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Synthesis of Lysozyme in T2-infected *Escherichia coli*

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ABSTRACT Synthesis of lysozyme started a little before 10 min after infection with T2H. The enzyme accumulated in the cells until lysis, but the accumulation was not an unique cause of lysis. Lysozyme synthesis was more sensitive to ultraviolet irradiation than that of early proteins, and was reactivated by multiple infection. Experiments using as a host bacterium an uracil- and arginine-requiring mutant of *Escherichia coli* were done to see if simultaneous synthesis of deoxycytidine triphosphatase, an early protein, and lysozyme might take place afer addition of the required substance to bacteria that had been infected in the absence of one of them. With limited uracil pool, deoxycytidine triphosphatase was synthesized but lysozyme was not. Fluorouracil rather increased synthesis of deoxycytidine triphosphatase and inhibited that of lysozyme.

Introduction

An enzyme which hydrolyzes cell wall of *E. coli* was found in a T2-infected bacterial culture, but not in an uninfected culture (Koch and Jordan, 1957), and was separated from phage particles (Koch and Weidel, 1956; Barrington and Kozloff, 1956). Koch and Dreyer studied it enzymaticaly and classified as a lysozyme (1957). Genetical investigation by Streisinger *et al.* (1961) proved that the synthesis of this enzyme is controlled by a single cistron on phage chromosome. This paper, partly reported preiously (Hershey *et al.*, 1960), will describe studies on the synthesis of lysozyme compared with that of deoxycytidine triphosphatase (dCTPase), which is synthesized within a first few minutes after phage infection (Kornberg *et al.*, 1959; Koerner *et al.*, 1960).

Materials and Methods

Strains. Wild type phage, T2H, and its mutant, *r1*, were obtained from Dr.

A. D. Hershey. Bacterial strains of *E. coli*, H and BB, and B6, a mutant requiring both arginine and uracil, were received from Dr. A. D. Hershey and Dr. I. Watanabe, respectively..

Infection. Cells in logarithmic phase at a desity of approximately 5 x 10^8/ml in TG medium (Hershey and Melechen, 1957) were infected at a multiplicity of about 5 at zero time unless otherwise stated. Uninfected cells at 5 min were less than 2 % of the input bacteria. Strain B6 was grown in TG supplemented with 20 μg/ml each of L-arginine and uracil, and was washed with TG without glucose before use.

Enzyme extract from culture. Enzyme synthesis was terminated by transfering 5 ml of culture into tubes containing 0.15 mg chloramphenicol (CM) in an ice bath. Cells were disrupted by sonication. Disruption of cells was more than 95 % complete as ascertained by microscopic observation. When the Nephelos method described below was used for measurement of lysozyme activity, NaCl was added to 0.3 M at this step to prevent the association of lysozyme and cell wall. Unbroken cells and cellular fragments were removed by centrifugation. 2-Mercaptoethanol was added to 0.1 M for assay of dCTPase.

Enzyme extract from phage particle. Phage particles purified by differential centrifugation were osmotically broken and the "ghosts" were removed by centrifugation. Disruption of particles by repeated freeze and thaw or treatment with $Cd(CN)_3$ released lysozyme from particles (Brown and Kozloff, 1957). Of those procedures, osmotic shock gave the highest enzyme activity.

Assay of lysozyme. Two methods were used, the Nephelos and the spot test method. The Nephelos method was described previously (Minagawa, 1961), and only one detail will be added here. Lysozyme activity is affected by the concentrations of NaCl (Brown and Kozloff, 1957). In our case its optimal concentration was 0.1 M. Cell wall and enzyme preparation, crude or partially purified by the XE 64 resin column, were mixed at various concentrations of NaCl, kept for 5 min and centrifuged in the cold.. No enzyme activity was found in the supernate at 0.1 M, but at higher and lower concentrations some or hole activity remained. The lost activity from the supernate was recovered by adjusting NaCl concentration of the precipitated part to 0.1 M. NaCl seemingly causes a reversible complex of the enzyme and cell wall. 0.3 M was sufficient to dissociate this association. When the extract was prepared without NaCl addition (TG contains 0.1 M NaCl), its activity was about 70 % of that of the extract prepared in the presence of 0.3 M NaCl; however this difference was not distinct when the spot test was used for the measurement.

