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Thermal Shock in a New Vibrio Phage.*† (19839)

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The effect of exposure to cold on bacteriophage was studied by d'Herelle(1) who found that young phase resists exposure to −180°C for 10 minutes while older phage does not. Rivers(2) demonstrated that a bacteriophage lytic for colon bacilli was inactivated by repeated freezing (−185°C) and thawing, and that the type of diluent influences the percentage of phase inactivated in this manner.

During the course of studies on heat inactivation of a newly isolated Vibrio phage, a sample of lysate was heated for 0.5 hour at 60°C then plunged into an ice bath (2°C) for 1 hour. There was a pronounced drop in titre after exposure to cold; more than 90% of the particles which survived heating at 60°C for 0.5 hour were inactivated during the period at 2°C. Experiments undertaken to investigate this phenomenon are described below.

Materials and methods. Materials and methods were the same as those described by Smith and Krueger(3). An ice bath main-

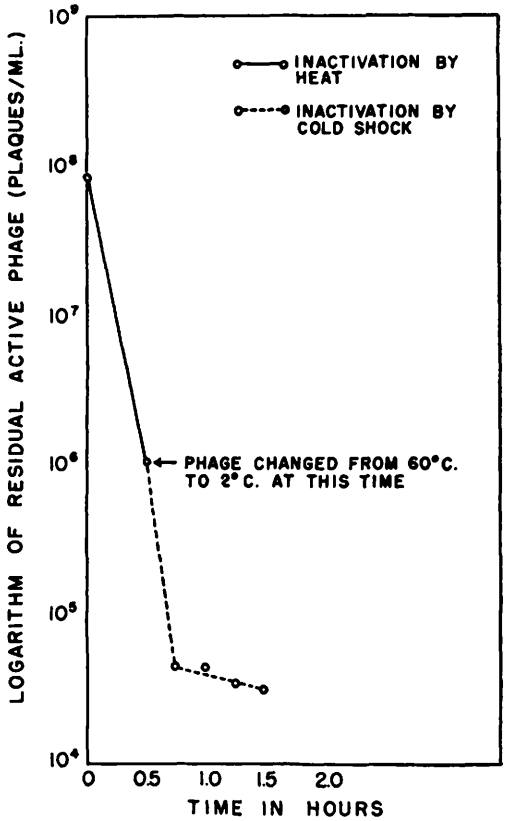

FIG. 1. Course of inactivation due to "Thermal Shock." Samples were titrated after various times at 2°C.

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TABLE I. Inactivation of Phage by "Thermal Shock."

Time and temp. of exposure				Titre of residual active phage in plaques/ml
hr	°C	hr	°C	
0				8.3×10^7
				1.2×10^8
		+ .25	2	4.5×10^4
.5	60	+ .5	2	4.3×10^4
		+ .75	2	3.3×10^4
		+ 1	2	3.2×10^4

tained at 2°C was used for exposing phage to the effects of cold.

Experimental results. To determine the course of the cold inactivation, phage lysate was heated at 60°C for 0.5 hour. The concentration of active phage remaining at this time was determined; samples of the phage were immediately plunged into the ice bath and at 15-minute intervals one was removed for titration. The greatest drop occurred during the first 15 minutes and comparatively little inactivation took place afterward, (Fig. 1, Table I).

To determine to what temperature phage must be heated before cold shock could be demonstrated, aliquots of phage lysate were held at temperatures from 50°C to 62°C. Samples were taken at 0.5 hour intervals from each of the temperatures used, plunged into the ice bath and kept there for 45 minutes. Samples held at 55°C and at temperatures lower than this were not affected by the sudden chilling. From 56°C to 62°C there was a gradual increase in the inactivation caused by "Thermal Shock", (Fig. 2, Table II).

To ascertain the period of heating required before inactivation by "Thermal Shock" occurs, phage lysate was heated at 60°C and at time intervals samples were plunged into the ice bath where they remained for 45 minutes. Determinations of phage remaining after the various intervals of heating and after subsequent exposure to the cold were made. Using as a criterion the percentage of phage inactivation brought about by "Thermal Shock" it is evident (Table III) that heating at 60°C for 12 minutes is necessary before significant loss due to cooling takes place. Continued exposure to 60°C up to 2 hours does not materially increase the percentage loss due to subsequent cooling.

