JOURNAL OF VIROLOGY, Jan. 1970, p. 45-50

Vol. 5, No. 1
Printed in U.S.A.

Multiplication of Bacteriophage P22 in Penicillin-Induced Spheroplasts of *Salmonella typhimurium*

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Received for publication 29 April 1969

The spheroplasts of *Salmonella typhimurium* (LT2) prepared by treatment with penicillin were capable of adsorbing phage P22 C_1. The normal multiplication of the phage took place, although the burst size was reduced to one-fourth of that in intact cells. Rate of incorporation of ^{14}C-thymidine into spheroplasts was increased several-fold on phage infection. Multiplication of C^+ also took place, but no lysogeny could be established in spheroplasts. Furthermore, spheroplasts prepared from cells lysogenized with wild-type phage, LT2 (C^+), and a temperature-inducible C_2 mutant, LT2(tsC_2), were not inducible. Unlike normal cells, both mitomycin C and actinomycin D interfered with the phage multiplication in spheroplasts. The spheroplast system offers great advantages in the study of the synthesis of nucleic acids and proteins in phage-infected LT2.

McQuillen (10) documented evidence on infection of bacterial protoplasts by phage particles. Weibull (21) demonstrated that protoplasts of *Bacillus megaterium* interact with the phage particles. It has been shown by quite a few workers (2, 13, 14, 21, 22) that bacteriophage development can proceed in cell wall-less protoplasts of gram-positive *B. megaterium* and *B. subtilis* provided that the cells are infected before conversion to protoplasts.

Osmotically sensitive "spheroplasts" produced by lysozyme treatment of *Escherichia coli* in sucrose broth are able to support growth of the T3 and T-even strains (4, 11). Spiegelman [cited by McQuillen (10)] observed that protoplasts of *E. coli* prepared by both penicillin and lysozyme methods adsorb T2. Every protoplast seemed infectible and the viral growth appeared to be normal. Kellenberger [cited by McQuillen (10)], however, found no growth of T2 in protoplasts prepared by phage action. Lederberg and St. Clair (5), definitively demonstrated that the spheroplasts of *E. coli* prepared by penicillin treatment can be infected with T3 and T7 and fully with T6, although they are relatively resistant to T1, T4, T5, and λ2. Taubeneck and Böhme (1, 20) also reported that penicillin-induced spheroplasts of *Proteus mirabilis* can be infected with bacteriophages.

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McQuillen (10), however, concluded that it cannot be said definitely whether the protoplasts of *E. coli* interact with intact bacteriophages. There is evidence that heated, osmotically shocked phage (18) and urea-treated phage (4) can infect lysozyme-treated *E. coli*. In these cases, it is most likely that naked deoxyribonucleic acid (DNA) infects the spheroplasts, and the possibility of attachment of phages to the receptor sites of the host is ruled out.

Phage-infected penicillin spheroplasts of *Salmonella typhimurium* have been used in the study of nucleic acid metabolism (3, 12). In the present communication, it is shown that P22 and its mutants infect these spheroplasts and that their multiplication takes place.

MATERIALS AND METHODS

S. typhimurium strain LT2, its *gal*$^-$ mutant, temperate phage P22 (C^+), clear plaque-forming C_1 mutant, and a strain of LT2 lysogenized with temperature-sensitive C_2, LT2(tsC_2), were obtained from M. Levine, Department of Human Genetics, University of Michigan, Ann Arbor, Mich.

^{14}C-thymidine was a preparation of Schwarz Bio-Research Inc., Orangeburg, N. Y. Crystalline penicillin G was a commercial preparation manufactured by Pfizer Private Ltd., Bombay, India. Actinomycin D was kindly supplied by K. C. Mezey of the Merck Sharp and Dohme Research Laboratories, Rahway, N.J. Mitomycin C was a gift from Y. Takagi, Kyushu University, Fukuoka, Japan. The antiserum against phage C_1 was prepared by infecting a rabbit with C_1

at intervals of 15 days for a period of 2.5 months, each time with 3 ml of a mixture containing 1.5 ml of phage (2×10^{11} particles per ml) and 1.5 ml of Freund's adjuvant (complete; Difco). The titer (K value) of the antibody was 640.