For the spot test the method was not standardized in earlier experiments but the method used later was as follows. A lysozyme sensitive clone obtained from strain BB was incubated in pepton broth at 37° overnight. 0.2 ml of the culture was suspended in 2 ml of melted agar broth, poured on hard agar plate, incubated at 37° for 4 to 5 hours, and cells grown were killed by exposing to chloroform vapour. Enzyme extract was diluted in a series with distilled water and from each one drop was spotted on the surface of the chloroform-killed bacteria,

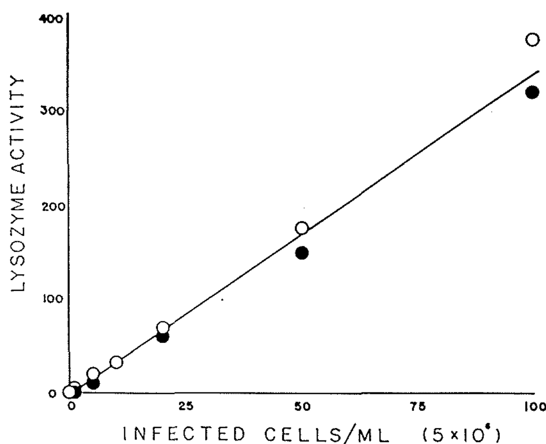

Fig. 1. Relationship between lysozyme activity by the spot test and a density of infected cells.

Approximately 5 x 10^8/ml cells of strain H were infected with T2 at a multiplicity of 5, diluted at 10 min to various densities as shown in abscissa, and aerated further for 50 min. Lysozyme activity in the cultures was determined as in Materials and Methods. Number of infected cells was titrated at 5 min after infection. Results of two independent experiments were plotted in the figure.

and incubated for 30 to 60 min at 37°. The reciprocal of the lowest dilution, at which a clear spot was seen against turbid background after the incubation, was defined as a spot test unit. The activity was measured at 2 or 5 unit intervals between 0 and 20 units, 10 between 20 and 100 units, and 20 at range more than 100 units. This method gave rather good reproducibility as shown in Fig. 1. Cells at a density 5 x 10^8 were infected, diluted to various densities with the culture medium at 10 min, and aerated further for 50 min. The enzyme activity in each tube in two independent experiments was plotted in the figure. It was proportional to densities of infected cells.

Assay for dCTPase. The method used was the same as that described by Kornberg *et al.* (1959), except that 0.01 ml of 0.1 M ATP was added to the reaction mixture to minimize nonspecific phosphatase activity. P^{32}-labeled dCTP was synthesized after Smith and Khorana (1958).

Ultraviolet light (UV) irradiation. A 10 ml sample of phage suspension, at a density 5 x 10^{11}/ml, in a 9 cm diameter petri plate was exposed to 15 w germicidal lamp (Toshiba) with constant shaking at a distance 80 cm.

Results

Lysozyme synthesis by intact phage. A suspension of strain H was divided in two parts; one part was infected with r^+ at a multiplicity of 3, the other with a mutant *r1* at a multiplicity of 6.5. Nine min after infection an aliquot of r^+ infected culture was superinfected with 10 times particles to ensure lysis inhibition. The culture was sampled at intervals and its lysozyme activity was measured. It was not detectable until 7 min after infection and just detectable at 10 min. After this period the activity increased linearly and extrapolation of the curve usually reached at 9 min (Fig. 2, A). The r^+ infected culture was centrifuged at 20 min. Only 5 % of the total ativity found in sonicated sample was in the

