STUDIES ON STREPTOCOCCAL BACTERIOPHAGES

I. Burst Size and Intracellular Growth of Group A and Group C Streptococcal Bacteriophages*

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Although the kinetics of viral replication have been carefully worked out for other systems, such as the *Escherichia coli* phage-host system (1–3), until recent years little attention has been devoted to similar studies in hemolytic streptococci and their bacteriophages. This relative lack of knowledge concerning streptococcal viral-host relationships can perhaps be traced not only to the complex growth requirements of the hemolytic streptococci but also to the need of special media for good plaque formation (4, 5).

The early reports of Kjems (6, 7) concentrated primarily on devising reproducible methods for the isolation of virulent and temperate bacteriophages in Group A streptococci. Selecting a virulent mutant of a temperate Group A bacteriophage (A12) he also performed one-step growth curve experiments obtaining a latent period of 65 min and an average burst size of 32 virus particles for this particular phage-host system. Of interest was his observation that a secondary release of viral particles occurred approximately 40 min following the initial burst, and Kjems suggested that this secondary release might be related to infection of other cocci in the streptococcal chain (8). More recently Friend and Slade (5), using a more defined medium, have reported essentially similar results for the latent periods of two other Group A streptococcal bacteriophages, A25 and A6. With respect to burst size, A25 bacteriophage consistently produced 32 virus particles, while the A6 bacteriophage varied from 30–70 plaque-forming units (PFU). Friend and Slade also noted a secondary burst of virus particles but demonstrated that this secondary release was abolished if the streptococcal chain was sonically disrupted into individual streptococcal cells prior to viral infection.

These reports have concentrated on viral replication in a few Group A bacteriophages, and no attempt has been made to study the viral events in a number of bacteriophages propagating in other streptococcal groups. In addition, the reports of Kjems and Friend and Slade were based on viral-host interactions in a strepto-

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coccal culture and did not accurately reflect viral replication in a singly infected coccus. Therefore the growth cycles of a number of Group A and Group C bacteriophages were reexamined, utilizing methods designed to maximize the probability that a single coccus in a given streptococcal chain would be infected by one phage particle.

The following report demonstrates that definite differences exist in the burst size and first intracellular appearance of Group A and Group C bacteriophages. Furthermore, variations in the time of the first extracellular appearance of these bacteriophages were also apparent, and, in general, the time needed for viral replication of Group C bacteriophages was shorter than that observed with Group A bacteriophages. During these studies it was noted that in addition to their viral inactivating effect, mercuric ions also had the ability to block intracellular viral replication at various points in the maturation cycle. The interruption of intracellular replication and maturation by mercuric ions and its reversal by reduced glutathione were used to study isolated events in streptococcal bacteriophage replication.

Materials and Methods

Bacterial Strains.—Streptococcal strains, 26RP66, $T25_3$, A590, and T12 used in this study were from The Rockefeller University collection. The Group C streptococcal strain C88 was kindly obtained from Dr. Eugene Fox.

Bacteriophages.—A25, A12, A6, and C1 phages were supplied by Dr. Richard M. Krause. ϕY and C343 bacteriophages were obtained from Dr. Eugene Fox. Stock phage lysates were prepared with the following propagating strains: strain $T25_3$ (A25 phage), strain T12 (A12 phage) and strain A590 (A6 phage), strain 26RP66 (C1 phage) and strain C88 (ϕY and 343 phages). All lysates were filtered through a Coors No. 2 candle filter and stored at 4°C until use.

Media.—Todd-Hewitt broth, a beef heart infusion medium, was prepared as described (9). This medium was used for the Group C streptococcal phage-host experiments. Dialysate medium was prepared as described by Wannamaker (10), with modifications by Zabriskie (11). This medium was the nutritive source for all Group A streptococcal phage-host experiments.

Preparation of Agar Plates.—Agar plates for the assay of bacteriophage were prepared as follows.

Group A system: 2.4% Difco agar was prepared in distilled water, the pH adjusted to 7.5, and sterilized in the autoclave. For each liter of agar medium, 500 ml of 2.4% of molten agar at 50°C was added to 500 ml of dialysate broth warmed to the same temperature. Sodium bicarbonate was not added for the preparation of plates. Agar medium for the soft agar layer was prepared in a similar fashion except that 1.2% agar in distilled water was used rather than 2.4% agar. Dialysate broth was added to the agar to make a final concentration of 0.6%.

