ACTION OF CHLORAMPHENICOL ON T-1 BACTERIOPHAGE

I. Inhibition of Intracellular Multiplication

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The complex sequence of events following infection of a susceptible bacterium with a bacteriophage which results in the formation of new virus is being elucidated gradually through the application of widely divergent techniques. However, despite the increasing knowledge concerning such matters, many basic questions remain unanswered. Foremost among these is the question of the biochemical mechanisms involved in the redirection of metabolic processes in the infected cell toward virus production. The present study presents a tool which may be useful for investigations along these lines.

The antibiotic, chloramphenicol, has been shown to inhibit strongly the synthesis of bacterial proteins without suppressing the formation of pentose and desoxypentose nucleic acids (Gale and Folkes, 1953; Wisseman *et al.*, 1953). The inhibition of adaptive enzymes previously reported for this drug (Hahn and Wisseman, 1951) has been interpreted as a special manifestation of the inhibitory effect on protein synthesis (Wisseman *et al.*, 1953). Other processes which include polysaccharide formation, phosphorylation, oxidation, and the utilization of adenosine triphosphate in enzymic reactions do not appear to be seriously affected (Wisseman *et al.*, 1953).

Observations on the action of an efficient inhibitor of protein synthesis introduced into a suspension of phage infected bacteria at specific stages of their developmental cycle may lead to information pertinent to the role of protein synthetic mechanisms in establishing and consummating the infection. Accordingly, in the present study the action of bacteriostatic concentrations of chloramphenicol was determined on different phases of the growth cycle of T-1 coliphage through application of standard techniques of bacteriophage study. The results indicate a marked inhibitory action on intracellular growth of the virus. The biochemical effects of the antibiotic on the phage system are the subject of another report.

MATERIALS AND METHODS

Stock cultures of *Escherichia coli*, strain B, on nutrient agar slants were stored at 5 C with monthly subculture. For experiments, the bacteria were grown in broth with aeration for 4 to 5 hours or until a concentration of about 10^9 organisms per ml was obtained, using an inoculum of approximately one part of an 18 hour broth culture to 100 parts medium. Dehydrated nutrient broth (Difco) containing 0.5 per cent NaCl and 1×10^{-3} M $CaCl_2$ was used as the growth medium and as diluent for all tests.

Bacteria-free stock T-1 bacteriophage suspensions titering 6×10^{10} infectious units per ml were prepared from lysates of broth cultures clarified by centrifugation followed by passage through an ultrafine sintered glass filter.

Methods for the titration of virus content, measurement of adsorption, and one step growth experiments in common use today have been documented in a comprehensive manner by Adams (1950). These techniques were applied essentially as outlined by him except for those modifications necessitated by the introduction of the antibiotic. These are indicated in the text.

EXPERIMENTAL RESULTS

Gross effects of chloramphenicol on the T-1 phage-*E. coli*, strain B, system were determined in preliminary screening experiments by observing the influence of the drug on lysis when added at different periods in the infection cycle. The course of lysis was followed turbidimetrically. Bacteriostatic amounts (10 to 100 μg per ml) of the antibiotic and about 10 to 20 phage particles per bacterium were employed. These experiments showed that lysis was completely inhibited when chloramphenicol was introduced into the system at about the same time as the

bacteriophage but that variable degrees of lysis took place when the drug was added later in the latent period.

Although they demonstrated a distinct effect of the antibiotic, observations dependent on lysis alone provided little information on the phase of the infection cycle affected and none on the actual yield of phage. Hence, in the experiments to follow, methods were employed which allowed more precise examination of the effect of the drug on the free phage particle, on the process of adsorption, and on the period of intracellular phage multiplication.

Effect of chloramphenicol on free phage particles. A direct virucidal action of chloramphenicol on free phage did not occur. For example, a virus suspension (1×10^6 per ml), free of bacteria, incubated at 37 C for one hour in the presence of 100 μg per ml of chloramphenicol showed no reduction in titer when assayed by the agar layer technique.

Effect of chloramphenicol on adsorption. The possible interference of chloramphenicol with adsorption of virus onto the bacterial cell was examined by two different methods, i.e., (1) by the measurement of the rate of disappearance of unadsorbed phage and (2) by determination of the proportion of surviving bacteria.

