THE THERMAL INACTIVATION OF T_4 AND λ BACTERIOPHAGE

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ABSTRACT Thermal inactivation of T_4 and λ bacteriophage shows two components of differing sensitivity are present. These cannot be interpreted as owing to nucleic acid and protein. One protein function—the inhibition of radiation-induced DNA degradation—is lost with quite different thermal kinetics. λ heated in the presence of DNase is more rapidly inactivated; λ is also protected by slow cooling after heat. These results suggest that the packing of the DNA in the head occurs so as to permit different degrees of thermal expansion in the outer coils. These can rupture the coat and this is one form of inactivation. Killed vaccines could be more safely made by heating in the presence of a nuclease followed by rapid cooling.

INTRODUCTION

Thermal inactivation studies of viruses have been of interest to us for many years. The process is often characterized by relatively simple kinetics, fitting the relation $\ln N/N_o = -k_1 t$ where N and N_o are the active and original virus activities, t is the elapsed time, and k_1 is a rate constant, and can be analyzed in terms of the theory of absolute reaction rates (1). It is also often true, however, that the kinetics are more complex; thus Woese (2) finds that the thermal inactivation of animal viruses is generally a two-component inactivation, meaning that there are two rate constants, each component following first-order kinetics. Experiments by Pollard and Woodyatt (3) indicated that the inactivation of T_1 phage has two components in proportions varying with temperature. Attempts to select populations of resistant and sensitive phage were never successful. Accordingly, we decided to study the kinetics of inactivation of phages T_4 and λ to see if the same phenomenon is found: it is. In addition we considered the possibility that the coiling of the DNA in the phage head could occur so that different base ratio segments were on the outside and that different relative expansions between protein and DNA could cause different ruptures of the protein coat, which would inactivate the virus. To test this we examined the loss of infectivity of λ in the presence of DNase and found a marked effect; we tested the inactivation of a component of the virus due only to protein (the inhibition of radiation-induced DNA degradation) and found quite different kinetics for it; and we observed that heating and slow cooling gave less inactivation. We therefore advance the hypothesis that one method of thermal inactivation of viruses is by differential expansion and cracking open of the protein coat.

TABLE I

A COMPARISON OF RELEVANT FEATURES OF T_4 AND λ BACTERIOPHAGES

Bacteriophage	DNA content	Gene arrangement	Nucleotide composition		Head size		Tail size	
					Width	Length	Width	Length
	g/particle		%		*mμ*		*mμ*	
T_4	2.5×10^{-17}	Permuted	A*	32.3	65	95	20	95
			T	33.3				
			G	18.1				
			HMC	16.5				
			C	0.0				
λ	1.2×10^{-17}	Not permuted	A	21.3	54	54	7	140
			T	28.6				
			G	22.9				
			HMC	0.0				
			C	27.1				

* A = adenine; T = thymine; G = guanine; HMC = 5-hydroxymethylcytosine; C = cytosine. The nucleotide composition is from Sinsheimer (5) and the head and tail sizes are from Stent (6).

In Table I we show the general properties of T_4 and λ phages. T_4 is bigger, contains more DNA, and also has hydroxymethylcytosine in place of cytosine. The adenine-thymine content of λ is less, meaning that its DNA should be harder to melt. The genome of T_4 is permuted; that of λ is not (4).

EXPERIMENTAL PROCEDURES

Table II shows the stock cultures and their origins. Phage preparations were obtained from lysates by spinning at 8000 *g* for 10 min in the presence of chloroform. This was repeated three times and then the phage supernatant was siphoned off the chloroform and stored in nutrient broth at 4°C.

Thermal Inactivation of Virus

For the experiments used to study thermal inactivation of the entire phage, 0.1 ml virus stock solution was added to 10 ml of 0.8% nutrient broth warmed to the desired experimental temperature. At different times 0.1 ml samples of the mixture were diluted into 10 ml of nutrient broth at room temperature. These were further diluted and plated.

For temperatures above 70°C where the reaction rate is rapid a "rapid transfer" method was used. 0.1 ml was added to 10 ml of nutrient broth at the high temperature and after the desired time interval the entire contents were rapidly transferred into beakers containing 90 ml nutrient broth at room temperature.

