Bacteriophages of L-Glutamic Acid-Producing Bacteria

Part VIII. Growth Characteristics of *Brevibacterium* Phages*

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The growth characteristics of phages were investigated with the four phages, active on *Brevibacterium lactofermentum*, which were selected from the respective serological groups, namely, P465 (group I), P468II (group II), Ap85III (group III) and P4 (group IV).

The adsorption rate of the phages, P465 and P468II, on the host bacteria was low, whereas that of the phages, Ap85III and P4, was higher. The adsorption rate constants for the four phages were respectively calculated at 2.02×10^{-10} , 1.87×10^{-10} , 4.32×10^{-10} and 3.15×10^{-10} cm³ per minute, at 30°C in G₅B₂ medium. With reference to the ionic environment for adsorption, the phages, P465 and Ap85III, specifically required either for Ca⁺⁺ or Mg⁺⁺; the phage P468II, for both; and the phage P4, for neither.

The growth characteristics of these phages were examined by the one-step growth experiment. The latent periods of the phages were 50, 53, 57 and 47 minutes, respectively; and the corresponding average burst sizes were about 98, 31, 145 and 126. The growth of the phage P4 was completely suppressed at above 34°C, although the host bacteria and the other three phages were capable of the full growth at that temperature.

INTRODUCTION

The first step in the growth cycle of phages is the adsorption onto their host cells. Since the step is the most accessible to observation, it has been more thoroughly studied than the subsequent steps. The studies on effects of environmental factors and of the physiological state of the host cell on the rate of phage adsorption have contributed greatly to understanding of the phage-and-host cell relationship. After adsorption of a phage to its host cell, a certain period of time elapses before the cell lyses, liberating phage progeny. The time interval in this is most conveniently measured by means of the one-step growth experiment as described by Ellis and Delbrück.¹⁾

With respect to the phages of L-glutamic acid-producing bacteria, Seto et al.²⁾ discussed briefly the growth of the phages, P1, P2, P3, P4 and P5, of *Mic. ammoniaphilum*. The previous data on the adsorption of phages to host cells and one-step growth experiments, in respect to the four phages from serological group I,³⁾ are unsatisfactory in accuracy, for above experiments had been carried out without using antiphage serum. This paper therefore takes them up again with more or less full account.

The present study was undertaken, first, to elucidate the aspects and the ionic requirements of the phages for adsorption to the host cell, *Brevibacterium lactofermentum*; and, second, to determine quantitatively by means of the

* This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, in April of 1966.

1) E. L. Ellis and M. Delbrück, *J. Gen. Physiol.*, **22**, 365 (1939).

2) S. Seto, T. Osawa and S. Yamamoto, *J. Agr. Chem. Soc. Japan*, **39**, 48 (1965).

3) T. Oki, T. Matsui, K. Akahoshi and R. Harada, *J. Agr. Chem. Soc. Japan*, **38**, 376 (1964); *Amino Acid and Nucleic Acid*, **9**, 53 (1964).

one-step growth technique two important characteristics of the phage multiplication, the latent period of intracellular growth and the burst sizes.

MATERIALS AND METHODS

Phages and bacteria. The four phages selected from the respective serological groups, P465 (group I), P468II (group II), Ap85III (group III) and P4 (group IV), and the host strains, YO2 for the first three and 468-5SM^r for the last, were used.⁴⁾

The host cells employed in the adsorption and one-step growth experiments were prepared as follows: 10% inoculum of the overnight host-bacterial culture in G-medium was added to a fresh G-medium, and the said culture was then incubated for 6 hours at 30°C. The cells in the logarithmic growth phase were harvested by centrifugation and resuspended in the adequate medium.

The dialysed phages as reported previously⁴⁾ were used to determine the ionic requirements for adsorption of the phages to the host cell.

Assay of phages and bacteria. The phages and bacteria were assayed by the same methods as described in the previous paper.³⁾ With phage P4, labile to temperature, the method was partially modified to prevent the phage from being exposed to high temperature; i.e., a diluted phage suspension (0.1~0.2 ml) was dropped on a basal layer medium, about 2 ml soft agar containing host cell was overlaid thereupon, and both were mixed well.

Antiphage sera. The anti-phage sera were prepared by the method described in the preceding paper.⁴⁾ The *K* values of the antisera against the above four phages were 15, 75, 50 and 150, respectively.

