THE ESTIMATION OF BACTERICIDAL ACTIVITY FROM EXTINCTION TIME DATA

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The most frequently used methods for the evaluation of bactericidal activity involve either the measurement of extinction times or the enumeration of surviving organisms after varying periods of exposure to the bactericide. In general, counting techniques are preferred, for they can provide information on the velocity of the reaction over a great range of the time-survivor curve, whereas extinction methods yield information at but one point of the reaction and concerning only the overall velocity of the complete bactericidal action. Furthermore, the accuracy and degree of reproducibility of estimates of bactericidal activity based on survivor counts are usually acknowledged to be superior to those based on extinction data, a belief which might be attributed to the paucity of replication, the use of inadequately small sampling intervals and the failure to take account of sampling variations in the usually employed experimental designs for extinction time determinations.

Counting methods possess some obvious disadvantages. They are tedious in performance, a consideration of importance in the routine testing of bactericides, and are not of universal application. Such methods could not be used, for example, where the test organisms themselves tend to aggregate into clumps or chains or where there is a tendency for the bactericide to agglutinate the organisms. Colony counts obtained from samples of agglutinated organisms would be meaningless. Hence, when there is a tendency for agglutination to occur during the bactericidal reaction, recourse must be made to methods other than counting.

The authors were presented with this problem when they attempted to evaluate the bactericidal activities of solutions of soaps. They found that solutions of potassium laurate agglutinated test organisms with which they were inoculated, the phenomenon being more marked with some organisms than with others. Microscopical examination revealed that a strain of *Bacterium coli* (N.C.T.C. No. 5933) was not seriously agglutinated by the soap, but *Pseudomonas pyocyanea* (N.C.T.C. No. 1999) was agglutinated to an extent which was considered to completely invalidate the use of counting methods.

In the face of this difficulty, a new method for the estimation of extinction times was adopted. It is the purpose of this communication to describe the method and to point out the advantages which it possesses over other methods for the determination of extinction data. The principal feature of the method is that of sampling the reaction mixture *immediately after mixing the organisms with the bactericide.* The samples are transferred to sterilised tubes, maintained at a controlled temperature, where the reaction is allowed to proceed until quenched by the

addition of a sufficient volume of sterilised broth to render the bactericide inactive. Other features of the method are its performance in replicate, the mean of several extinction times so obtained being utilised: the use of a dropping pipette for sampling the reaction mixture in place of the more usual platinum loop; and the use of relatively narrow limits—between 1/5 and 1/10 of the anticipated death times—for the exposure time intervals.

During the development of the method several different concentrations of phenol were used as the bactericidal solution. Phenol was selected because its behaviour as a bactericide has been extensively studied and it was known to yield reproducible results when subjected to other methods of evaluation. If the assessment of activity of phenol by the proposed technique accorded with that revealed by numerous established and accepted techniques, then the method could be assumed to be satisfactory.

METHOD

(i) *Cultivation of Test Organisms.* The strain of *Bacterium coli* (N.C.T.C. No. 5933) was maintained by freeze-drying. At monthly intervals a freeze-dried culture was opened and transferred to a slope of peptone agar. After 24 hours' incubation, four "sub-master" slopes were prepared from this "master" slope, and each day for 14 days slopes were inoculated from a "sub-master" slope. The slopes so prepared were used in the experiments from the fourth to the fourteenth day, when a fresh "sub-master" slope was introduced.

The peptone agar was prepared by gelling a peptone broth, described by Needham[1], with 2 per cent. of bacteriological agar. The broth contained 1 per cent. of "Oxoid" peptone and 0·5 per cent. of sodium chloride, the solution being adjusted to *p*H 7·3 by the addition of sodium hydroxide.

(ii) *The Bacterial Suspension.* The 24-hour growth of the organisms was washed from the surface of 3 agar slopes with a few ml. of sterilised quarter strength Ringer's solution, as recommended by Wilson[2] and used by Berry and Michaels[3]. The suspension thus obtained was centrifuged at 2000 r.p.m. for about 1 minute in order to precipitate small fragments of agar removed from the slopes and then was lightly shaken with sterilised glass beads to ensure complete dispersion of the organisms. The volume of the suspension was adjusted to contain about 2000×10^6 *Bact. coli* per ml., a density corresponding to that found in a 24-hour culture of Needham's broth. This adjustment was made with the aid of a photo-electric absorptiometer.

