THE EFFECT OF TEMPERATURE ON THE FORMATION OF T1 AND T2r BACTERIOPHAGE

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ABSTRACT A very rapid variation of yield of T1 and T2r bacteriophage in *E. coli* at slightly higher than normal temperatures has been observed. T1 phage will develop at 41.2°C but not at 41.7°C. By infecting cells grown on lactose and, therefore, induced to contain beta galactosidase, a technique which indicates when cells have become leaky was worked out. This method shows that at elevated temperatures the enzymatic attack on the cell wall continues to go at a faster rate, while completion of the phage goes more slowly. Thermal constants are given for the processes. Cells at higher temperature, grown on P^{32} medium develop incomplete particles capable of combining with phage antibody. This suggests that the process affected by the early leakiness of the cells is the completion of the virus protein coat. Supplementing the medium with casamino acids, phosphate, and ATP causes the "rescue" of phage particles by aiding the formation of the coat. This can be achieved several minutes after the cells have become leaky and may form a useful system for the study of phage development in the presence of analogs.

INTRODUCTION

Among the problems of basic interest to biophysicists is the problem of the development of structure and form in the living cell. One very elementary, and so presumably simple, system to study, which might conceivably serve as a model for some aspects of more complicated systems, is the process of assembly of a virus. While a great deal is known about the component parts of various kinds of viruses, and indeed great detail about the genetics of some of them, the means by which the parts of a virus actually become assembled in the host cell are only known speculatively, if at all. One method of attempting to gain some insight into the process at work is to subject the cells, which have been infected and so are developing fresh virus, to variations of temperature. The detailed study of the response of the virus-host system to temperature offers some promise because there have been recent advances in the understanding of the action of heat on biologically active

DNA, and one can hope that the more complicated system will show analogous behavior which would be suggestive of a way to make a start in understanding.

In prosecuting such studies on the bacteriophage-bacterial system, we found that there is a very remarkable temperature dependence of the yield of virus. Cells at 41.2°C will permit T1 bacteriophage to develop in a small but definite yield, while at 41.7°C there is not only no yield, but also an actual inactivation of the phage. At both these temperatures uninfected cells readily grow and divide. This paper gives an account of a series of investigations designed to shed light on the processes at work. We conclude that the rapid loss of yield as the temperature becomes higher is due to the presence of two processes involved in the completion and escape of phage. The escape process is the enzymatic attack on the cell wall by lysozyme produced by the phage infection: the completion involves the formation of complements of DNA, the appropriate protein coat, and the assembly of the two. Our findings indicate that the normal time course of these processes, in which the cell wall disruption occurs after complete phage have been made, is altered because the enzymatic attack on the cell wall goes faster at higher temperatures, while the development of the phage itself goes more slowly. The two processes thus become "uncoupled," with drastic results.

There have been several studies of the effect of temperature on the formation of bacteriophage. Luria (1943) commented briefly on the failure of phage production at temperatures at which bacteria are quite able to grow and divide. Maaloe (1950) observed the effect of shifting temperature from low to high on the yield of T4r phage. He suggested that metabolic rates imposed different demands on virus formation and lysis. More quantitative analysis was given by Weis-Benzton, Maaloe, and Rasch (1950). Buzzell, Trkula, and Lauffer (1954) observed that the host virus complex is very sensitive to heat at 47°C. The temperature sensitivity of the capacity to support phage growth was found by Pollard (1959) to be more sensitive to temperature than the formation of bacterial colonies. The subject of the formation of viruses in general has been treated theoretically by Lwoff and Lwoff (1962*a*) and an interesting summary of work on animal viruses as related to temperature is given by Lwoff (1962*b*).

While the work to be described was in progress Groman and Suzuki (1962) showed that there is a rapid variation in the yield of lambda phage at temperatures above the optimum for phage growth, and concluded, from various combinations of temperature in the growth cycle, that the process of lysis of the cell wall occurs earlier than that of the completion of the phage and so diminishes the yield. In this paper we bring direct evidence to show that the process suggested by Groman and Suzuki is in operation, and give quantitative measurements on the processes of lysis and of phage completion which enable estimates of the average burst size to be made. In addition, we show that the two processes of lysis and completion can become separated at low temperatures.

In the case of T1 and T2 bacteriophages we also observe that the process of lysis of the cell wall before completion of the phage causes an inactivation of the bacteriophage. We suggest that this is due to the leakage from the cell of components necessary for the formation of the protein coat. We also show, that for the case of T1, and hence probably for T2 also, that supplement to the medium can modify the inactivation process and permit completion of the phage to occur.

EXPERIMENTAL METHOD

Cells of *Escherichia coli* (ATCC No. 11303) were used as host cells. T1 phage of the strain used in previous work at Yale University and obtained originally from Dr. Demerec at Cold Spring Harbor, and T2r obtained from Dr. M. F. Mallette of the Department of Biochemistry at The Pennsylvania State University were used. Preliminary studies showed that with 5×10^8 cells per ml 10 per cent attachment occurred in 1 minute in a broth suspension.

For the single-step growth curves involving the counting of phage released, bacteria at 5×10^8 per ml and phage at 10^4 per ml were combined in broth suspension at 37°C for 1 minute. 0.1 ml of the mixture was then diluted into 10 ml chilled broth and centrifuged for 3 minutes in a Sorvall angle centrifuge to bring down the bacteria. The supernatant fluid was poured off, and the pellet, which contains attached cells, with excess cells was resuspended in 20 ml of broth maintained at the appropriate temperature. At various intervals, 0.5 ml samples were withdrawn and plated on agar plates against *E. coli* bacteria. The procedure in the case of T2r was similar, except that the broth contained 5 gm per liter added NaCl to facilitate attachment.