Media. G-medium as previously reported⁴⁾ was used for growing bacteria and phages. The media used for the adsorption of the phages to their host cell and for the one-step growth experiments were as follows: G₅B₂ or G₅B₅₀ medium consisting of 50 g glucose, 8 g urea, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 4 g corn steep liquor, 2 mg Mn⁺⁺, 2 mg Fe⁺⁺, 100 μg thiamine·HCl, 2 or 50 μg biotin, respectively, and 1,000 ml distilled water; and S medium consisting of 100 g glucose, 8 g urea, 1 g KH₂PO₄, 1 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 100 μg thiamine·HCl, 2 μg biotin,

2 g casamino acid and 1,000 ml distilled water. These media were autoclaved for 15 minutes at 115°C. Dilutions of the phages, and experiments on the ionic requirements in the phage adsorption followed the routine procedures with the dilution buffer (0.01 M Tris-HCl buffer containing 5 g NaCl, 0.05 g MgSO₄·7H₂O, 0.05 g CaCl₂·2H₂O and 0.1 g casein in 1,000 ml, at pH 7.4).

Adsorption. The procedures for the experiments on the adsorption and the one-step growth are comparable to those given by Adams.⁵⁾ The bacterial suspension (about 5×10⁸ cells/ml in G medium) was brought to 30°C, when the phages were added to give a final concentration of 5×10⁵ plaque-forming units (PFU) per ml, and both were mixed well. A 0.1 ml samples of the mixture was removed at the 5-minute intervals and immediately diluted to 1:100 with chilled dilution buffer to prevent further adsorption. The diluted samples were then centrifuged at 10,000 r.p.m. for 5 minutes at 0°C to separate unadsorbed free phages from the adsorbed. The aliquot of the supernatant fluid was accurately assayed on the unadsorbed free phages. The adsorbed phages were estimated by the difference between the initial and the free phage titers at the sampling time.

Another procedure which determined the number of infected bacteria was carried out by using antiphage serum. In the preliminary test, it was confirmed that the antiphage serum had no effect upon the ability of infected bacteria to produce plaques, provided the antiserum was diluted so that its concentration on the plate did not affect the plaque formation.

The multiplicity of infection was 0.001, as above. After mixing bacteria with the phages, 0.1 ml of the mixture was removed at one time and at the 5-minute intervals and diluted to 1:10 in the antiphage serum by an approximate dilution as calculated from the *K* value, to inactivate 99% of the free phages in 5 minutes. After 5 minutes, the sample was further diluted to remove the antiserum, and was plated to determine the number of infected bacteria, which, at each interval, was subtracted from the total phage input in the adsorption tube, to give the amount of the remaining unadsorbed phage.

The adsorption rate constant, *K* (cm³ per min), was calculated from the following equation.⁶⁾

5) M. H. Adams, "Bacteriophages," Interscience Publishers, N. Y., 1959, p. 466.

6) A. P. Krueger, *J. Gen. Physiol.*, **14**, 493 (1931).

4) T. Oki, T. Matsui and A. Ozaki, *This Journal*, **31**, 861 (1967).

$$K = 2.3/(B)t \times \log P_0/P$$

in which P_0 stands for phage titer at zero time; P , for the phage not adsorbed at a specific time (t); (B), for concentration of bacteria by number of cells per ml. Any deviation from the routine techniques is given in the results.

One-step growth experiment. The detailed account of the one-step growth experiment is as follows: A one-tenth ml of a phage solution containing 1×10^7 PFU per ml was mixed with 0.9 ml of bacterial suspension containing 1×10^8 cells per ml (M.O.I. = about 0.01). After adsorption of the phage in 10 min, 0.5 ml of the mixture was added to 4.5 ml of diluted antiserum, and 99% of the unadsorbed free phages was neutralized for 5 min, at 30°C. The neutralized mixture was then diluted to 1:10 in medium, and, immediately after that, 1 ml of the diluted solution was added to 9 ml of a fresh medium in the first tube. Successively, 1 ml from the first tube was added to 9 ml of another fresh medium in the second tube. These tubes were incubated at 30°C in the monod-shaker, and 0.1 ml samples from the first and second tubes were plated by the double-layer, technique, at 2-to-5-minute intervals. After incubation at 30°C overnight, the plaques were counted.

FIG. 1. Adsorption of Four Phages onto Host Bacteria.

- × P465 (host strain: YO2)
- P468II (")
- Ap85III (")
- Δ P4 (" : 468-5SMr)

Incubation was carried out at the P/B ratio of 0.001 in G_5B_2 medium at 30°C.