(iii) *Preparation and Inoculation of the Bactericide.* The solutions of phenol used in the experiments were prepared in water distilled from a heavily tinned still fitted with an all-glass condensing system. Water from the same still was also used in the preparation of the nutrient media and the Ringer's solution. 5 ml. quantities of the phenol solutions were introduced into 60 ml. glass-stoppered "Pyrex" bottles, which were placed for at least 20 minutes in a water bath maintained at $20° \pm 0·1°$ C.

The inoculation of the phenol solutions with the suspension of *Bact. coli* was at first performed using a 1 ml. graduated pipette. The pipette was

rinsed by drawing the suspension into it and rejecting several times. 0·2 ml. was then added to the bactericide solution, which was agitated by gentle rotation for 10 seconds in order to effect uniform distribution of the organisms throughout the solution. It was afterwards considered preferable to make the addition of the suspension by means of a dropping pipette, adding 10 drops, which was equivalent to approximately 0·18 ml.

(iv) *Sampling the Reaction Mixture.* As soon as possible after mixing the organisms with the bactericide, a portion of the reaction mixture was withdrawn into a dropping pipette, which was clamped vertically and fitted with a rubber teat. The standard dropping pipettes were prepared by the method described by Withell[4]. Six drops of the reaction mixture, delivered from the pipette at one second intervals, were transferred to each of a series of sterilised aluminium-capped test-tubes, which had been immersed in a water bath maintained at 20° ± 0·1° C. for at least 20 minutes. Care was taken that the drops fell upon the bottom of the tube. The tubes were replaced in the water bath immediately after inoculation.

The reaction between the bactericide and the test organisms was allowed to proceed in the tubes at the controlled temperature. After predetermined time intervals, tubes were removed from the water bath and the reaction was quenched by the addition of 5 ml. of sterilised broth. Immediately after quenching, each tube was placed in a water bath maintained at 37° C., and at the end of the experiment the tubes were transferred to an incubator and examined for evidence of growth after 3 days. The importance of incubating the tubes immediately after the addition of broth was proved when preliminary tests, with as many as 20 replicate experiments performed on one day, showed quite clearly that tubes left in the laboratory for an hour or so before being transferred to the incubator gave an extinction time estimate considerably shorter than did experiments in which the tubes were immediately incubated. It appears that the bactericide damages a proportion of the organisms without killing them and that rapid transferrence to a favourable environment at optimal growth temperature allows the organisms to make good their recovery before death supervenes. A large volume of literature has appeared on the errors inherent in methods of evaluating bactericides, but little importance has been attached to the above factor.

Illustration of the Method Using Solutions of Phenol

The experiments were performed with 8 different concentrations of phenol, one experiment with the greatest possible replication being performed on one day with one concentration of phenol. It might be objected that it would be fundamentally more sound to perform a number of experiments with several different phenol concentrations but with very limited replication during one day. However, such a procedure would tend to obscure between-replicates variability, for the variations observed by performing many experiments on different days would probably have been attributed to day-to-day variations in the susceptibility of the test organisms. Unpublished results since obtained from these laboratories indicate that, on the evidence of determinations of mean single survivor

times with the same concentration of phenol performed over many days, day-to-day ("subculture") variations are small relative to the standard error of an estimate of the extinction time. Variations between "master" cultures were found to be significantly greater than those within "master" cultures.

Typical results are set out in Tables I and II, which show the extinction times of *Bact. coli* in 1·0 per cent. and 1·15 per cent. w/v phenol respectively. The lowest extinction time observed in 1·0 per cent. phenol was 60 minutes

TABLE I

DEATH TIME OF *Bact. coli* IN 1·0 PER CENT. W/V PHENOL SOLUTION

Experiment	Time in minutes								Estimated death time Minutes
	40	50	60	70	80	90	100	110	
1	+	+	+	+	+	−	−	−	90
2	+	+	+	−	−	−	−	−	70
3	+	−	+	−	−	−	−	−	70
4	+	+	+	−	−	−	−	−	70
5	+	+	+	−	−	−	−	−	70
6	+	+	−	+	−	−	−	−	80
7	+	+	+	−	+	−	−	−	90
8	+	+	+	−	−	−	−	−	70
9	+	+	−	−	−	−	−	−	60
10	+	+	−	−	−	−	−	−	60
11	+	+	+	−	−	−	−	−	70
12	+	+	−	+	−	−	−	−	80
								TOTAL	880
								MEAN DEATH TIME	73·3 minutes

