THE RATE OF BACTERIOPHAGE INACTIVATION BY FILTRATES OF ESCHERICHIA COLI CULTURES

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INTRODUCTION

The inactivation of bacteriophage by substances from susceptible bacteria has been studied by Burnet (1) and Freeman (2). Its inactivation by antisera has been studied by Andrewes and Elford (3) and Burnet, Keogh, and Lush (4). Recently Ashenburg *et al.* (5) reported that saline solutions of gum arabic, starch, or glycogen inactivated an anti-*Klebsiella pneumoniae* phage.

We have studied in more detail the rate of phage inactivation by culture filtrates of the susceptible bacteria in order to determine the dependence of the process on phage and inhibitor concentrations.

EXPERIMENTAL

The phage inactivating solution was prepared from culture filtrates of the susceptible strain of *coli* (B_1) previously used in this laboratory (6). The organisms were grown in synthetic medium of the composition given by Delbrück (7). With adequate aeration, these cultures attained a maximum plate count at 48 hours of more than 5×10^9 organisms per cc. 16 liter cultures were incubated for 8 days with aeration. The cells were then spun out in a Sharples supercentrifuge and the clear supernatant concentrated in vacuo at 35°C. to about one-tenth its volume. This material was then dialyzed in cellophane sausage casing against running tap water for 48 hours. The remaining solution was then clarified in the centrifuge and further concentrated in vacuo at 35°C. It was again dialyzed, clarified in the centrifuge, and passed through a Seitz filter. The final product had a pH of 6.5, contained 2.5 mg./cc. total solids, and represented a 35 to 1 concentrate of the initial filtrate of the 8 day bacterial cultures. This concentrated, dialyzed bacterial filtrate is called "filtrate" or simply "*F*" below.

Diluted bacteriophage suspensions were prepared by 100-fold dilution in distilled water of fresh filtered phage lysates of susceptible *coli* cultures. The suspension then contained 7×10^6 phage particles per cc. as determined by the plaque counting method previously described (6).

Suitable dilutions in nutrient broth of the phage inactivating filtrate were brought to temperature. To 0.9 cc. of such a dilution of *F* was added 0.1 cc. of a dilution in broth of stock phage adjusted to contain about 3×10^5 phage particles per cc. This mixture

was incubated at the selected temperature and 0.1 cc. samples removed from time to time, the amount of active phage remaining being determined by plaque counts.

Each experimental determination of active phage corresponded usually to counts of two or four plates, containing a total of 100 to 400 plaques. Thus the sampling error lies between 5 and 10 per cent. The efficiency of plating (6) may change in the course of the inactivation. The data on the later stages of the inactivation reaction cannot be interpreted quantitatively until this point is determined.

Rate of Phage Inactivation at 0°C.

A progressive decrease in phage assay was noted with all concentrations of F tried (Fig. 1). This decrease was logarithmic in all cases until 95 per cent of the phage had been inactivated, after which the reactions became slower. The reaction is therefore first order with respect to phage, as was indicated by Burnet (4) for several phages active against *B. dysenteriae.*

The rate is not proportional to the concentration of inactivator but nearly proportional to the square root of this value. The rate of phage inactivation may thus be expressed by the equation

$$-\frac{d \log (P)}{dt} = k_0(F)^{\frac{1}{2}}$$

where (P) is the phage concentration, (F) the concentration of filtrate preparation, the undiluted material being assigned the arbitrary value unity, and k_0 is a constant. Table I shows the values of k_0 calculated from this equation, using the rates obtained graphically, for inactivator concentrations from 0.001 to 0.04. At concentrations lower than these, the results become uncertain and variable.

The slower rate of inactivation of the last fraction of phage is discussed below. That this decrease in rate did not result from exhaustion of inactivator substance is shown in the experiment plotted in Fig. 2. The inactivation of phage was permitted to proceed until 95 per cent inactivation had been accomplished. Then more phage was added to the reaction vessel and its rate of inactivation followed. As the figure shows, the second quantity of phage was inactivated at the same rate as the first batch, indicating that the concentration of inactivator had not appreciably decreased.

Rate of Phage Inactivation at 37°C.