RESULTS AND DISCUSSION

Effect of medium on adsorption of phages.

The adsorption curves of the four phages in G_5B_2 medium at 30°C are shown in Fig. 1. The phages, P4 and Ap85III, were adsorbed on the host bacteria at the rate of more than 70 per cent in 15 minutes; while the phages, P465 and P468II, were slowly adsorbed at the rate of 50 per cent in 5 minutes and about 65 percent in 15 minutes. The adsorption of P465 in this experiment was slightly slower than that indicated in the previous paper;³⁾ it is considered, however, to be conditional differences on which the experiments have been proceeded.

The adsorption rate of the phage particles was found to be roughly proportional to the concentration of the phage and bacteria. Considering the fact that the number of added bacterial cells greatly exceeds the number of the phage (M.O.I.=0.001) in these experiments, the adsorption process follows the formula of the first order reaction with respect to the concentration of the phage remaining, unadsorbed, in the medium. In this case, the K values for the phages, P465, P468II, Ap85III and P4, were calculated to be 2.02×10^{-10} , 1.87×10^{-10} , 4.32×10^{-10} and 3.15×10^{-10} cm^3 per minute, respectively. On the other hand, the K values vary in a wide range with various types of medium used (Table I). From the above results, the complete medium like G_5B_2 is definitely better than the defined

TABLE I. EFFECT OF MEDIUM ON ADSORPTION RATE CONSTANT OF PHAGES

Phage	Medium	$K (\times 10^{-10})$			
		S (defined media)	G_5B_2	G_5B_2 plus 0.01% CaCl_2	Bouillon
P465		0.72	2.02	4.62	1.68
P468II		0.74	1.87	3.12	1.49
Ap85III		1.00	4.32	7.15	2.70
P4		2.13	3.15	2.98	2.48

Phage-host mixture ($P/B=0.001$) was incubated in the monod-shaker at 30°C.

The K value is an average of at least two data.

medium for the adsorption of the phages, P465, P468II and Ap85III; and the adsorption of the phages was accelerated by the supplement of 0.01 per cent CaCl_2 to G_5B_2 medium. On the contrary, the adsorption of the phage P4 was similar in all these media. Therefore, it is supposed that those three phages require the ionic environment or organic cofactors for adsorption, as described in many reports.⁷⁻¹⁴⁾

With respect to the adsorption of the phages of L-glutamic acid-producing bacteria, Seto et al.²⁾ discussed that the phages, P1, P2, P3, P4 and P5, were adsorbed onto *Mic. ammoniaphilum*-A at the K values of 5.2 to 7.2×10^{-10} cm^3 per minute. These adsorption rate constants are a little larger than those of *Brevibacterium* phages.

Effects of inorganic and organic cofactors on adsorption rate constant (K). Effects of inorganic and organic cofactors on adsorption of the phages, P465, P468II and Ap85III, were investigated. Experiments were made using the dialysed phages to avoid the contamination of monovalent or divalent cation. The dialysed phages were diluted respectively with 0.01 M Tris-casein (0.01%) buffer (pH 7.5) containing to 10^{-2} M to 10^{-3} M monovalent or divalent cations, 0.2% casamino acid (vitamin-free, Nissui Seiyaku Co.), 5% vitamin mixture* and 100 $\mu\text{g}/\text{ml}$ tryptophan, each to contain 3×10^5 PFU per ml; the diluted phages were each mixed with bacterial suspension at the M.O.I. of 0.001. Then, unadsorbed free

TABLE II. EFFECT OF ADSORPTION COFACTORS ON STABILITY OF DIALYSED PHAGE IN TRIS-CASEIN BUFFER

Cofactor	Added conc.	Survival %		
		P465	P468II	Ap85III
KCl	0.01 M	89.5	91.5	67.8
NaCl	0.01	100.8	97.4	85.1
$(\text{NH}_4)_2\text{SO}_4$	0.01	92.5	84.5	87.7
CaCl_2	0.01	104.7	100.5	90.1
MgCl_2	0.01	96.9	97.2	93.4
MnCl_2	0.001	86.3	104.5	89.0
FeSO_4	0.001	0	0	0
ZnSO_4	0.001	70.5	96.8	90.4
CoSO_4	0.001	94.7	101.5	81.4
CuSO_4	0.001	33.6	48.5	23.6
BaCl_2	0.001	98.9	100	93.5
Tryptophan	100 $\mu\text{g}/\text{ml}$	98.0	97.1	83.8
Casamino acid	0.2%	99.3	94.9	96.3
Vitamin mixture	5 %	99.1	97.3	86.6
Control:				
Tris-casein buffer		100	100	100

Incubation: Shaken for 30 min. at 30°C in 0.01 M Tris-casein (0.01%) buffer (pH 7.5) containing respective cofactor.