For experiments designed to study the time at which the cells became open, the method used involved the use of cells which contain the enzyme beta galactosidase. In the ordinary case, the cell membrane and cell wall act to keep this enzyme within the cell and thus, when cells are mixed with the substrate, the enzyme cannot act, and there is only a very small background effect, probably due to a small fraction of cells which are imperfect as to their membranes and cell walls. On the other hand, when cells are permeable, as regards the substrate, the reaction takes place to an appreciable extent. The substrate used is orthonitrophenyl β-D-galactopyranoside, usually referred to as ONGP. The normal yellow color of nitrophenol is not observed when the molecule is coupled to the galactose. The enzyme can break the linkage and when this occurs a yellow color appears, which is quantitatively related to the amount of enzyme action on the substrate.

For experiments of this nature, cells were grown overnight on Roberts C minimal medium (2 gm NH_4Cl, 6 gm Na_2HPO_4, 3 gm NaCl, 62 mg $MgCl_2 \cdot 6H_2O$, 80 mg Na_2SO_4, 5 gm lactose per liter), with lactose as carbon source. In the morning a fresh culture was grown to 5×10^8 per ml, centrifuged as before, washed with 5 ml broth, and after recentrifugation, resuspended in broth. T1 or T2 was added at a concentration of 10^8 per ml, allowed to attach for 1 minute, and the mixture chilled and centrifuged. The pellet was resuspended in broth at the desired temperature, and at the necessary intervals 1 ml was removed and added to 0.2 ml KCN (0.05 M) in an ice-cold test tube. 1/10 ml was diluted in C minimal medium (salts only, no carbon source) and plated against *E. coli* bacteria, and 0.8 ml were used to assay for beta galactosidase. The 0.8 ml was added to 2.5 ml of phosphate buffer and substrate, allowed to act for 10 minutes at

30°C, and the reaction stopped with N/10 Na_2CO_3 at that time. The optical density of the yellow solution was read in a spectrophotometer at 420 mμ wavelength.

The requirement of labeling cells with phosphorus imposes a need for a medium of growth in which the buffer is not composed of phosphate, for this overwhelms the radioactive phosphorus and requires the use of huge amounts of radioactivity. A buffer, which is fairly satisfactory is tris(hydroxymethyl)aminomethane, and, following Roberts, a medium was made up as follows: NH_4Cl, 2 gm; NaCl 4 gm; KCl 1 gm; Tris, 7 gm; $MgCl_2 \cdot 6H_2O$ 62 mg, $Na_2SO_4 \cdot 10H_2O$, 182 mg per liter. The pH was adjusted to 7.2 with dilute HCl, and phosphate added as Na_2HPO_4 in very small amounts which were adjusted largely by trial and error to give adequate levels of radioactivity in the culture. Actual limiting phosphate is about 1 mg per liter for about 10^8 cells per ml, and this forms a lower limit which can be used.

RESULTS

Single-Step Growth Experiments. Fig. 1 shows a series of curves indicating the way in which phage multiplication and release occur as a function of time, for a series of temperatures. It is not easy to show, on a single graph, all the data for all the experiments. The scatter for the lowest temperature was the great-

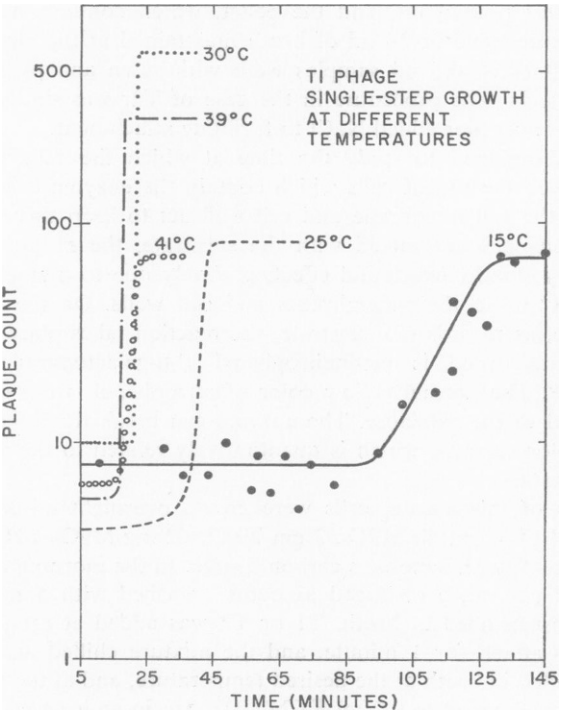

FIGURE 1 The variation of phage yield in single-step growth experiments as a function of temperature. It can be seen that there is an increase until 39°C and a drop above that temperature.

est and these data are shown. The figure shows, on careful examination, that there is a considerable effect of temperature on the latent period and also on the yield of virus in the range from 15° to 30°C, followed by a relatively temperature-insensitive range from 30° to 40°C. As represented on this graph by the line at 41°C, there is a very slight lengthening of the latent period and a reduction of the yield. In view of this not very dramatic behavior, the process which takes place in a range of less than a degree, just above these values, is very startling. The results are shown in Fig. 2. The behavior at 40.77° and 40.92°C fits with the presence of a development and yield of phage, of lower value and at a slightly delayed time, but at 41.25° there is no yield at all, and in fact the infected cells, which presumably contain phage, actually lose the ability to release even the phage with which they are infected, which means that presumably somewhere in the process something is effectively inactivating the virus. To show this more clearly, at a slightly higher temperature a higher initial number of infected cells was plated. The process of the loss of phage at 41.77°C is very marked. Thus, in an interval of no more than 0.35°C the pattern has changed from an ability to produce a burst to a steady loss of infectious centers.

