction rates are different in the lag period or in the period beyond.

To ascertain whether the relative rates were different at different stages in the reaction, all the results have been graphically analysed. Each separate time-survivor curve was drawn by joining the experimentally determined mean counts by straight lines. The curve produced was divided at points to correspond to various percentage deaths, i.e. 0–10, 10–20, 20–30, etc., percentage deaths until the observations were finished. From such time-survivor curves the time required for any particular stage of the reaction (i.e. 0–10 percentage deaths, or 40–50 percentage deaths) can be estimated. If this time is inserted in the formula

$$k = \frac{1}{t} \log_{10} \frac{N}{n},$$

the reaction rate (k) can be calculated in stages over the reaction. For example, for 0–10 percentage deaths the formula is

$$k = \frac{1}{t} \log_{10} \frac{100}{90},$$

and for 40–50 percentage deaths $k = \frac{1}{t} \log_{10} \frac{60}{50}$.

Each experiment has been analysed in this way, and the mean reaction rates for the whole series of experiments have been calculated by finding the mean of the individual reaction rates. These figures have been used for another purpose in a previous paper (Withell, 1942), and the reader is referred to that paper for further details of the method of calculation. From these figures of the mean death-rates at different stages of the

Table 3. *The ratio of the death-rates of a* Micrococcus *exposed to* 0·05% *para-chlor-meta-cresol solution and* 0·5% *phenol solution at successive stages of the reaction*

	Percentage deaths							
	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80
Relative rate	1·20	1·54	1·36	1·39	1·28	2·00	3·06	1·67

reaction the ratio of the death-rates with phenol and para-chlor-meta-cresol can be computed, and it can be seen whether this ratio is constant over successive stages of reaction. The results of this calculation are shown in Table 3. In each case the rate of death in the para-chlor-meta-cresol solution is divided by the corresponding rate in the phenol solution. These results are calculated from six duplicate experiments where the death-rate was followed to 80% deaths or beyond. This table shows that the ratio of the death-rates at different stages of the reaction are not constant. The figures show a reasonable agreement up to 50 percentage deaths, but for 50–60 and 60–70% deaths the ratio shows a sharp

increase. This fact can be appreciated better from Fig. 2, where the mean curves are drawn from the same set of figures that were used to calculate the mean relative reaction rates. It is therefore difficult to select any arbitrary limit within which to compare the reaction rates. If both solutions had caused the organisms to die at a constant rate, the relative rates of death would have been the same, over the whole course of the reaction, Phelps had assumed that all organisms died in every solution at a constant rate, and if this assumption had been true, his suggestion would have been useful; but the existence of the lag phase complicates matters, for the lag phase is likely to vary in extent, and consequently affect the relative reaction rates. This means that it is difficult to use relative mean k's for comparing bactericidal solutions, but this fact does not affect Phelps's (1911) and Watson's (1908) conception of n, the concentration coefficient, and θ the

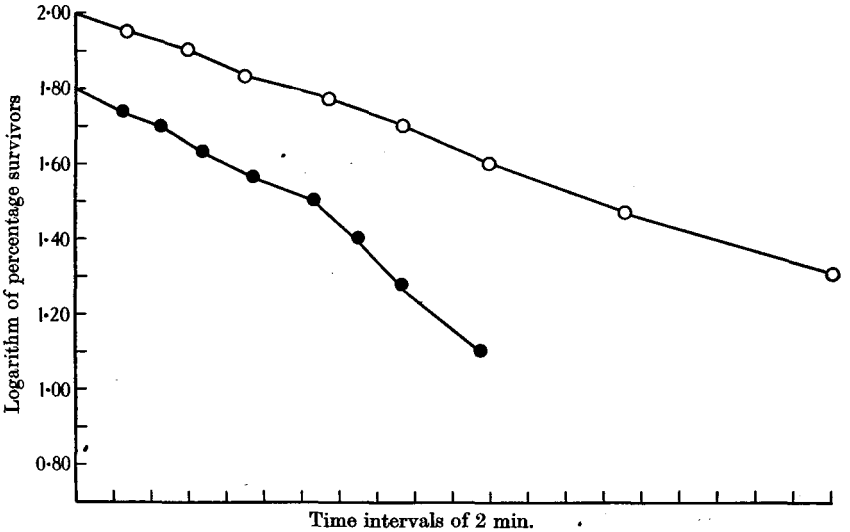

Fig. 2. The mean rate of death of a *Micrococcus* in phenol 0·5% (upper curve) and in 0·05% para-chlor-meta-cresol (lower curve). It will be seen that if the rates are compared, different values will be obtained according to the extent of the reaction covered by the figures.

