THE EVALUATION, FROM EXTINCTION DATA, OF THE INACTIVATION OF BACTERIOPHAGE BY CHEMICAL AGENTS

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The inactivation of coliphage T6r by phenol has been examined using an extinction method and the reproducibility of the results shown to be acceptable. Two methods of interpreting the results are described and their relationship discussed. A linear relationship has been found between log extinction time and log phenol concentration for extinction times between 2 and 200 min.

MANY investigations of the inactivation of phages by chemical agents have been made using plaque counting methods.

These studies have been mainly concerned with the dynamics of the inactivation process but not with complete inactivation, i.e. sterilisation.

A few investigations (Hunter and Whitehead, 1940; Klein, Kalter and Mudd, 1945; Deutsch and Rohr, 1955) have used end-point or extinction methods but the procedures gave extinction time estimates of low precision and were used for qualitative comparisons of the viricidal activity of the agents.

Extinction methods have been widely used with antibacterial agents but many of the methods can be criticised as giving extinction time estimates which lack precision and reproducibility. One exception is when the analysis devised by Mather (1949) is applied to the results obtained by methods similar to that described by Berry and Bean (1954). This method has been adapted for the investigation of the viricidal action of phenol and other chemical agents on a coliphage.

EXPERIMENTAL

Bacterial host. The bacterial host was a laboratory strain of *Escherichia coli* which was sensitive to the phage. The organism was grown at 37° in peptone water (1·0 per cent Oxoid peptone and 0·5 per cent sodium chloride in distilled water; pH 7·0) or on peptone agar (peptone water with $2\frac{1}{2}$ per cent shredded agar). Cultures were maintained on peptone agar slopes stored at room temperature and subcultured monthly. Cultures in peptone water were initiated by subculture from a stock slope and maintained by daily serial subculture for up to 6 days; the cultures were normally used after 18 to 30 hr. incubation. In peptone water cultures the viable count reached 4×10^8 organisms per ml. in 18 hr. without aeration and 4×10^9 organisms per ml. with active aeration, the counts being performed by the surface viable method of Miles and Misra (1938).

Bacteriophage. The phage used was a coliphage obtained from the School of Hygiene and Tropical Medicine, London. It had the cultural

characteristics of the strain classified as T6 by Demerec and Fano (1945) in the "r" (rapidly lysing) form described by Hershey (1946). The phage was cultivated by a modification of the method described by Hershey, Kalmanson and Bronfenbrenner (1943) in which peptone agar plates were surface seeded with a mixture of *E. coli* and phage in a ratio which gave barely confluent lysis when the plaques were fully developed. The phage particles were harvested by flooding each plate with 3 ml. of sterile distilled water, allowing them to stand for 20 to 30 min., decanting the suspension formed and removing bacteria and debris by centrifuging (7,000 r.p.m. for 20 min.) and filtering the supernatant through 5/3 sintered glass. This procedure gave 2·2 to 2·6 ml. of phage suspension per plate; the phage titre varying from 6 to 9 $\times$ 10^{10} infectious particles per ml. In addition to the phage, the lysates contained peptone and sodium chloride extracted from the solid medium and soluble degradation products from the host cells. The solid content of the lysates, determined by evaporation to constant weight over silica gel, was 1·54 per cent and the sodium chloride content, by the assay of the British Pharmacopoeia, 1958, was 0·50 per cent. The same values were obtained for sterile peptone water and the lysates were therefore considered to be equivalent to suspensions of phage in peptone water.

All phage inocula used were taken from a single stock suspension stored at 5°. Plaque counts performed on the stock at intervals gave a mean count of 4·06 $\times$ 10^{10} phage particles per ml. with 95 per cent confidence limits of $\pm$0·29 $\times$ 10^{10}. The method of plaque counting used was based on that described by Williams-Smith (1951, 1953) and details of the modifications used will be reported in a subsequent communication.

The constancy of titre, plaque morphology, host specificity and rate of lysis of broth cultures of the host were accepted as evidence of absence of significant variation in the phage.