The temperatures were measured with a good mercury in glass thermometer and, as concerns relative temperatures, are accurate. The times of the experiment were short enough to permit watching thermometers continually and keeping the

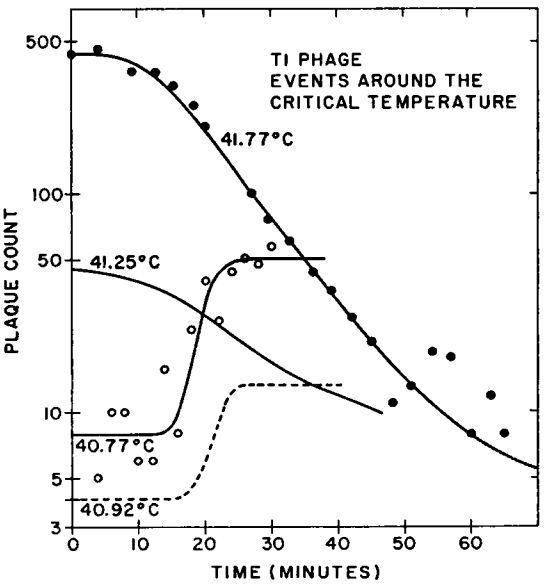

FIGURE 2 Single-step growth experiments in a narrow temperature range from 40.77° to 41.77°C. The process changes from one of growth and burst to a process of inactivation in a temperature interval of one-half degree.

water bath steady by vigilance. However, absolute calibration to the accuracy of relative measurements was not made. We estimate that an absolute value uncertainty of 0.2°C is possible. The significant feature is in the relative values.

In order to see whether the sensitive part of the process is early or late, we held infected cells at 37°C for varying lengths of time before raising the temperature to 42°C where the inactivation process takes place. We found that cells which were held in this way for approximately two-thirds of the latent period and then raised in temperature went on to produce a virus yield. If transfer was made before that, no burst occurred. We also examined cells which were infected, allowed to develop at 42°C, and prematurely caused to open artificially. This was done by holding them in the refrigerator overnight in 0.05 M KCN and 4 M glycine, a technique which causes artificial lysis of infected cells. No free phage were observed. Check experiments at 30°C, where the development of phage is normal, indicated that this method revealed the presence of free phage 6 minutes before the end of the normal latent period. The temperature of 30°C was chosen because the latent period is somewhat longer than that at 37°C and might be in rough agreement with any possible development of phage at 42°C.

These two experiments indicate that the failure to produce phage is not due to the failure of the process of cell wall disruption, but rather is concerned with the failure to make completed phage.

Temperature Sensitivity of Phage Grown at Various Temperatures. Recent experiments in our laboratory have shown that the temperature inactivation of T1 phage is a two component process. Phage particles of different sensitivity seem to be present in each phage culture. If it were true that the phage which was made at higher temperatures was assembled differently so as to select one or the other component, the proportion of sensitive and insensitive phage particles might be expected to change for cultures developed at high and low temperatures. To test this hypothesis we grew phage lysates of *E. coli* at 30°, 39°, and 41.3°C, this last being the highest temperature at which we could obtain a lysate. A lysate, which takes several hours to produce, can be obtained at a very slightly higher temperature than the highest temperature for phage yield in a one-step growth experiment. The phage were freed of bacteria by centrifuging, and two samples of them were exposed to 74° and 68°C temperatures in a water bath. Surviving fractions of each were plotted at various times. The results are shown in Fig. 3. There is no difference in the sensitivity which can be seen to be significant. Thus, there seems to be no selection imposed on the culture by the conditions of growth over the range of temperature from 30° to 41.3°C. This suggests that those phage particles which are assembled at the higher temperature are, at least by this criterion, perfect particles. The stress of heat seems to permit all or none in the process of formation of phage.

Time of Opening of Cells. The technique of using lactose-grown cells,

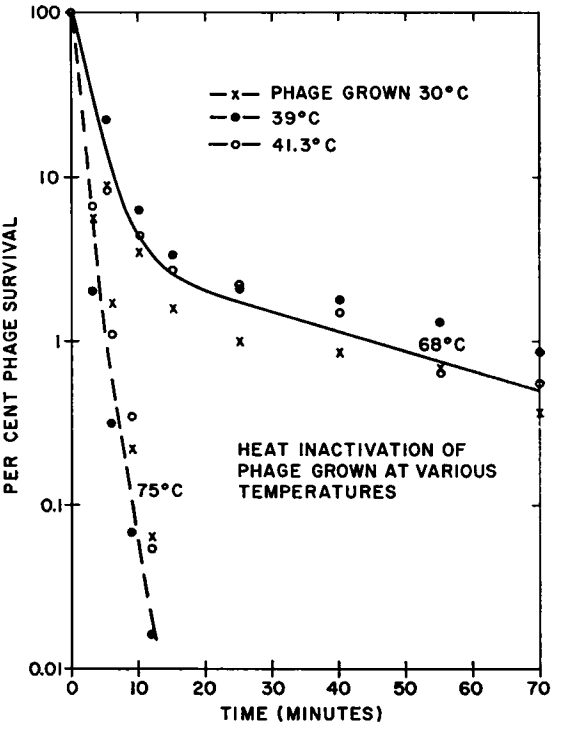

FIGURE 3 Temperature sensitivity of phage grown at three different temperatures. There are two components, one more heat resistant than the other. The proportion of the two is not markedly affected by the temperature of growth.

containing beta galactosidase, was applied to study the degree to which cells become open during the process of phage development at higher than normal temperatures. The normal behavior of cells in this regard is shown in Fig. 4. Cells infected at 37°C and sampled at short time intervals were observed both for phage yield and for the ability to cause the appearance of yellow color when mixed with the substrate. It can be seen that there is a marked increase in color as the phage are released, and that at this optimum temperature the two processes are clearly taking place at the same time. Control experiments with uninfected cells showed very little color, less than 5 per cent of the color produced by the phage-infected cells.