Group C system: Plates were made in a similar fashion except that the Todd-Hewitt broth was used in place of dialysate medium. The soft agar layer was prepared by the addition of a 1.2% concentration of proteose-peptone beef heart infusion agar to Todd-Hewitt broth to make a final concentration of 0.6%.

Plating and Counting Bacteriophage.—The soft agar layer method of plating phage and streptococcus was used (12). Phage dilutions were always made in the type of broth used

for the growth of the particular streptococcus. 0.2 ml of these phage dilutions were added to 1.7 ml of the 0.6% agar.

Incubation.—All phage plates of the Group A system were incubated in a candle jar at 37°C for 18 hr. Plates for the Group C system were incubated at 37°C for 18 hr without the use of a candle jar.

Optical Densities.—All OD readings were carried out in 10 × 75 mm test tubes at 650 mμ in a Coleman Junior Spectrophotometer.

Indicator Strains.—

Group A system: An 18 hr culture of strains T25$_3$, T12, or A590 in dialysate broth were centrifuged and resuspended in fresh dialysate medium to an OD of 0.04–0.06. 0.1 ml of this suspension was added to the 1.9 ml soft agar tubes at the time of plating.

Group C System: An 18 hr culture of strain 26RP66 or C88 in Todd-Hewitt broth was centrifuged and resuspended to one-third its original growth volume with fresh Todd-Hewitt broth. 0.1 ml was used in the soft agar at the time of plating.

Preparation of Phage-Associated Lysin.—Group C streptococcal phage-associated lysin was prepared by the method of Zabriskie and Freimer (13).

Bacteriophage Antibody.—The bacteriophage antisera for use against the A25 phage and the C1 phage were prepared according to the outline by Adams (12). 1–2 ml amounts of filtered phage lysates of A25 phage containing 1×10^9 PFU per ml and C1 phage containing 5×10^9 PFU per ml were injected intravenously into rabbits daily for 5–7 days. Animals were bled 10 days after the last injection.

EXPERIMENTAL

Single Cell–Burst Experiments.—In order to study the events of viral replication in the hemolytic streptococcus, a chaining organism, certain modifications of the classical single cell burst techniques (3) had to be instituted. First, in order to circumvent the problem of multiply infected cocci in a streptococcal chain, the ratio of virus particles to streptococcal chains was adjusted so that the probability of one virus particle infecting one chain was maximal. Secondly, secondary bursts of those cocci infected following the burst of the primarily infected coccus were prevented by "quick freezing" of the host-phage mixture at the end of the viral cycle and then plating the contents of each tube immediately upon defrosting.

A typical streptococcal single cell burst experiment was performed as follows: 0.1 ml of an 18 hr culture of the Group A streptococcal strain grown in dialysate medium was inoculated into 10 ml of warmed dialysate medium. This mixture was incubated at 37°C for 2 hr yielding a count of 5×10^7 chains/ml as determined by the pour plate method. Group A bacteriophages were then added to the cells at a multiplicity of infection which ranged from 0.6 to 0.25 PFU per chain and incubated for 15 min at 37°C to assure complete adsorption of phage to bacteria. The suspension was then diluted in cold broth so that the final dilution mixture contains 0.5–0.8 chains of varying length per milliliter in a total of 100 ml of dialysate broth. In the final suspension of 0.5–0.8 chains per ml the number of infected chains was estimated to range from 0.1–0.6 per ml. 1-ml aliquots were dispensed quickly (using a Cornwall Pipetting Unit[1]) into 100 tubes and incubated further (1¾ hr for the A phage system and 60 min for the C phage system) at 37°C. At the end of the incubation period, the tubes were quick

[1] Made by Becton-Dickinson & Co., Rutherford, N. J.

frozen in a dry ice and alcohol bath to stop any further multiplication of phage which may have reinfected the other cocci in the chain. The tubes were then defrosted individually and the contents plated using the soft agar technique. Control tubes containing sterile phage suspensions were found to be unaffected by the freezing and thawing process.