TABLE II

DEATH TIME OF *Bact. coli* IN 1·15 PER CENT. W/V PHENOL SOLUTION

Experiment	Time in minutes								Estimated death times minutes
	12	14	16	18	20	22	24	26	
1	+	+	+	+	+	−	−	−	22
2	+	+	+	−	−	−	−	−	18
3	+	+	+	−	+	−	−	−	22
4	+	+	+	+	−	+	−	−	24
5	+	+	+	+	−	−	−	−	20
6	+	+	+	+	−	−	−	−	20
7	+	+	−	+	−	+	+	−	26
8	+	+	+	+	−	−	−	−	20
9	+	+	+	+	+	−	−	−	22
10	+	+	+	−	+	−	−	−	22
11	+	+	+	+	−	−	−	−	20
12	+	+	+	+	−	−	−	−	20
13	+	+	+	−	+	+	−	−	24
14	+	+	+	+	+	+	−	−	24
15	+	+	+	−	−	−	−	−	18
16	+	+	+	−	−	−	−	−	18
17	+	+	−	−	−	−	−	−	16
18	+	+	−	+	−	−	−	−	20
								TOTAL	376
								MEAN DEATH TIME	20·9 minutes

and the highest 90 minutes, whilst in 1·15 per cent. phenol the times were 16 and 26 minutes respectively. Thus with both of these solutions the maximal extinction time for an individual replicate estimation was approximately 150 per cent. of the minimal estimated time. Similar ratios

of maximal to minimal times were observed with the other concentrations of phenol examined. For each phenol concentration the mean extinction time was calculated by dividing the sum of the individual extinction times by the number of replicate determinations performed. In deciding the individual extinction times a convention was adopted that if no growth was obtained after a given period of exposure in all replicates, then any growth obtained thereafter was neglected.

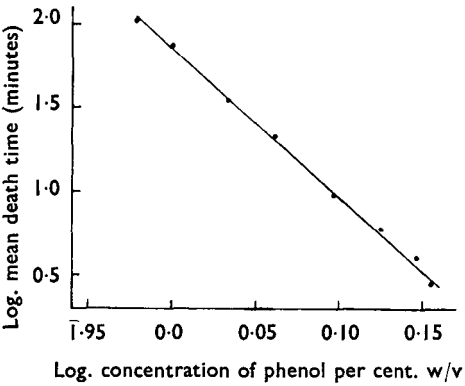

FIG. 1. The relationship between mean death times of *Bact. coli* and concentrations of phenol.

Table III records a summary of the experiments performed with the eight concentrations of phenol, and Figure 1 shows the relation between concentrations and extinction times plotted on a logarithmic scale. The

TABLE III

THE DEATH TIMES OF *Bact. coli* IN AQUEOUS SOLUTIONS OF PHENOL

Phenol concentration per cent.	Log phenol concentration	Mean death time ($\bar{x}$)	Log. mean death time	Number of replicate determinations	Standard deviation	Coefficient of variation
		Minutes				
0·952	1̄·9780	104·5	2·0155	11	6·578	6·32
1·000	0·0000	73·3	1·8653	12	9·427	12·85
1·080	0·0333	34·6	1·5391	15	5·306	15·30
1·150	0·0607	20·9	1·3201	18	2·512	12·03
1·250	0·0969	9·6	0·9823	20	1·624	16·91
1·333	0·1248	5·9	0·7723	12	0·576	9·73
1·400	0·1461	4·0	0·6020	20	0·623	15·81
1·428	0·1547	2·8	0·4471	15	0·160	5·71

obvious close approximation to a linear relationship over the range of concentration used is in good agreement with the results described by Phelps[5] and by many other workers in more recent years.