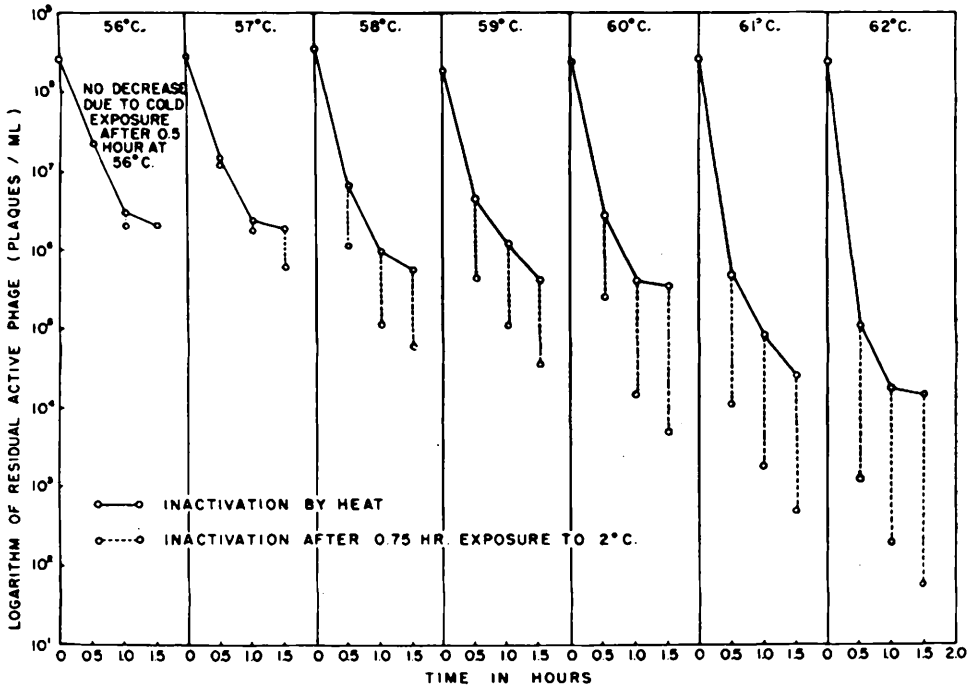

FIG. 2. Effect of time and temperature of exposure on percentage of inactivation by "Thermal Shock."

TABLE II. Inactivation of Vibrio Bacteriophage by Heating at Temperatures from 56°C to 62°C (incl.), and Effect of Temperature of Preheating on Inactivation by "Thermal Shock."

Time of exposure Heated hr		°C	Titre of residual active phage in plaques/ml—temp. of heat exposure 56°C	%	57°C	%	58°C	%	59°C	%	60°C	%	61°C	%	62°C	%
0			2.9 x 10^8	—	2.8 x 10^8	—	3.4 x 10^8	—	1.9 x 10^5	—	2.4 x 10^8	—	2.7 x 10^8	—	2.6 x 10^8	—
.5			2.4 x 10^7	—	1.6 x 10^7	—	6.8 x 10^6	—	4.5 x 10^6	—	2.8 x 10^6	—	5 x 10^5	—	1.2 x 10^5	—
.5	+	.75	2.5 x 10^7	0	1.53 x 10^7	4	1.3 x 10^6	80	4.5 x 10^5	90	2.6 x 10^5	90	1.2 x 10^4	97	1.4 x 10^3	99
1			3.1 x 10^6	—	2.4 x 10^6	—	9.5 x 10^5	—	1.3 x 10^6	—	4.03 x 10^5	—	8.6 x 10^4	—	1.8 x 10^4	—
1	+	.75	2.23 x 10^6	28	1.9 x 10^6	21	1.23 x 10^5	87	1.1 x 10^5	92	1.65 x 10^4	93	1.9 x 10^3	98	2 x 10^2	99
1.5			2.1 x 10^6	—	1.9 x 10^6	—	5.7 x 10^5	—	4.2 x 10^5	—	3.6 x 10^5	—	1.7 x 10^4	—	1.68 x 10^4	—
1.5	+	.75	2.1 x 10^6	0	6.1 x 10^5	63	6 x 10^4	89	3.5 x 10^4	92	5.1 x 10^3	97	5 x 10^2	97	6 x 10^1	99+

% = percentage of remaining phage inactivated by cold exposure.