Protein Moiety Inactivation: Inhibition of DNA Degradation

In *Escherichia coli* B_{s-1} ionizing radiation induces rapid and nearly complete degradation of the DNA. This degradation is inhibited by phage ghosts (7) which have been treated by DNase and hence is because of the protein coat. In order to observe the effect of heat on this inhibi-

TABLE II

THE ORIGINS OF THE STOCK CULTURES USED

Virus	Source	Host cell	Source
T_4	S. Person*	*E. coli* B	ATCC 11303
	W. Ginoza*	*E. coli* B_{s-1}	S. Person
		E. coli W602	W. Ginoza
T_4amB22	S. Person	*E. coli* K12CR63	S. Person

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tion process the DNA of the cells were labeled with thymine-^{14}C by the method of growth in the presence of deoxyadenosine (8). Cells were grown in Roberts' C minimal salts (NH_4Cl, 2 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 3 g; $MgCl_2$, 10 mg; Na_2SO_4, 26 mg) with 0.5% glucose and 0.5 μg/ml of deoxyadenosine at 37°C. Thymine-^{14}C (specific activity 100 μCi/mM) was diluted to a specific activity of 0.05 μCi/ml. When the cells had grown to a concentration of 10^8/ml nonradioactive thymine was added at 20 μg/ml of culture. The cells were allowed to grow for another 30 min to "chase" the label. They were then centrifuged at 6000 *g* for 3 min, washed with medium containing cold thymine, centrifuged again, and resuspended in phage attachment medium (0.8% nutrient broth plus 0.5% NaCl) warmed to 37°C. Samples were then bubbled with oxygen, given 21 krad gamma radiation using a Gammacell 200 (Atomic Energy of Canada Ltd., Ottawa, Ont.), and transferred back to 37°C and aerated. 1.0 ml samples were taken at intervals and added to 1 ml cold 10% trichloroacetic acid (TCA). After 30 min the mixture was filtered, and the filter was dried and counted in a Geiger-Mueller counter. For the action of phage, the same procedure was followed, with the addition of phage at 10 min after infection. The same procedure was followed with heated phage. The multiplicity of infection was approximately 3.

Deoxyribonuclease on λ Phage

Pancreatic DNase I (Worthington Biochemical Corp., Freehold, N.J.) was used at a concentration of 200 μg/ml. Small test tubes containing 1.4 ml nutrient broth were warmed to the desired temperature; then 0.4 ml DNase and 0.2 ml phage were added. After the desired time at the temperature chosen, the mixture was transferred to a small beaker with 18 ml nutrient broth with 0.02 M Mg at room temperature. Incubation at 37°C followed for 1 hr, and then the samples were diluted and plated.

Where longer exposures to heat were called for, in which case the DNase itself becomes inactivated, additions of DNase were made at 1½ min intervals. In each case an equivalent volume of water was substituted for the DNase in the control case.

Thermal Inactivation with Slow Cooling

The same procedure was adopted for the time of heating at the desired temperature. However, this was followed by transferring the whole tube to a water bath 10°C lower in temperature for 1–5 min, and subsequently allowing the tube to cool to room temperature before diluting and plating.

Thermal Constants

From these data, using the theory of absolute reaction rates, some values for the enthalpy and entropy of activation can be derived. These are sometimes useful for comparison. Table III gives such values.

TABLE III

SOME VALUES FOR ENTHALPY AND ENTROPY OF ACTIVATION

Process	Enthalpy	Entropy
	kcal/mole	*cal/mole per K°*
T_4 plaque-forming ability		
(Fast component)	79	161
(Slow component)	86	183
λ plaque-forming ability		
(Fast component)	76	155
(Slow component)	83	173
T_4 ability to inhibit degradation	135	

