THE INTRACELLULAR GROWTH OF BACTERIOPHAGES

II. The Growth of T3 Studied by Sonic Disintegration and by T6-Cyanide Lysis of Infected Cells*

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Daughter T4 particles can be recovered from infected bacteria by poisoning their metabolism during development and adding an excess of the virus T6 to induce them to lyse prematurely (1). However, since the yields of virus depend on which poison is used, it seemed desirable to develop and test some radically different method for arresting metabolism and disrupting infected cells. For arresting metabolism it was noted that the simple procedure of chilling infected cells would have the advantage of slowing down almost all reactions in the cells rather than blocking only certain enzyme systems. Furthermore, chilling does not induce infected cells to lyse as certain poisons do (1).

For disrupting the cells, the mechanical action of intense sonic vibration could be used. Unfortunately, the large, even numbered viruses—including T4, the subject of the previous study—proved to be quickly inactivated in sonic fields; but the small viruses, T1, T3, or T7 are resistant to treatments which disintegrate large proportions of their host cells (2).

The procedure of chilling and then vibrating T3-infected cells proved to be satisfactory for the liberation of intracellular virus. This procedure was then carefully compared with the T6-cyanide method previously used for T4. It was found that the two methods yield essentially the same number of daughter T3 virus particles and that these particles appear well before chilled control cells begin to lyse. The results of this study have been briefly described and discussed in many places (3–5). The details will be reported in the present paper.

Materials and Methods

The phage T3 was chosen for study because of its resistance to sonic vibration and because we had at our disposal a strong anti-T3 serum prepared and kindly supplied

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to us by Dr. M. H. Adams. The media used and the host strain of *Escherichia coli*, B/r/1, have been described previously (1). As before, all virus assays were done by the agar layer technique, making use of the mutant B/6 on which the T6 in the lysing medium does not form plaques. In addition to T6, the lysing medium contained 0.01 M KCN. A number of changes in technique were, however, made necessary by the results of some preliminary experiments described in the following sections.

Sonic treatment was given to 40 ml. samples in the stainless steel cup of the Type R-22-1 oscillator kindly supplied by the Raytheon Manufacturing Company. Before use in an experiment the cup assembly was autoclaved, while between runs in a given experiment, contaminants were eliminated by rinsing the cup thoroughly with boiling water. Then, while a sample was being treated, iced water was circulated through the jacket of the cup. In this way the temperature of the samples was kept below 7°C. during treatment.

Experiments

Effects of Cyanide and T6 on Bacteria Infected with T3.—In order to find out whether the T6-cyanide method is applicable to T3-infected bacteria, a series of four experiments was done by the procedure previously used with T4. Growth at 30°C. rather than 37°C. was studied to lengthen the latent period from 13 minutes to the more convenient time of about 20 minutes.

The results shown in Fig. 1 are quite analogous to those obtained with T4 (1). When the lysing medium is added to the infected bacteria before 12 minutes of their latent period have elapsed, practically no active virus particles can be recovered, not even the infecting ones. When the lysing mixture is added at times after 12 minutes, however, an increasing number of daughter particles is recoverable.

The action of cyanide alone on T3-infected bacteria is also similar to its action on T4-infected cells. Early in the latent period cyanide alone fails to lyse T3-infected bacteria, but it slowly inactivates the cells as producers of daughter virus particles. In the T3 case, too, 0.01 M cyanide added in the second half of the latent period induced rapid clearing of turbid cultures of infected bacteria.

The Blocking of T3 Growth at Lowered Temperatures.—A series of experiments was performed to see at what low temperatures the increase of intracellular T3 would be effectively blocked. T3-infected cells were incubated at 30°C. until each contained, on the average, 1.1 to 2.2 daughter particles of T3. At this point 0.8 ml. samples of growing culture were placed in each of a series of tubes and incubated at 5 different temperatures. After 1.5, 10, 30, and 60 minutes of incubation an aliquot was removed from each sample and diluted into lysing medium at 30°C. Assays of the lysed cells were made after 60 minutes in the lysing medium when phage liberation was judged to be complete from the earlier experiments.