DISCUSSION

After the performance of only a few experiments it became obvious that the results of any one determination were by no means perfectly reproducible. In a number of replicate determinations there was always a scatter of the end-points. This is a factor which has not been stressed sufficiently in reports of extinction data, and may well be due to the fact that many workers have sampled their reaction mixtures at intervals of similar duration to those specified in the Rideal–Walker or Food and Drugs Administration methods. Such sampling time intervals as 2·5, 5, 7·5 and 10 minutes or 5, 10 and 15 minutes constitute a large proportion of the anticipated death time and will tend to obscure any between

replicates variations. In the present series of experiments the time intervals between samples was reduced to between 10 and 20 per cent. of the expected extinction time. It is the use of these narrow sample intervals which serves to account for the sampling variations at the extreme end of the time-survivor curve and which gives the method here described a greater degree of accuracy than all previous commonly used extinction methods.

Withell[6] has shown that simultaneous death does not occur when bacteria are introduced into a disinfectant solution. The logarithms of the resistance of the individual organisms to the bactericide are distributed normally: towards the end of the disinfection process only the most highly resistant organisms remain alive. These organisms of high resistance represent only a very small proportion of the initial inoculum and the number remaining viable decreases only slowly with time, especially in the more dilute disinfectants. Thus a sample removed towards the end of the bactericidal reaction may or may not contain a survivor, although there may be many survivors in the bulk of the solution. The between-replicates variation, estimates of which have been expressed as coefficients of variation for each phenol concentration in Table III, can therefore be explained in terms of variation in resistance among the last survivors and variations in sampling. Provided this is realised, and the structure of the experiment be so designed that the variation may be estimated, there appears to be no *prima facie* reason as to why the extinction method should not provide valid and reproducible data.

Previous methods of determination of extinction times have usually involved sampling the reaction mixture by means of a platinum loop. It is well known that, even in the hands of experienced operators, the volume of reaction mixtures sampled by means of a loop may vary widely from a mere film across the loop to a large pendant drop. For a given solution, the sample volume withdrawn with a loop depends upon the angle at which the loop is withdrawn from the liquid, upon effects of heating when the loop is repeatedly sterilised, and upon the skill of the operator. Estimates of the variations between samples delivered by means of dropping pipettes have been given by Withell[4] and, in the case of pipettes fitted with needles, by Cook and Youssef[7]; these reveal that the variations in drop volumes from different pipettes, where samples of 10 or 20 drops are delivered, is unlikely to exceed 2 per cent.

The method of sampling employed possesses another advantage. Owing to the small number of survivors remaining towards the end of the disinfection process, the possibility of obtaining a representative sample of the reaction mixture at this point will be increased by taking as large a sample as possible, the only limitation to the sample size being the convenience of the volume of broth required to render the bactericide inactive by dilution. In practice, the 6-drop samples withdrawn from the reaction mixture correspond to a volume exceeding 0·1 ml., and hence are greater than those withdrawn by means of a loop. The sampling error, of course, would be minimised by taking several simultaneous samples. This was not possible in the experimental procedure adopted,

but it was possible to take similar samples from a number of experiments performed under identical conditions.

The relatively simple experiment which has been outlined will yield a mean extinction time estimate representative of a high percentage mortality of the inoculum. A more accurate statistical treatment of the authors' results has been described by Mather[8], who has calculated the mean single survivor time with respect to a sample volume of the reaction mixture, together with estimates of sampling variation and standard error of an estimate. However, for most practical purposes the simple calculation of the mean extinction time may be considered adequate.

Summary

1. A new method for the determination of extinction time data is described.

2. The method has been employed for the disinfection of *Bact. coli* by aqueous solutions of phenol over a range of concentrations and death times ranging from 2 to over 100 minutes.

3. The use of extensive replication, short sampling intervals, and constant sample volumes withdrawn immediately after inoculation of the bactericide are among the chief features of the technique.

4. Estimates of the mean extinction time obtained from the data are of reproducibility within limits sufficiently close to invalidate other extinction time methods, and are of comparable value with those obtained by the use of any other techniques.

5. The method has been satisfactorily applied to systems containing water-insoluble phenols solubilised in solutions of soaps (Berry and Bean[9]) where clumping of the test organisms might completely invalidate other methods of estimation of bactericidal activity.

References

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