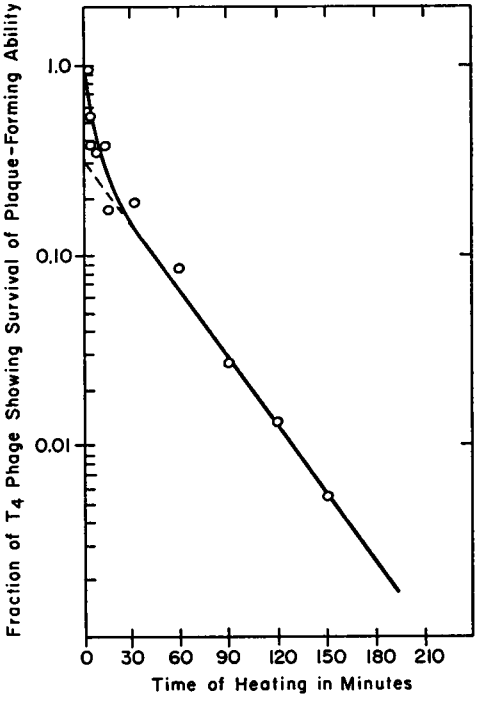

FIGURE 1

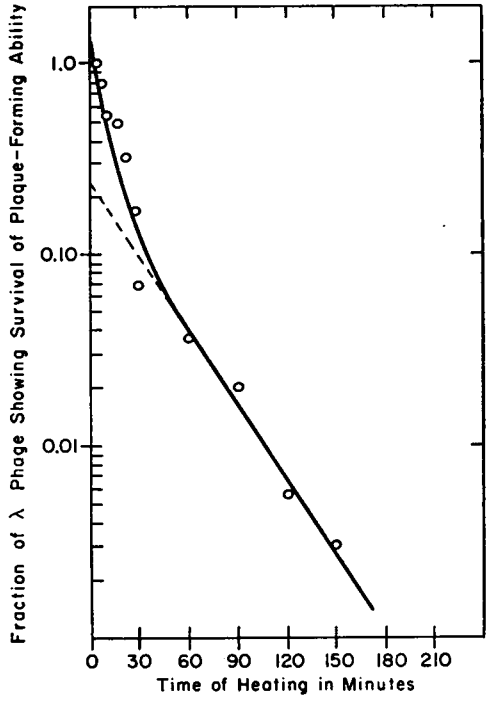

FIGURE 2

FIGURE 1 Thermal inactivation of T_4 phage at 65°C. Survival of plaque-forming ability as a function of time was determined by plating experiments using *E. coli* B as the host bacteria.

FIGURE 2 Thermal inactivation of λ phage at 65°C. *E. coli* W602 was the host cell.