TABLE III. Increase in Inactivation by "Thermal Shock" as the Time Interval of Preheating at 60°C Is Increased.

Min. exp. at 60°C	Min.	°C	Titre residual active phage in plaques/ml	% inactivation
0			3.4 x 10^8	—
3			2.5 x 10^8	—
3	+ 45	2	2.14 x 10^8	14
6			1.2 x 10^8	—
6	+ 45	2	9.4 x 10^7	21
9			2.5 x 10^7	—
9	+ 45	2	2.22 x 10^7	11
12			2 x 10^7	—
12	+ 45	2	2.6 x 10^6	87
15			1.25 x 10^7	—
15	+ 45	2	2.1 x 10^6	83
30			2.8 x 10^6	—
30	+ 45	2	2.6 x 10^5	91
60			4 x 10^5	—
60	+ 45	2	1.7 x 10^4	95
90			3.6 x 10^5	—
90	+45	2	5.1 x 10^3	97
120			1.7 x 10^5	—
120	+45	2	1 x 10^3	99

Investigation of the possibility that this phenomenon might be ascribed to the presence of 3% NaCl was carried out. Phage was resuspended in 1% aqueous yeast extract containing concentrations of sodium chloride ranging from 5% to 0.

Aliquots of each salt concentration were heated at 60°C for 0.5 hour, plunged into the ice bath immediately and held there for 45 minutes. The data tabulated in Table IV indicate that inactivation due to "Thermal Shock" occurs in the presence of sodium chloride in all of the concentrations used, and also in the presence of yeast extract solution to which no electrolyte has been added. "Thermal Shock" also occurred when phage was resuspended in M/15 phosphate buffer pH 7.9 containing 3% sodium chloride and was treated in the manner already described. This was not the case if the suspending fluid consisted of M/15 phosphate buffer at pH 7.9. Samples were plunged into the ice bath after being heated both at 50°C and at 53°C for several different time intervals and no inactivation due to chilling could be observed. Here resistance to heat inactivation was considerably reduced so that the use of the usual time intervals and temperatures was not feasible.

The evidence so far establishes the limits of temperature and time of heating necessary

TABLE IV. Effect of NaCl on Inactivation by "Thermal Shock." Phage was heated at 60° C for 0.5 hr and chilled (2°C) for .75 hr. The menstruum for suspending phage was 1% yeast extract aqueous containing various concentrations of NaCl.

% NaCl	Titre of phage in plaques/ml before treating	Titre residual active phage in plaques/ml after 0.5 hr at 60°	Titre residual active phage in plaques/ml after 0.5 hr at 60°C +0.75 hr at 2°C	% inactivation by "thermal shock"
5	2×10^8	2.69×10^6	1.46×10^5	94
4	1.63×10^8	1.14×10^6	1×10^4	99
3	2.4×10^8	2.8×10^6	2.6×10^5	90
2	1.16×10^8	1.62×10^6	9.9×10^4	94
1	4.26×10^7	6.9×10^5	1.49×10^4	97
.5	4.5×10^7	1.9×10^6	3.2×10^5	83
.25	2×10^7	5.7×10^4	6×10^5	89
.125	1.9×10^7	4.4×10^4	2×10^5	95
0	1.7×10^8	1.6×10^5	6×10^5	96

to prepare the phage particle for inactivation by "Thermal Shock". This phenomenon is not directly dependent upon any one factor in the suspending fluid. However, a suspending fluid which protects the phage from heat inactivation so that it can be held at the necessary temperature for the required time appears to be obligatory.