The point indicating 100% viable, for the lower curve, has been shifted down to permit the representation of two lines on one graph.

temperature coefficient, providing a more logical method is used for the primary comparison of bactericidal efficiency.

I have demonstrated in a previous paper (Withell, 1942) that the lag phase, observed when the logarithm of survivors is plotted against time, is a manifestation of the distribution of resistances of the organisms. The distribution of resistances of bacteria has been shown to be approximately normal when measured by the logarithm of the survival times. The same is often observed in curves showing the variability of larger organisms, in their resistance to different doses of drugs (Gaddum, 1933). That is, the logarithm of the survival times is, nearly always, more normally distributed than the survival times themselves. The slope of the normal ogive may vary when organisms are exposed to lethal agents of different natures or strengths. When the slope of the normal curve varies, then the shape of the time-survivor curve plotted against arithmetic units is very often different, a constant rate, a lag phase, or a decreasing rate being shown, depending on the value of λ (the standard deviation of the logarithms of the survival times) and the

strength of the lethal agent. A better appreciation of the response of the organism to any lethal agent can be obtained by using a logarithmic time scale, for if percentage response in expressed as probits and plotted against the logarithm of time, the normal distribution of the logarithms of the survival times is obvious. Approximately sigmoid curves result, for example, when percentage survivors are plotted against the logarithm of time. Thus an arithmetic time scale may demonstrate an artificial difference in the response of the organisms to different bactericides, for it takes no account of the normal distribution of the logarithm of the survival times, and therefore the shape of the arithmetic time-survivor curve is not always a suitable medium for judging the comparative efficiency of bactericides. The use of a logarithmic time scale is considered in the next section.

(*b*) *The comparative time for* 50 *percentage response by the organism to the lethal agent*

Methods of biological assay are commonly designed so that the lethal dose for 50% response is calculated. The ratio of L.D. 50 for two solutions is the ratio of the activity of those solutions. I propose that the method be used in the estimation of bactericides, and that instead of estimating L.D. 50 the time for 50% deaths be calculated. This time could conveniently be designated L.T. 50. Relative values of L.T. 50 for two bactericides would indicate relative efficiency. The most convenient way to estimate the ratio L.T. 50 is to convert percentage deaths into probits and plot probits against the logarithm of time.

Table 4. *Death-rate of a* Micrococcus *in* 0·5% *phenol solution*

Time	Mean count	No. of organisms	Percentage deaths	Probit	Time	Mean count	No. of organisms	Percentage deaths	Probit
0	82	102,600	—	—	10	43	53,800	45·5	4·88
3	72	90,070	12	3·84	15	30	37,540	63·3	5·34
4	64	80,070	21·5	4·21	25	20	12,510	87·8	6·16
5	57	71,320	30	4·48	41	7	4,381	95·7	6·72

The probit is a method expressing percentage response. Gaddum (1933) introduced the term normal equivalent deviation (N.E.D.) with a unit of one standard deviation, and used N.E.D. as a method of expressing percentage response to any stimulus. Bliss (1938) gives a convenient table for the calculation of probit values from percentage response. Details of the use of probits in bacteriological work are given by Withell (1942).

The usefulness of expressing percentage response in this way is that when plotted against the logarithm of time a straight line results if the variation in response is distributed normally when plotted in this way. I have shown that this is approximately true for a large number of organisms, both vegetative and sporing organisms, when exposed to a wide variety of lethal agents. Gaddum (1933, 1940) has shown that the same relationship holds for the variation in response of experimental animals to different doses when the logarithm of the dose is used. Table 4 shows the data required for constructing a probit-logarithm of time graph. Percentage response can be conveniently converted to probits by a table given by Bliss (1938).

Fig. 3 shows the result of one experiment with the phenol and para-chlor-meta-cresol solution, where the percentage responses of the organisms have been converted into probits and plotted against the logarithm of time. The relative activity of the two solutions is given by the anti-logarithm of the distance Y measured on the x axis (logarithm of time).

In Table 5 (*a*) will be found the results of a series of comparative experiments where L.T. 50 has been compared for phenol 0·5% and para-chlor-meta-cresol 0·05% using an aerial *Micrococcus* as test organism. Table 5 (*b*) shows similar data for *Bact. coli*.