Apparatus. For the measurement of small volumes, dropping pipettes were used as described by Cook and Yousef (1953). When calibrated gravimetrically the pipettes were found to deliver a mean single drop volume of 1/58 ml. of water and 1/63 ml. of peptone water. The 95 per cent. confidence limits of the mean volume of 6 drop samples was approximately 0·2 per cent of the mean. Automatic tilt measures* were used to measure 20 ml. volumes of media. The volume delivered by these measures, as determined by weighing 10 separate volumes of water delivered by each of 3 measures, was 19·98 $\pm$ 0·04 ml. (P = 0·95).

Solutions of Phenol, Analar, were prepared immediately before use by diluting 5 per cent stock solutions which were stored in the dark for up to one month. All solutions were prepared using sterile distilled water.

Inhibition of Phage Growth

A serial tube dilution method was used to determine the bacteriostatic concentration of phenol for the bacterial host in peptone water and the

* "E-mil Brand", Kipps measures, manufactured by H. J. Elliott, Ltd., Glamorgan

rate of mass lysis in phage cultures containing sub-bacteriostatic concentrations of phenol. The bacteriostatic tests were performed in triplicate and each culture was inoculated with approximately 4×10^6 viable cells per ml. of culture medium. In the phage inhibition tests, 5 replicate cultures were prepared for each concentration of phenol tested and inoculated with approximately 4×10^6 viable host cells and 1×10^4 phage particles per ml. of culture. Both types of culture were incubated at 37° and their opacities periodically compared visually with those of control cultures containing no phenol. The phage tests were repeated using cultures with phage inocula averaging $1{\cdot}5 \times 10^8$ and 0·15 particles per ml. of culture and containing the maximum concentration of phenol previously shown to permit an approximately normal rate of growth of the host.

Method of Evaluating Phage Inactivation

Ten ml. of the phenol solution under test was pipetted into a glass-stoppered tube of approximately 20 ml. capacity and placed in a water-bath at 25 ± 0·05° for 30 min. to allow the temperature of the solution to equilibrate with that of the bath. The phenol solution was then inoculated with 6 drops (0·095 ml.) of the phage suspension from a dropping pipette and the mixture immediately shaken vigorously for 20 sec. Approximately 1 ml. of the mixture was drawn into a sterile dropping pipette and 6-drop samples of it delivered into a number of empty sterile test-tubes whose temperature had previously been equilibrated at 25° by storage in the bath for 30 min. and which were immediately returned to the bath. Normally 9 to 11 such samples were distributed.

The time of contact of the phage with the phenol was measured from the moment the first drop of phage inoculum entered the solution. At suitable intervals of time each reaction tube was withdrawn from the bath and 20 ml. of peptone water added to it from a flask fitted with an automatic tilt measure. After mixing, 6 drops of an 18–25 hr. peptone water culture of *E. coli* were added from a dropping pipette, the tube was again shaken and placed in a water-bath maintained at 37°. The tubes were incubated at 37° and their opacity compared with that of control cultures after 3 to 4 hr., 18 to 24 hr., 2 days, 3 days and 4 days. The control cultures contained the same concentration of phenol and received the same host inoculum as the diluted reaction mixtures but contained no phage.

The presence of surviving infective phage particles was shown by visible lysis in the incubating cultures. The reliability of the periodic examination of the cultures in detecting active phage was confirmed by placing loopfuls of reaction mixture cultures which had been incubated for 4 to 7 days, on the surface of peptone agar plates previously surface seeded with host cells. On incubating these plates, a uniform lawn of bacterial growth was produced except where loopfuls of culture had been placed. There, a more dense region of bacterial growth resulted but, where the culture contained active phage, a clear halo of lysis surrounded this region.