At this temperature phage inactivation followed the logarithmic curve for only about the first 50 per cent, after which the inactivation became slower. Furthermore, the relationship of initial rate to F concentration is different; the rate is nearly proportional to the concentration of F, not its

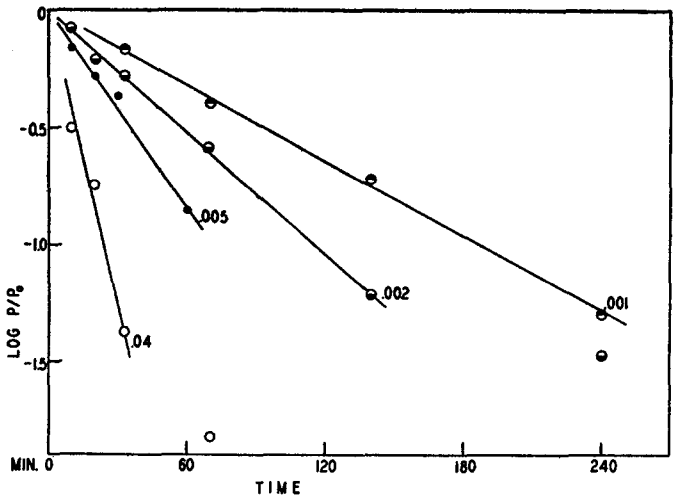

FIG. 1. The influence of F concentration on the rate of phage inactivation at 0°in broth. The numbers adjacent to the curves refer to the concentration of filtrate in the reaction mixture in arbitrary units.

TABLE I

The Influence of F Concentration on the Rate of Phage Inactivation at 0°C. in Broth

(F)	$\frac{-d \log (P)}{dt}$	k_0
0.04	0.042	0.21
0.005	0.015	0.21
0.002	0.009	0.20
0.001	0.006	0.19

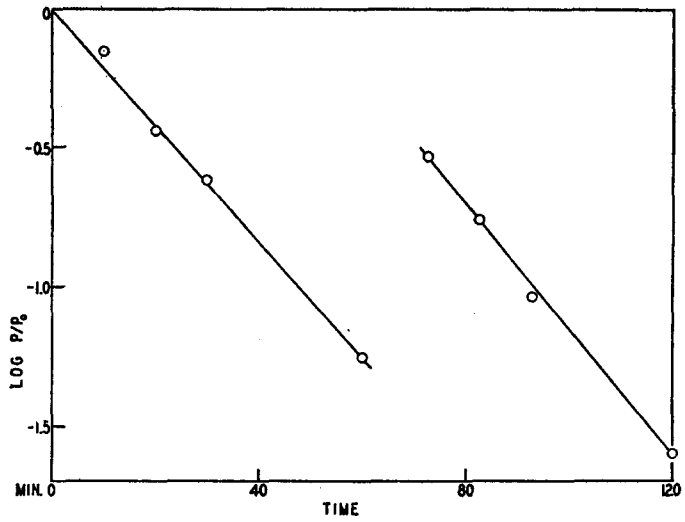

FIG. 2. The inactivation of a second portion of phage, after the inactivation of 95 per cent of a first portion, demonstrating the presence of excess inhibitor. The second portion of phage was added at 65 minutes.

square root. Fig. 3 shows the course of the inactivation reaction for three F concentrations, and Table II shows the corresponding values of the reaction rate constant, k_{37}, calculated from the equation

$$-\frac{d \log (P)}{dt} = k_{37}(F)$$

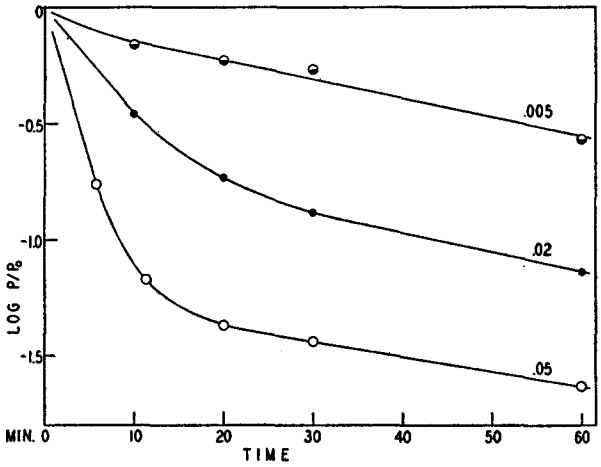

FIG. 3. The course of phage inactivation by filtrate at 37°C. in broth. The numbers adjacent to the curves refer to the concentration of filtrate in the reaction mixture in arbitrary units.

TABLE II

The Influence of F Concentration on the Rate of Phage Inactivation at 37°C. in Broth

(F)	$\frac{-d \log (P)}{dt}$	k_{37}
0.05	0.15	3.0
0.02	0.05	2.5
0.005	0.016	3.2

Clearly, the rates are here proportional to the concentration of F, not $F^{\frac{1}{2}}$ as at 0°C. Similar experiments at intermediate temperatures showed that the initial rates were proportional to (F) at 20°C., but became proportional to $(F)^{0.76}$ at 6°C.