This experiment was repeated at a variety of temperatures. Results at 15°, 25°, 42°, and 45°C are shown in Fig. 5. The data at 42° and 45°C indicate that cells become open very early after infection and that the drop in the count of infectious centers corresponds rather well with the increase in color. In order to determine what is the significance of the ability of the enzyme to attack the substrate, *i.e.* whether the substrate gets *in* or the enzyme gets *out* of the cells, we measured the fraction of enzyme activity found in cells which had been held at 42°C, which

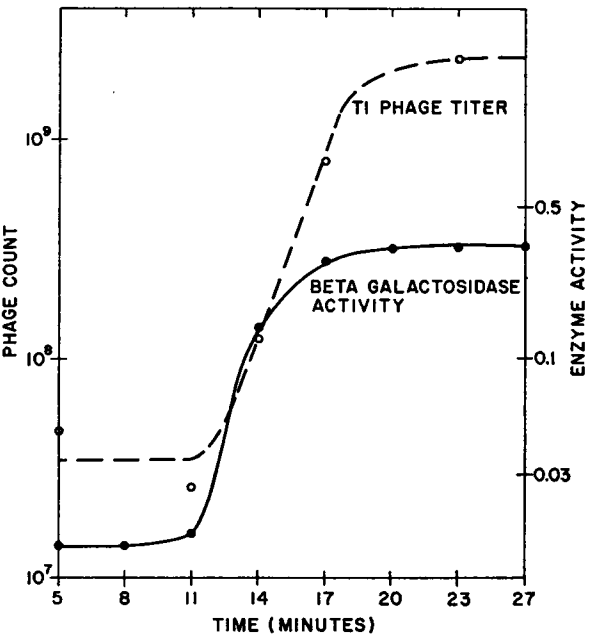

FIGURE 4 Release of beta galactosidase activity and phage by lactose-adapted cells at 37°C. The two processes, one representative of lysis and the other of maturation, go together.

resided in the pellet formed after low speed centrifugation, as compared with the enzyme activity in the supernatant medium. The activity in the medium is presumably due to enzyme release, while that in the pellet of cells is due to substrate which can penetrate the cell wall and so develop activity with enzyme which has not actually escaped the structure of the cell. The results are shown in Table I.

It can be seen that while there is an early access to the interior of the cell on the

TABLE I

COMPARISON OF BETA GALACTOSIDASE ACTIVITY IN SUPERNATANT (RELEASED FROM CELLS) WITH THAT IN THE PELLET (HELD IN CELLS)

Time of incubation at 42°C	Relative activity in pellet	Ratio of activity in supernatant to that in pellet
min.		
4	1.2	0.0
8	1.7	0.0
12	2.7	0.05
16	3.0	0.15
20	3.4	0.23

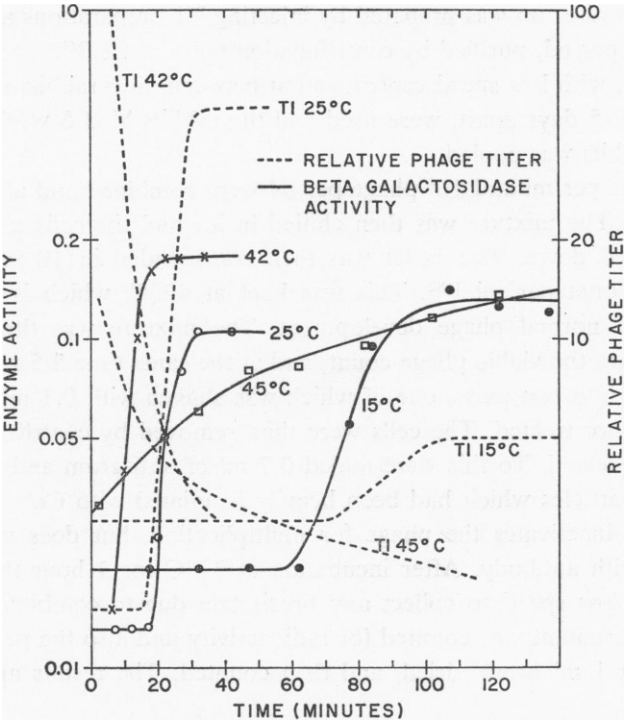

FIGURE 5 Release of beta galactosidase activity and phage growth at various temperatures. The two do not go together above 42° and at 15°C. At high temperatures the enzyme activity starts very early, indicating leaky cells. At low temperatures the enzyme activity appears slightly before the mature phage.

part of the substrate, ONGP, the release of the relatively large beta galactosidase molecule does not occur until later.