Initial titer of dialysed phage was 3×10^5 PFU per ml.

phages and infected bacteria were assayed after shaking for 10 minutes at 30°C for adsorption, and the adsorption rate constant, K , was calculated.

Inactivation of free phages by various salts, vitamin or amino acids, which were used as cofactors to study the effect on the adsorption of the phages, was previously tested under the same condition as in adsorption; and the results are given in Table II. The other cofactors except FeSO_4 and CuSO_4 scarcely affected on the free phages, but, because both FeSO_4 and CuSO_4 inactivated free phages considerably, adsorption rate constant was calculated from the number of infected bacteria. Table III shows that for the three phages the various cofactor supplements had significant effect on the adsorption rate constants. The adsorption rate constants, K , of the phages, P465 and Ap85III, increased remarkably in a

7) A. Gratia, *Compt. Rend. Soc. Biol.*, **133**, 702 (1940).

8) P. M. Rountree, *J. Gen. Microbiol.*, **5**, 673 (1951).

9) T. T. Puck, A. Garen and J. Cline, *J. Expt. Med.*, **93**, 65 (1951).

10) L. W. Barksdale and A. M. Pappenheimer, *J. Bacteriol.*, **67**, 220 (1954).

11) E. B. Collins, F. E. Nelson and C. E. Parmelee, *ibid.*, **60**, 533 (1950).

12) M. Delbrück, *ibid.*, **56**, 1 (1948).

13) T. F. Anderson, *ibid.*, **55**, 637 (1948).

14) T. F. Anderson, *J. Cellular Comp. Physiol.*, **25**, 17 (1945).

* It consists of Ca-pantothenate, riboflavin, thiamine-HCl, *p*-aminobenzoic acid, pyridoxine-HCl, pyridoxal-HCl, folic acid, biotin, niacin, adenine, guanine, uracil and xanthine.

TABLE III. EFFECT OF COFACTORS ON ADSORPTION OF PHAGE IN *Br. lactofermentum*
No. 2256—DIALYZED PHAGE SYSTEM

Cofactor	Conc.	Adsorption rate constant $K (\times 10^{-11})$		
		P465	P468II	Ap85III
K ⁺ (KCl)	0.01 M	0.03	0	0.44
Na ⁺⁺ (NaCl)	0.01	0.33	0	1.28
NH ₄ ⁺ ((NH ₄) ₂ SO ₄)	0.01	0.17	0	0.77
Ca ⁺⁺ (CaCl ₂)	0.01	16.4	0.31	7.45
Mg ⁺⁺ (MgCl ₂)	0.01	9.84	0.12	4.67
Ca ⁺⁺ and Mg ⁺⁺	0.01 each	12.3	1.19	5.95
	0.001 "	18.7	2.11	8.10
Mn ⁺⁺ (MnCl ₂)	0.001	0.33	0.12	0.51
Fe ⁺⁺ (FeSO ₄)	0.001	0.26	0	0.11
Zn ⁺⁺ (ZnSO ₄)	0.001	0.23	0	0.58
Co ⁺⁺ (CoSO ₄)	0.001	0.53	0	0.15
Cu ⁺⁺ (CuSO ₄)	0.001	0.40	0	0.27
Ba ⁺⁺ (BaCl ₂)	0.001	0.10	0	0.22
Tryptophan	50 μ g/ml	0.14	0	0.41
	100 "	0.08	0	0.29
Casamino acid	0.2 %	0.54	0	0.51
Vitamin mixture	5 %	0.07	0	0.31
Ca ⁺⁺ and Mg ⁺⁺ , 0.001 M each plus tryptophan	100 μ g/ml	—	3.34	—
or casamino acid	0.2 %	—	4.94	—
or vitamin mixture	5 %	—	3.56	—
Control: Tris- casein buffer		0.14	0	0.62
S medium		5.77	5.85	8.55

Adsorption was carried out for 10 min. at 30°C in 0.01 M Tris-casein (0.01%) buffer (pH 7.5) containing respective cofactor.