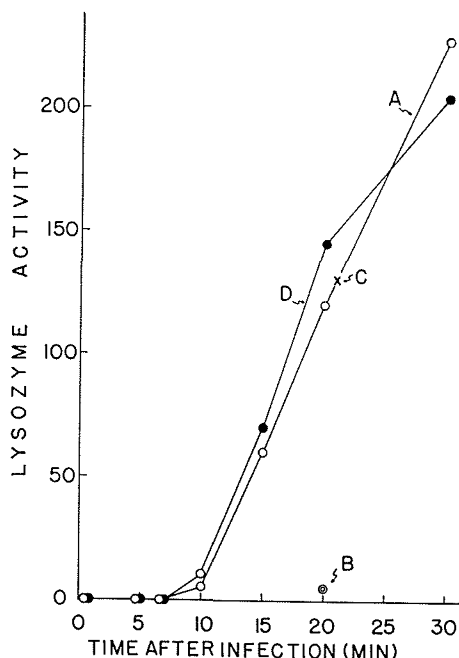

Fig. 2. Time course of lysozyme synthesis.

Cells were infected with r^+ at a multiplicity of 3 (curve A), or with *r1* at a multiplicity of 6.5 (curve D). At intervals culture was sampled and its lysozyme activity was nephelometrically determined after sonication. One part of r^+ infected culture was superinfected with r^+, at a ratio phage to bacteriam 10, at 9 min to insure lysis inhibition (point C), and another part was withdrawn at 20 min and centrifuged to estimate the enzyme activity outside cells (point B). Lysozyme activity was expressed as units/10^3 infected cells.

supernate(Fig. 2, B). Lysozyme activity in a chloroform lysate 2 hours after infection was compared to that of phage particles purified from 2 hours culture. which was measured as in Materials and Methods and calculated under the same particle density basis in the lysate. The lysate contained roughly 200 times as high activity as the shockate. The activity in superinfected culture (Fig. 2, C) was close to the unsuperinfected. Data not shown in the figure indicated that the enzyme was formed in the same way with infection at low multiplicity (input ratio of phage to bacterium, 0.1) and at high multiplicty (input ratio, 5).

In cells infected with *r1* the enzyme activity increased similarly as in those infected with r^+ at first 20 min, but thereafter the rate decreased (Fig. 2, D), probably due to starting of cell burst. Since Streisinger *et al.* (1961) proved that lysozyme synthesis in infected cells is controlled by phage gene, *e*, the increase of the enzyme activity must be increase of the newly synthesized enzyme. These results indicate that synthesis of lysozyme starts a little before 10 min after infection, continues and accumulates till cell bursts and its accumulated amount is not a single direct cause of the lysis.

Lysozyme synthesis by UV irradiated phage (*UV phage*). Lysozyme synthesis was inhibited by UV irradiation of the phage particles. Table 1 summarizes the results in which phage was inactivated to 1×10^{-3} or 10^{-4}. For multiple infection 5×10^8 cells and 2.5×10^9 particles were mixed and for single infection 2.5×10^9 cells and 5×10^8 particles were mixed at time zero. At 20 and 40 min enzyme

synthesis was stopped and its activity was measured. With both infections UV inhibited the synthesis. There was a remarkable difference, however, in single infection with UV phage there was no enzyme synthesis even at 40 min, whereas

Table 1. Lysozyme synthesis by UV phage.

Survival of phage	Phage/bacterium at infection	Lysozyme activity	
		20 min	40 min
1	0.2*	134	290
$1X10^{-3}$	0.2*	0	3
$1X10^{-4}$	0.2*	0	0
1	5**	120	—
$1X10^{-3}$	5**	63	—
$1X10^{-4}$	5**	22	—

Bacteria were infected with intact or UV phage and sampled at 20 and 40 min after infection. Extract was prepared and lysozyme activity was deterimined by the Nephelos method as shown in Materials and Methods (units/10^3 infected cells).
* ; 5 x 10^8 phage particles and 2.5 x 10^9 cells were mixed at infection.
**; 2.5 x 10^9 phage particles and 5 x 10^8 cells were mixed at infection.

in multiple infection the enzyme was detectable even at 20 min, the amount being lower at higher doses of irradiation. This is contrast to synthesis of early proteins, which is resistant to UV (Flaks *et al.*, 1959; Delihas, 1961). Table 1 also shows that the multiplicity of infection with intact phage does not affect the time at initiation and amount of the enzyme synthesized.