The frequency distributions of plaque counts of six single cell burst experiments with three Group A bacteriophages and three Group C bacteriophages have been examined. The summarizing statistics of these distributions are presented in Table I. In each experiment 100 plates were examined. While there was substantial variability in the proportion of positive plates in experiments using different propagating strains, much less variation was noted in the three experiments in which the same propagating strain (26RP66) was used.

Expressed on a per streptococcal chain base, the frequency distributions of

TABLE I

Summary of Distribution of Plaque Counts

Streptococcal group	Bacteriophage	Propagating strain	No. of plates with counts	Mean burst* size	Median burst size	Range‡	SD
A	A25	$T25_3$	23	20.13	12	1–93	20.58
A	A6	A590	86	37.06	20	2–101	37.27
A	A12	T12	69	17.80	13	1–52	15.92
C	C1	26RP66	33	145.63	136	1–350	146.10
C	ϕY	26RP66	49	19.98	10	1–73	20.19
C	C343	26RP66	45	24.44	13	1–100	24.72

* The average burst size for each bacteriophage is obtained by dividing the total number of plaques counted by the number of positive plates.

‡ The range of counts refers to the largest and the smallest number of plaques counted in individual plates.

burst size, for those plates in which plaques were observed, were found to be nonsymmetric, unimodal with a very long tail to the right. This type of distribution, exemplified by the burst size experiments with A25 phage, is illustrated in Fig. 1. In Table I the mean burst size for each bacteriophage has been calculated by dividing the total number of viral plaques by the number of positive plates. The results indicate that there is considerable variation in the burst sizes of these streptococcal bacteriophages. However, when the median burst size of the phages are calculated, the results are quite similar regardless of the phage or propagating strain. The sole exception was the C1 bacteriophage in which the burst size was 10–14 times that observed for the other members. Since the median burst size is that value that divides the frequency distribution in half, it is perhaps a better summarizing statistic to use in these experiments since it is not heavily weighted by some of the extreme values of these counts.

It is of interest to note that the standard deviation of burst size for these distributions is approximately equal to the respective mean burst size.

Intracellualr Phage Growth Eexperiments.—Since the results of the single cell burst experiments indicated marked variations in the number of virus particles produced by Group A and Group C bacteriophages, the time of intracellular appearance of viral particles and the latent period in certain of these bacteriophages was investigated next. For the purposes of comparison, a Group A

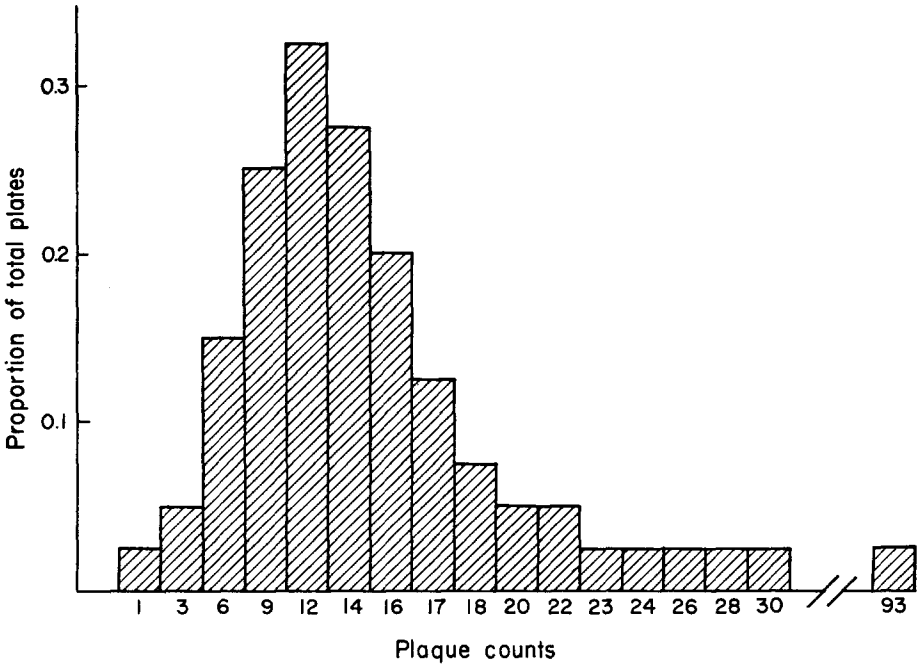

FIG. 1. Frequency distribution of the burst size of A25 bacteriophage. Proportion of total plates refers to the percentage of positive plates containing a specified number of plaque counts.

streptococcal bacteriophage, A25, (low phage yield) and a Group C bacteriophage, C1, (high phage yield), were selected for these studies.