Plaque assay of the phage. Plaque assay was carried out in the usual way on agar plates seeded with LT2. For measuring lytic induction, eosine methylene blue (EMB)-galactose agar plates were prepared as described by Smith and Levine (17). The *gal*$^-$ strain was used as the plating bacterium. The clear plaques that appeared after incubation for 12 to 14 hr at 37 C indicate the lysed cells. Subsequently, the incubation was continued at 25 C for 24 hr. Lysogenized cells, which are *gal*$^+$, appeared as fluorescent colonies surrounded by halos of lysis, whereas normal cells appeared as ordinary colonies only.

Preparation of spheroplasts. The spheroplasts were prepared by the method described by Spizizen (19). LT2 was allowed to grow overnight in Penassay Broth (PB) medium. The PB contained, in 1 liter: beef extract, 1.5 g; yeast extract, 1.5 g; peptone, 5 g; dextrose, 1 g; NaCl, 3.5 g; K_2HPO_4, 3.68 g; and KH_2PO_4, 1.32 g. A sample (30 ml) of the culture grown overnight was diluted to 130 ml with 100 ml of PB containing 20% sucrose, 0.2% $MgSO_4 \cdot 7H_2O$, and 10^3 units of penicillin G per ml. The diluted culture [optical density (OD) = 0.15, measured at 640 nm in a Leitz colorimeter and containing 1.3×10^8 cells/ml] was allowed to grow for 2 hr at 35 C. Within this period, the OD increased from 0.15 to 0.30 and the cells were converted to spheroplasts as observed under the microscope after staining. The cells became completely spherical and osmotically fragile. The extent of contamination with intact cells, as determined by plating after dilution with water, was less than 0.01%. The spheroplasts so formed were collected by centrifugation at 10,000 $\times$ g for 10 min at 0 C and then were washed three times with 50 ml of 20% sucrose [pH adjusted to 7.0 with tris(hydroxymethyl)aminomethane-hydrochloride (2×10^{-3} M)]. The spheroplasts were usually suspended in 100 ml of PB containing 20% sucrose. The OD of this suspension was about 0.35, as measured at 640 nm. Protein was determined by the Folin-Ciocalteau method of Lowry et al. (9).

Measurement of adsorption of phage particles. Samples (0.05 ml) of phage suspension (5.7×10^7 particles per ml) were added to 5 ml of suspensions containing various amounts of cells and spheroplasts. The mixtures were immediately shaken and kept at 28 C. Portions (0.05 ml) were removed at requisite times and shaken vigorously with 5 ml of 0.85% NaCl containing a few drops of chloroform. Chloroform-treated samples were further diluted, if necessary, and plated to determine the number of unadsorbed phage particles.

Determination of burst size. The burst size was determined by single-step-growth and by single-burst experiments. In the single-step-growth experiment, the spheroplasts of cells were suspended in PB containing 20% sucrose (3.5×10^8 spheroplasts per ml). To a sample of spheroplast suspension (0.9 ml), NaCN (final concentration, 2×10^{-3} M) was added. This was followed by 0.1 ml of a phage suspension at 7×10^8 phage per ml to give a multiplicity of infection (MOI) of 0.2. The mixture was kept at 35 C for 5 min to allow adsorption of phage particles. Samples were diluted with PB containing 20% sucrose. One sample was centrifuged at once, and a portion of the supernatant fluid was plated to determine the number of unadsorbed phages. Other samples were incubated at 35 C, and samples were plated at requisite intervals to determine the number of phage particles produced.