Effect of fluorouracil (FU) and uracil (U)-starvation on the enzyme synthesis. Aronson reported (1961) that FU used together with thymidine did not inhibit phage DNA synthesis, but inhibited phage coat protein synthesis. If so, FU must affect formation of early enzymes, at least some of early enzymes pertinent to phage DNA synthesis, but may inhibit lysozyme synthesis.

Washed B6 cells were aerated in TG containing argine and U in one tube, and in TG containing arginine and 20 μg/ml FU in the other tube. After 10 min of aeration phage was added and the activity of dCTPase and lysozyme was measured at intervals as shown in Fig. 3a. Synthesis of dCTPase in U-medium took place as reported by the other investigator (Kornberg *et al.*, 1960), it started within a few minutes after infection, though it is not drawn in the figure, and almost stopped after 15 min. In FU-medium the synthesis continued over this period and stopped after longer incubation. Therefore more dCTPase was synthesized in FU. On the other hand lysozyme synthesis was strongly inhibited by this agent. It is, however, obscure if shut-off of dCTRase synthesis in FU is resulted from the same cause with that in U (or normal infection), since in FU cell lysis started at around 30 min.

As shewn in Fig. 3b, in U-starved cells which had been aerated for 10 min

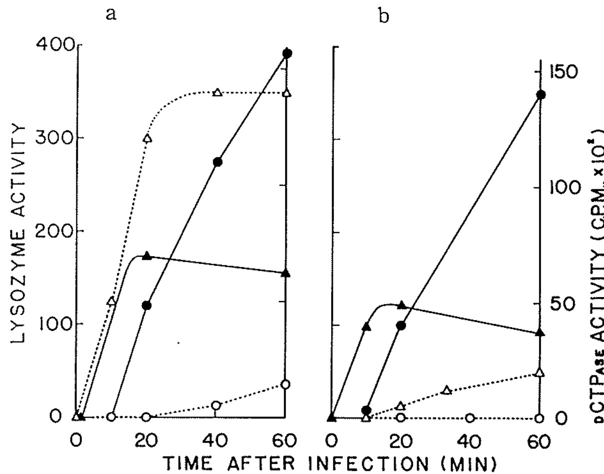

Fig. 3a. Effect of FU on synthesis of lysozyme and dCTPase. An aliquot of washed B6, requiring arginine and uracil, was aerated in TG containing both requirments (20 μg/ml each) and another in TG containing arginine, FU (20 μg/ml) and thymidine (20 μg/ml), for 10 min and infected. Lysozyme activity was measured by the spot test. Solid lines; enzyme activities in U-media, broken lines; enzyme activities in FU-media, circles ; lysozyme activity (spot test units), and triangles ; dCTPase activity (counts/min).

Fig. 3b. Effect of uracil starvation on synthesis of lysoyzme and dCTPase.

An aliquot of washed B6 cells was aerated in TG containing both requirements (20 μg/ml) and another in TG containing arginine only for 10 min and infected. Solid lines; enzyme activities in U-media, broken lines ; enzyme activities in U-less media, circles; lysozyme activity (spot test units), and triangles; dCTPase activity (counts/min).

before infection, dCTPase synthesis occured with much less rate when compared to normal medium and continued during the period of experiment and lysozyme synthesis was not detectable. After 30 min aeration in the absence of U, neither enzymes were synthesized. Therefore FU can partially replace U to induce early protein synthesis, involving dCTPase, but inhibits lysozyme and coat protein synthesis.

Gros *et al.* (1960) reported that FU is incorporated into mRNA and leads to synthesis of modified enzyme. We have compared the sensitivity to higher temperature and NaF of dCTPase whch was synthesized in the presence of U or FU. No difference has been observed so far.

Fig. 3b suggests that dCTPase or early protein is synthesized by utilizing limited intracellular U, and consequently this exhaustion does not allow lysozyme

synthesis. B6 cells were starved for U by aerating in TG containing arginine for 20 min, and infected. At the infection U was added. At time 0, 2, 4, 8, and 12 min aliquots were poured on the crushed frozen medium and centrifuged to remove U. Pellets were resuspended in TG containing arginine only and aerated for 30 min. U was added to one part of 12 min treated culture as a control. Infective centers were titrated in those cultures and no significant difference was found. The activity of dCTPase in all of the U treated cultures was the same as the control, while lysozyme activity was 0 and 1/4 of that of the control in 0 and 4 min treated culture, respectively.