The experimental procedure was as follows: 0.1 ml of strains $T25_8$ (propagating strain for A25 phage) or 26RP66 (propagating strain for C1 phage) were inoculated into 10 ml of the appropriate broth and incubated at 37°C until a concentration of 5×10^7 colonies per ml, as assayed by the pour plate method, was reached. The culture was centrifuged and the organisms resuspended in 5 ml of a phage lysate containing 5×10^8 PFU/ml. This suspension was then incubated for 5 min at 37°C. At this time 5 ml of a phage antiserum, appropriately diluted in broth to neutralize 90% of the phage particles within 5 min, was added to the mixture and reincubated for another 5 min. At this time a 30-fold dilution of this suspension

was made into fresh broth (prewarmed to 37°C) in order to stop any further action of the phage antiserum. This "sampling tube" was then incubated at 37°C for the duration of the experiment.

At 3-min intervals, 0.1 ml aliquots were removed from the sampling tube, diluted 1:5

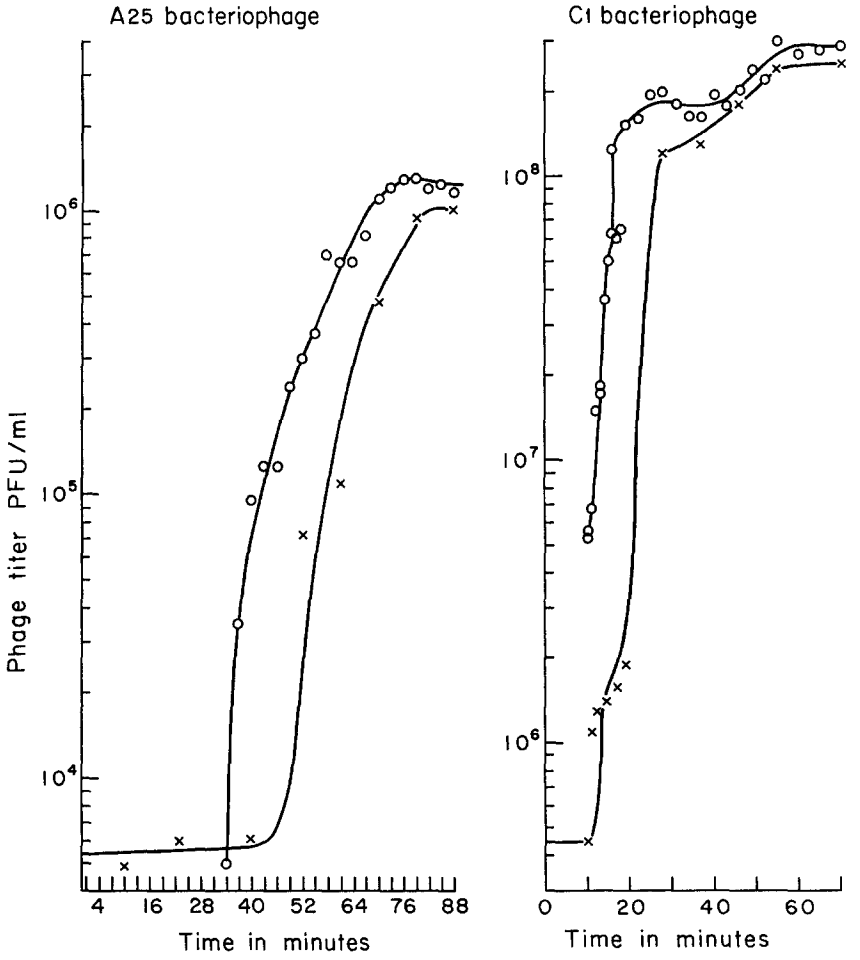

FIG. 2. Intracellular (O——O) and extracellular (X——X) growth of curves of A25 and C1 bacteriophages.

into M/15 phosphate buffer pH 6.6 containing 0.001 M 2-mercaptoethanol and quickly added to an equal amount of activated Group C streptococcal phage lysin. (The concentration of the phage lysin was a critical factor in these studies since the addition of undiluted phage lysin in the test system destroyed the bacterial lawns used for viral assay. In general, a 1:2 or 1:4 dilution of the centrifuged lysin preparation proved to be satisfactory for most experiments and lysed 90% of the streptococcal cells within 1 min.) The test mixture was then

incubated for 6 min at 37°C at which time samples were removed; 10-fold dilutions of these aliquots were prepared and then plated by the soft agar layer technique. The number of plaques counted in each sample was a reflection of the intracellular growth cycle of the phage.