In the single-burst experiment, 5 ml of spheroplast suspension (3×10^8 spheroplasts per ml) was infected with C_1 at an MOI of 1. Adsorption for 5 min in the presence of 2×10^{-3} M NaCN was allowed. Then the suspension was diluted in PB containing 20% sucrose to yield 2 spheroplasts per ml. Samples (0.5 ml) were incubated at 37 C for 90 min, mixed with soft agar, and plated as usual to determine the number of phage particles. When each spheroplast had to be infected for burst size determination, the infection was carried out at an MOI of 10, and the excess unadsorbed phages were neutralized by the addition of C_1 antiserum (0.025 ml).

Pulse-labeling of C_1-infected spheroplasts with ^{14}C-thymidine. The spheroplasts obtained from 130 ml of cell culture were suspended in 50 ml of 40% sucrose (0.88 mg of protein per ml). A 15-ml amount of this suspension was diluted with an equal volume of a synthetic medium containing, in 1 liter: K_2HPO_4, 21 g; KH_2PO_4, 9 g; sodium citrate $\cdot 5\frac{1}{2} H_2O$, 0.94 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 2 g; and glucose, 4 g. The diluted suspension was shaken at 37 C for 15 min and then was infected with C_1 at an MOI of 10. The ^{14}C-thymidine incorporating activity before and after infection was checked in the following way. To a 1-ml sample of the suspension collected at the requisite time intervals 0.02 ml of 10^{-4} M ^{14}C-thymidine (6.0×10^7 counts per min per μmole) was added. After 90 sec, the incorporation was stopped by adding 0.4 ml of ice-cold 17% trichloroacetic acid containing 2.5 mg of nonradioactive thymidine per ml. After 30 min in ice, the precipitate was collected on a membrane filter (Millipore Corp., Bedford, Mass.) and washed four times, each time with 5 ml of ice-cold water. The membrane filters were then placed in planchets, dried, and counted in a gas-flow counter of the Bhabha Atomic Research Centre, Bombay, India.

Conversion of LT2(tsC_2) to spheroplasts and high-temperature induction. Strain LT2(tsC_2), which is inducible at 42 C, was grown at 25 C (generation time being 60 min instead of 30 min at 37 C) and converted to spheroplasts at 25 C by treatment with penicillin as described above. The complete conversion took about 4 hr, instead of the 2 hr required at 37 C. To 5 ml of spheroplast suspension (10^5 spheroplasts per ml), 0.02 ml of C_1 antiserum (K = 640) was added to neutralize the small number of phages liberated by induction at 25 C. Then the spheroplast suspension was exposed to 42 C, and samples (0.1 ml) were removed at desired intervals and plated on EMB-galactose plates with LT2 *gal*$^-$ strain as the indicator bacterium (17). In the control experiment, normal cells of LT2(tsC_2) were grown at 25 C and then exposed to 42 C, and induction was measured in the same way.

RESULTS

Kinetics of adsorption of phage C_1 to spheroplasts. Before studying the multiplication of phages in spheroplasts, the adsorption of phages on the spheroplasts was measured. The ratio of the number of unadsorbed phage particles to the total number of phage added has been plotted against time (Fig. 1). For comparative purposes, adsorption kinetics to normal cells have also been presented. Linear rates of adsorption were observed in both the cases. Further, the rate of adsorption increased with increase in the number of cells or spheroplasts, as expected. It is, however, interesting to observe that there was practically no difference in the kinetics of adsorption of the phage particles by the normal cells and the spheroplasts. Apparently, phage attachment sites on the walls of the cells of *S. typhimurium* remain unaffected on conversion of the cells to spheroplasts by penicillin treatment.

Determination of burst sizes of C_1 and C^+. The penicillin spheroplasts were found to be capable of carrying out the process of multiplication of the phages. The latent period and the burst size were determined by a single-step-growth experiment (Fig. 2). The burst size of C_1 in spheroplasts was reduced to one-fourth of that in normal cells. The latent period, however, remained uneffected. Essentially identical results were obtained when spheroplasts were infected with C^+ at an MOI below 1.