Results obtained by both methods (figure 1) indicate that adsorption is not prevented by even high concentrations of chloramphenicol, and that the rate of adsorption in systems containing drug does not differ remarkably from that in an uninhibited control. In the experiments illustrated, the antibiotic was added to the bacterial suspension immediately prior to the virus. Identical results were obtained when the drug was mixed with the bacteria 15 minutes before the phage was added.

Intracellular phage multiplication. The following experiments demonstrate the effect of the drug on intracellular phage growth.

(a) *One step growth studies.* The effect of the drug introduced at different stages in the latent period of single infection cycles was studied through application of the one step growth method. The basic procedure will be given in some detail in order that the experiments in which chloramphenicol was introduced at different phases and for different lengths of time may be understood more readily.

The virus and bacteria were mixed together to give final concentrations of about 5×10^6 per ml and 5×10^7 per ml, respectively, and, after an adsorption period of five minutes, the mixture was diluted 1:10 in antiphage serum. Five minutes were allowed for the serum inactivation of the free phage, 99 per cent being inactivated in this time. Further dilutions (about 1:250) were made then to the "first growth tube" (FGT), the dilution being chosen to reduce the serum concentration to an ineffective level and to provide a reasonable number of plaques from samples removed during the latent period. A "second growth tube" (SGT) was prepared also by diluting an aliquot of the first growth tube 1:100 in order to allow measurement of the increase in infectious centers attendant with lysis of the bacteria. All of these procedures were carried out at 37 C.

The entire procedure from mixing the phage and bacteria to preparation of the first growth tube and second growth tube was carried out in 11 minutes. Assays for the number of infectious centers were made from the first growth tube and second growth tube at this time and at subsequent one or two minute intervals until lysis was complete and the stationary phase was attained. Intervals between samples were then lengthened. Samples were plated in triplicate; the plaques were counted after six hours of incubation at 37 C. The one step growth curve was constructed by plotting against time the relative plaque count, obtained by dividing the actual plaque count corrected for dilution by the average count in the latent period. The resulting curve demonstrated the length of the latent period, the rise period, the stationary period, and the average burst size.

In the first series of experiments, a bacteriostatic concentration of chloramphenicol (10 μg per ml) was added to infected bacteria at different intervals during the latent period. Figure 2 presents the results of some of these tests. Curve 1 illustrates the sequence of events in the control experiments which contained no drug.

Curve 2 illustrates the effect of chloramphenicol when it was added at the time that the phage and bacteria were mixed and also was maintained in a concentration of 10 μg per ml in the solutions used to prepare the various dilutions tested. It is evident that no burst or increase in infectious centers occurred under these conditions. Identical results were obtained in a similar type of experiment when the drug was first added five minutes