At 6-min intervals, 1.0 ml aliquots were also removed from the sampling tube, centrifuged to sediment the bacteria, and the supernatants assayed for the presence of free phage particles. The number of plaques contained in these samples were a record of the extracellular appearance of mature phage particles.

The results of these experiments are summarized in Fig. 2. It can be seen that the first intracellular appearance of A25 phage in Group A streptococcal strain $T25_3$ occurs at approximately 34 min and increases logarithmically up to 75 min after infection. In comparison the Group C bacteriophage makes its first intracellular appearance before 10 min and reaches its peak at approximately 25 min. There is also a marked difference between the latent periods of these two bacteriophages. Whereas the latent period of A25 phage extends to approximately 45 min with extracellular phage release continuing to about 80 min after phage infection, the first extracellular appearance of C1 bacteriophage occurs as early as 12 min after infection. Furthermore, the cycle of this particular bacteriophage is completed within 30 min.

The fact that the intracellular growth line of the C1 bacteriophage does not coincide with the curve denoting the appearance of extracellular phage can be explained by the conditions of the experiment. Since 5 min are required for complete adsorption and an additional 5 min are utilized in the neutralization of any free phage particles, the first sample is obtained 10 min after the mixture of phage and host cells. The appearance of a 10-fold increase in the number of intracellular phage particles at the first sampling suggests that a small percentage of the total streptococcal population has produced mature phage particles within the first 10 min of phage infection. These results were reproducible in several experiments.

Blocking Intracellular Phage Production with Mercuric Ions.—During their studies on the inactivation of Group A and Group C bacteriophages by mercuric ions, Kessler and Krause (14) noted that mercury-treated phages, while retaining their ability to adsorb to the cell surface, were unable to complete the viral cycle. While the authors suggested that this blocking action of mercuric ions occurred primarily at the cell surface, it was conceivable that mercury also exerted an effect at the intracellular level of replication. To test this hypothesis preliminary experiments were performed in which the inactivating effect of mercuric ions on phage and streptococci were tested with varying concentrations of mercuric ions in the medium. As an outgrowth of these studies it was found that when phage and streptococci were allowed to interact in the presence of 2.5×10^{-6} M Hg^{++}, a concentration which did not result in inactivation or death of either phage or host, no phage progeny were produced. Since these studies suggested that mercury may be exerting an effect on intracellular viral

replication, it was also possible that this inactivating effect might easily be reversed by the introduction of known reducing agents (14). Accordingly experiments were designed in which mercuric ions were introduced at known points during the viral cycle and then tested for the reversibility of viral inactivation by the subsequent introduction of reduced glutathione.

In a typical experiment, 1 ml of a 2.5×10^{-5} M solution of $HgCl_2$ in broth was added to 9.0 ml of Group A streptococcal strain $T25_3$ at a concentration of approximately 5×10^7 chains/ml. This was incubated at 37°C for 10 min, at which time 1.0 ml of A25 phage at 1×10^7 PFU/ml was added to the 9.0 ml of the mercury-treated cells. 30 min after the addition of the A25 phage, the mixture was divided into two equal portions. A solution of reduced glutathione at a final concentration of 1×10^{-2} M was added to one portion; plain broth to the other. Both samples were then kept at 37°C for the duration of the experiment. At timed intervals 0.1 ml aliquots were removed from each sample and diluted into 9.9 ml of broth. 1 ml from these dilutions were centrifuged and the supernatants plated by the soft agar techniques.

As can be seen from Fig. 3, the portion exposed to the reducing agent exhibited progeny phage release whereas the unreduced portion did not. Since these results indicated that mercury was blocking some metabolic pathway necessary for phage growth or release, experiments were designed to determine at what points in the viral cycle mercury exerted its inactivating effect.