Experiments with P^{32} Tracer and T1 Antibody. It is possible to see whether the failure to make complete phage occurs only at the stage of DNA multiplication, or also at the stage of protein coat assembly, by observing the combination of phosphorus-labeled material emerging from the cells with specific T1 antibody. Antibody will combine with protein coat material, but not with the DNA, and combination with antibody occurs even if the protein coat is not fully assembled. Thus, any appreciable combination of phosphorus-labeled material with antibody indicates material which, at any event, has some of both components present. If such material is not infectious, it is presumably incomplete in some way. To conduct these experiments, it is necessary to check how much completion has taken place independently of the abnormally early opening of the cells at the higher temperatures. To do this, we artificially opened some of the cells by the simple technique of shaking samples for 2 minutes with 0.1 ml of chloroform.

The rabbit antiserum was prepared by injecting T1 preparations at a titer of 10^{10} virus particles per ml, purified by centrifugation twice at 28,000 RPM in the Spinco ultracentrifuge, with low speed centrifugation between, into rabbits subcutaneously. Five injections, 5 days apart, were used and the rabbits bled 5 weeks later. Serum from three rabbits was pooled.

5×10^8 cells per ml and 10^8 phage per ml were combined and allowed to attach for 2 minutes. The mixture was then chilled in ice and the cells plus the infected cells centrifuged down. The pellet was then resuspended in 10 ml Tris medium with 500,000 CPM per ml P^{32}. This was kept at 42°C, which is just above the temperature of normal phage development. The mixture was titered at various times to measure the viable phage count, and at the same time 2.5 ml samples were taken, divided into two parts, one of which was shaken with 0.1 ml of chloroform and the other not treated. The cells were then removed by centrifugation and the supernatant collected. To this were added 0.2 ml of antiserum and 10^9 non-radioactive phage particles which had been heavily irradiated with Co^{60} gamma rays, a process which inactivates the phage for multiplication, but does not prevent the combination with antibody. After incubation at 37°C for 1 hour the mixture was centrifuged at low speed to collect any precipitate due to combination with antibody. The supernatant was counted for radioactivity and also the pellet, which was resuspended in 1 ml broth, dried, and then counted. The results appear in Table II.

TABLE II

COMBINATION OF ANTIBODY WITH P^{32}-LABELED MATERIAL IN INFECTED CELLS GROWN AT 42°C AND OPENED ARTIFICIALLY

	Supernatant, not artificially opened		Pellet, cells not artificially opened	Supernatant, artificially opened		Pellet, cells opened
Time	Titer	P^{32}	P^{32}	Titer	P^{32}	P^{32}
min.						
0	1.3×10^7					
30	4.7×10^6	8900	930		19,800	980
45	1.5×10^6	4600	990	5×10^5	13,600	1250
60	3.0×10^6	6700	790	2×10^5	15,000	1290
120	1.0×10^6	3100	530	8×10^4	8,900	1610

In the case where the cells are not artificially opened, the count of viable infectious centers, the phage titer, goes down, as does the amount of P^{32} which does not combine with antibody and also that which does combine. On the other hand, the effect of chloroform seems to reduce still more the amount of phage which can form plaques in the assay, while releasing considerably more phosphorus into the supernatant. Most of this does not combine with antibody, but an increasing frac-

tion *does,* as shown by the increase in the counts in the pellets, which corresponds to material which will combine with the specific antibody to T1. The fact that the phage titer goes steadily down in this case indicates that the particles which combine with antibody are not complete and viable phage. This leads us to conclude that there is some DNA synthesis which must continue at 42°C and that some DNA-protein combination *short of* the formation of viable phage must occur which steadily increases in amount at 42°C.

Effect of Crystalline Egg White Lysozyme on Cells at Different Temperatures. Since the change in lysis at 42°C and above might be due to a greater sensitivity of the cell wall to the action of lysozyme, experiments were carried out to see if a difference is apparent. Cells grown in lactose were exposed to lysozyme (0.2 mg/ml) for the times indicated in Table III. Temperatures of 37°C (normal behavior) and 45°C (above normal growth behavior) were used, and controls lacking lysozyme were also examined. The cells were then assayed for beta galactosidase as described previously with the results indicated in Table III.

TABLE III

EFFECT OF LYSOZYME ON CELLS AT DIFFERENT TEMPERATURES

	37°C		45°C	
Time	Lysozyme	Control	Lysozyme	Control
min.				
5	0.040	0.030	0.060	0.025
15	0.055		0.088	
30	0.062	0.045	0.100	
60	0.095		0.142	
75	0.125	0.080	0.152	0.040

The amount of color release is shown for various conditions.

It can be seen that the color produced, which is a measure of the enzyme activity, is actually released earlier and at a faster rate from cells at 45°C. This affords a very natural explanation for the early leakiness of cells at high temperatures. The relation of the cell wall, as substrate, to this enzyme is clearly temperature-dependent and goes faster at higher temperatures. Thus, the more rapid lysis of the cell wall is in accord with enzyme kinetics. What is more surprising is the fact the rate of phage assembly is slower at higher temperatures. It should be pointed out that egg white lysozyme is not necessarily similar to T1 lysozyme.