RESULTS

Inhibition of Phage Growth

The host strain of *E. coli* grew in the presence of 0·2 per cent but not in 0·25 per cent phenol. However, in 0·2 per cent phenol and all concentrations tested down to 0·025 per cent the rate of growth was slower than in the absence of phenol. Using phage cultures, mass lysis occurred in all concentrations of phenol permitting growth of the host cells but in those concentrations giving slow host growth, lysis was correspondingly delayed. No evidence of the selective inhibition of the phage was found. In 0·025 per cent phenol, lysis occurred at a normal rate and infection of the cultures by the smallest phage inocula used could be detected. In these cultures, mass lysis was obvious after 7 hr. incubation and their opacity remained less than that of the control host cultures for at least 26 hr. In the cultures with larger phage inocula, lysis was obvious within 2 hr. but after 12 hr. incubation their opacity equalled that of the control host cultures.

Phage Inactivation

In most of the reaction mixture cultures containing active phage, lysis was apparent after 18 to 24 hr. incubation. An earlier examination was necessary to detect lysis in those cultures where large numbers of phage had survived exposure to the phenol. In such cultures, lysis of the sensitive host cells and growth of the resistant cells was rapid and after 18 hr. incubation their opacity equalled that of the control cultures. The inspections at 2, 3 and 4 days were made since a number of cultures showed lysis only after 2 days incubation. In some experiments the cultures were examined at daily intervals for up to 7 days but in no case was lysis obvious after longer than two days incubation.

The result of a single test for the inactivation of the phage has been expressed as the Inactivation Time which is the time of contact of phage and phenol after which no active phage can be detected in the reaction mixture. A minimum of 5 replicate determinations was considered necessary to give a reliable estimate of the Inactivation Time, the mean of the individual tests being calculated and expressed as the Mean Inactivation Time (M.I.T.). In any one group of replicate tests the time intervals at which the samples of reaction mixture were diluted were selected so that, within the 9 to 11 samples taken from each reaction mixture, at least one sample contained active phage and at least the last two samples diluted show no phage activity. Provided these conditions were satisfied the time intervals were made as short as possible so as to achieve maximum precision in the M.I.T. estimates. Each M.I.T. determination involved a number of preliminary experiments to obtain an approximate value. The required distribution of samples containing active phage was found in most instances with time intervals of approximately 1/8 to 1/10 of the expected M.I.T.

A typical set of results, obtained from three separate determinations of the M.I.T. of 3 per cent phenol for the phage, are shown in detail in Table I.

TABLE I

THE DETERMINATION OF THE MEAN INACTIVATION TIME (M.I.T.) OF 3 PER CENT PHENOL FOR COLIPHAGE T6r AT 25°

Experiment	Replicate test	Contact time (min.) 16	20	24	28	32	36	40	44	48	Inactivation time (min.)
I	1	+	−	+	−	−	−	−	−	−	28
	2	+	−	+	+	−	−	−	−	−	32
	3	+	−	+	−	−	+	−	−	−	40
	4	+	+	+	−	+	−	−	−	−	36
	5	+	+	+	+	−	−	−	−	−	32
	6	+	+	−	−	−	−	−	−	−	24
										M.I.T.	192/6 = 32·0
II	1	−	−	−	+	−	−	−	−	−	32
	2	+	−	−	−	−	−	+	−	−	44
	3	+	+	+	+	+	−	−	−	−	36
	4	+	+	−	+	−	−	−	−	−	32
	5	+	+	−	−	−	−	−	−	−	24
	6	+	+	−	+	−	−	−	−	−	32
										M.I.T.	200/6 = 33·3
III	1	+	+	+	+	+	−	+	−	−	44
	2	+	+	+	+	−	−	−	−	−	32
	3	+	+	−	−	−	−	−	−	−	24
	4	+	+	+	−	+	−	−	−	−	36
	5	+	+	+	+	−	−	−	−	−	32
	6	+	+	+	+	−	+	−	−	−	40
+ = active phage present; − = no phage activity.										M.I.T.	208/6 = 34·7

Reproducibility of the Method

The level of confidence with which the M.I.T. can be taken as an estimate of the inactivation of phage by phenol is shown by the results of a series of replicate determinations of the M.I.T. for five different concentrations of phenol. These experiments are summarised in Table II.