9 ml of log phase Group A streptococcal strain $T25_3$ at 5×10^7 chains/ml was added to 1.0 ml of a 5×10^7 PFU/ml concentration of A25 phage and incubated at 37°C. At timed intervals of 10, 20, 30, and 40 min, 0.9 ml aliquots were removed and added to 0.1 ml of a 2.5×10^{-5} M concentration of $HgCl_2$ in broth. Incubation of these samples were then continued until a total elapsed time of 70 min from the time of phage-host interaction had been reached. At timed intervals during the experiment 0.1 ml aliquots of these samples were removed, diluted 1:100, centrifuged to sediment the cells, and the supernatants then titered by the soft agar technique. At the end of the 70 min incubation period, 0.1 ml aliquots were removed from the 10, 20, 30, and 40 min samples and diluted into 0.9 ml of activated phage-associated lysin. These samples were incubated at 37°C for another 10 min to assure complete lysis of the infected cells. 0.1 ml of each of the samples was then removed, diluted, and plated by the soft agar technique.

It can be seen in Fig. 4 that if mercury is introduced within the first 20 min of phage-host interaction no further production of mature virus particles occurs. This is true even though the infected cells are incubated well beyond the normal latent period for the A25 phage before being lysed (see Fig. 2). However, when mercury is introduced at 30 and 40 min, times in which intracellular phage are known to be present, then increasing amounts of mature virus particles are released following lysis of the cells. Thus the number of phage particles found intracellularly during the viral cycle is directly proportional to the time at which the mercury was added to the phage-host system; i.e., more bacteriophage being found the later the mercury is added.

In order to test the reversibility of this blocking action a similar experiment

was performed in which 1×10^{-2} M-reduced glutathione was introduced at 70 min in place of the phage enzyme. The mixture was incubated for an additional 60 min during which time samples were removed and tested for phage content. It was found that in the presence of reduced glutathione the viral cycle was completed. In addition, the release of mature virus particles was directly related to the time at which mercury was added to the system. Lysis occurred first in

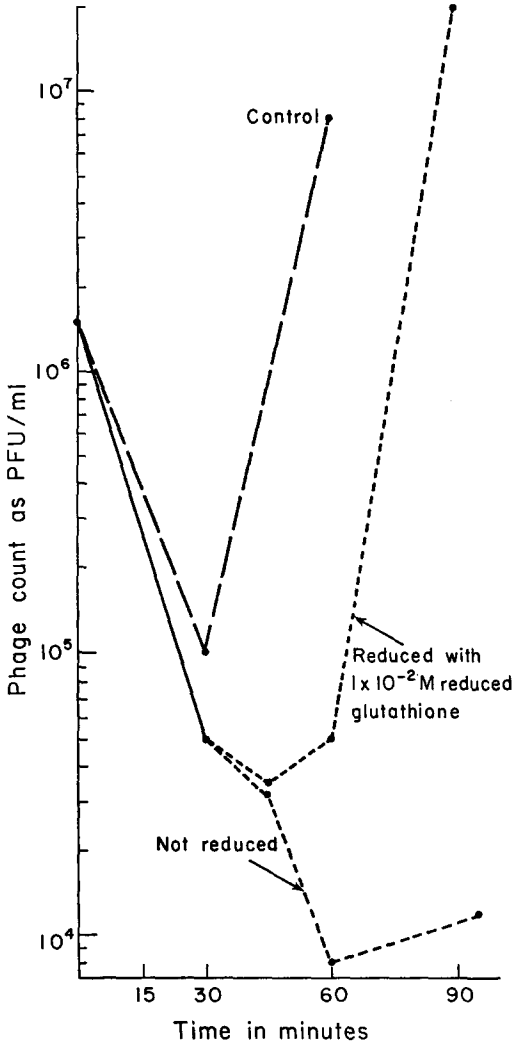

FIG. 3. Adsorption of A25 phage to mercury-treated Group A cells (solid line) and reactivation with reduced glutathione as compared with nonreduced cells, (diverging dotted lines). Control line refers to the normal one-step growth curve.