The results are shown in Fig. 2 in which the ratio of the titer of each sample to its 1.5 minute titer is plotted as ordinate against the time at which the aliquot

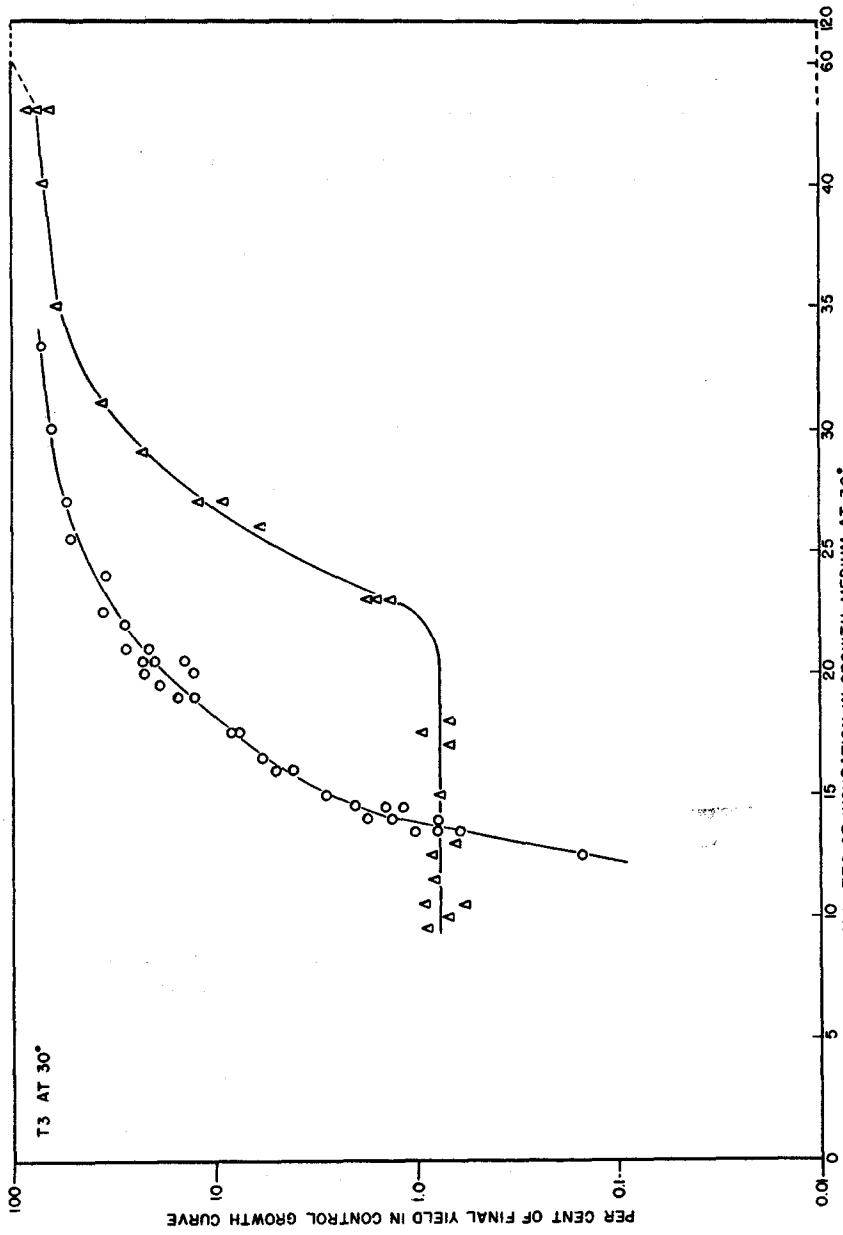

FIG. 1. The liberation of intracellular T3 as a function of the length of time the virus-host complexes have had to develop before the addition of the T6-cyanide mixture used to lyse them (circles), compared with the control one-step growth curve for T3 (triangles). It may be seen that T6-cyanide liberates virus from the cells when it is added well before normal lysis begins and that the same ultimate yields are obtained.

was diluted into the lysing medium. Lines connecting the points for a given sample thus represent the virus growth at the indicated temperature insofar as the T6-cyanide technique can be used to measure it. The dotted curve depicts the increase of T3 at 30°C. as determined from Fig. 1. It is seen that the intracellular growth of T3, at least as determined by the T6-cyanide method, stops between 9.6 and 6.3°C. Since these low temperatures did not appear to inactivate virus-host complexes early in the latent period nor to induce lysis