The burst size was also determined by single-burst experiments as described above. At an MOI of 1, only 9 of the 40 plates showed plaques. The totals for these plates are 61, 51, 38, 24, 8, 7, 154, 5, and 2. The average burst size calculated from the Poisson distribution was 31. Another experiment was performed in the same way but at an MOI of 10. In this case, the unadsorbed phages were neutralized with antiserum. Of the 40 plates, 22 could be scored for plaques. The totals for these plates are 67, 108, 3, 17, 74, 50, 2, 90, 12, 3, 41, 22, 18, 23, 7, 1, 26, 20, 195, 176, 17, and 15. The burst size calculated from the Poisson distribution was 31.

Another experiment was designed to ascertain whether premature lysis of the spheroplasts by osmotic shock during the eclipse period leads to loss of infective centers. A single-step-growth infection with phage C_1 was carried out in the usual way, but with dilutions in 20% sucrose and

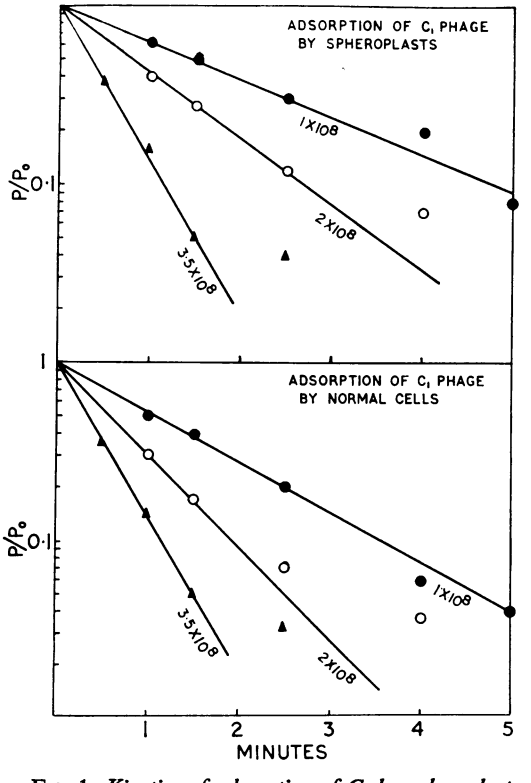

FIG. 1. *Kinetics of adsorption of C_1 by spheroplasts of LT2 (top) and by normal cells (bottom). The concentrations of cells and spheroplasts (number of particles per milliliter of adsorption mixture) are indicated in the figure.*

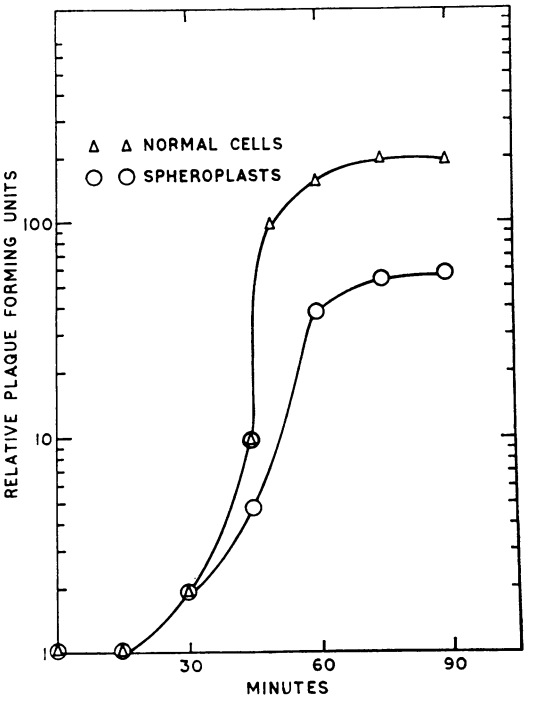

FIG. 2. *Single-step growth of C_1 in normal cells and spheroplasts of LT2.*

in water. The spheroplasts were infected at an MOI of 5, and unadsorbed phages were neutralized by antiserum (Fig. 3). When dilution in water was carried out before the end of the eclipse period, only 5% infective centers were obtained compared to that with dilution in 20% sucrose. The final yields of phage particles obtained by both methods of dilution were practically the same. Similar experiments with normal cells (separate dilutions with 20% sucrose and water) showed no difference in the single-step growth curve. This clearly demonstrates the osmotic fragility of the penicillin-treated cells of LT2 and their infection with C_1.

