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Factors influencing organism-bacteriophage populations

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NOTE TO USERS

FACTORS INFLUENCING ORGANISM-BACTERIOPHAGE POPULATIONS

by

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Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

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INTRODUCTION

Commercially, bacteriophage is important in bacterial processes wherever the infection of the host culture by the phage can adversely affect the production of useful products by the host. Cultures of lactic streptococci are used for converting lactose into lactic acid, which in turn plays an important role in the manufacturing processes for a number of dairy products. The successful production of various types of cheeses, butter cultures and cultured milks may be prevented by the failure of proper acid development. Probably the most common cause of slow acid production is bacteriophage. In light attacks by bacteriophage the bacteria may multiply rapidly enough to produce sufficient acid to complete the process; however, the time required usually is somewhat longer than normal. In severe attacks, the organisms may be destroyed almost completely in the very early part of the process, with the result that acid production may cease entirely.

Measures recommended for control of bacteriophage consist of sanitation, rotation of cultures and special care in handling cultures. These procedures have been effective only to a limited extent. Frequent outbreaks which cause rather severe monetary losses to the industry still are encountered.

Recent studies have brought forth much useful information regarding the fundamental aspects of the organism-

bacteriophage relationship. The purpose of this investigation was to study certain factors influencing the organism-bacteriophage populations with the belief that the results might have an important bearing upon the solution of the bacteriophage problem.

REVIEW OF LITERATURE

The review of literature in this thesis has been confined to those references dealing specifically with the subject of bacteria-bacteriophage populations in the case of organisms of the lactic streptococcus group, except where explanations for some of the phenomena encountered were available in published material concerning other bacteriophage-organism combinations. Many general review articles, from which a good summary of information on bacteriophages may be obtained, have appeared. The early reviews include those of d'Herelle (1926), Hadley (1928), Bronfenbrenner (1928), Burnet (1934), Krueger (1936) and Delbrück (1942). More recent reviews include those of Flu (1946), Craigie (1946), Delbrück (1946), Anderson (1946), Price (1948), Cohen (1949), Fong (1949), Lauffer et al. (1949) and Anderson (1950). Most of these reviews include a lengthy list of references.

Unfortunately, a good general review dealing with bacteriophage active on the group of lactic streptococci has not been published, but for a comprehensive survey of the subject, one may consult the papers by Nelson (1936), Nelson et al. (1939), Johns and Katznelson (1941), Whitehead and Hunter (1941, 1946, 1947), Meanwell (1941), Nichols (1945), Nichols and Ineson (1947), Nichols and Hoyle (1949), Babel (1946, 1947), Greene and Babel (1948), Turner (1948), Parmelee et al. (1949),

Collins (1949), Wilkowske (1949), Bennett (1950) and Overcast (1950) and the references listed by these authors.

Babel (1948) conducted studies to determine the relationship between the number of Streptococcus lactis organisms used to inoculate given lots of milk and the bacteriophage titers of the milks when the initial inoculations with the bacteriophage were kept constant. He found that as the amount of inoculum was decreased from 1.0 to 0.001 percent, the final concentration of bacteriophage produced in the milks decreased. When the initial inoculations of S. lactis were kept constant, and amounts ranging from 10^{-1} to 10^{-7} ml. of the bacteria-free filtrates active against the culture were used, the final bacteriophage titers of the milk remained the same.

Turner (1948) made a similar but more detailed study of the effect of varying the concentration of organism and bacteriophage upon the multiplication of the organism and the bacteriophage. In single trials using one combination of organism-bacteriophage, the bacteriophage concentration was held relatively constant at approximately 10 particles per ml., while the plate count of the organism was varied, levels of 1,000, 10,000 and 1 million per ml. being used. In other trials using the same organism-bacteriophage combination, the organism concentration was held constant at a count of approximately 100,000 per ml. and the bacteriophage concentrations used were 1,000, 10,000 and 1 million particles per ml. Results of this study showed that increasing the cell concentra-

tion shortened the bacteriophage lag period, while increasing the bacteriophage concentration resulted in a decreased bacterial population at the time of lysis. This author stated that additional studies would be necessary before any very general conclusions on the effects of such variations could be drawn.

