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Inactivation of a bacteriophage by chemical antibacterial agents

A. M. COOK AND W. R. L. BROWN

An extinction method has been used to examine the inactivation of coliphage T6r by a number of chemical antibacterial agents. A marked difference in the effect of concentration on inactivation efficiency has been shown for different agents, chloramine-T and formaldehyde having concentration exponents of approximately 2 and 3 respectively; crystal violet, cetrimide and phenol having concentration exponents of approximately 11, 13 and 15 respectively. It is suggested that a low concentration exponent is associated with inactivation of the phage by an effect on the protein coat of the particle and a high concentration exponent with an effect on its internal structure.

AN extinction method of examining the inactivation of phage has been previously described (Cook & Brown, 1963). This method has now been applied to a study of the effect of a number of chemical antibacterial agents on a coliphage.

Experimental

Bacteriophage and bacterial host. The phage used was a coliphage with the cultural characteristics of strain T6r (Demerec & Fano, 1945; Hershey, 1946) and the bacterial host a laboratory strain of *Escherichia coli* sensitive to the phage. The methods and media used for cultivating the host and preparing phage stocks were those previously described (Cook & Brown, 1963). A single phage stock suspension, stored at 5°, was used throughout the experiments on phage inactivation. The titre of this stock, as determined by periodic plaque counts using a modification of the method described by Williams-Smith (1951, 1953), was $4{\cdot}06 \pm 0{\cdot}29 \times 10^{10}$ (P = 0·95) phage particles per ml.

Antibacterial agents. From a number of preliminary experiments, 5 antibacterial agents showing relatively high viricidal activity against phage were selected. The substances used were cetrimide and crystal violet of the British Pharmacopoeia 1958, phenol (Analar) and formaldehyde solution (Analar, 36·83% CH_2O), and chloramine-T (British Drug Houses Laboratory Regent). Experimental solutions of chloramine-T and formaldehyde were prepared daily as required, those of the other agents were prepared immediately before use by diluting stock solutions stored in the dark at ambient room temperature (20–25°). All solutions were prepared using sterile distilled water.

Inhibition of phage growth. The bacteriostatic activity of each agent for the bacterial host was determined using a serial tube dilution method. The effect of sub-bacteriostatic concentrations of the agents on the rate of mass lysis in phage cultures was examined by the same method using phage inocula of various titres (Cook & Brown, 1963).

From the School of Pharmacy, University of London, 29/39 Brunswick Square, London, W.C.1

Inactivation of extracellular phage. The method used was that previously described in detail (Cook & Brown, 1963), and consisted, essentially, of adding a standard phage inoculum (4×10^8 phage particles per ml of reaction mixture) to a solution of the agent under test, immediately removing a number of samples of the mixture, diluting these samples with peptone water after suitable time intervals, inoculating the dilution with bacterial host cells and incubating the cultures at 37°. The presence of surviving infective phage was shown by visible lysis in the incubating cultures. The time of contact of the phage and antibacterial agent after which no infective phage can be detected is now defined as the Extinction Time.

Each agent was used in a series of ranging tests until the concentration range giving conveniently measurable Extinction Times was established. The Mean Extinction Time (M.E.T.) for each of several concentrations in this range was then determined, each estimate of M.E.T. being based on not less than 5 replicate determinations. (The term "Mean Extinction Time" is now preferred to "Mean Inactivation Time", used previously, Cook & Brown, 1963).

In all tests the reaction mixture sample volume taken was 6 drops (approximately 0·1 ml) from a standardised dropping pipette. To ensure dilution of the antibacterial agent to a concentration which would permit the detection of small numbers of infective phage particles, the samples were normally diluted with 20 ml of peptone water. In the tests on formaldehyde 100 ml volumes of peptone water were used to achieve the required dilution.

Results

Inhibition of phage growth. The inhibitory action of the antibacterial agents on the growth of the bacterial host is shown in Table 1.