Effects of mitomycin C and actinomycin D on the multiplication of C_1. It is well known that mitomycin C interferes with the duplication of DNA. It has been observed by Sekiguchi and Takagi (15, 16) that high levels of mitomycin C (100 μg/ml) interfere with the multiplication of T2r phage in *E. coli* and produce defective phage particles. Mitomycin C has, however, no effect on the multiplication of C^+ in LT2 (6, 7). Therefore,

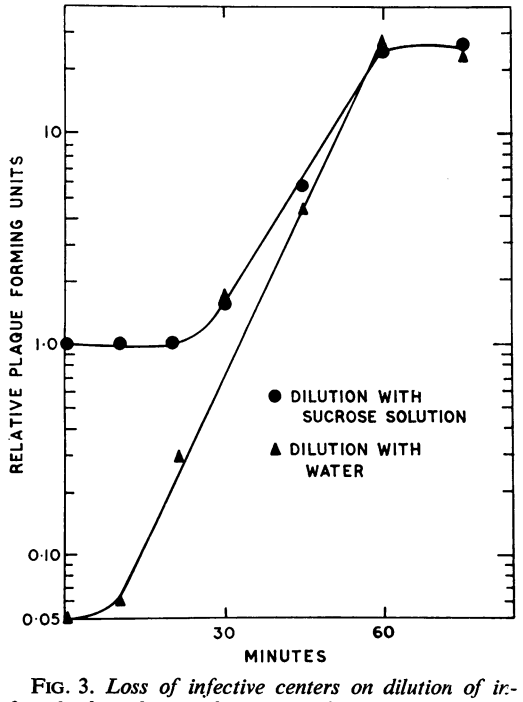

FIG. 3. *Loss of infective centers on dilution of infected spheroplasts with water. In the single-step-growth experiment, the spheroplasts were infected with C_1 at an MOI of 5. After allowing 5 min for adsorption, 0.01 ml of C_1 antiserum was added to neutralize the unadsorbed phages. After allowing 5 min more for complete neutralization, suitable dilutions were carried out with 20% sucrose and with water only. The remaining procedure was the same as in Fig. 2.*

it was of interest to study the effect of mitomycin C, if any, on the multiplication of C_1 in penicillin spheroplasts of LT2. Mitomycin C, even at a low concentration (5 μg/ml), did not allow the production of infective phage particles (Table 1). It is not known whether multiplication was interfered with or defective phage particles were produced. A further indication that mitomycin C affects the multiplication of phage is the finding that mitomycin C added at various times (up to 20 min) after phage infection did not allow the multiplication of phage (Table 2), but, when added after 30 min, had no effect at all.

That the spheroplasts are not being destroyed or made incapable of supporting the multiplication of phage in the presence of mitomycin C was confirmed by the fact that the number of infected centers produced in the presence and absence of mitomycin C (10 μg/ml) was the same. Further, the preincubation of the spheroplasts with mitomycin C for a period of 30 min before infection did not affect the production of infected centers. Apparently, the effect of mitomycin C occurs during the multiplication of the phage.