Effect of arginine starvation on enzyme synthesis. It was known that some amino acid requiring mutants of *E. coli* do not synthesize RNA in the absence of the amino acid (Gros, 1960). Stent and Brenner (1961) have found a gene, *RC*, affecting this phenomenon. When the bacteria have RC^{str}, they cannot synthesize RNA in the absence of required amino acid, but when they have RC^{rel} they can synthesize RNA. We have observed that synthesis of RNA with a base composition similar to phage DNA does occur in both types of infected mutants in the absence of the amino acid. If this RNA included RNA for early and late proteins (and if the rate of translation from mRNA into protein were the same), it would accumulate in the absence of the amino acid and the corresponding proteins would be simultaneously synthesized after addition of the amino acid.

B6 cells, starved by 30 min aeration in TG containing U, were infected, aerated further for 15 min, and centrifuged to remove U. Pellet was resuspended in TG and devided in two aliquots, one supplemented with U and arginine, the other with arginine only. After those treatment synthesis of dCTPase and lysozyme started in a way similar to the normal, except that the rate of lysozyme synthesis decreased in the medium containing arginine only after 20 min, probably due to exhaustion of available U. This would imply that mRNA for early and late protein synthesis are not formed simultaneously in the absence of protein synthesis or mRNA for late protein is translated only after formation of some early protein.

Effect of chloramphenicol (CM). Since CM stops protein synthesis but allows to synthesize phage RNA in infected cells, pre-treated as well as post-treated (Okamoto *et al.*, 1962), we can see the effect of the antibiotic on the initiation of enzyme synthesis.

CM (50 μg/ml) was added to culture 5 min before infection, and 5 and 10 min after infection, and removed by centrifugation 15 min after the addition. Pellets were resuspended in fresh warmed TG, aerated, and the enzyme activity was measured (Fig. 4). In the figure zero time was chosen when pellets were resuspended. Ordinates of corresponding curves should not be compared each other, since a number of enzyme forming cells could not be estimated in different treatments. Whenever CM was added, synthesis of dCTPase started with a similar rate immediately after removal of CM. When the two curves of dCTPase synthesis (A' and B') were compared, however, there were some differences; when CM was added after infection the enzyme activity was higher at

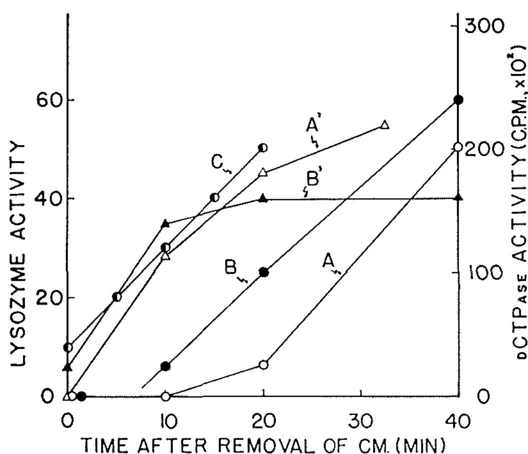

Fig. 4. Effect of CM on the enzyme synthesis. Cells of H in a logarithmic phase were collected, washed and resuspended in TG. CM (50 μg/ml) was added 5 min before infection (curves A and A'). 5 min (curves B and B') and 10 min (curve C) after infection and removed by centrifugation after 15 min contact. Pellets were resuspenden in warmed fresh TG and the enzyme activity was measured at intervals after sonication. In the figure zero time was chosen when pellets were resuspended. Curves A, B, and C ; lysozyme activity (spot test units), and curves A' and B' ; dCTPase activity (counts/min).

zero time and the synthesis stopped earlier.