Additives to the Medium Which Will "Rescue" the Incomplete Phage. In view of the evidence that the leakiness of the cells is causing a problem in the completion of phage, a view supported by the antibody experiments just described, we endeavored to see if ways could be found which could modify the cells to permit

completion of the phage at abnormally high temperatures. After several attempts, involving such things as trying to pack the cells close together, growth on agar, and high sucrose concentration, we found that a medium containing those factors which should increase protein synthesis produced a marked recovery from the process of inactivation. The medium, which we designate as "rescue" medium was 8 gm/liter casamino acids, 0.4 gm/liter adenosine triphosphate, 6 gm/liter Na_2HPO_4, 3 gm/liter KH_2PO_4. The time course of a single step growth curve for three experimental conditions is shown in Fig. 6. The full line shows the number of infectious centers (either free phage or infected cells)observed at various times at 42° and 43°C (dashed line) where the "rescue" medium was present from the start. The interrupted line shows the effect of late addition of "rescue" medium (15 minutes) at 42°C. In all cases a recovery is apparent. The fact that "rescue" can be affected after 15 minutes suggests the interesting possibility that cells which are already somewhat leaky, and hence more accessible to outside substances, can be "persuaded" to complete phage in spite of this. Some exploitation of this system should be possible.

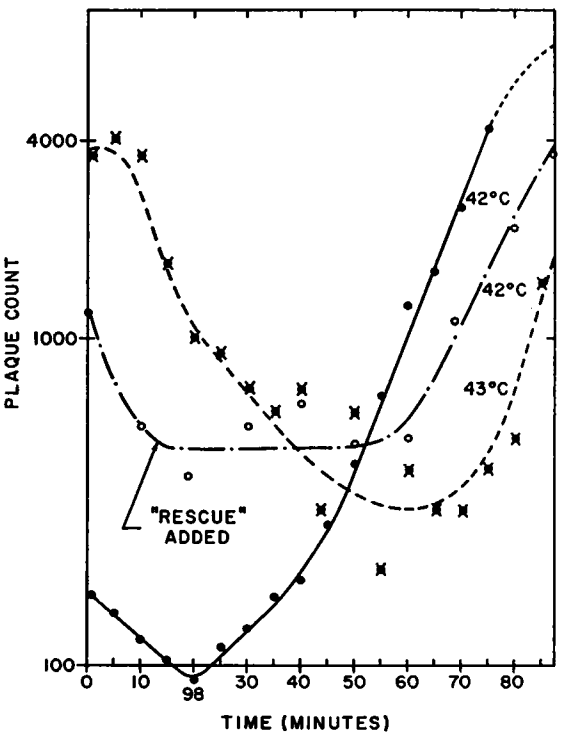

FIGURE 6 The development of phage at high temperatures in the presence of medium containing casamino acids, ATP, and phosphate. There is a definite "rescue" of the phage. If the "rescue" medium is added after 15 minutes the behavior shown by the open circle plot is seen.

Summary of Kinetic Data. Rather a large number of observations of single step growth curves for T1 and T2r phage were made, and a correspondingly large number of beta galactosidase activity measurements were also made. In order to present the impression gained from these findings we give, in Table IV, a summary of rate constants for four processes at a variety of temperatures. The rate constants for beta galactosidase activity with T1 and T2r infection were determined, in the first place, by observing the time at which 50 per cent of the activity had developed and taking the reciprocal of this time, in minutes, to be the rate constant and, in the second place, by an analysis of the spread in release time by a method described later.

Since our experiments show that phage release is strongly affected by the premature opening of cells, the rate constants for phage release are not so easy to measure. We made the assumption that, for T1 and T2r there is a "true" burst size of 200 in each case, and calculated, from the enzyme release data what the correction to the observed phage release curve would be assuming there were no premature release of phage. Thus, we used the small yield figures at high and low temperatures to provide the initial part of the phage release curve and estimated the rest by allowing for the cut-off which was determined by the enzyme release. The method can be understood better by consulting Fig. 7. This is a schematic plot

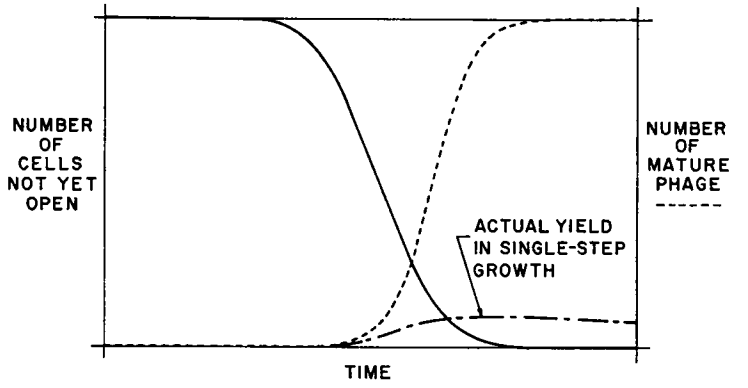

FIGURE 7 Schematic representation of the statistical process involved in a single-step growth experiment. The phage can only mature from cells which have not opened. The opening is distributed according to a probability integral and so is the maturation. The product of the two measures the number of mature phage in unopened cells.

of the two factors at work in causing phage release. The continuous line shows the statistical distribution of cells not yet open and thus able to support phage growth, and the dashed line is the distribution of the number of mature phage, assuming no interference with their completion. Only those phage completed in unopened cells will become viable and this amount is shown as the double dashed line. By

using the observed enzyme activity data, the fraction of unopened cells can be estimated at any time, and the observed phage release scaled up to give an estimate of the expected rate of phage completion. Thus, at the time of 50 per cent enzyme activity, the correction on the observed phage number is 2. By this rather approximate method, a reasonable idea of the kinetics of phage development can be obtained. These figures are given in Table IV.

The significance of the sixth and seventh columns is given later.

Some data on the growth rate of uninfected cells are also included for comparison. These were measured by increase in turbidity of the cell culture.

If the figures in the table are consulted, it can be seen that for bacterial growth and phage formation there is a marked drop in the rate constant at temperatures above 40°C. For the process of lysis, as measured by the color reaction for beta galactosidase, the drop occurs at higher temperatures. This accounts for the premature lysis at high temperatures.