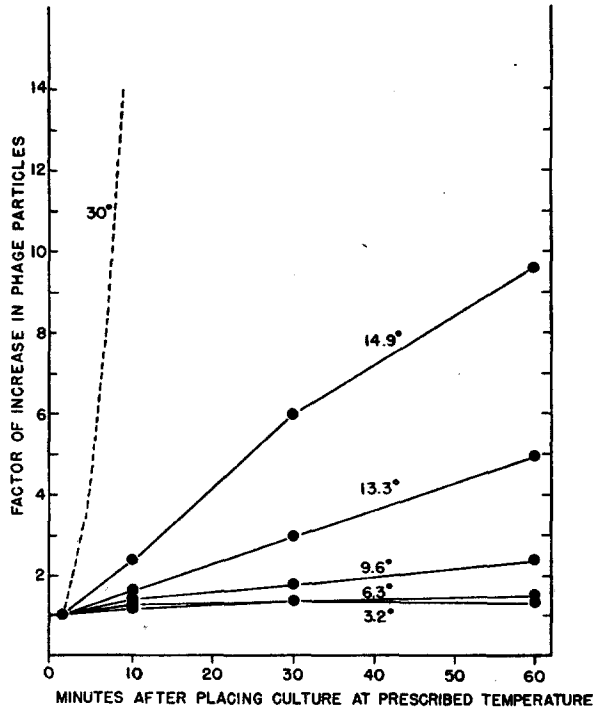

FIG. 2. The increase of T3 liberated by T6-cyanide from infected cells incubated for various times at low temperatures. It is seen that the increase stops between 6.3 and 9.6°C.

late in the period, the chilling technique was judged to be suitable for storing cultures.

The Sonic Liberation of T3 from Infected Bacteria.—A number of preliminary experiments were done to see whether sonic vibration would actually liberate T3 from infected cells before they would normally have lysed. In Table I are presented the design and results of an experiment in which a cooled mixture of infected cells and unadsorbed, serum-inactivated phage was treated with sonic vibration. The number of active T3 particles increased rapidly and approached

a limit after about 5 minutes' treatment. The parallel control culture of infected cells in which growth was held static at low temperatures showed no increase in titer during the 60 minute period during which the experimental sample was being vibrated. With one notable reservation it therefore appeared likely to us that sonic disruption of the cells had indeed liberated daughter T3 particles contained within them.

The reservation in our conclusion lay in the fact that there were about 20 times as many serum-inactivated T3 particles as there were infected bacteria

TABLE I

The Liberation of Intracellular T3 by Sonic Disruption of Infected Bacteria

Procedure—Ten ml. of actively growing host cells (B/r/1 at 10^8/ml.) were centrifuged down and resuspended in 1 ml. of growth medium. At time $t = 0$, 2×10^{10} T3 particles in 1 ml. were added and allowed 2 minutes at 30°C. for adsorption on the cells. At this point adsorption was stopped and the free phage inactivated by diluting the mixture 1:40 in growth medium containing 0.001 part of anti-T3 serum. At time $t = 8$ minutes the mixture was further diluted 1:70 in growth medium at 30°C. to stop the action of the antiserum. At $t = 18$ minutes 1 ml. of this growth mixture was placed in 39 ml. of iced growth medium to stop growth. Five ml. of the iced mixture were held in ice as the static control while 30 ml. were placed in the cooled cup of the sonic vibrator. The contents of the vibrator cup and of the static control were assayed at intervals with the following results:

Time of assay	Length of sonic treatment	Concentration of plaque-forming particles	
		In vibrator cup	In static control
min.	*min.*		
20.5	0.5	8×10^3	
23	3	1.5×10^4	
25	5	1.7×10^4	
30	10	1.8×10^4	
40	20	1.9×10^4	
42	0	—	3.5×10^3
50	30	1.9×10^4	
80	60	1.2×10^4	
82	0	—	2.7×10^3

in the sample tested. The seemingly remote possibility therefore existed that the rise in the titer of T3 during sonic treatment could have been due to the sonic disruption of bonds between T3 and attached antibody molecules. When this possibility was tested directly (6), it was found that the serum-inactivated T3 is indeed reactivated in sonic fields, but at a much lower rate than could have accounted for the rise in titer observed in the experiment of Table I. Nevertheless, the reactivation phenomenon made it desirable in precise work to replace the use of antiserum for eliminating free phage by the method of centrifuging, washing, and resuspending infected cells in the cold.