TABLE 1. GROWTH OF *E.COLI* IN THE PRESENCE OF ANTIBACTERIAL AGENTS (IN PEPTONE WATER AT 37°)

Antibacterial agents	Maximum concentration permitting		Minimum concentration inhibiting growth in all replicates (M)
	approximately normal rate of growth (M)	growth in all replicates after 48 hr (M)	
Cetrimide*	$2{\cdot}7 \times 10^{-6}$	$5{\cdot}5 \times 10^{-6}$	$2{\cdot}7 \times 10^{-5}$
Chloramine-T	$8{\cdot}9 \times 10^{-4}$	$2{\cdot}5 \times 10^{-3}$	$2{\cdot}8 \times 10^{-3}$
Crystal violet	$1{\cdot}0 \times 10^{-6}$	$2{\cdot}0 \times 10^{-5}$	$5{\cdot}0 \times 10^{-5}$
Formaldehyde	$3{\cdot}3 \times 10^{-4}$	$1{\cdot}2 \times 10^{-3}$	$1{\cdot}8 \times 10^{-3}$
Phenol	$2{\cdot}7 \times 10^{-3}$	$2{\cdot}1 \times 10^{-2}$	$2{\cdot}7 \times 10^{-2}$

* Molarity calculated assuming sample to be pure cetyltrimethylammonium bromide

Mass lysis occurred in phage cultures containing all concentrations of the antibacterial agents permitting growth of the host cells. Where the concentrations of the agents were low enough to permit an approximately normal rate of growth of host cells, mass lysis occurred at a normal rate but in those concentrations giving a slow host growth rate lysis was correspondingly delayed.

Inactivation of extracellular phage. The results of the later experiments on the inactivation of extracellular phage are illustrated in Fig. 1. The results for phenol have previously been reported in detail (Cook & Brown, 1963) but have been summarised and included here for comparison.

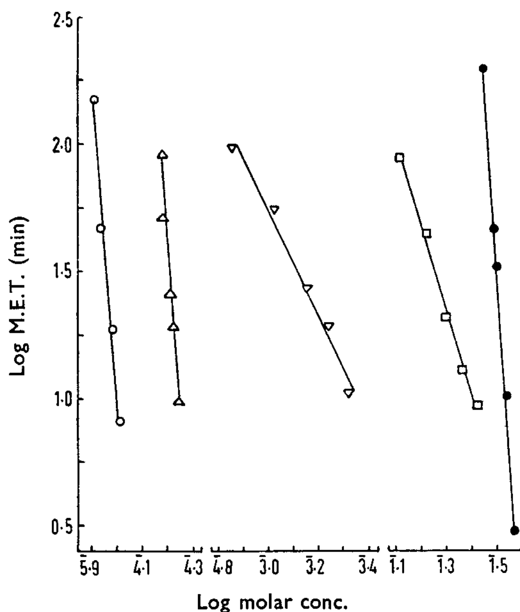

FIG. 1. Relation between concentration of antibacterial agent and Mean Extinction Time (M.E.T.) for the inactivation of coliphage T6r (25°). Cetrimide △—△; Chloramine-T ▽—▽; Crystal Violet ○—○; Formaldehyde □—□; Phenol ●—●.

For each antibacterial agent, the regression of log M.E.T. upon log molar concentration was shown to be linear (Table 2). The slope of such a regression represents the concentration exponent (n) which can be defined (Watson, 1908) by the expression,

TABLE 2. REGRESSION OF LOG M.E.T. UPON LOG MOLAR CONCENTRATION FOR THE INACTIVATION OF COLIPHAGE T6r

Antibacterial agent	Correlation coefficient	Regression coefficient	Standard deviation of regression coefficient
Cetrimide ..	0·9972	− 13·28	0·57
Chloramine-T ..	0·9896	− 2·00	0·17
Crystal violet ..	0·9964	− 10·85	0·65
Formaldehyde ..	0·9966	− 3·35	0·16
Phenol	0·9986	− 15·20	0·47

(The regression coefficient here equals the concentration exponent, n.)

$$n = \frac{\log t_2 - \log t_1}{\log c_1 - \log c_2}$$

where c_1 and c_2 are concentrations giving M.E.T. of t_1 and t_2. The calculated value of the regression coefficients (or slopes) are shown in Table 2 together with their standard deviations.