The individual estimates of the M.I.T. for each concentration of phenol are the results of separate experiments carried out at various times during a period of 3 months.

TABLE II

THE MEAN INACTIVATION TIME (M.I.T.) OF VARIOUS CONCENTRATIONS OF PHENOL FOR COLIPHAGE T6r (25°)

Concentration of phenol (per cent w/v)	Contact time interval (min.)	Number of replicate tests in each determination	Range of inactivation times (min.)	M.I.T. (min.)	Mean M.I.T. (min.)	95 per cent confidence limits of mean M.I.T. (min.)
3·50	⅓	5	2⅓–4 2⅓–4 2⅓–4	3·04 3·20 2·75	3·00	±0·57
3·25	1	5	9–13 9–12 8–11	10·8 10·6 9·4	10·3	±1·80
3·00	4	6	24–40 24–44 24–44	32 33 35	33·3	±3·79
2·90	5	5	25–60 30–60 40–50	47 50 45	47·3	±6·25
2·66	20	5	180–240 180–240	204 208	206	±25·4

Estimation of the Mean Single Survivor Time

The results of the experiments referred to in Table II were subjected to the analysis devised by Mather (1949) which permits the calculation of the Mean Single Survivor Time (M.S.S.T.) and the standard error of the estimations. The M.S.S.T. is defined as the time at which there is, on the average, one surviving organism per sample volume. The analysis of the results is summarised in Table III.

TABLE III

ESTIMATION OF MEAN SINGLE SURVIVOR TIMES (M.S.S.T.) FROM THE REGRESSION BETWEEN TIME OF CONTACT AND LOG (— LOG PROPORTION OF NEGATIVE SAMPLES). COLIPHAGE T6r EXPOSED TO PHENOL AT 25°

Phenol concentration (per cent w/v)	Visual estimate of M.S.S.T. (min.)	Calculated estimates			
		First approximation		Second approximation	
		M.S.S.T. (min.)	Standard error	M.S.S.T. (min.)	Standard error
3·50	2·03	2·12	0·18	2·12	0·17
3·25	7·45	7·25	0·39	7·35	0·36
3·00	23·60	23·16	1·22	23·33	1·06
2·90	36·75	36·54	2·09	36·75	2·06
2·66	124·5	119·6	15·5	121·2	14·0

Correlation between M.I.T. *or* M.S.S.T. *and Concentration of Phenol*

The regressions of log M.I.T. and log M.S.S.T. against log phenol concentration were analysed by standard statistical methods as described by Brownlee (1949). The correlation coefficients were 0·9985 and 0·9988 respectively with 3 degrees of freedom and the correlations were therefore highly significant. The calculated regression coefficient for log M.I.T. against log phenol concentration was −15·140 and the residual variance about the regression line was 0·002. For log M.S.S.T. against log phenol concentration, the corresponding values were −14·659 and 0·001. The variance of the difference between the regression coefficients was 0·383 from which $t = 0{\cdot}777$ with 6 degrees of freedom which corresponds to a probability between 0·4 and 0·5.

A linear relationship therefore exists both between log M.I.T. and log phenol concentration and between log M.S.S.T. and log phenol concentration. The regression lines representing these relationships are parallel over the time range of 2 to 200 min., and the mean ratio of M.I.T. to M.S.S.T. over this time range is 1·439.

DISCUSSION

Any study of the inactivation of phage by a chemical agent must include an investigation of the possible inhibition, by the agent, of the growth of the host and of the phage in cultures of the host. It is then essential that, when testing for phage surviving at the end of the inactivating process, the agent is neutralised chemically or is diluted to a concentration which not only will permit the growth of the host cells but also permit the infection of the host by small numbers of phage particles. This point does not appear to have received the attention it merits in previous investigations.