Effect of Salt on the Inactivation

The inhibitor substance does not pass through a dialysis membrane and is probably a large molecule. Surface forces can be expected to play an important rôle in these reactions. These surface forces can be readily changed by changes in electrolyte concentration. The course of the phage

inactivation in the absence of significant amounts of electrolytes was investigated, by diluting both phage and inactivator in distilled water instead of broth. The effects of the addition of various concentrations of sodium chloride were also studied.

The addition to the broth used as the diluting medium of 25 per cent NaCl stopped the inactivation reaction. No significant decrease in phage occurred in 2 hours incubation at 37°C. in a filtrate concentration of 0.1. Without salt, at this (*F*) concentration, the phage would have been 90 per cent inactivated in less than 5 minutes. Similarly when tested at 0° this concentration of salt practically stopped the inactivation. On the other hand, salt concentrations of 0.5 per cent were found to increase the rate of inactivation. A 2.5 per cent concentration slightly decreased the rate of inactivation. In distilled water dilutions, where the only electrolytes were the impurities in the filtrate and phage, the inactivation did not proceed at an appreciable rate. Incubation of the phage without (*F*) under the same conditions of temperature and salt concentration resulted in no change in the amount of phage. This is contrary to a statement by Gratia (8) who attributed an inactivation of phage in a lysate with added salt to a direct action of the salt on phage. We believe that this effect of moderate salt concentrations in his case may have been not on phage directly but due to salt accelerated inactivation of phage by substances from the lysed bacteria.

The effects of salt concentration led us to test whether phage which had been inactivated by *F* could be regenerated by agents known to be effective dissociating agents. High concentrations of various salts, or low concentrations of soaps, were added to solutions in which phage had been inactivated with *F*, but without the slightest indication of the reactivation of any of the inactivated phage. These reagents alone had no effect on active phage, in any concentrations used.

Phage Inactivation by Common Polysaccharides

The inactivation of phage by several common polysaccharides was recently reported by Ashenburg *et al.* (5). We have confirmed their results with gum arabic and starch, with our phage, and have found in addition that inulin and acetylated gum arabic also will inactivate our *coli*-phage. The concentrations of starch or gum arabic needed to attain reasonable rates of inactivation (50 to 100 mg. per cc.) are greatly in excess of those required in the case of the specific substance from the bacteria. The preparations of inhibitor substance (*F*) used by us contained 2.5 mg. total solids per cc. Additional chemical work on this substance, still in progress,

shows that considerably more than half of this must be considered impurity. Therefore, it may be safely concluded that concentrations of inhibitor of less than 0.002 mg. per cc. produce a rapid inactivation of phage (Fig. 1).

At 0°C., the inactivation with starch is too slow to be measured. The course of the reaction at 37° with starch is similar to that with the bacterial inactivator. The decrease in phage is logarithmic at first, becoming slower in the later stages. This rate is proportional to the starch concentration. This is especially interesting in view of the high specificity of culture filtrates as inactivating agents, a specificity which parallels the adsorption of phage by the corresponding bacteria (1).

DISCUSSION

The interpretation of these data in terms of a reaction mechanism is complicated by several factors. The absolute concentration of inhibitor substance is unknown, and the relative molecular concentrations of phage and inactivator cannot be determined. Furthermore, this phage has not been isolated in high enough concentrations to make it feasible to study the course of the reaction in the presence of excess phage instead of excess inactivator. The change with temperature in the dependence of the rate on F concentration makes the consideration of temperature coefficients difficult. For example, at very low F concentrations, the inactivation at 0° is faster than that at 37° for the same concentration of inactivator.

We shall consider first only the initial rates, reserving for later discussion the slower rates attained after inactivation of the main fraction of phage. The assumption of the following series of reactions accounts for the observations in a simple way:

$$b + b \rightleftharpoons bb \tag{1}$$

$$P + b \rightleftharpoons P{:}b \tag{2}$$

$$P{:}b \rightarrow X \tag{3}$$

The symbol $P{:}b$ represents a complex of phage and inactivator in which the phage retains temporarily full activity, while X represents the inactivated complex. "b" is the molecular species of the inactivator substance which combines with phage and "bb" is a dimolecular form which is inert toward phage. Reactions (1) and (2) are equilibria, and are rapid and reversible, and reaction (3) is the rate controlling irreversible step leading to inactivation of phage. Equilibrium (1), from our data, must be temperature sensitive, being pushed to the left by an increase in temperature. According to these reactions, phage is monovalent with respect to "b" in this inactivation process.