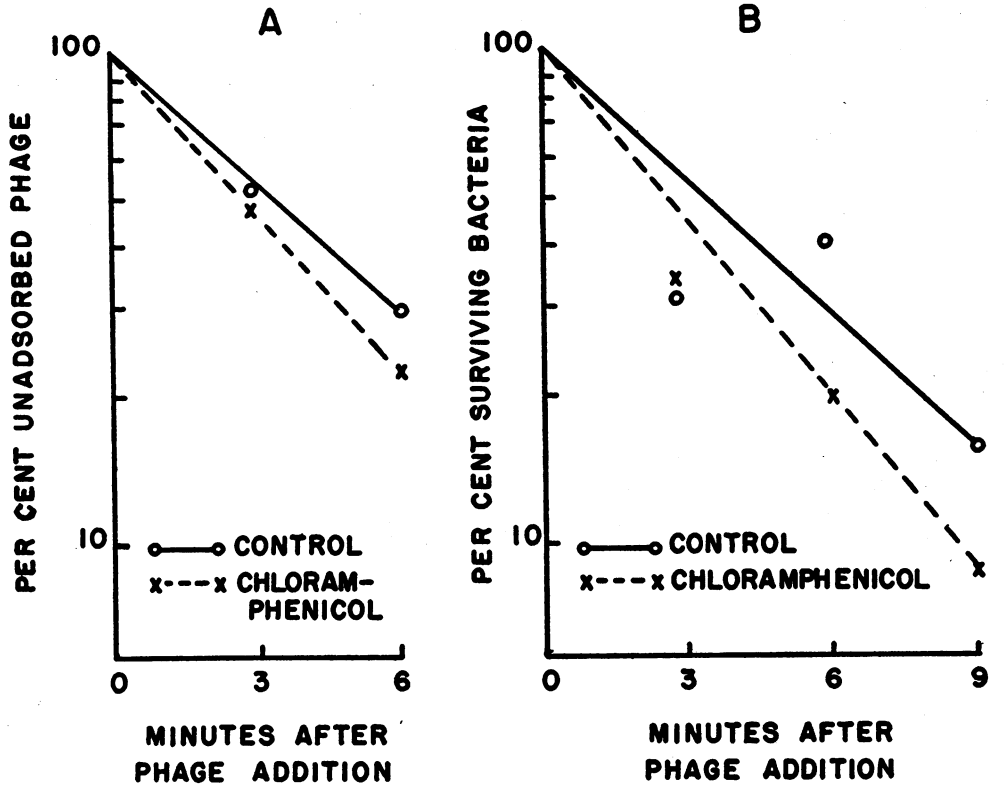

Figure 1. Adsorption of T-1 bacteriophage onto *Escherichia coli*, strain B, in the presence and absence of chloramphenicol.

A. *Method of disappearance of free phage.* Chloramphenicol (final concentration 100 μg per ml) and then T-1 phage (final titer 1×10^8) were added to a suspension of *E. coli*, strain B (1×10^8 per ml), incubated at 37 C. Assays were made on aliquots (diluted immediately 1:100) drawn 3 and 6 minutes later to determine (1) total infectious centers and (2) unadsorbed phage remaining in supernatant after centrifugation in cold to remove bacteria and adsorbed phage. Log per cent unadsorbed phage is plotted against time.

B. *Method of adsorption by proportion of surviving bacteria.* Chloramphenicol (final concentration 100 μg per ml) and then T-1 phage (final titer 1×10^8 per ml) were added to a suspension of *E. coli*, strain B (1×10^7 per ml), incubated at 37 C. Aliquots taken 3, 6, and 9 minutes later were greatly diluted and then assayed on agar plates previously spread with anti-T-1 serum to protect uninfected bacteria. Log per cent surviving bacteria are plotted against time.

after virus and bacteria were mixed (i.e., it was first encountered in the serum inactivation tube).

Curve 3 presents data from experiments in which chloramphenicol was introduced late in the latent period, i.e., it was not present during the periods of adsorption and serum inactivation but it was present in the "first" and "second" growth tubes. Thus, the events occurring in the first 10 minutes of the latent period were allowed to proceed without inhibition by drug. In contrast to the previous experiment, a burst occurred at the usual time under these conditions; the yield of phage, however, was reduced markedly.

In each of the experiments just described, the chloramphenicol, once introduced into the system, was maintained at a constant concentration through all subsequent manipulations until time of plating when it was diluted to a noninhibitory level. In contrast to these experiments, others were performed in which chloramphenicol was present in effective concentrations only for short intervals within the latent period. The fourth curve illustrates such an experiment. The drug, which was added five minutes after the phage, was permitted to act for only five minutes. Then, its concentration was reduced to a noninhibitory