Since gram-negative organisms are insensitive to the action of actinomycin D, it was expected that the antibiotic would not interfere with the multiplication of C_1 in LT2. But spheroplasts are known to be sensitive to actinomycin D. Therefore, the effect of various concentrations of actinomycin D on the multiplication of C_1 in spheroplasts was studied (Table 1). Actinomycin D at a concentration of 5 μg/ml had no effect on phage multiplication, a concentration of 10 μg/ml had a partial effect, and a concentration of 25 μg/ml produced complete inhibition. To study the

TABLE 1. *Effects of various concentrations of mitomycin C and actinomycin D on the multiplication of C_1 in spheroplasts*[a]

Concn of antibiotic	Burst size (plaque-forming units)	
	Mitomycin C	Actinomycin D
μg/ml		
0	36	36
1	19	
2	12	
5	2	34
10	2	12
25	2	2
50	1	2
75		1

[a] The experimental procedure was the same as that in the burst size determination described in Materials and Methods, except that various amounts of mitomycin C or actinomycin D were added as indicated.

TABLE 2. *Effects of mitomycin C and actinomycin D added at various times after infection on the multiplication of* C_1[a]

Time after addition of C_1	Mitomycin C		Actinomycin D	
	Normal cells	Spheroplasts	Normal cells	Spheroplasts
min				
	140	25	160	34
0	110	1	135	2
10	100	1	145	5
20	120	4	160	3
30	110	26	150	12

[a] The experimental procedure was the same as that in the burst size determination described in Materials and Methods, except that mitomycin C (10 μg/ml) or actinomycin D (50 μg/ml) was added at the indicated time after infection with C_1. Values express burst size (plaque-forming units).

effect of actinomycin D added at various times after phage infection, a concentration of 50 μg/ml was chosen (Table 2). It is clear that in case of normal cells actinomycin D at this concentration (and even at higher concentrations tested) had no effect on the multiplication of C_1, whereas actinomycin D added to spheroplasts up to 20 min after infection blocked phage development completely. Even after 30 min, addition of actinomycin D produced partial blocking.

Pulse-labeling of C_1-infected spheroplasts with ^{14}C-thymidine. The pulse-labeling was carried out as described in Materials and Methods. The rate of pulse-labeling of uninfected spheroplasts was more or less constant throughout the period of the experiment, whereas in phage-infected samples the rate of incorporation of thymidine increased about threefold, indicating the accelerated rate of DNA synthesis (Fig. 4). This is apparently due to multiplication of phage DNA in the spheroplasts.

Lysogenization and lytic induction of spheroplasts. As already stated, at a low MOI, C^+ can multiply like C_1 in spheroplasts as in normal cells, although the burst size is reduced. It was, however, observed that, even at an MOI of 10 to 20, no lysogeny was established with C^+, and the spheroplasts lysed after 60 to 75 min. This was not a case of lysis from without, as phage multiplication took place. Since no lysogeny could be established in spheroplasts, lysogenized normal cells were converted to spheroplasts by penicillin treatment and then plated to determine whether phage production could be induced. No plaques could be scored. Even treatment of these spheroplasts with low concentrations of mitomycin C (<1 μg/ml) did not lead to induction. Absence of active growing condition in spheroplasts may be responsible for noninduction.

Since LT2(tsC_2) is inducible at 42 C but grows normally at 25 C (8), it was grown and converted to spheroplasts by treatment with penicillin at 25 C. (This conversion at 25 C took much longer time than that at 37 C.) These spheroplasts were then exposed to 42 C. The results in Table 3, indicate that an insignificant number of spheroplasts were induced, even after 40 min of exposure to 42 C. Under these conditions, about 70% of the normal lysogenic cells were induced. It should be mentioned here that the spheroplasts remained