TABLE IV

Kinetics of Release of Beta Galactosidase and T1 Phage

Temperature	1/Kelvin temperature (inverse degrees)	Time of onset	Plateau time	Rate from midpoint	Rate factor from spread	Ratio
°C		*min.*	*min.*	*min.*$^{-1}$		
Beta galactosidase						
15	3.54×10^{-3}	62	97	1.32×10^{-2}	2.3×10^{-3}	6
25	3.36×10^{-3}	17	32	4.54×10^{-2}	9.7×10^{-3}	5
37	3.23×10^{-3}	10	18	8.0×10^{-2}	2.5×10^{-2}	3
39	3.21×10^{-3}	10	21	7.2×10^{-2}		
42	3.18×10^{-3}	5	21	9.1×10^{-2}	1.4×10^{-2}	6
45	3.14×10^{-3}	11	120	2.2×10^{-2}	9.7×10^{-4}	23

Temperature	1/Kelvin Temperature (inverse degrees)	Time of onset	Rate constant
°C		*min.*	*min.*$^{-1}$
T1 phage			
15	3.54×10^{-3}	87	8.5×10^{-3}
25	3.36×10^{-3}	27	3.5×10^{-2}
30	3.31×10^{-3}	23	5.9×10^{-2}
37	3.23×10^{-3}	11	7.4×10^{-2}
39	3.21×10^{-3}	12	7.4×10^{-2}
40.78	3.19×10^{-3}	16	4.6×10^{-2}
40.93	3.185×10^{-3}	18	2.9×10^{-2}

(*Table continued on following page*)

TABLE IV (*concluded*)

Kinetics of Release of Beta Galactosidase and T2r Phage

Temperature	1/Kelvin temperature (inverse degrees)	Time of onset	Plateau time	Rate from midpoint	Rate factor from spread	Ratio
°C		*min.*	*min.*	*min.*$^{-1}$		
Beta galactosidase						
15	3.54×10^{-3}	120	–	–		
25	3.36×10^{-3}	44	66	1.8×10^{-2}	0.076	0.24
30	3.31×10^{-3}	34	40	2.7×10^{-2}	0.22	0.12
37	3.23×10^{-3}	20	27	4.3×10^{-2}		
37	3.23×10^{-3}	17	25	4.8×10^{-2}	0.25	0.19
42	3.18×10^{-3}	2	42	4.6×10^{-2}	5.5×10^{-2}	0.90
42.2	3.175×10^{-3}	1	62	3.2×10^{-2}		
45	3.14×10^{-3}	1	122+	1.6×10^{-2}		

Temperature	1/Kelvin temperature (inverse degrees)	Time of onset	Plateau time	Rate from midpoint
°C		*min.*	*min.*	*min.*$^{-1}$
T2r Bacteriophage				
15	3.54×10^{-3}	120	–	–
25	3.36×10^{-3}	36	62	2.0×10^{-2}
30	3.31×10^{-3}	32	40	2.8×10^{-2}
37	3.23×10^{-3}	17	25	4.5×10^{-2}
42	3.18×10^{-3}	62	122	1.1×10^{-2}
42.2	3.175×10^{-3}	80	140	9.0×10^{-3}

Rate Constants for Cell Growth

Temperature	1/Kelvin temperature (inverse degrees)	Rate constant
°C		*min.*$^{-1}$
15.0	3.54×10^{-3}	2.17
20.0	3.41×10^{-3}	2.33
25.0	3.36×10^{-3}	7.10
30.0	3.31×10^{-3}	10.00
38.4	3.21×10^{-3}	10.00
39.8	3.20×10^{-3}	14.00
44.5	3.14×10^{-3}	2.00

In the range where the rate constant increases with temperature, it is possible to describe the variation in the rate constant by the simple relation:

$$k_1 = Ae^{-\Delta H^*/RT}$$

where k_1 is the reaction rate, A is a constant, R is the gas constant, ΔH^* is the enthalpy of activation. Table V shows the values which are obtained.

The range where the rate constant falls with increasing temperature is the range in which the uncoupling of the process of lysis of the cell wall and of the process of phage completion is most apparent. For the case of T1 infection, it can be seen that at 42°C the rate of action of the lysing mechanism is increasing in speed while at 40.93°C the process of phage completion has markedly slowed down. The same

TABLE V

ACTIVATION ENTHALPIES FOR VARIOUS PROCESSES

Process	Enthalpy
	cal/mole
T1 development	2.4×10^4
Release of beta galactosidase by T1 infection	2.1×10^4
T2 development	2.0×10^4
Release of beta galactosidase by T2 infection	2.0×10^4

is also true for T2r. Thus, the suggestion made by Groman and Suzuki regarding premature lysis seems to be right.

Uncoupling of Phage Completion and Cell Wall Lysis at Low Temperatures. Table IV shows that at 15°C, for T1 the rate of lysis of the cell wall occurs appreciably ahead of the rate of phage completion. This explains the low yield of T1 at low temperatures. This uncoupling is not observed for T2r where the rates go closely together.

Statistical Analysis of the Relation Between Cell Opening and Maturation. A very simple hypothesis can be used to interpret the data. It is supposed that the attached host virus complexes represent a population of cells in which the process of cell opening is distributed according to a probability integral and that the maturation of phage is distributed according to a probability integral with a different rate. The idea can be seen from Fig. 7. The continuous line represents the number of cells which are not yet open and the dashed line the number of mature phage per cell assuming no cell opening can occur. The product of the number of cells unopened and the number matured gives a measure of the number which will actually appear in a single step growth experiment. This is indicated by the line as shown.

