

The Use of Lysozyme in Studies With a Bacterial Virus

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In the field of virology available evidence points toward the probability that the cells, in which multiplication occurs, provide metabolic organization required for virus synthesis, and that the virus, in some way, supplies a directive influence. Cells of *B. coli* infected with T₂ bacteriophage are incapable of normal growth and division though their metabolism continues at a high rate (1). This metabolism is now directed toward synthesis of nucleic acids and proteins which eventually become a part of the multiplying virus.

Although little information is available concerning the processes which occur in biological synthesis of nucleic acids and proteins, conditions necessary for a number of biological synthetic reactions have recently been ascertained. Since a number of these can be demonstrated in cell-free media, the hope is nurtured that virus multiplication in cell-free media may be possible. From time to time reports have been published that this has been accomplished (2). None of these claims have as yet been substantiated by other groups. It is evident, however, that the discovery of conditions permitting virus reproduction in the absence of cells would make available an important new tool for virus work. For this reason it is desirable that this possibility be reinvestigated from time to time as additional knowledge concerning viruses and concerning the biochemistry of cellular metabolism becomes available.

The experiments reported in this paper gave no indication of reproduction of bacteriophage. The presentation of this work appears justified in order that undue repetition by others may be avoided, and in the hope that the considerations which led to the choice of conditions for these experiments may be suggestive for further fruitful experimentation.

A medium containing all possible cellular components appeared to offer the best opportunity for success. An attempt was made to choose conditions which would:

(a). Provide a method of cell lysis in which intracellular constituents (enzymes, proteins, etc.) would not be denatured or destroyed. For this purpose lysozyme, which presumably acts only on the cell wall, was chosen. It was consequently necessary to choose a lysozyme-sensitive organism which serves as a host for a virus. *Staphylococcus aureus* strain K₁, and P₁ bacteriophage* were chosen to fill this requirement.

* The *Staphylococcus aureus* K₁ and bacteriophage P₁ were obtained from Dr. A. P. Krueger.

(b). Maintain a high concentration of constituents in order that dissociation of enzyme-coenzyme combinations would be minimized.

(c). As far as possible avoid autolysis. In many of the experiments sufficient lysozyme was added to give considerable lysis in a brief period. Phage was always added to the lysate as promptly as possible. Cooling was also used to decrease the rapidity of autolytic reactions.

During the course of the work, in personal conversation with Dr. Krueger, we learned of his experiments with the same organism, phage, and with lysozyme in which an average increase of phage of 180 percent was observed. Dr. Krueger very kindly informed us of the conditions of his experiments (3). The first four experiments in Table I are typical of a larger series which we have conducted. No significant increase of phage was observed.

In these experiments one percent of egg white was added to a bacterial culture containing $1-2 \times 10^9$ cells per ml. and lysis was allowed to proceed for twelve minutes. Cells which had not been lysed were removed by filtration through a super-cel pad prepared according to the method of Krueger, Scribner, and Brown(4). The cell-free filtrates were collected in a chilled flask, inoculated with phage, and held at 5° for thirty minutes. Phage assays were made by the plaque count method of Hershey, Kalmanson and Bronfenbrenner (5).

Experiments 5 (a) and (b) (cf. Table I) were conducted in similar manner with addition of 0.85% and 2.3% NaCl respectively to the lysates before addition of phage. Utter, Krampitz, and Werkman (6) had reported that addition of sodium chloride to lysed preparations of *Micrococcus lysodeikticus* made possible the demonstration of enzymatic activities which were not observable in salt-free lysates.

In experiment 6 pyruvate, Mg^{++} , $PO_4^{=}$, sodium chloride, potassium chloride, adenosinetriphosphate, and cytochrome c were added to the lysate before addition of phage. Several workers have recently reported observations that cellular preparations maintained in media containing these substances are able to carry out a number of complex enzymatic oxidations (7).