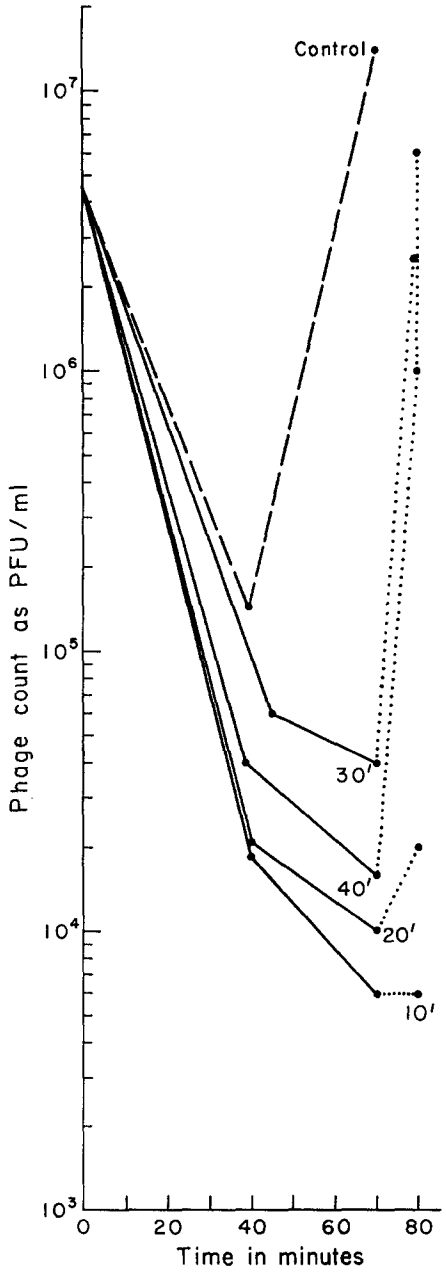

FIG. 4. Blocking of further intracellular phage production following the addition of mercury at various times after phage infection (10, 20, 30, and 40 minutes). Dotted lines indicate the number of intracellular phage particles present at the times at which mercury was added. Control line refers to the normal one-step growth curve.

those samples in which mercury was added late in phage multiplication and last in those in which mercury was added soon after phage infection. These results clearly demonstrate that the introduction of mercury at any given point during the eclipse or latent period of the A25 phage stops any further maturation of the phage. This inhibition can be reversed at any time by the addition of reduced glutathione.

DISCUSSION

Using the one-step growth curve method, Kjems (7) and more recently Friend and Slade (5) reported that the average burst size of Group A streptococcal bacteriophages was 32. Their results in general agree with the data reported in this study when figures for the mean burst size are used. However, when median burst size figures are substituted (see Table I), then the burst size of Group A bacteriophages is considerably less than that reported by the other authors. In a similar fashion, the discrepancies in the mean burst size for the two Group A bacteriophages, A25 and A6, reported by Friend and Slade (5) may, in part, be explained by the data here concerning the relationship between the mean burst size and its variation—that is, the larger the mean burst size, the larger the standard error of the mean.

While the one-step growth curve experiments of Kjems and Friend and Slade give figures for the burst size of streptococcal bacteriophages, it must be emphasized that their results were obtained from thousands of phage-infected bacteria and are therefore only an indication of the average number of phage particles produced. In contrast, the single cell burst technique utilized in this paper renders it possible to study the events of phage multiplication in individually infected streptococci. The fact that the samples were immediately frozen after the latent period and rapidly plated individually after defrosting was a crucial point in this technique since it prevented either reinfection or burst of those cocci adjacent to the infected cell. Thus the avoidance of secondary bursts previously noted by Kjems (7) and Friend and Slade (5) permitted an estimate of the virus particles in single-infected cells.

The data concerning the proportion of positive plates and the burst size of streptococcal bacteriophages are, in part, a function of the joint and conditional distribution of three variables. These variables may be summarized as follows: (*a*) the number (and distribution) of streptococcal chains with at least one infected cell in aliquots drawn from the total pool; (*b*) the distribution of chain lengths (number of cells per chain) for a given propagating strain; and (*c*) the number of infected cells per chain in a sample.

A positive plate will be obtained if at least one chain with one infected cell is included in the aliquot drawn from the total pool. The number of streptococcal chains with one or more infected cells included in such samples is a variable which follows the Poisson distribution. The observed proportion of positive plates is a function of the mean or expected number of chains with at least one

infected cell included in a sample taken from the pool. Using experimental data then, it is possible to estimate the probability that one or more than one chain with one or more infected cells is included in a given sample.