Comparison of the slopes of the regressions of log M.E.T. upon log molar concentration for different agents indicated that they could not be considered parallel (Table 3).

TABLE 3. COMPARISON OF REGRESSION COEFFICIENTS FOR THE REGRESSION OF LOG M.E.T. UPON LOG MOLAR CONCENTRATION

Comparing regression coefficients of:	Variance of difference in regression coefficients	t	Degrees of freedom	Corresponding probability
Cetrimide and phenol	0·6222	3·092	6	0·02 to 0·05
Cetrimide and crystal violet . .	0·8504	2·860	5	0·02 to 0·05
Phenol and crystal violet	0·6009	7·283	5	about 0·001
Chloramine-T and formaldehyde . .	0·06729	20·11	6	less than 0·001

Discussion

In the experiments on the inhibition of mass lysis in phage cultures, no evidence of the selective inhibition of phage growth was found with any of the antibacterial agents tested. The selective inhibition of phage growth reported by Graham & Nelson (1954) for lactic streptococcus phages, was not found to occur with coliphage T6r as tested here.

The reduced rate of growth of host cells and the consequent reduction in the rate of lysis in phage cultures containing concentrations of antibacterial agents approaching bacteriostatic concentrations has a decided significance in the present work. The level of dilution of the reaction mixture samples in the tests for phage inactivation was selected so as to ensure the reduction of the concentration of antibacterial agents to one which had been shown to permit a normal rate of growth of host cells and mass lysis by phage. Under these conditions small numbers of surviving infective phage can be readily detected.

The 5 antibacterial agents examined can be arranged in ascending order of efficiency in inactivating extracellular phage as phenol, formaldehyde, chloramine-T, cetrimide and crystal violet.

The most interesting feature, however, of the results is the difference found in the effect of changes of concentration on their activity, expressed as the concentration exponent (n).

The validity of using n as a basis for speculation on the mechanisms of the inactivation of bacteria by bactericides has in the past, been the subject of some argument (Rahn, 1945). Much of the early criticism of this use of n was based on its variability, resulting largely from the use of imprecise methods of determination such as those used by Tilley (1939) on whose results Rahn's arguments were largely based. It can now be accepted that, provided the determination of n is sufficiently precise, significant variations in values of n between the action of different

bactericides on one organism at least gives an indication that the mechanism of inactivation in each case is different. It has been concluded from the present work that the value of n has an equal significance in the inactivation of phage by antibacterial agents.

If this significance of n is accepted the 5 antibacterial agents examined in detail fall into 2 general groups; chloramine-T and formaldehyde with relatively low values of n (approximately 2 and 3 respectively) in one group, and crystal violet, cetrimide and phenol with values of n greater than 10 (approximately 11, 13 and 15 respectively) in the other. It should be noted that, within each group, the slopes of the regression from which the values of n were derived have been found to show significant departure from parallelity when tested statistically. The possibility therefore exists that there are differences in the mechanism of inactivation among the members of each group.

The question which now arises is, can any similarity be found between the possible general mechanisms of inactivation of phage by the substances in each group?