The probability integral is the integral of the normal distribution corresponding to either the number of cells opening per second or the number of phage per cell maturing per second (dN/dt). If t_0 is the mean time of opening; t an arbitrary time;

h_1, a parameter which measures the spread in rate of opening, being least when h_1 is large; N_0 the total phage per cell, we have

$$\frac{dN}{dt} = \frac{N_0 h_1}{\sqrt{\pi}} e^{-(t-t_0)^2 h_1^2}$$

Thus, a plot of the amount of beta galactosidase released *versus* time should be the integral of the above equation. This can readily be verified by plotting on "probability paper" and such plots are shown in Fig. 8. The straight-line relation can be taken to verify the normal distribution and the slope is a measure of h_1. This parameter has the dimensions of number divided by time and can be thought of as being a measure of the amount of cell structure to be lysed divided by the average time to do the lysing. Its value and the rate deduced from the value of the mean time to

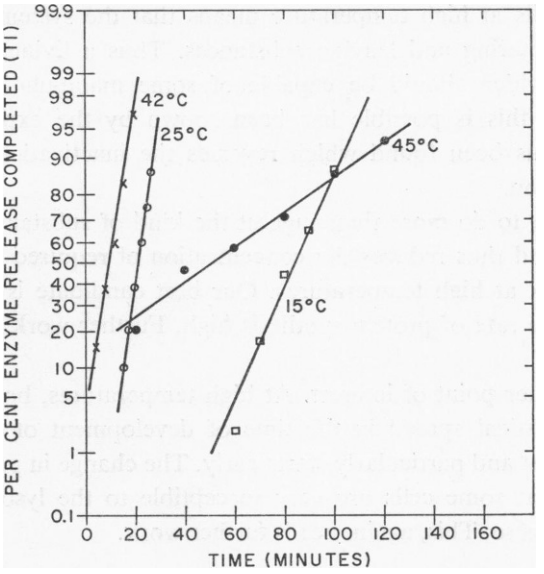

FIGURE 8 The per cent of beta glactosidase activity developed *versus* time plotted on a "probability" scale. The slope of the line is a measure of the spread and the time to reach 50 per cent a measure of the rate of lysing. It can be seen that at 45°C the rate is slow but the spread is very great. It is partly this increase in spread which prevents the maturation of phage.

lyse, as directly measured, should be closely related. In Table IV values of h_1 are given in the last column. It can be seen that a reasonable correlation between the rate constant deduced from the midpoint and the values of h_1 is observed except for the 45°C data for T1 and 42°C for T2 where the spread is altogether too large. For T2 at 45°C the spread was so great that this analysis could hardly be applied. Thus, the sharpness of cut-off of formation of mature phage as the temperature is

increased is partly due to the fact that cells are, on the average, lysing too early and *also* partly due to the increased spread at higher temperatures. The increased spread is interesting because it suggests that some cells are opened very fast by lysozyme at 45°C and some take much longer.

DISCUSSION

The development of bacteriophage at temperatures above normal is a good example of the disruption of a complex system which needs to have several processes occurring together in time. The system is unusually simple in that the two major processes can be studied separately and the kinetics can be shown to diverge—that of lysis continuing to go faster and that of phage completion going more slowly as the temperature is increased.

The system is of interest for another reason. The early occurrence of leakiness in the infected cells at high temperature means that the system is abnormal with respect to both entering and leaving substances. Thus a living, synthesizing, unit has been found which should be capable of some manipulation with regard to metabolites. That this is possible has been shown by the experiments in which special medium has been found which reverses the inactivation of the virus and causes its completion.

We are not able to do more than suggest the kind of substance which leaks out of infected cells and thus reduces the concentration of required material inside the host virus complex at high temperatures. Our best candidate is the transfer RNA needed to keep the rate of protein synthesis high. Further work is needed to prove this hypothesis.

There is one other point of interest. At high temperatures, both for T1 and T2r infection, the statistical spread in the time of development of bata galactosidase activity is very great and particularly starts early. The change in pattern is so marked that it suggests that some cells are very susceptible to the lysozyme activity and others are much less so. This, again, needs further work.

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REFERENCES

BUZZELL, A., TRKULA, D., and LAUFFER, M. S., 1954, Thermal inhibition of a bacterium bacteriophage system, *Arch. Biochem. and Biophysics*, **49**, 234.

GROMAN, N. B., and SUZUKI, GRACE, 1962, Temperature and lambda phage reproduction, *J. Bact.*, **84**, 431.

LURIA, S., 1943, Dissociation of the growth of bacterial viruses and their host by means of temperatures above optimum, *J. Bact.*, **46**, 575.

LWOFF, A., and LWOFF, M., 1962*a*, Evenements Cycliques et molecules metastables, *J. Theoret. Biol.*, **2**, 48.

LWOFF, A., 1962*b*, The thermosensitive critical event of the viral cycle, *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 159.

MAALOE, O., 1950, Some effects of changes of temperature on intracellular growth of the bacterial virus T4r, *Acta. Pathol. Microbiol. Scand.*, **27**, 680.

POLLARD, E., 1959, *Proc. 1st Nat. Biophysics Conf.*, 1957, 683.

WEIS-BENTZON, M., MAALOE, O., and RASCH, G., 1952, An analysis of the mode of increase in number of intracellular phage particles at different temperatures, *Acta. Pathol. Microbiol. Scand.*, **30**, 243.