As to lysozyme synthesis there was an ordinary or longer lag period was required, when CM was added before infection. The lag was shortened but still distinct when CM was added 5 min after infection. It disappeared completely when CM was added 10 min after infection. Because CM inhibits protein synthesis almost completely within an half min after added (Koch and Hershey, 1959), some protein synthesized after infection plays an important role to initiate lysozyme synthesis, being in contrast to synthesis of dCTPase. This result also suggests that sequential synthesis of phage related protein stopped at the time when CM was added, and resumed at the time when CM was removed.

Discussion

Apparent correlation of cessation of early enzyme synthesis and onset of late protein synthesis has been observed by many investigators. Present experiments also are in the case, showing that with infection of cells partially U-starved or

in FU medium the shut-off of dCTPase was retarded and lysozyme synthesis was strongly suppressed. This will mean RNA formed after infection is pertinent directly or indirectly to shut-off of dCTPase synthesis. As to this phenomenon there are several posturates; 1) late protein synthesis or late protein synthesized represses early enzyme synthesis (Luria, 1962), 2) new phage DNA plays some role in shut-off of the synthesis (Luria, 1962; Sekiguchi and Cohen, 1964), or 3) an early functioning represses synthesis of early enzymes. At present we have no critical evidence to discriminate those posturates. However infection in the presence of FU and thymidine, which permits phage DNA synthesis, continued dCTPase synthesis in the present experiment and infection of restricted bacteria with many DNA-negative *amber* mutants of T4 did not continue the synthesis (unpublished data). These seem to make direct role of new DNA in the phenomenon less possible. Our recent finding that only a double mutant of gene 44 and 62 allowed contineous synthesis of dCTPase as UV phage did may support the last posturate.

Strict order of synthesis of early protein (deoxycytidylate hydroxymethylase) and late protein (tail fiber) was observed to be maintained, when Ebisuzaki (1963) studied the process recovering from inhibition of tryptophan analogs by tryptophan. Results by Sekiguchi and Cohen (1964), whose experiments were carried out independently on the similar basis of the idea by using T6 and a thymine, uracil and histidine requiring bacterial mutant, are consistent to ours. In our experiment with a small U pool only dCTPase was synthesized, but lysozyme was not. This suggests RNA corresponding to those enzyme is also sequetially synthesized. They obtained clear evidence showing RNA synthesized in the absence of the requring amino acid, histidine, was functional. Post addition of the amino acid synthesis of early enzymes started with higher initial rate. In our case, however, the difference of the initial rates was obscure after post addition of arginine. This might be due to difference of the strain used.

Addition of CM earlier than 5 min after infection did not change the order of the protein synthesis after removal of the antibiotic. It might be posturated that CM destroyed preparatory system for lysozyme synthesis. This is, however, ruled out by the fact that when CM was added at 10 min and removed at 25 min, lysozyme was immediately synthesized. This lead us to assume that the protein which is synthesized between 5 and 10 min after infection plays some role in initiation of lysozyme synthesis.

Single infection with UV phage did not form measurable amount of lysozyme, but multiple infection formed some amount of the enzyme. (Table 1). In the latter case DNA synthesis was measured by incorporation of H^3-thymidine. There was no DNA synthesis within an experimental error (1/3 of a phage equivalent DNA/infected cell). This might be understandable since gene doses injected in a cell under condition where DNA synthesis is suppressed and some factor or step for DNA synthesis play an important role in lysozyme synthesis (to be published).

Ebisuzaki (1966) has recently compared UV sensitivity of early and late

protein synthesis in cells infected with T4 v^{+} and a mutant v (equivalent to T2) at a lower multiplicity and concluded that late protein synthesis was more sensitive than early protein synthesis with both strains, and also showed unexpectedly large target size of the lysozyme gene. This might be due to nucleotide sequence of the lysozyme gene itself, or to that preceeding synthesis or reaction is required for starting of lysozyme synthesis. Presence of CM sensitive reaction before initiation of tne lysozyme synthesis and the fact, that the lysozyme gene functions immediately after superinfected 10 min after primary infection but it functions only after a lag when superinfected at 5 min (to be published), prefer the latter hypothesis.

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