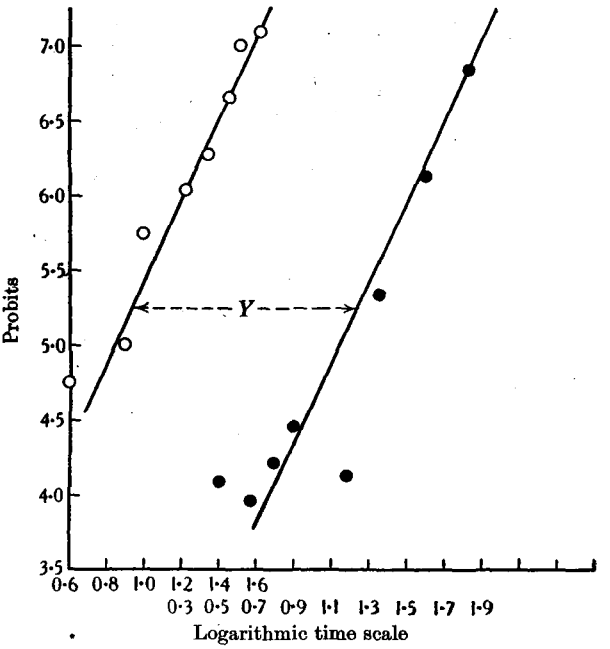

Fig. 3. Rate of death of a *Micrococcus* in phenol 0·5% (dots) and in 0·05% para-chlor-meta-cresol (open circles). Percentage response converted to probits and plotted against the logarithm of time. The two lines are approximately parallel, and an estimation of the comparative activity of the two solutions is given by the antilogarithm of the distance Y measured on the time axis.
(Upper time scale—para-chlor-meta-cresol; lower time scale—phenol.)

Table 5 (*a*). *Ratio of times for* 50% *deaths of a* Micrococcus *immersed in phenol* 0·5% *and para-chlor-meta-cresol* 0·05%

Exp.	Conc. of organisms per mil. in phenol 0·5%	Conc. of organisms per mil. in para-chlor-meta-cresol 0·05%	Ratio of L.T. 50
224	102,600	257,000	1·41
229	178,800	140,600	2·18
233	420,000	375,300	2·00
236	267,000	262,000	1·33
241	247,000	287,000	1·48
242	311,600	297,000	1·10
245	337,800	364,000	1·32
247	312,400	303,800	1·95
249	$37{\cdot}6 \times 10^6$	$36{\cdot}7 \times 10^6$	1·10
			Mean = 1·54

Table 5 (*b*). *Data for* Bact. coli

269	369,700	291,000	1·15
262	17×10^6	21×10^6	1·12
			Mean = 1·135

All the counts from which these figures were derived will be found in a previous paper (Withell, 1942, Appendix II), but not all the counts recorded in that paper have been used in calculating these ratios. I have rejected any comparative experiment where the first count with either bactericide showed less than 50% survivors. Details of the tech-

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nique used to make each duplicate experiment as nearly as possible identical will also be found in previous papers (Withell, 1938, 1942).

The mean ratio of the activity of phenol 0·5% and para-chlor-meta-cresol 0·05% when compared against a *Micrococcus* was 1·54. For *Bact. coli* the only experiments which satisfied the conditions of the first counts in each bactericide above 50% gave a value of 1·15 with approximately 330×10^3 organisms per mil., and 1·12 with 20×10^6 organisms per mil.

If *all* the experiments recorded in the previous paper (Withell, 1942) are used the mean ratio using the *Micrococcus* is 1·8 (10 experiments) and for *Bact. coli* is 1·10 (4 experiments).

Discussion

When Madsen & Nyman (1907), Krönig & Paul (1897) and Chick (1908, 1910, 1912) made the first observations on bactericidal action by following the course of the reaction by viable counts, a method of comparing bactericides by the slope of the line when the logarithm of the percentage survivors was plotted against time, appeared obvious. This was because in many cases an exponential time-survivor curve was obtained and the rate of reaction often appeared constant. Since that time it has been shown that an exponential time-survivor curve is only one of the types of bacterial response to lethal agents (Henderson Smith, 1921, 1923; Withell, 1938, 1942), but quite apart from this fact the methods in current use have avoided the labour necessary for estimating percentage survivors, by using 'end-point' method and have consequently sacrificed accuracy. 'End-point' methods have a number of disadvantages.