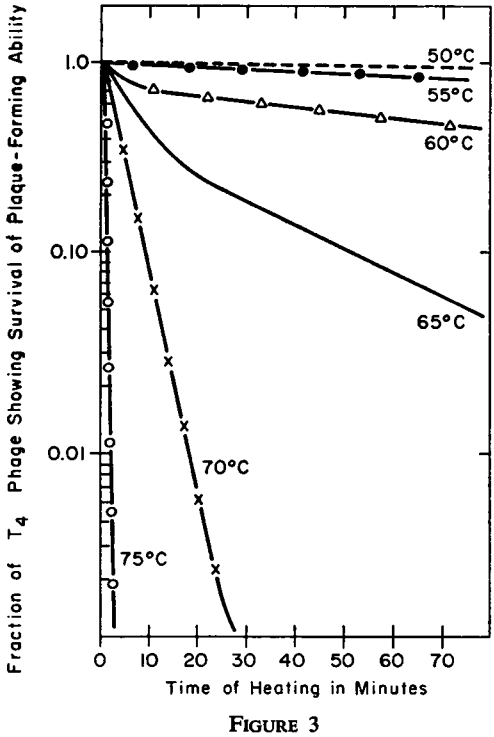

FIGURE 3

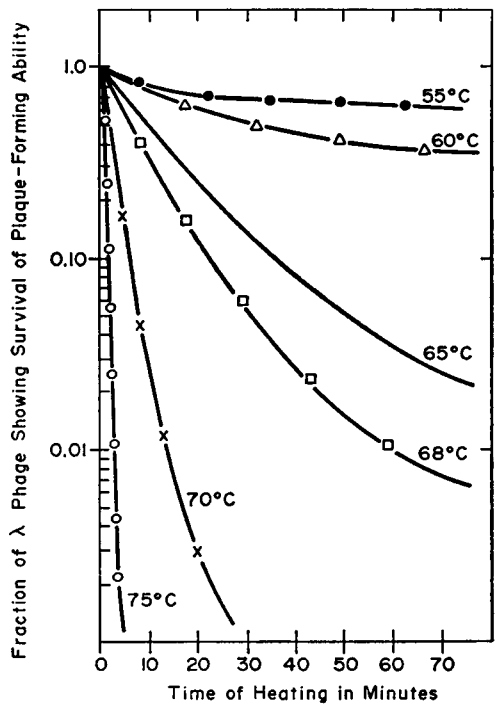

FIGURE 4

FIGURE 3 Thermal inactivation of T_4 phage at various temperatures. Survival of plaque-forming ability was determined by plating experiments using *E. coli* B as the host bacteria. Experimental points are not shown; the data are approximately as seen in Fig. 1.

FIGURE 4 Thermal inactivation of λ phage at various temperatures. Experimental points are not shown; the data are approximately as seen in Fig. 2.

EXPERIMENTAL RESULTS

Two sample curves showing the thermal inactivation of T_4 and λ are shown in Figs. 1 and 2. Both were observed at 65°C; both show the two-component appearance. In Figs. 3 and 4 are shown the curves found at various temperatures. In order to avoid confusion on the page the experimental points are not always given. The two-component aspect is less apparent at high temperatures. In Fig. 5 can be seen the data for one single experimental series in which the inhibition of DNA degradation is studied. It is apparent that within 10 min after the addition of unheated phage the loss of ^{14}C label in the TCA-precipitable fraction (undegraded DNA) is checked, and that this ability to inhibit is lost as the phage are heated. By taking the difference in per cent degradation between the situation without any phage and that for unheated phage as 100% the fraction of degradation-inhibiting ability can be estimated. This can be compared with the loss of plaque-forming ability and the result is seen in Fig. 6. Quite clearly the effect of heat is not as drastic, nor does it show