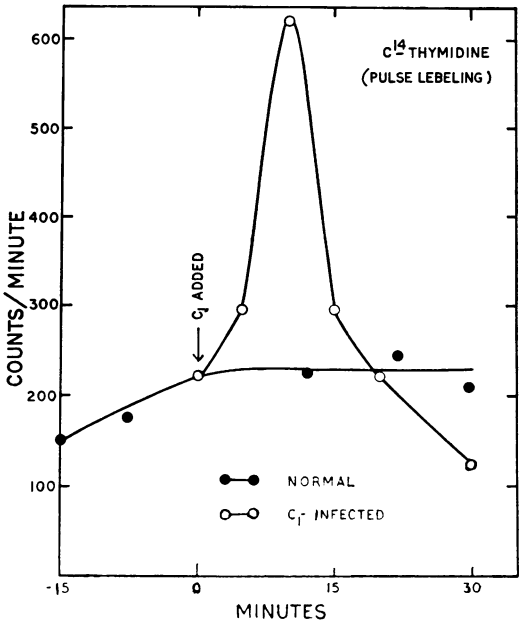

FIG. 4. *Pulse labeling with ^{14}C-thymidine of the noninfected and infected spheroplasts of LT2.*

TABLE 3. *Induction of cells and noninduction of spheroplasts of LT2(tsC_2)*

Time after exposure to 42 C	No. of cells induced	No. of spheroplasts induced
min		
0	0.05×10^5(5)[a]	5.7×10^2 (5.7×10^{-3})
5	0.10×10^5(10)	7.4×10^2 (7.4×10^{-3})
10	0.32×10^5(32)	10.0×10^2(10.0×10^{-3})
15	0.62×10^5(62)	11.0×10^2(11.0×10^{-3})
20	0.71×10^5(71)	12.0×10^2(12.0×10^{-3})
30	0.76×10^5(76)	13.0×10^2(13.0×10^{-3})
40	0.71×10^5(71)	12.0×10^2(12.0×10^{-3})

[a] Numbers in parentheses indicate per cent of induced cells calculated on the basis of 10^5 cells (or spheroplasts) present per milliliter.

unchanged on exposure to 42 C, and the multiplication of both C^+ and C_1 took place in spheroplasts at this temperature after infection.

DISCUSSION

The spheroplasts of LT2 prepared by penicillin treatment retain the properties of the intact cells in terms of adsorbing phages and acting as hosts for phage multiplication. The damage to the cell wall structure must be limited. Dilution of the spheroplasts with water instead of 20% sucrose during burst size determination (Fig. 3) and pulse-labeling with ^{14}C-thymidine (Fig. 4) clearly favor multiplication of phage in the spheroplasts. Though the latent period and the time of lysis in spheroplasts remain the same as in intact cells, the burst size is reduced to one-fourth of that in normal cells. The fragility of the spheroplasts may be responsible for this, although it has been observed that the induction of lysozyme in spheroplasts is delayed by 20 min in comparison with that in intact cells (*unpublished data*).

The sensitivity of phage multiplication in spheroplasts to both actinomycin D and mitomycin C is highly interesting. Though the host is sensitive to mitomycin C, phage multiplication in normal cells of LT2 is known to be insensitive to mitomycin C (6, 7). Actually, mitomycin C is an excellent lytic inducer. Phage multiplication in spheroplasts, however, is quite sensitive to mitomycin C. It is not known whether noninfective phage particles are produced in this case. In the case of actinomycin D, however, the host itself is insensitive to the antibiotic, so it is not expected to affect the multiplication of phage in normal cells. This was found to be true. Spheroplasts, however, are known to be affected by actinomycin D; therefore, it is not surprising that the phage multiplication in spheroplasts was inhibited by actinomycin D. The sensitivity of phage multiplication in spheroplasts to both the antibiotics may be conveniently utilized for studies on phage-specific DNA or ribonucleic acid synthesis.

Neither lysogenization nor lytic induction could be carried out in spheroplasts. This is most probably due to the absence of active growing conditions in spheroplasts.

Enzymatic studies with the spheroplast-phage system are in progress in this laboratory with the hope of making visible certain changes which are less evident in the intact cell-phage system. Unlike preinfection and subsequent conversion to spheroplasts in the case of lysozyme treatment, the penicillin spheroplasts can be directly infected and thus offer certain distinct advantages in enzymatic and allied studies.

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