(1) It is difficult to estimate any variation in sensitivity of the organism to the two bactericides. This is a fundamental disability, because if the sensitivity of the organism varies in the two solutions, *no* end-point method will give consistent results. The most convenient way to demonstrate a difference in sensitivity is to use probit-logarithm of time graphs. If a comparative counting experiment, which follows the rate of death of a suspension of organisms in two bactericides, gives probit-logarithm of time lines which are parallel, the coefficient which expresses the relative efficiency of the bactericides will be the same whether estimates are made at 10, 50 or 99 percentage response. In other words, the time chosen for the test will not influence the coefficient by end-point or counting methods. This is the case with the series of experiments recorded in this work and is illustrated by Fig. 1. This fact could not have been appreciated if end-point methods had been used; so that when the current standard tests are performed the question of sensitivity variation is not considered.

Fig. 1 indicates why sensitivity variation is important in the estimation of bactericidal activity.

When a standard time of 15 min. (the Rideal-Walker Test) is used, the results are often entirely arbitrary, and indeed the coefficients are sometimes vastly different when different times are used. This is well indicated by Chick & Martin (1908, Table III) for mercuric chloride. The phenol coefficient for this substance was 13·6 at 2·5 min., but was about 550 when the standard time was 30 min. A single end-point method will give no information on this fundamental objection to the present tests. A single counting experiment *will* give the information required, most conveniently, by a consideration of probit-logarithm-of-time graphs.

(2) The 'end-point' is not necessarily sterility, but absence of viable organisms in

the sample removed and diluted with broth. This sample is small, and a varying percentage of viable organisms may be still alive when the broth yields a negative result. In other words, end-points are difficult to determine. This is well shown by Thaysen (1938) who could not obtain consistent results with the Rideal-Walker test in the preliminary experiments with *Bact. typhosum* and phenol. His experiments show that a varying and very small percentage of organisms is left, when most of the organisms are dead. It is for this reason that it is practically impossible to estimate the time for completion of the reaction.

In the more exact methods of physical chemistry, when it is desired to determine the rates of reactions at different concentrations, e.g. when determining the 'order' of a reaction, end-point methods are not generally used. The usual way is to find by experiment the time when the reaction is half completed. Similarly with radioactive substances the half-life period is chosen as a characteristic of a particular element. In comparing the death-rates of organisms bacteriological workers would do well to bear in mind the impossibility of accurate estimation of 'end-points'.

Thus end-point methods necessitate the standardization of a time, and whatever time is chosen the results will be arbitrary. Further, at any standard time the results will vary amongst themselves, because end-points are difficult to determine. It is doubtful whether the standardizing of the various details of the Rideal-Walker and Chick-Martin tests by the British Institute of Standards has done any real service to the study of germicidal activity. It has, rather, emphasized and accentuated the value of these tests in the eyes of both scientific workers and the public, when such value is indeed questionable.

There is one other point that can conveniently be discussed at this juncture, namely, that the value of the phenol coefficient is obtained from one concentration of phenol and test bactericide. Such a value applies *only to the concentrations compared.* The phenol coefficient is, however, applied to the pure test bactericide and the implied suggestion is that this value is a constant for this substance irrespective of its concentration. The effect of increase of concentration, or dilution of a bactericide, is determined by its concentration exponent n' (Watson, 1908; Tilley, 1939). Chick (1930, Table III) has tabulated values of n' for different bactericides. Each chemical has its own value of n', and many of the chemicals in Chick's Table III have different values of n' from phenol. This means, for example, that a disinfectant may be twice as efficient as phenol when measured by the Rideal-Walker test and compared to a 1 in 100 phenol solution. If the phenol strength was 1 in 50 in the test the relative efficiency of x would not necessarily be the same, and any different values obtained would depend on relative values of the concentration exponent n'. It appears to me to be fair comment on the present methods to say with Topley & Wilson (1938, p. 129), 'There is no doubt that the present tests are highly unsatisfactory'.

The series of determinations of comparative bactericidal activity described in this paper were determined by following the death-rates of the organism by viable counts. This counting method has certain fundamental advantages over 'end-point' methods: (1) There is no question of any standard time being fixed for the comparison of activity. The comparative value obtained is therefore not an arbitrary value depending on the time chosen as standard. (2) The method is free from the difficulty associated with the determination of end-points.

There remains the consideration of how the viable counts and relative death-rates are to be interpreted. If Phelps's (1911) suggestion is followed and the reaction rates compared, the results in Table 2 show the degree of accuracy attained. As a straight line is not always obtained when the logarithm of survivors is plotted against time, the calculation of relative rates is complicated, for the relative reaction rates vary over the course of the reaction. This fact could, however, be neglected in comparing bactericides and the mean reaction rates compared, whether or not lag period is observed. All methods of interpretation of bacteriological time-survivor curves are likely to offer difficulties if an arithmetic time scale is used for the reasons discussed above.