Further experiments were conducted in an attempt to determine the mechanism involved in the reaction. A search was made for a hypothetical substance which might be formed under the experimental conditions employed to account for phage inactivation. An aliquot of phage lysate was heated at 60°C for 0.5 hour, one portion (A) was plunged into the ice bath immediately, and a second portion (B) was similarly chilled after the addition of unheated phage lysate. If an inactivator were present it was expected that the unheated phage would be inactivated as well as that which had been heated and that the loss of phage per ml would be greater in portion B than in A. As can be seen from Fig. 3a, this did not happen.

Again, an aliquot of phage lysate was heated at 60°C for 0.5 hour, plunged into an ice bath immediately and held there for 15 minutes. Untreated phage lysate was added, and the mixture of previously treated and untreated

	Titre of active phage in plaques/ml
(a) 0 time	1.6×10^8
(b) after 0.5 hr at 60°C	1.8×10^6
A. (b) held at 2°C, 0.5 hr 8.0×10^4 loss 1.7×10^6 plaques/ml	B. (b) $+1 \times 10^6$ phage particles/ml 3×10^8 (b) after 0.5 hr at 2°C 2.09×10^6 loss 1×10^8 plaques/ml

FIG. 3a. Experiment to Detect the Presence of an Inactivator. Phage lysate was heated at 60°C for 0.5 hr. One aliquot was plunged into the cold for 0.5 hr. Untreated phage was added to a second aliquot before it was plunged into the cold. The loss of phage particles was not greater in the sample to which the untreated phage had been added indicating that the cold inactivation is not due to the presence of an inactivator formed during the heating period.

	Titre of active phage in plaques/ml
(a) 0 time	1×10^8
(b) after 0.5 hr at 60°C	1.6×10^6 (loss due to heating 98%)
(c) b. held at 2°C 0.5 hr	1.5×10^5 (loss of remaining phage due to cold exposure 91%)
(d) c. + untreated phage lysate 1.5×10^7 phage particles/ml	1.8×10^7
(e) d. held at 0.5 hr at 60°C	1.86×10^6 (loss due to heating 90%)
(f) e. held at 2°C 0.5 hr	9.9×10^4 (loss of remaining phage due to cold exposure 95%)

FIG. 3b. Experiment to Detect the Presence of an Inactivator. Phage lysate was heated at 60°C for 0.5 hr. Untreated phage was added to the treated lysate. This mixture was heated and cooled in the same manner as the original sample. The percentage loss of phage particles was not greater in the mixture than in the original lysate suggesting that the cold inactivation is due to a change in the physiological state of the phage particle rather than to an inactivator.

TABLE V (a). Experiment to Detect Possible Role of Reactivation in "Thermal Shock."

Time and temp. of exposure							Titre residual active phage in plaques/ml	%*
hr	°C	hr	°C		hr	°C		
0							1.5×10^8	--
							1.3×10^6	—
		+ .5	2				3×10^4	97
		+ .5	20	L†			2.3×10^4	98
		+ 1	20	"			2.2×10^4	98
		+ 1.5	20	"			2.2×10^4	98
		+ 2	20	"			2.2×10^4	98
.5	60	+ 2.5	20	"			2.2×10^4	98
		+ 3	20	"	+ .75	2	2.3×10^4	98
		+ .5	30	D			3.2×10^4	98
		+ 1	30	"			2.7×10^4	98
		+ 1.5	30	"			2×10^4	98
		+ 2	30	"			2.1×10^4	98
		+ 2.5	30	"			2.2×10^4	98
		+ 3	30	"			2.3×10^4	98

* Percentage remaining phage lost due to chilling.
† L = light; D = dark.

phage lysate was then heated and chilled as was done with the original sample. The course of inactivation of the mixture followed closely that of the original lysate. (Fig. 3b).

These two experiments gave no indication that the hypothetical inactivator is involved in production of "Thermal Shock". Also the fact that "Thermal Shock" occurs in the presence of widely different suspending fluids makes the existence of such a substance seem improbable.

The possibility of aggregation of phage particles in the cold was next considered. Samples of phage lysate were treated by "Thermal Shock". After this, one half were shaken and mixed violently while the other half were mixed gently. Plaque counts of all the samples gave no evidence of significant differences in titre. If aggregates were present they were not broken up by the procedure employed.