B/P ratio: 0.001

concentration (10^{-2} M) of either Ca⁺⁺ or Mg⁺⁺, and the other cofactors did not affect. However, the adsorption of the phage P468II increased in a concentration (10^{-2} to 10^{-3} M) of both Mg⁺⁺ and Ca⁺⁺, and, by adding tryptophan, amino acids or vitamins besides above cofactors, it further increased. Accordingly, from the data it is obvious that the phages, P465 and Ap85III, have specific requirement for either Ca⁺⁺ or Mg⁺⁺; and the phage P468II requires both of them for the adsorption onto the host cell. This is important from a practical standpoint. In many cases the cationic

requirement is known to involve a step subsequent to adsorption,¹⁵⁾ and, in the case of the phage T5, the step is definitely penetration of the phage DNA into the host cell.^{16,17)} In addition to the ionic requirements, there is one case of a relatively specific requirement for an organic cofactor for adsorption, as described on the tryptophan-requiring strains of the phage T4.^{12,13)}

Effect of temperature on adsorption of the phages, P465 and P4. Effect of temperature on the adsorption of the phages, P465 and P4, was investigated. The experiments were carried out at various temperatures ranging from 25°C to 40°C in nutrient broth, and the K value was calculated from the unadsorbed free phages assayed 10 min. later. The results are given in Table IV. Both P465 and P4 were adsorbed at the maximum velocity at 32°C, but the adsorption of P4 was characteristic at the temperatures above 35°C.

The K value calculated from unadsorbed free phages differs quite from that from number of infected bacteria, i.e., the former is the largest at 40°C whereas the latter nearly zero, because adsorbed P4 failed to form the

TABLE IV. EFFECT OF TEMPERATURE ON ADSORPTION

Temperature (°C)	$K (\times 10^{-10})$	
	P465	P4
25	1.10	1.85
28	1.69	2.29
30	2.03	2.13
32	2.31	2.20 (0.88)
35	1.89	1.17 (0.05)
40	0.87	5.76 (0)

Adsorption was carried out for 10 min. at 30°C in nutrient bouillon.

P/B ratio: 0.001

Number in parentheses shows the K values calculated from the number of infected bacteria.

15) M. H. Adams, "Bacteriophages," Interscience Publishers, N. Y., 1959, pp. 141 & 234.

16) R. Wahl, *Ann. Inst. Pasteur*, **72**, 73 (1946).

17) S. E. Luria and D. L. Steiner, *J. Bacteriol.*, **67**, 635 (1954).

plaque above 35°C. This probably means that at the temperature over 35°C unadsorbed free P4 is inactivated, or that P4 adsorbed onto the host cell causes the abortive infection on a stage of the multiplication, or that both may occur. In fact, as described in previous

paper¹⁸⁾ that P4 is labile to temperature, free phage P4 was stable below 36°C but rapidly inactivated at 40°C (Fig. 2). Although the effect of temperature on the multiplication of above phage will be discussed later, its plaque-forming ability was suppressed at above 32°C as shown in Fig. 3, when the plates, which were double-layered with phages and the host cells, were incubated at various temperatures, whereas the host bacterium and the phage P465 grew.

Furthermore, investigation on the effect of the host cell, i.e., the B₂ cells grown in biotin-deficient medium (20 µg/l) and the B₅₀ cells grown in biotin-sufficient medium (50 µg/l), on the phage adsorption were made. A large amount of evidence has indicated that the action of biotin would be closely related to the metabolism of fatty acids, to the cellular

FIG. 2. Effect of Temperature on the Free P4 Phage.

Incubation was carried out in nutrient bouillon (pH 7.4).

FIG. 3. Effect of Incubation Temperature of Plate on Plaque Formation.

Doubled-layered plates were incubated at various temperatures for 20 hours.

FIG. 4. One-Step Growth Curves of Four Phages.

Incubation: Shaken at 30°C in G₅B₂ medium after adsorption for 10 min. at 30°C. M.O.I.=0.01

- × P465
- P468 II (●: M.O.I.=5)
- △ Ap85III
- P4

18) T. Oki, T. Matsui and A. Ozaki, This Journal, **31**, 1040 (1967).

permeability and the structure of cell wall in glutamic acid-producing bacteria.¹⁹⁻²⁴⁾ However there was little difference in the adsorption rate constants between the B₂ and B₅₀ cells. That is, the *K* value of the phage P465 was 2.78×10^{-10} on the B₂ cells and 2.94×10^{-10} on the B₅₀ cells.