The methods used here have attempted to maximize the probability that one and only one cell in any given chain will be infected by one phage particle. However, streptococcal chain length is a variable and the probability that a given chain has none, one or more infected cells is related to chain length; i.e., the probability of finding at least one infected cell in a chain of length eight is greater than that probability for a chain of length four in a given pool.

The number of infected cells per chain is assumed to follow a binomial distribution. Under this model, the probability of any given cell being infected is assumed to be constant. Using experimental data, then, it is possible to estimate the probabilities of obtaining a sample that contains a chain of given length with one or more infected cells. The observed variation in the distribution of phage counts for a given propagating strain and bacteriophage may be a function of the inclusion of more than one chain with at least one infected cell in the sample and/or the inclusion of a single chain with more than one infected cell.

The expression of burst size on a *per chain* basis used here is subject to these components of variation which should be controlled if this measure is to be expressed on a *per cell* basis. Further work is currently being undertaken in these laboratories in order to permit the precise specification of the several distributions considered here, and to estimate the effects of these components of variation on the proportion of positive plates and on the estimates of burst size. It is hoped that these investigations will permit the precise estimation of of burst size on a *per cell* basis.

The fact that the average burst size of the A25 phage was 12 PFU per infected cell as compared with the C1 phage which produced 136 PFU sheds some light on the results obtained in recent electron microscopy studies of Group A and Group C bacteriophages. Morgan's[2] inability to observe A25 phage particles within the streptococcal cell whereas Cole (15) reported the presence of C1 phage aggregates, might be explained by the differences in burst size obtained with these two phages.

The intracellular growth studies of Group A and Group C bacteriophages were of interest since they demonstrated that the eclipse period of 34 min for the A25 phage is more than three times that noted for the C1 phage. Since the eclipse is that part of the latent period during which the materials for bacteriophage progeny are being produced, one might have expected more mature phage particles in the A25 phage system. Yet, the number of C1 phage particles released was 10 times that observed with A25 phage. These results suggest that the mechanism for A25 phage production is either more complex than that for

[2] C. Morgan. Personal communication.

the Group C system or that the synthesis of virus units in the A25 phage-host system is faulty.

Another interesting aspect of these studies of intracellular phage growth was the observation that the first intracellular appearance of the C1 phage occurred as early as 10 min after infection. This coincides with the first intracellular appearance of the C1 phage-associated enzyme as reported by Fox and Wittner (16). The excessive amount of this enzyme coupled with the fact that its production begins at a point in time closely associated with the completed virus suggests that the enzyme is intimately associated with the replication of virus particles.

The use of mercuric ions in high concentration as a means of inactivating phage has been known for many years (Krueger and Baldwin (17), Kessler and Krause (14)). However, little is known concerning the effect of low concentrations of mercuric ions on the intracellular production of phage progeny. Experiments in this report illustrate the fact that mercuric ions at concentrations which do not inactivate A25 bacteriophage nor kill Group A streptococci produce a profound effect when added to a phage-host mixture of these organisms. The effect is primarily at the intracellular level and the studies clearly demonstrate that mercuric ions have the ability to block phage maturation at any stage of the eclipse or latent period. This blockage can be maintained for a number of minutes, then quickly reversed by the addition of reduced glutathione. The ability of these ions to stop viral production at various points during the latent period and its easy reversibility by glutathione offers an important tool in studying the precise events of the viral latent period in streptococcal phage-host interactions.

SUMMARY

Evidence has been presented that the burst size in the Group A and Group C streptococcal phage-host systems are in general similar producing approximately 13 phage particles per infected coccus. The exception was the C1 phage which produced 10 times more virus particles than all the other phages tested. The eclipse period for the A25 phage-host system was found to extend for 34 min, while the C1 phage were found as early as 10 min after infection. Conclusive evidence has been presented indicating that mercuric ions at 2.5×10^{-6} M concentration have the ability to halt intracellular phage production at any point during the infective cycle of A25 bacteriophages. This blocking action can then be quickly reversed with the addition of reduced glutathione with subsequent completion of the viral cycle.

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