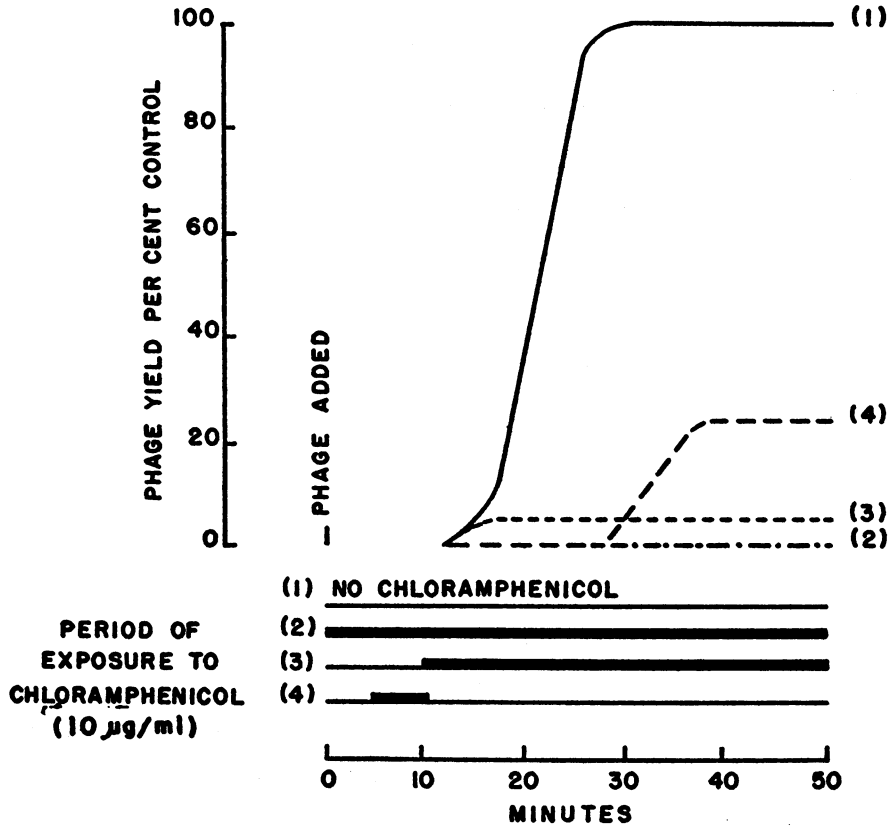

Figure 2. Phage yield and latent period in one step growth experiments in the presence of chloramphenicol added in different phases of the growth cycle.

level by greatly diluting the suspension. Under these conditions, a burst occurred but it was delayed and the yield of phage was generally somewhat reduced. Similar results were obtained when the drug was present for only the first five minutes of the latent period.

(b) *Sonic vibration.* In order to exclude the possibility that chloramphenicol interfered with lysis of infected cells rather than inhibiting phage multiplication, studies were undertaken on infected cells which were disrupted by sonic vibration according to the methed of Doermann (1947–1948) during a one step growth experiment. An aliquot taken simultaneously with the usual sample was pipetted into a chilled tube containing sufficient sodium cyanide (final concentration, 0.01 M) to prevent further growth. The inhibited sample was subjected to sonic vibration[1] for two minutes, after which plaque counts were made.

[1] Raytheon 9 kc magnetostriction sonic vibrator.

In experiments without drug, sonic vibration permitted demonstration of an increase in phage titer a short time before normal lysis occurred (*see* figure 3). These findings are in agreement with those of Doermann (1947–1948) and Anderson and Doermann (1952) which show the appearance of infectious intracellular phage some time before normal lysis. In chloramphenicol inhibited cells, sonic vibration failed to cause any increase in phage titer which could be interpreted as accumulation of previously undetected intracellular virus. Thus, chloramphenicol appears to prevent the actual formation of infectious phage particles within the bacteria.

Effect of chloramphenicol on the growth and survival of E. coli, strain B, under conditions of the phage experiments. Chloramphenicol exhibits primarily a bacteriostatic action on some organisms (Wisseman *et al.*, 1950), but on others it exerts a distinct bactericidal action (Gray, 1952; Alexander *et al.*, 1949). Since interpretation of