The action of formaldehyde on extracellular phage has been extensively studied and, while the mode of action has not been fully elucidated, the consensus of opinion is that the phage is inactivated by the combination of the formaldehyde with the protein coat of the phage particle. Sauerbier (1960) has suggested that formaldehyde inactivates T1 phage, not by protein damage but by reacting with the phage deoxyribonucleic acid (DNA) and the demonstration by Mutsaars (1957a, b, 1959) that multiplicity reactivation occurs in formaldehyde inactivated coliphage-N also implies DNA damage. On the other hand it was clearly demonstrated by Hershey & Chase (1952) that T2 coliphage, inactivated by formaldehyde, is still adsorbed on to its host but the phage DNA is not released. A similar reaction was reported by Bourgaux (1957) for coliphage-N treated with formaldehyde. It has further been clearly established (Staehelin, 1958; Berns & Thomas, 1961; Grossman, Levine & Allison, 1961) that formaldehyde will not react with amino-groups involved in strong hydrogen bondings as is the case in DNA. That formaldehyde forms an unstable combination with phage is indicated by the ease with which formaldehyde inactivated T-group phages are reactivated by storage in the presence of histidine (Heicken & Spicher, 1959). Such an unstable combination implies that the formaldehyde combines with some part of the protein coat of the phage.

No attempts have been made to investigate the mechanism of action on phage of compounds liberating chlorine. Indeed, little information is available on the mechanism of action of these compounds on bacteria although it seems clear that "chlorine compounds" including the chloramines, attack bacteria through the undissociated hypochlorous acid formed by the interaction of the liberated chlorine with water (Marks, Wyss & Strandskov, 1945; Hadfield, 1957; Brazis, Leslie, Kabler & Woodward, 1958). Chlorine is known to have an intense reactivity with proteinous material (Sykes, 1958) and it seems likely that the site of the attack on phage by chloramine-T is the protein coat.

Both formaldehyde and chlorine react strongly with organic matter so that the inactivation time estimates for these two agents will be particularly affected by the presence of peptone in the phage inoculum. The effect will be to make the M.E.T., determined experimentally, longer than the true inactivation time. This effect will be most pronounced with low concentrations of the agents so that the value of n obtained will be greater than the true value although the size of the effect is a matter for speculation. Since the arguments on the significance of the values of n found here are based on the smallness of n for formaldehyde and chloramine the effect, in any case, does not invalidate the argument.

The mode of inactivation of phage by crystal violet has not been investigated but it seems likely, from the known affinity of crystal violet for DNA (Stearn, 1930; Mirsky & Ris, 1951; Graham & Nelson, 1954) that it occurs by combination with phage DNA. That ability to combine with free DNA does not necessarily confer the ability to produce marked inactivation of intact phage is shown by the acridines, for which combination with free DNA has been proved (Peacocke & Skerret, 1956; Luzzati, Masson & Lerman, 1961) but which have little or no effect on free phage (Hotchin, 1951).

The prevalent theory of the mode of action of the quaternaries (including cetrimide) on bacteria is that their main effect is to cause an increase in the permeability of the cytoplasmic membrane and a lethal loss of intracellular constituents (Salton, 1951; Stedman, Kravitz & King, 1957). This effect has been suggested by Gilby & Few (1957) to be caused by the interaction of the compounds with phospholipids in the cytoplasmic membrane. The action of phenol on bacteria has been shown to be very similar to that of cetrimide (Gale & Taylor, 1947; Maurice, 1952; Stedman & others, 1957).

It is therefore suggested that both cetrimide and phenol act on phage in a similar manner by affecting the association of the protein coat of the phage with the DNA (without necessarily causing complete dissociation of the two) or by denaturing both the protein coat and the "internal protein" which has been shown by Spizizen (1957) to play an essential part in initiating phage multiplication within the host cells.

It is suggested that, when the inactivation of a phage by an antibacterial agent has a low value of n, the agent is attacking the protein coat of the particle. When the value of n is high then the attack is on the internal structure of the phage, either by combination with the DNA, as in the case of crystal violet, or by affecting the association of the DNA with the protein content of the phage, as with cetrimide and phenol. The determination of an accurate value for n may be a useful preliminary indication of the site of action of an antibacterial agent on a phage.

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