Taking these reactions as descriptive of the mechanism and with the assumptions stipulated above, a rate expression can be derived. The equilibrium constants for reactions 1 and 2 are respectively

$$K_1 = \frac{(bb)}{(b)^2} \tag{4}$$

$$K_2 = \frac{(P \cdot b)}{(P)(b)} \tag{5}$$

The total concentration (all forms) of inactivator is

$$(F) = (b) + 2(bb) \tag{6}$$

The rate of phage inactivation is

$$-\frac{d(P)}{dt} = k'(P \cdot b) \tag{7}$$

which by substitution from (5) becomes

$$-\frac{d(P)}{dt} = k'K_2(P)(b) \tag{8}$$

Substituting for (b) from (4) and (6) gives

$$-\frac{d(P)}{dt} = \frac{k'K_2}{4K_1}(P)\left(-1 \pm \sqrt{1 + 8K_1(F)}\right)$$

When the inactivator is chiefly in the "bb" form, K_1 is large and the rate expression becomes

$$-\frac{d(P)}{dt} = k_0(P)(F)^{\frac{1}{2}} \text{ where } k_0 = \frac{k'K_2\sqrt{2}}{2K_1^{\frac{1}{2}}}$$

which is the observed relationship at 0°C. When the inactivator is largely in the dissociated "b" form, the rate expression is substantially equation (8)

$$-\frac{d(P)}{dt} = k_M(P)(F) \text{ where } k_M = k'K_2$$

which is the observed relation at 37°C.

Although the equations assumed above lead in this way to rate expressions which agree with the experimental findings, this does not prove their reality. However, they permit discussion of the observations on definite terms.

The influence of salt on the course of the reaction may be described as an influence on reaction (2) in which a minimal electrolyte concentration is necessary to permit the combination of P and b, and high concentration

dissociates the $P \cdot b$ complex, preventing the inactivation by reaction (3). The precipitin reaction provides an analogy. Here, the presence of moderate amounts of electrolytes is necessary for the combination of antigen and antibody to proceed, whereas in strong electrolyte solutions, the combination does not occur (9).

After a part of the phage has been inactivated the rate decreased (see Fig. 3), indicating that a fraction of the phage particles was more slowly inactivated than the remainder. Schlesinger (10), and Delbrück (11) reported that in the adsorption on live and dead bacteria, there is a fraction of the phage which is less reactive than the remainder. This variation in adsorption rate of different fractions of the phage cannot explain the results obtained in the filtrate inactivation. At 0°C. the phage inactivation was first order until after inactivation of more than 95 per cent of the phage (see Fig. 1) while at 37°C. the rate was first order during the inactivation of about 90 per cent when $(F) = 0.05$, and about 65 per cent when $(F) = 0.02$ (see Fig. 3). Similar results were obtained in 2.5 per cent saline solutions.

From these results, and those previously appearing in the literature (3 and 4) it appears that there are two competing processes, one inactivating phage (the logarithmic part of the inactivation curve) and the other producing "partly inactivated phage." The second process, if more temperature sensitive than the first, would result in logarithmic inactivation over a greater range at 0° than at 37°C. The inactivation of the "partially inactivated phage" might occur by (*a*) reversal of the second process to permit inactivation by the first mechanism, or (*b*) a direct inactivation of the partially inactivated phage.

These considerations lead to the view that phage inactivation by filtrates is more than simply combination of phage with inhibitor substance, and that the extent of phage inactivation is not a direct measure of the extent of this combination. This view is supported by the observation of Andrewes and Elford (3) that phage ceases to pass through a membrane filter immediately after mixing with antiserum, indicating in this case the formation of a phage-antibody complex still retaining phage activity. In this regard, phage is similar to catalase (12) and urease (13), where the combination of the enzyme and anti-enzyme does not result in complete loss of activity.

SUMMARY

1. The rate of inactivation of an anti-*coli* phage by filtrates of cultures of the homologous bacteria has been studied.

2. The inactivation rate at 37°C. is proportional to phage concentration and filtrate concentration.

3. At 0°C. the rate of phage inactivation becomes proportional to the square root of the filtrate concentration.

4. A reaction scheme to account for these observations is suggested and discussed.

5. This *coli*-phage is also inactivated by relatively large concentrations of soluble starch, inulin, gum arabic, and acetylated gum arabic.

6. The inactivation is markedly influenced by salt concentration, being rapid at moderate salt concentrations and slow at high or extremely low salt concentrations.

7. The inactivated phage cannot be regenerated by high salt concentrations, or by soaps.

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