TABLE II

Design of Experiments Comparing Sonic and T6-Cyanide Methods for Estimating Intracellular T3

Time	Operation	Purpose
min.		
−10	1. Centrifuge 20 ml. of actively growing culture of B/r/1 at 5 × 10^7/ml. and resuspend bacteria in 1 ml. of growth medium	To concentrate host cells so adsorption will be more rapid in step 3
−2	2. Aerate 1 ml. of bacteria (now 1 × 10^9/ml.) at 30°C.	To resume respiration and initiate stirring
0	3. Add 3.5 × 10^8 T3 particles in 0.05 ml. of growth medium	To permit adsorption of T3 on host cells
2 Stop clock	4. Add 9 ml. iced growth medium. Centrifuge in cold, discard supernatant, wash pellets in iced growth medium, and resuspend bacteria in iced growth medium	To stop adsorption and development. To eliminate unadsorbed T3 particles
2 Start clock	5. Add 0.1 ml. of resuspended bacteria to 4.9 ml. of growth medium at 30°C. to make Growing Culture	To allow development of T3-host complexes to be resumed
3, 6, 9, etc.	6. At 3 min. intervals withdraw 0.2 ml. of Growing Culture and add to 3.8 ml. iced medium to make a series of Static Cultures	To stop development of samples of Growing Culture for storage until subsequent treatments
3.5,	7. At 10 min. intervals add 2 ml. from a Static Culture to 38 ml. iced growth medium. Assay for T3	To determine infective centers for control
13.5, 23.5, etc.	Treat the remainder sonically for 5 min., assay debris for T3	To determine infective centers after sonic treatment
4, 14, 24, etc.	8. At 10 min. intervals add 0.1 ml. from a Static Culture to 1.9 ml. iced lysing medium and keep in cold for 12 min. Place in 30°C. bath and assay for T3 50 min. later	To determine infective centers after T6-cyanide treatment
5	9. Add 0.1 ml. from Growing Culture to 1.9 ml. iced growth medium, centrifuge in cold, and assay supernatant liquid	To determine unadsorbed phage in Growing Culture

Comparison of Sonic and T6-Cyanide Methods for Liberating Intracellular T3 Particles.—In Table II is given in some detail the protocol for experiments designed to compare the two methods with each other and with the control. Each operation in the procedure is given, together with the time at which it was performed and the purpose which it served.

The results of the assays from two such experiments are given in Figs. 3 *a* and 3 *b*. Here the concentrations of plaque-forming particles are plotted on a logarithmic scale against the lengths of time the infected cells had had to develop at 30°C. before they were chilled in step 6 of Table II.

DISCUSSION

As seen from the results plotted in Figs. 3 *a* and 3 *b* the assays of the controls remain constant for about 18 minutes after the formation of the virus-host complex and then rise by a factor of 150 in the next 9 minutes. The constancy of the initial period is due to the fact that when an intact complex is plated on agar its action and that of the virus particles contained within it are localized on the plate so that each complex is capable of forming only a single plaque, no matter how many daughter virus particles it may have contained. When the complexes begin to lyse at 18 minutes, however, the daughter particles from each lysed cell become separated from each other in the liquid growth medium so that when it is plated each free daughter particle is able to form a plaque. The number of plaques obtained for the control at any time is thus equal to the number of unlysed complexes plus the number of particles liberated from those complexes which have lysed. Eventually, when all the complexes have lysed, an average of 150 particles is liberated per cell. While the titer is rising, however, we cannot determine the yield per cell without knowing how many complexes have lysed at a given time.

The results of breaking up complexes with the T6-Cyanide mixture or with sonic vibration are in essential agreement with each other, but quite different from the control. In the early stages of development T6-cyanide destroys 99 per cent of the plaque-forming capacity of the suspensions, the residual 1 per cent being equivalent to the amount of free phage which was not removed when the complexes were washed. Five minutes of sonic vibration destroys only about 90 per cent of the total activity, the remaining activity probably being due to complexes which escaped disruption. The important conclusion to be drawn here is that the T3 particles which infected the cells were not recovered by either treatment—a strong suggestion that the infecting particles are somehow committed to the particular cells which had adsorbed them in such a way that they cannot successfully infect another cell when given an opportunity to do so on an agar plate. It seems likely that a virus particle changes its state when it is adsorbed on a cell—indeed, since after adsorption we have as yet no way of distinguishing between particle and cell, we should more properly speak of the

combination between the two as a "virus-host complex" since it has properties present in neither the phage nor the host cell (4, 5).