It is also conceivable that this phenomenon may represent a situation in which denaturation and reversal of denaturation or photoreactivation are involved. In this case the titres obtained after the samples have been iced would represent the true values for the course of heat inactivation. The difference between the titres obtained from a sample before and after icing would be a measure of the number of phage particles which had become reactivated due to incubation at 30°C or exposure to light before being chilled.

To test this possibility an experiment was performed as follows: Aliquots of lysate were held at 60°C for 0.5 hour. One was chilled immediately and a series of 6 aliquots was kept at 30°C in the dark incubator. One of these was plunged into the ice bath at each half-hour interval during a period of three hours. Another series of 6 aliquots after being heated, was held at room temperature (20°C) and directly exposed to the fluorescent light which was the customary light source in the laboratory. One of these samples was plunged into the ice bath at each half-hour interval over a period of three hours. If reactivation takes place under either of these sets of conditions, no further loss in titre would be expected on chilling, (Table Va & b). However the typical drop occurred; according to this evidence once the phage particle becomes vulnerable to inactivation by "Thermal Shock" it remains so for a period of at least 3 hours.

These results would be expected if the lysate contained two kinds of phage, one more thermostable than the other and at the same time susceptible to inactivation by cold. Attempts to isolate such a phage from among survivors after heat treatment were unsuccessful(3).

Discussion. The phenomenon of inactivation by "Thermal Shock" refers to the decrease in phage titre due to sudden chilling and is in addition to that which occurs during preliminary heating. The conditions of tem-

TABLE V (b). Control Experiment for Table V (a) to Detect Effect of Standing at 20°C and at 30°C on Phage after It Has Been Heated at 60°C for .5 Hr.

Time and temp. of exposure				Titre residual active phage in plaques/ml
Hr	°C	Hr	°C	
0				9.9×10^7
.5	60			6.7×10^6
		+1	20 in L*	6.3×10^5
		+2		6.1×10^5
		+3		6×10^6
		+1	30 in D	6.9×10^5
		+2		7.4×10^5
		+3		7.7×10^5

* L = light; D = dark.

perature and time required for its occurrence were established. The differences in titres found to obtain between lysates that had been heated and those which had been heated and chilled could be explained only by postulating a loss of active phage when previously heated phage is plunged into the cold bath, *i.e.*, there is no evidence that reactivation of inactivated phage occurs under the conditions of the experiment.

As the time and temperature of the heat treatment is increased the change in titre of the phage when chilled is increased. This is further evidence in favor of a cold inactivation for if reactivation were occurring we would expect the opposite effect. When phage particles are inactivated by heat it may be assumed that the denaturation becomes more permanent as time and temperature of exposure are increased. Reactivation then would become decreased with longer exposure to higher temperatures.

It could be assumed that we are dealing with a mixture of phages, one of which is both thermoresistant and labile to cold. Isolates from phage suspensions previously exposed to heat (and to heat and cold) did not confirm this concept.

If "Thermal Shock" is actually a loss phenomenon in which final exposure to cold somehow causes the inactivation of phage, this step could be ascribed to: a) the development of an inactivator or b) a change in the physiological state of the phage particle brought about by heating and rendering it very susceptible to exposure to low temperatures. Since we have not been able to secure any experimental evidence suggesting the existence of an inactivator the second explanation appears to be the more acceptable of the two. There is no experimental basis for deciding at this time whether thermal shock: a) actually disrupts the phage particles, b) produces changes in the particle such that it cannot be adsorbed to the host cell, or c) alters the phage enough to prevent its participation in the complex reactions requisite for reproduction of phage without destroying its capacity for union with the bacterium.

Summary. 1. The phenomenon of "Thermal Shock" is described. Phage, which was partially inactivated by heating at 60°C for 0.5 hour, showed further inactivation when plunged into an ice bath (2°C). 2. Temperature and time conditions of heating to prepare the phage for inactivation by "Thermal Shock" have been defined. 3. Evidence is presented that "Thermal Shock" is a result of change in the physiological state of the phage particle brought about by pre-heating and does not involve a masked reactivation.

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