In devising an extinction time technique for use with phage, two other problems arise, that of lysis of the host by inactivated phage and multiplicity reactivation of the phage. The test applied to each sample of the reaction mixtures at the end of an inactivation period was intended to show simply whether or not infective phage had survived. The number of host cells added to the diluted reaction mixture samples was approximately equal to the number of phage originally present in the sample. While the cultures were examined at relatively infrequent intervals, cultures from samples giving increasing time of contact of the phage with a given concentration of phenol showed, in general, an increase in lysis time corresponding to that to be expected from decreasing numbers of phage in the inocula. The tests therefore gave no indication that inactivated phage particles were adsorbed to and lysed the host cells or that multiplicity reactivation occurred.

A very few reaction mixture sample cultures showed a pronounced delay in lysis, clearing of the culture being apparent only after 2 days incubation. The phenomenon was rare and occurred only in cultures from samples quenched at times approaching the final inactivation time, when the numbers of infective particles present were small. In these cultures, the rate of lysis was much slower than that shown by small phage inocula in the presence of sub-bacteriostatic concentrations of phenol and no explanation of the effect can be offered until more data is available.

In the present experiments, simple dilution of the reaction mixture samples was relied upon to overcome inhibition of the growth of the host or surviving phage. The reaction mixture samples were made relatively large to minimise sampling errors and the concentrations of phenol required to inactivate the phage were high in relation to the concentrations inhibiting its growth. The volumes of peptone water used to dilute the samples were, therefore, larger than those commonly used in bacterial extinction tests.

The phage titre of the inoculum was standardised by plaque counts but the survival of the phage exposed to phenol was estimated by its ability to grow in fluid culture. Justification for this comparison can be found in the close agreement previously shown between plaque counts and dilution end-point counts (Hershey and others, 1943; Kleczkowski and Kleczkowski, 1951).

To obtain a precise estimate of the inactivation time of the phage by various concentrations of phenol the contact time interval used in the tests was short in relation to the final inactivation time. A wide variation was found, however, between individual estimates of the inactivation times, the range between maximum and minimum values obtained from 5 to 6 replicate estimations commonly being from 50 to 75 per cent of the mean estimate. This variation is an indication of the distribution of resistance to inactivation between individual phage particles in the phage inoculum. The use of a 5 or 6 fold level of replication was found to give a mean estimate of the inactivation time which adequately reflected the distribution of resistance and the reproducibility of which was therefore

acceptable. In Table II, the 95 per cent confidence limits of the mean M.I.T.'s are about 10 to 20 per cent of the mean. It should be noted that the estimates were made at intervals over a period of some months so that the confidence limits incorporate the errors due to variations in all aspects of the tests.

The successful application of the analysis of Mather (1949) to the results is evidence that the distribution of surviving phage particles between the samples towards the end of the inactivating process is the same as that found amongst bacteria, the distribution being Poissonian. Interpretation of the results of phage inactivation tests in terms of the M.S.S.T. is therefore possible. Although the cycle of computations is too complex for routine use, the standard errors of the estimations have been calculated for the present results, and it has been found that the value of the M.S.S.T. estimated visually from the plot of contact time against log (—log proportion of negatives) falls within the calculated limits of error from both the first and second approximation. The reliability of this visual estimate is only acceptable when 10 or more replicate determinations have been performed using a contact time interval of 1/8 to 1/10 of the expected M.I.T. (or 1/6 to 1/7 of the M.S.S.T.).

Interpretation of extinction time data by means of the M.S.S.T. undoubtedly gives the most accurate interpretation of these data but it requires a high level of replication to have significance.

It has been shown that the relationship of the M.I.T. to the M.S.S.T. is constant over a wide range of extinction times. This is considered to indicate that at a high level of replication, the interpretation of the results in terms of the M.I.T. is as reliable as their interpretation in terms of the M.S.S.T. At a lower level of replication, estimates of the M.I.T. have been shown to have confidence limits of 10 to 20 per cent of the mean.

If these limits of accuracy are considered acceptable, the examination of the effect of chemical agents on phages may be carried out using 5 or 6 replicate determinations and the results expressed as the M.I.T. If a higher degree of reproducibility is required then a higher level of replication must be used and the results may then be best expressed as the M.S.S.T.

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