It remains to discuss the second method of interpreting the results—the use of L.T. 50 as a measure of bactericidal efficiency. When an arithmetic time scale is used the results of comparisons of the reaction rates have been shown to vary with the stage of the reaction used for the comparison. This appears to me to be a fundamental disability of methods using an arithmetic time scale. If a logarithmic time scale is used it has been shown that the logarithmic survival times are approximately normally distributed (Withell, 1942). This brings out the similarity between bacteriological assays and other pharmacological assays, for Gaddum (1933) has shown that a logarithmic dose scale is often more useful in biological assay than an arithmetical dose scale, particularly when the variation of the animals is large. I have shown that λ, for bacteria, is, in most cases that I have calculated (Withell, 1942, Table 12), larger than the animal variation (Gaddum, 1933). If we assume that the logarithm of the survival times of bacteria is normally distributed, then bactericidal tests can be formulated on this basis in exactly the same way that well-tried biological assay processes on animals are formulated. The time for a 50% response of the test animal organism is very generally used in biological assay work and has been shown to be a useful criterion for the comparison of the activity of two solutions (Gaddum, 1933, 1940; Bliss, 1941; Burn, 1937). Because a determination of L.T. 50 in bactericidal assays involves counting, it avoids the difficulties associated with end-point methods, and if a logarithmic time scale is used any difficulties associated with the arithmetic time scale are also eliminated.

The most convenient way to determine the L.T. 50 is to use probit-logarithm-of-time graphs (Bliss, 1941) when the standard deviation of logarithmic survivor times (λ) can also be calculated. If the two bactericides give markedly different values of λ the assay is not likely to be accurate. For phenol 0·5% and para-chlor-meta-cresol 0·05% the lines are approximately parallel (i.e. λ are approximately equal). I have evidence which I hope to give in a later paper that different concentrations of phenol and para-chlor-meta-cresol give probit-logarithm of time lines, which are also parallel lines.

It is not to be expected that any method, depending on a response of organisms to bactericidal agents, is likely to be particularly accurate. The 'normal' curve which expresses the distribution of the logarithmic survivor times for bacteria is much flatter than similar curves for higher animals. The slope of this curve controls to some extent the accuracy of the method, and the logarithmic standard deviation for bacteria is generally larger than that of higher organisms, and therefore the error of the assay is likely to be greater with bacteria than with the higher animals.

No value, analogous to the phenol coefficient, is given to para-chlor-meta-cresol from the work in this paper. The values that are put forward are the comparative activities of the two solutions. Additional information can be gained by determining the concen-

tration coefficients of both phenol and para-chlor-meta-cresol. This can be done for phenol, for example, by comparing L.T. 50 for different strengths of phenol solutions. When n' for each substance is found, the relative efficiency of the bactericides can be calculated at any concentration (Watson, 1908; Tilley, 1939). Watson (1908) showed that concentration and time of disinfection of any bactericide are related by the general formula $C^{n'}t = \text{constant}$, where C is the concentration, n' is the concentration exponent, and t the time of disinfection. If t is transferred to the value of the time for 50 percentage response, then the difficulty of end-points is avoided and the sense of the equation preserved.

If the temperature coefficient, θ (Chick, 1908, 1910; Phelps, 1911), is determined for two bactericides, it is possible from n and θ and the relative activity of the two substances at one pair of concentrations, to calculate the relative activity of these bactericides at any concentrations and temperatures. For example, with *Bact. coli* as a test organism the relative activity of phenol 0·5% and para-chlor-meta-cresol 0·05% was 1·135 at 20° C. If n and θ are estimated separately for each chemical, it is possible to calculate the ratio of the activity of each substance at any concentration and temperature. If these observations are repeated, using a sporing organism, the calculation can be repeated and relative values obtained for sporing and vegetative forms.

Throughout this work the *relative* value of the two bactericides is estimated and discussed. There has been no attempt to derive an absolute value for any substance. The reason for this can be appreciated from Table 2, where the great difference in the absolute rates of reaction in the series of experiments is indicated.

Such information as n and θ and the ratios of L.T. 50 of one particular pair of concentrations would give much more information about bactericides than the present tests. The determinations would involve more work than the present standard tests in current use, but the results of these determinations would be sound and logical values, which can be determined in practice without undue difficulty, and which are supported by the demonstration that the logarithms of the survivor times are normally distributed.