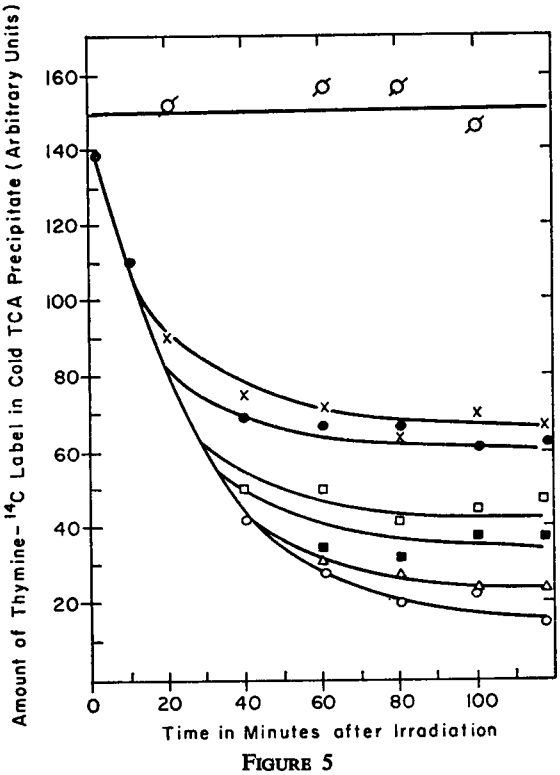

FIGURE 5

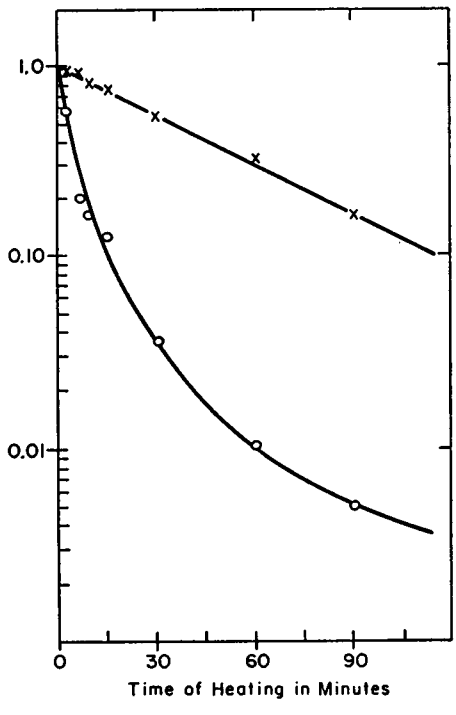

FIGURE 6

FIGURE 5 Inhibition of DNA degradation in irradiated *E. coli* B_{s-1} cells at 37°C by T_4 phage previously heated at 65°C for various lengths of time. Control unirradiated ∅; irradiated plus unheated phase ×; 13 min heat ●; 30 min heat □; 60 min heat ■; 90 min heat △; no phage ○.

FIGURE 6 Comparison of survival of T_4 phage plaque-forming ability (○) at 65°C with survival of ability to inhibit DNA degradation in the irradiated host cell (×). Survival of plaque-forming ability as a function of time was determined by plating experiments. Survival of ability to inhibit DNA degradation was calculated from Fig. 5. These two curves are quite different.

the two-component feature. Similar conclusions are reached for heating at 69.5°C. The data for four temperatures are shown in Fig. 7. It can be seen that the range of temperature in which the inhibiting ability is lost is less than that for the loss of infectivity.

The Effect of DNase

Heating T_4 in the presence of DNase showed very little effect. One reason for this may be found in the relative inactivation rates of the virus and the enzyme. DNase is itself sensitive to heat in this temperature range. On the other hand, λ which inactivates faster at lower temperatures, showed a marked effect. The difference may reflect the nature of the protein coat in the two cases. In Fig. 8 we show the effect of

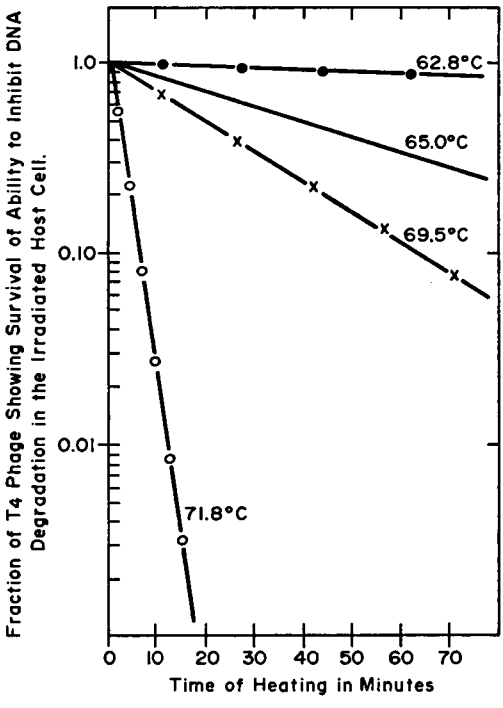

FIGURE 7

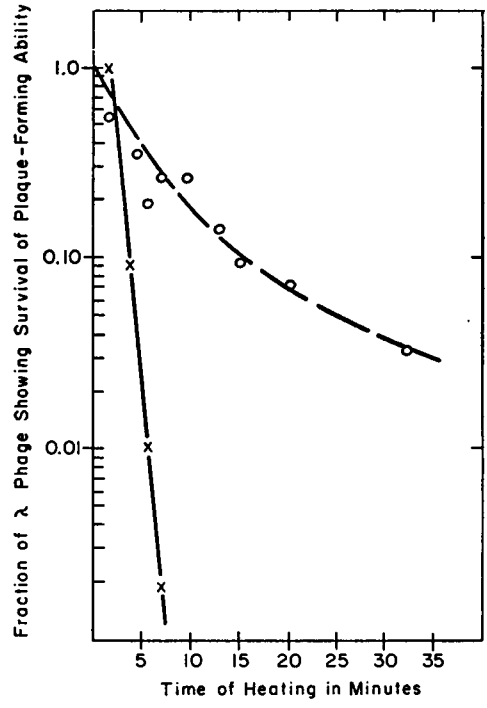

FIGURE 8

FIGURE 7 Temperature dependence for the survival of T_4 phage ability to inhibit DNA degradation in the irradiated host cell. Comparison with Fig. 3 shows the amount of in-inhibited DNA degradation. The number of plaque-forming phages is also quite different both in span of temperature and in single vs. bimodal curves. Experimental points are not shown; the data are approximately as seen in Fig. 6.