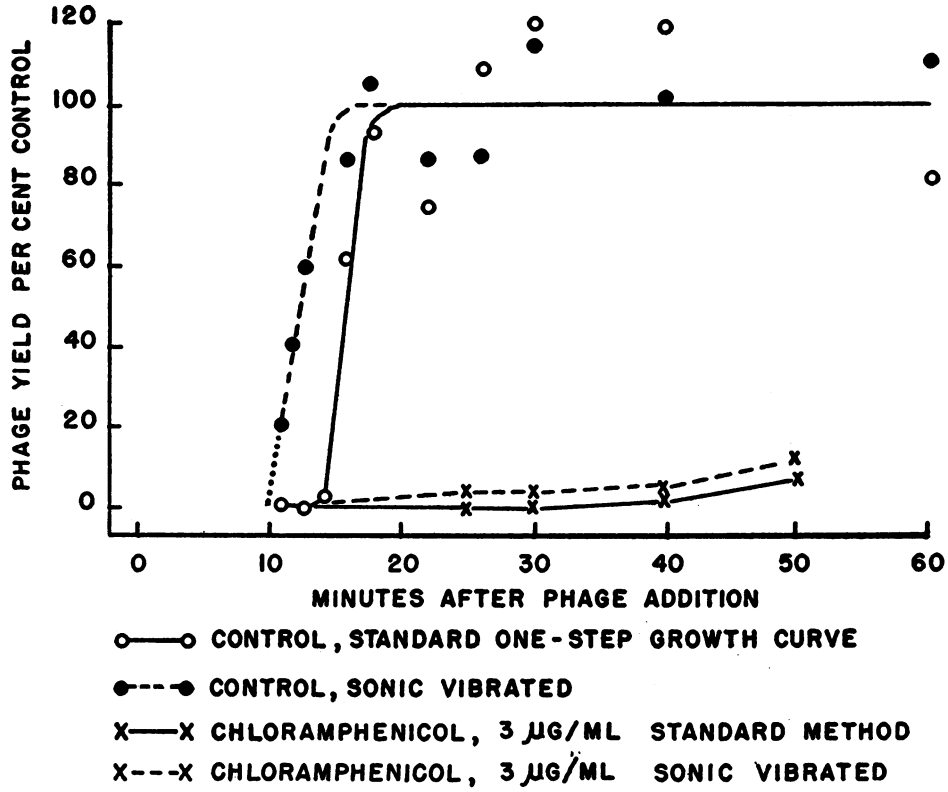

Figure 3. Phage yield after artificial disruption of host cells by sonic vibration in control and chloramphenicol inhibited one step growth experiment.

the action of chloramphenicol on bacteriophage production requires knowledge of the degree of survival of drug treated uninfected bacteria which have been grown and tested under conditions identical with those of the virus studies, a number of experiments were performed in order to elucidate this matter. Results of these studies are presented in figure 4 in which each curve represents the average of three separate experiments. It is seen that at a chloramphenicol concentration of 10 µg per ml there was no appreciable lethal effect of the antibiotic for the first 30 minutes of incubation and only a moderate decrease in viable cell count at 60 minutes. On the other hand, chloramphenicol in a concentration of 100 µg per ml caused a rapid fall in viable cell count which began after incubation of only 15 minutes. Since most of the experiments which demonstrated inhibition of virus formation were performed with chloramphenicol concentrations of 10 µg per ml or less and since the normal latent period for the phage is 13 minutes, simple killing of the host cell cannot account for the action of the drug on phage multiplication.

Comparison of bacteriostatic and virustatic concentrations of chloramphenicol. The concentration of chloramphenicol required to inhibit bacteriophage production appears to be of the same order of magnitude as that which inhibits bacterial growth. A comparison of per cent phage yield measured by the one step growth method performed in the presence of graded low concentrations of the antibiotic (0.5, 1.0, 2.0, 3.0, and 5.0 µg per ml) and the growth of uninfected bacteria determined turbidimetrically in the same concentrations of the antibiotic is presented in table 1.

The tabular data show that the increasing but still subbacteriostatic levels of antibiotic have an essentially parallel inhibitory effect on the multiplication of phage in infected cells and on the growth of the uninfected bacterial cells. Furthermore, the concentrations required to elicit complete virustasis and bacteriostasis are both in the range of 3.0 to 5.0 µg per ml.

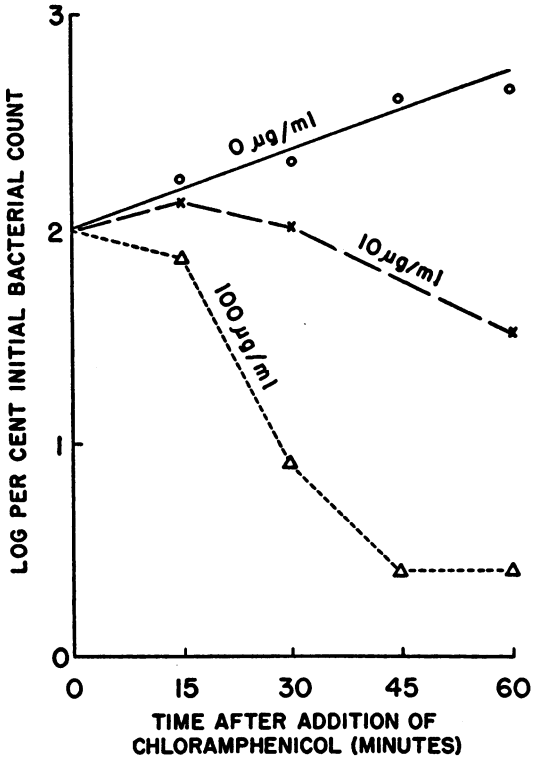

Figure 4. Effect of chloramphenicol on growth and survival of uninfected *Escherichia coli*, strain B, under conditions obtaining in one step growth experiments.

Nutrient broth containing 0, 10, and 100 μg per ml chloramphenicol and about 5×10^7 log phase cells per ml was incubated at 37 C. Aliquots were removed periodically for estimation of viable cell count by the serial dilution plate count technique.