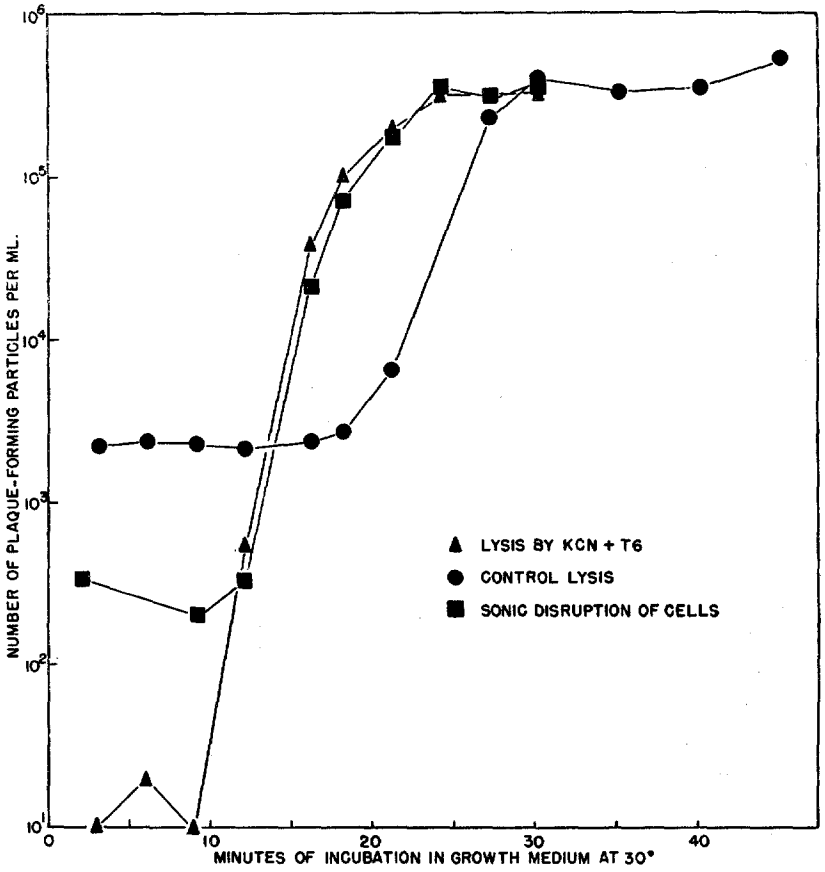

FIG. 3 *a*

FIG. 3 *a* and 3 *b*. The results of two experiments (see Table II for details) comparing the number of plaque-forming particles after treatment with T6-cyanide (triangles) and after sonic vibration (squares) of developing T3-host complexes. It is seen that the two methods yield essentially the same amounts of intracellular virus.

Between 12 and 27 minutes of development, disintegration of the complexes yields ever increasing numbers of daughter T3 particles. It is clear that these particles exist in the complexes well before they begin to appear in the controls. It is interesting, however, that 5 minutes' sonic treatment consistently liberates fewer daughter particles than T6-cyanide does. This may be due to the fact that the sonic treatment did not disrupt all the complexes or that it inactivated

some of the virus. On the other hand, a small proportion of the maturing particles may have reached the stage in which, although their completion could not be blocked by cyanide, they were still susceptible to sonic vibration. While it is significant, the difference between the yields of virus obtained by the two