FIGURE 8 Comparison of survival of λ phage plaque-forming ability at 70°C when DNase is added (×) in concentrations of about 200 μg/ml and when no DNase (control ○) is added. Equal amounts of water were added to keep the dilutions equal.

heating λ at 70°C in the presence of DNase. The presence of the enzyme markedly increases the amount of inactivation. Similar, but not quite so dramatic, effects were observed at 75°C.

The Effect of Slow Cooling

Fig. 9 shows the two inactivation curves obtained when the virus was rapidly cooled and when it was permitted to reach room temperature relatively slowly. It can be seen that the slow component of the two-component inactivation curve is markedly accentuated. Similar results were obtained at 65°C.

DISCUSSION

The experimental results indicate that the kinetics of the loss of plaque-forming ability for T_4 do not correspond to the loss of one protein function at all. They also

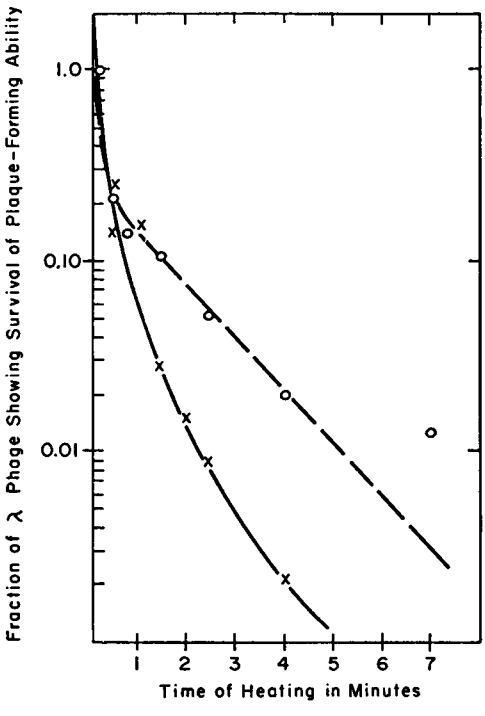

FIGURE 9 Thermal inactivation of λ phage at 75°C utilizing the technique of slow cooling (○) compared to the technique of rapid cooling (×).

indicate that any one preparation of virus must have two populations, one of which is sensitive and one less so. The populations are not stable in the sense that selection of the resistant fraction leads to a resistant population, but rather that there is always a fraction that is sensitive in any one virus preparation. It was suggested that the packing of the DNA could be such that the outer layers could readily expand in some cases and not in others, the difference being due to the local excess of adenine-thymine pairs. The question arises as to why this expansion should inactivate the virus. In the case of T_4 this present work does not suggest an answer. On the other hand, in the case of λ, the two facts that exposure of the heated phage to DNase causes sharply increased loss of infectivity and that annealing diminishes the loss suggest that the expansion of the DNA can cause a rupture of the protein coat and that this can render the phage vulnerable to attack from agents outside, notably nucleases. The process of annealing permits the coat to return to a normal shape.

We therefore advance, as one reason for the presence of more than one component of inactivation, the concept that the DNA is packed inside the head randomly, or at least not wholly uniformly, and that this packing gives a population of resistant or sensitive cells dependent on the base ratio in the part of the DNA that is packed on the outside and hence able to expand. This suggestion was previously advanced (9) in a slightly different form.

One of the problems of preparing killed vaccines is the presence of a component that is resistant so that the vaccine is dangerously alive. Our work would suggest

that one useful addition to the technique would be to heat the virus in the presence of the appropriate nuclease and to cool very rapidly after heating.

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