TABLE 1

Comparison of inhibitory effect of graded low chloramphenicol concentrations on bacteriophage production and on growth of uninfected bacteria

CHLORAMPHENICOL	PHAGE YIELD* (PER CENT CONTROL)	BACTERIAL GROWTH† (PER CENT CONTROL)
μg per ml		
0.0	100	100
0.5	38	50
1.0	10	19
2.0	4	5
3.0	1	0
5.0	0	0

Both systems incubated for 1 hour at 37 C.
* One step growth method.
† Turbidimetric method.

DISCUSSION

Chloramphenicol strongly suppressed the growth of T-1 bacteriophage in its host organism, *E. coli*, strain B. Complete inhibition of phage multiplication occurred at about the same minimal antibiotic concentration as that which just inhibited growth of the uninfected bacterium. The drug had no direct virucidal action and did not appear to interfere with adsorption of the phage onto the host cell.

Intracellular phage production was suppressed promptly by chloramphenicol introduced at any time in the latent period. New phage production did not appear to progress beyond the stage of development attained at the time the drug was added. On the other hand, the effect of the antibiotic on lysis was different depending upon the time of its introduction. Thus, if the drug was introduced early in the latent period, lysis was inhibited completely. Furthermore, artificial disruption by sonic vibration of such inhibited cells gave no evidence that the phage had multiplied within the cells. In contrast, the addition of chloramphenicol to the test system late in the latent period, i.e., 10 minutes after mixing, was followed by a different sequence of events. In this case, lysis was not inhibited; it occurred at the usual time (13 minutes). New phage particles were demonstrable, but the yield was small and approximated the number of infectious particles found to be present at 10 minutes in the uninhibited system by artificial lysis.

The action of the drug in the early part of the latent period was reversible since a delayed burst occurred upon dilution of the drug to an ineffective concentration. The yield of phage was usually less, but not markedly less, than that of an untreated control. The response to chloramphenicol in this type of experiment was the same if the drug was present at the time of mixing of the phage and bacteria, and for an interval of time corresponding to the first five minutes of the latent period, or if it was introduced five minutes after mixing and was present only for the second five minute interval.

Chloramphenicol, then, exerts an inhibitory action on phage production regardless of the time of its addition in the latent period. It does not appear to interfere with processes leading to lysis of the bacterium once these have been initiated or have progressed beyond a critical point.

Subbacteriostatic amounts of chloramphenicol, as employed by Edlinger (1951), permit continued phage growth at a reduced rate. Lysis, once initiated, is insensitive to drug inhibition as indicated above. Subbacteriostatic amounts of chloramphenicol added to a suspension of infected bacteria late in the latent period may permit the completion of a sufficient number of phage particles to give the impression that this phase of the latent period is relatively insensitive to the drug, as reported by Edlinger (1951). However, when completely inhibitory concentrations of drug are employed, it is clearly evident that the late phase of the latent period is also sensitive to the antibiotic.

The action of chloramphenicol on phage production resembles in many respects that of 5-methyl tryptophan (Cohen, 1949). Both compounds, added early in the latent period, inhibit phage production and prevent lysis. Furthermore, in both instances the inhibition is reversible. On the other hand, proflavine (Foster, 1948) inhibits phage production but does not prevent lysis from occurring at the usual time. All three compounds, when added late in the latent period, permit lysis with the recovery of approximately that number of new infectious particles demonstrated to be present at the time of drug addition by artificial lysis.

If chloramphenicol was introduced early in the latent period and was not removed, there was a slow but progressive decline with time in the number of infectious centers. However, it is of interest that all attempts to recover viable bacteria through drug treatment of infected cells were unsuccessful.

SUMMARY

Bacteriostatic concentrations of chloramphenicol inhibit the intracellular growth of T-1 bacteriophage in *Escherichia coli*, strain B. The drug has no direct virucidal action and does not prevent adsorption of the phage onto the bacterium.

Chloramphenicol interrupts phage maturation at any stage in the latent period. A bacteriostatic concentration of the antibiotic added early in the latent period inhibits lysis and prevents the formation of new infective phage particles. In the presence of the same drug concentration added late in the latent period, lysis occurs at the usual time. However, the number of phage particles released on lysis approximates the number completed at the time the drug was introduced.

Chloramphenicol inhibition of the early phases of the latent period is partially reversible.

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