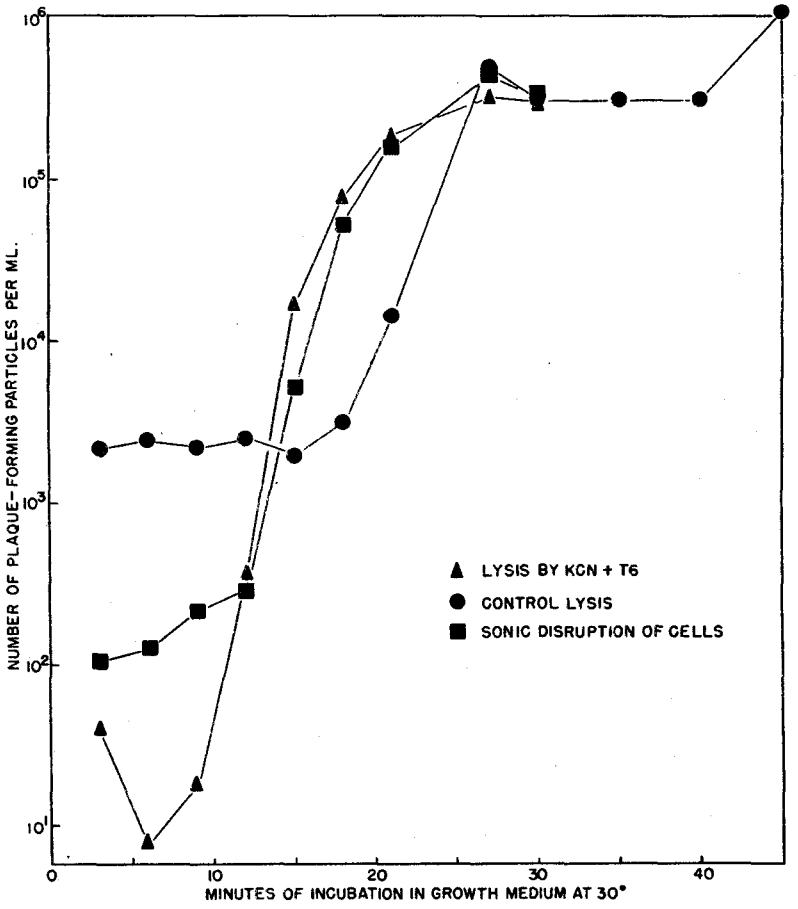

FIG. 3 *b*

methods is not great and gives one confidence in the T6-cyanide method as applied to the extraction of daughter virus particles from cells infected with T3.

When one recalls that T3 and T4 are unrelated viruses, one is struck by the similarity between the behavior of the T3-host complexes studied here and that of the T4-host complexes previously studied: they are similar both in their reactions to cyanide alone and in the manner in which daughter particles appear in the debris of cells which have been prematurely lysed by the T6-cyanide

mixture. These results suggest that, insofar as cyanide and T6 reveal it, the mode of intracellular production of these two unrelated viruses is similar. Since the T6-cyanide method alone would have permitted one to make an essentially correct estimate of intracellular T3 particles, one is led to believe that it gives a valid count of intracellular T4 particles also.

Since both of these methods give essentially the same result, their relative usefulness depends largely on the purpose of the experiment in which they are to be used. The sonic method is advantageous when it is desirable to avoid introduction of foreign phage or cyanide into the culture. On the other hand, it cannot be used with the larger viruses, T2, T4, and T6, which are destroyed by the sonic energy required to disrupt bacteria. Since the genetic material and information now available are largely restricted to these viruses, genetic applications must, at present, use the T6-cyanide technique. A further disadvantage of the sonic method is that the number of samples treated in a given time interval is restricted and the size of the samples must be comparatively large. The usefulness of the T6-cyanide technique will be illustrated in the third paper of this series (7) in which analysis is made of the intracellular phage population of individual bacteria, and in which intracellular aspects of genetic recombination are investigated.

In some ways our results are in agreement with the results of Beumer and Beumer-Jochmans (8) who found in general that newly formed virus-host complexes have the sensitivity of the host to sonic vibration rather than that of the phage: if the phage (*e.g.*, Twort) is sensitive and the host (*e.g.*, staphylococcus Ew) is resistant, the complex is resistant; if the phage (*e.g.*, PF) is very sensitive and the host (*e.g.*, *coli* 4R) is sensitive, the complex has the sensitivity of the host; if the phage (*e.g.*, pfi) is resistant and the host (*e.g.*, *coli* 4S) is sensitive, the complex is sensitive. However, with the bacteria at low concentrations they recovered resistant phage particles from complexes of resistant phage and sensitive host. It seems likely in the light of our experiments that the particles liberated in these last experiments may have been daughter particles rather than the infecting particles as they supposed. Unfortunately, the authors did not report the latent periods of the phages they studied nor the lengths of time the complexes had to develop before they were chilled and subjected to sonic vibration.

SUMMARY AND CONCLUSION

The growth of the virus T3 has been followed by breaking up the complexes it forms with host cells at various stages in their development and then assaying the debris for active virus particles. Two independent methods for breaking up cells were used: sonic vibration and lysis by the T6-cyanide method previously used for the study of the growth of T4.

During the first half of the latent period both treatments, as well as cyanide

alone, destroyed the capacity of the complexes for producing daughter virus particles. Furthermore, the infecting particles could not be recovered from them during the first half of the latent period.

After the complexes had had 12 minutes of incubation at 30°C. both methods freed daughter virus particles from them in numbers which increased steadily with time until, near the end of the rise period, the normal burst size was reached. In general the agreement between the two yields is so good that one may conclude that both methods liberate quantitatively the mature daughter T3 particles which exist in the complexes before normal lysis occurs.

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