The figures given here for the relative activities of the two solutions are not comparable with the Rideal-Walker coefficients where *Bact. typhosum* is used as a test organism. The *Micrococcus* and *Bact. coli* were used in this work because they gave reliable figures in counting experiments and formed no clumps in the bactericides. Rapps (1933) gives the R.W. coefficient of para-chlor-meta-cresol as 13·3 and mentions other observers' figures of from 40·0 to 140. Klarmann's figure (1933) is 30·5 and Heading (1937) mentions 25·0 as the correct value.

SUMMARY AND CONCLUSIONS

The Rideal-Walker and Chick-Martin tests depend on end-point methods for comparing bactericides. End-point methods have a number of disadvantages: (1) It is difficult, if not impossible, to estimate end-points accurately. (2) The nature of the test demands a standard time. The results at any standard time will be arbitrary. If the sensitivity of the organisms varies in the two solutions tested, the coefficients obtained will vary with the time chosen as standard. It is impossible to estimate the manner in which the survivor times of the organism are distributed, by end-point methods, and therefore the standard tests in current use do not ascertain whether the result is arbitrary or not. This can only be appreciated if the tests are repeated using different times.

Apart from the difficulties associated with end-points, the coefficients obtained in the present tests are calculated from one concentration of phenol and one concentration of test bactericide. This is also an arbitrary value, for the relative activity of two bactericides will depend on relative values of the concentration exponent, n, for each bactericide; if both phenol and the test bactericide have identical values of n the coefficients will be the same at all concentrations which are compared, but if values of n are different then different coefficients will be obtained for every different concentration compared. As a corollary, unless the value of n for the test bactericide is the same as that of phenol, it is illogical to transfer the phenol coefficient obtained at one concentration to the pure substance, for this procedure implies that the phenol coefficient applies to any concentration of the test bactericide. This is only true when the concentration coefficients of phenol and test bactericide are the same. The standard methods offer no value for n.

All these difficulties can be avoided if the rate of death is followed in both phenol and test bactericide, by viable counts. Two methods of interpreting the results are discussed:

(1) The comparison of reaction rates, calculated by the formula

$$k = \frac{1}{t} \log_{10} \frac{N}{n}.$$

This method is complicated by the occurrence of lag period at the start of the reaction between organism and bactericide. This lag period varies in extent in different experiments (often extending up to 50% of the deaths) and makes the interpretation of the results difficult. The lag period has been shown to be a manifestation of the distribution of logarithmic survival times (Withell, 1942), and is a function of the standard deviation of the logarithmic survivor times (λ). This fact is taken into account in method (2).

(2) The use of the time required for 50% response as a method of comparing bactericides. This time has been symbolized L.T. 50 and is analogous to the lethal dose for the 50% response (L.D. 50) widely used in biological assay. If a logarithmic time scale is used, the logarithmic survival times are found to be approximately normally distributed and consequently probit-logarithm of time lines are straight and in the case of phenol 0·5% and para-chlor-meta-cresol 0·05% these lines are parallel. In this case a logarithmic time scale means that the *comparative* times for all percentage responses are equal. In cases where the probit-logarithm-of-time lines are not parallel this would indicate that the standard is unsuitable for the test, for *any methods* of assay based on a use of a standard to which the organism gives a different response-time relation from that which they give to the test substance or bactericide, are likely to give arbitrary results.

L.T. 50 has been chosen because of the simplicity of the idea underlying it, and because a similar function, L.D. 50, has been used with success in biological assay work. There is also a solid mathematical backing for the use of L.D. 50 in assays involving biological response.

It is suggested that by means of L.T. 50, phenol and the test bactericide can be compared in the following way:

(*a*) determine the relative speeds of action of the two solutions at any convenient concentrations;

(*b*) determine the concentration coefficient (n') of both phenol and test bactericide by means of comparative L.T. 50 values;

(*c*) determine the temperature coefficient for each substance by similar methods.

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From these observations the relative efficiency of the two bactericides can be calculated at any temperature and concentration. If two sets of figures are obtained, one with a vegetative organism, and the other with a sporing organism, a great deal of information can be summarized. It has been shown that an 0·05% solution of para-chlor-meta-cresol is approximately one and a half times as efficient as an 0·5% phenol solution (against a *Micrococcus*). The comparative figure is about 1·0 when *Bact. coli* is used.

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