Experiments have also been conducted varying from the above in other respects. If phage reproduction is directly associated with enzymatic manifestations of the virus or the host, such enzymatic activity probably should occur more readily at 36° than at 5° . For this reason use of the higher temperature was also investigated. In addition, in many experiments crystalline lysozyme has been used with a somewhat longer period of lysis of host cells. Removal of unlysed cells was accomplished by use of a Berkfeld filter. Experiment 1, Table II represents one of the experiments of this group illustrating variation in the time of lysis. It seemed possible that the period of cellular invasion by phage might be eliminated by use of lysates. In this event multiplication might occur in a shorter interval than that observed with intact cells. In experiment 2, the effect of varying periods of incubation of phage and lysate was studied. It will be noted that no indication of multiplication was noted at either short or longer intervals.

TABLE I. Incubation of Bacteriophage in Bacterial Lysates.

Expt. No.	Amt. Lysis 12 Min. (1/100 Egg White)		Phage Added	Phage Found After 30 Min. at 5°		Egg White in Saline
				Bacterial Filtrate	Bacterial Lysate	
1.	From	To	2.0×10^6	2.4×10^6	2.8×10^6	1.1×10^6
2.	1.2×10^9	5.3×10^8	1.5×10^6	1.4×10^6	1.6×10^6	3.9×10^5
3.	1.4×10^9	8.5×10^8	5.3×10^5	4.2×10^5	3.9×10^5	7.5×10^4
4.	1.2×10^9	5.0×10^8	5.0×10^4	6.1×10^4	6.0×10^4	1.3×10^6
5.	1.5×10^9	6.4×10^7	1.9×10^6	1.4×10^6	1.6×10^6	1.6×10^6
(a)	1.8×10^9	6.9×10^8				
(b)						
6.	1.0×10^9	9.4×10^7	9.2×10^5	1.6×10^6	1.8×10^6	6.9×10^5
				5.9×10^5	5.7×10^5	

TABLE II. Incubation of Bacteriophage in Bacterial Lysates

	Time Lysis	Amt. Lysis	Phage Added	Phage Found After 10 Mins. at 36°		
				Bacterial Filtrate	Bacterial Lysate	Egg White in Saline
1.	4 hrs.	8.4 x 10 ⁹	4.0 x 10 ⁵	7.3 x 10 ⁵	2.2 x 10 ⁵	3.1 x 10 ⁵
	2 hrs.	7.4 x 10 ⁸				
	30 min.	8.4 x 10 ⁹ 2.4 x 10 ⁹ 8.4 x 10 ⁹ 4.2 x 10 ⁹	4.0 x 10 ⁵ 4.0 x 10 ⁵ 4.0 x 10 ⁵	4.2 x 10 ⁵ 3.7 x 10 ⁵	2.4 x 10 ⁵ 5.6 x 10 ⁵	
2.	12 min.	1.1 x 10 ⁹ 3.6 x 10 ⁸	1.0 x 10 ⁶	(10 min.) 9.2 x 10 ⁵ (30 min.) 1.2 x 10 ⁶ (75 min.) 8.5 x 10 ⁵ (120 min.) 7.8 x 10 ⁵	7.4 x 10 ⁵ 7.2 x 10 ⁵ 9.0 x 10 ⁵ 7.0 x 10 ⁵	8.9 x 10 ⁵ 8.3 x 10 ⁵ 6.1 x 10 ⁵ 7.3 x 10 ⁵
3.	2½ hrs.	(a. cell free) 3.0 x 10 ⁴		6.0 x 10 ⁴	7.0 x 10 ⁴	
	2½ hrs.	(b. 2.5 cells per ml.) 3.0 x 10 ⁴		5.7 x 10 ⁴	1.2 x 10 ⁵	
	2½ hrs.	(c. 2,500 cells per ml.) 3.0 x 10 ⁴		3.0 x 10 ⁴	3.1 x 10 ⁴	
4.	4 hrs.	(unfiltered) 1.9 x 10 ⁹ 3.9 x 10 ⁸	8.4 x 10 ⁵	2.7 x 10 ⁵	5.4 x 10 ⁵	
	4 hrs.	2.8 x 10 ⁹ 1.9 x 10 ⁸	2.2 x 10 ⁵	a) 2.7 x 10 ⁵ b) (3 mg./ml. of ATP) 1.8 x 10 ⁵	1.9 x 10 ⁵ 2.0 x 10 ⁵	2.9 x 10 ⁵ 2.4 x 10 ⁵