Determination of latent period and burst size by means of one-step growth experiments. Typical one-step growth curves of the four phages are presented in Fig. 4. The curves gave the values for the latent period, the average burst size and the rise period, as given in Table V. Among the four phages, differences were notable in both the average burst size (31~160) and the rise period (24~48 min.), but in the latent period the difference of values (47~59 min.) was very small. The resulting

TABLE V. BURST SIZE AND LATENT PERIOD OF FOUR PHAGES BY ONE-STEP GROWTH EXPERIMENTS

Phage		Latent period (min.)	Rise period (min.)	Burst size
P465	B ₂	50	48	98
	B ₅₀	51	39	100
P468II		53	29	31
	M.O.I.=2	58	35	80
	M.O.I.=5	59	38	160
Ap85III		57	24	145
P4		47	40	126

Each value is an average of five experiments.

Incubation: Shaken at 30°C in G₅B₂ medium after adsorption for 10 min. at 30°C.

M.O.I.: 0.01

B₂: B₂ cell (grown in biotin-deficient medium, 2 μg/l)—G₅B₂ medium system.

B₅₀: B₅₀ cell (grown in biotin-sufficient medium, 50 μg/l)—G₅B₅₀ medium system.

19) W. Trager, *J. Bacteriol.*, **56**, 195 (1948).

20) I. Shiio, S. Otsuka and M. Takahashi, *J. Biochem.*, **51**, 56 (1962).

21) M. Shibukawa and T. Ohsawa, *This Journal*, **30**, 750 (1966).

22) K. Takinami, Y. Yamada and H. Okada, *This Journal*, **31**, 223 (1967).

23) K. Kono, Y. Iijima, T. Oki, N. Miyachi and A. Ozaki, *Amino Acid and Nucleic Acid*, **11**, 99 (1965).

24) I. Shiio, S. Otsuka and N. Katsuya, *J. Biochem.*, **53**, 333 (1963).

values (average burst size and latent period) for P465 in these data (Table V) are remarkably different from the results given in the previous data.³⁾ This may be due to unsatisfactory procedural conditions in experiments; i.e., in the previous report,³⁾ antiphage serum was not used and the number of experiments was limited only to a few. Seto et al.²⁾ estimated the latent period as 40 to 140 minutes and the burst size 15 to 150 on five phages of *Mic. ammoniaphilum* under their conditions. It seems that there is great variety in the latent period and the average burst size among the phages of L-glutamic acid-producing bacteria.

Delbrück²⁵⁾ and Heden²⁶⁾ indicated that the latent period and the burst size are strongly affected by the physiological state of the host cell. However, in the one-step growth experiments with P465, the physiological dif-

FIG. 5. Effect of Temperature on Multiplication of Phages P465 and P4.

Incubation: Shaken at indicated temperature (●: 30°C, ○: 34°C, △: 36°C) in G₅B₂ medium after adsorption for 10 min. at 30°C.

-----: P465 ———: P4

25) M. Delbrück, *Biol. Revs. Cambridge Phil. Soc.*, **21**, 30 (1946).

26) C. Heden, *Acta. Pathol. Microbiol. Scand.*, Suppl. No. 88. (1951).

ference of the host cells between the B₂ and B₅₀ cells has not influenced upon both the latent period and the burst size, but only the rise period was shortened in the biotin-sufficient cells (Table V). With the phage P468II, the average burst size varied with the multiplicity of infection; that is, 30 with the single infection, 80 with M.O.I. 2 and 160 with M.O.I. 5, but the latent period did not change.

Effect of temperature on the latent period and the burst size. The one-step growth experiments were carried out at the temperatures of 30, 34 and 36°C with the phages, P465 and P4. The results are given in Fig. 5. Although the temperature generally affects the latent period of the generation time of bacteria,^{1,27)} the latent period of the phage P465 was scarcely influenced at 36°C. On the contrary, the growth of the phage P4 was completely suppressed even at 34°C, although the

host bacteria was capable of the full growth at the same temperature. This conformed with the results given in Fig. 3, in which the plaque-forming ability of the phage P4 was extremely suppressed at above 32°C, and it may be explained by such a temperature sensitivity of phages as was examined with *ts* mutant, which was developed by Campbell²⁸⁾ and Edgar et al.^{29,30)}

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27) M. Hongo, T. Miyamoto and A. Murata, This Journal, **30**, 500 (1966).

28) A. Campbell, *Virology*, **14**, 22 (1961).

29) P. S. Edgar, G. H. Denhardt and R. H. Epstein, *Genetics*, **49**, 635 (1964).

30) P. S. Edgar and I. Lielansis, *ibid.*, **49**, 649 (1964).