It seemed possible that rapid destruction of enzymes or other labile materials necessary for phage reproduction might account for failure to obtain phage increase. Continuing lysis of a few cells might provide a supply of such materials. In experiment 3, the three parts represent bacterial filtrates to which (a). no cells were added; (b). 2-3 cells per ml. were added; and (c). 2500 cells per ml. were added to the lysate prior to addition of phage. In experiment 4 the filtration to remove cells was omitted. No significant increase in phage was observed. Similarly addition of adenosinetriphosphate to provide a source of energy for synthetic reactions was without effect.

Thus, to date we have not been successful in demonstrating significant increase in phage in lysozyme-lysates.

Cohen (8) has recently obtained evidence consistent with the interpretation that intracellular phage formation may proceed at a constant rate starting at the time of infection and terminating with lysis of the cell. In contrast, the work of Latarjet (9) indicated that the formation of phage was not completed until just before lysis. It occurred to us that lysis of infected cultures by lysozyme during the "constant period" might provide evidence bearing on this point, for demonstration of an increase in phage would strongly indicate formation of additional phage before the usual time of lysis.

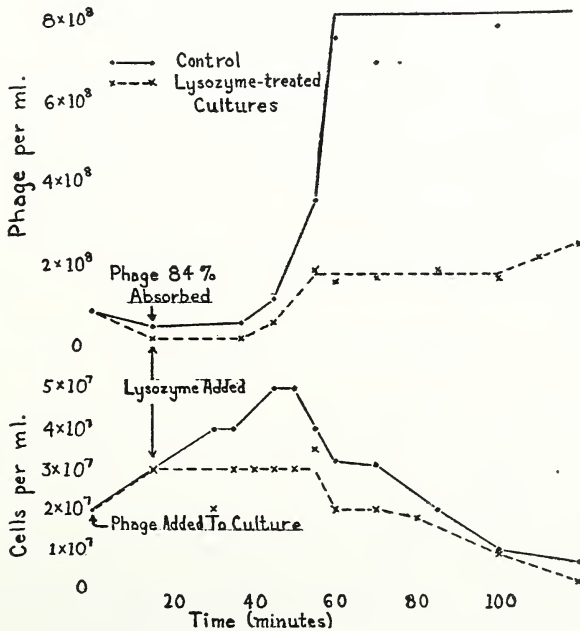


Fig. 1. Effect of addition of lysozyme to *Slophyllococcus aureus* K^1 infected with P_1 phage.

Young (2½ hr.) cultures of *Staphylococcus aureus* K₁ in nutrient broth were infected with phage P₁. After 6-15 minutes egg white was added to experimental tubes and an equal volume of broth to the controls. The cultures were incubated at 37° with aeration, and aliquots were removed at intervals for assay. Figure I illustrates the findings in a representative experiment. The phage increase in control and lysozyme-treated cultures occurred at the same time, although a smaller increase was repeatedly observed with the latter. Efforts were made to determine whether lysozyme acted on infected cells. Unfortunately, a considerable portion (10-90 percent) of the bacteria were uninfected in spite of the fact that the phage to bacteria ratio was as high as 25 to 1. No conclusions were possible.

Use of lysozyme failed to provide evidence of phage increase prior to the normal time of lysis. The decrease in burst size in comparison with control cultures suggests the following possibilities: (a). lysis of some infected cells at a stage at which phage is incompletely formed and is therefore inactive, (b). destruction by lysozyme of some materials necessary for phage formation, or (c). action on cell walls, thereby changing their permeability and affecting the supply of nutrients to the cell.

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