Collins (1949), in studying the relationship of the nutrition of S. lactis to bacteriophage proliferation, found that a close relationship appeared to exist between the nutrition of organisms of the S. lactis group and the multiplication of their homologous bacteriophages. Specific mention was made of the fact that soluble calcium, while of no detectable importance to organism growth, seemed to be required for the multiplication of many bacteriophages.

A detailed study of the influence of pH on proliferation of S. lactis bacteriophage was made by Overcast (1950). Using five organism-bacteriophage combinations, he found that the optimum pH for lactic streptococcus bacteriophage proliferation was at about pH 6.5, although considerable proliferation occurred over a pH range of 5.4 to 7.5 for all strains. He found little correlation between the sensitivity of the organisms to high or low pH levels and the sensitivity of the homologous bacteriophage.

In studying changes in bacteriophage and S. lactis populations, Nelson and Parmelee (1949) found that bacteriophage proliferates at a greater relative rate than the sensitive

S. lactis at temperatures favorable for bacteriophage. The optimum temperature for both was 32° C. Each bacteriophage proliferation curve showed a lag phase, a logarithmic growth phase and a maximum stationary phase. The maximum level of bacteriophage was approximately 10^9 bacteriophage particles per ml., irrespective of strain, host organism and some variation in temperature.

The action of bacteriophage on a sensitive culture usually results in the origin of secondary growth organisms. For many years, the phenomenon of secondary growth has been observed by various workers. Gratia (1921) observed that, after subjecting a culture of Bacillus (Escherichia) coli to the action of bacteriophage on an agar slant, unusual vitreous colonies were found. Transplanted material from these colonies gave growth which possessed great resistance to the lytic agent. Dessicated material surrounding the vitreous colonies gave no growth in broth. The author found that merely allowing the normal culture to age also was a method of artificial selection of resistant bacteria.

Later d'Herelle (1922) noted that staphylococcus cultures in broth medium, when acted upon by bacteriophage, showed a clearing after 5 hours but later became cloudy again. He correctly attributed the recurrence of bacterial growth to the presence of resistant organisms which the bacteriophage was unable to lyse.

Burnet (1929) was among the first of those who favored

the idea of spontaneous origin of bacterial mutants. This idea later was abandoned in favor of the theory that the virus acts as a selective agent which permits the mutant type to develop but does not induce the production of the resistant bacterial variant.

In her investigations concerning hemolytic streptococci, Evans (1934) found that secondary cultures resulting from the action of bacteriophage, whether derived from secondary colonies on lysed agar plates or from secondary growth in broth cultures, usually were resistant to lysis by the bacteriophage in the presence of which such secondary growth had occurred.

The mechanism for the origin of virus-resistant bacteria was established by Luria and Delbrück (1943). Their analysis of the distribution of the numbers of virus-resistant bacteria in a series of similar cultures of a virus-sensitive strain of E. coli validated the hypothesis that the resistant bacteria arise by mutation of the sensitive cells independently of the action of the virus. The virus, therefore, in destroying the sensitive cells serves as a selective rather than a causative agent.

Whitehead and Cox (1935, 1936), in their work with cheese cultures, had hoped to take advantage of the appearance of resistant secondary organisms in cultures by developing strains from the secondary organisms which would be immune to bacteriophage attacks. These authors concluded that secondary organisms were temporarily resistant but gradually became susceptible

to the bacteriophage with which they were produced, and that they were subject to attack by other strains of bacteriophage. Similar observations were reported by Nelson (1936), Whitehead and Hunter (1937), Nelson et al. (1939) and Anderson and Meanwell (1942).

Ford and Babel (1950) found that secondary growth organisms resulting from the action of bacteriophage on a culture of S. lactis appeared to be similar to the parent culture in morphology, colony characteristics and ability to ferment sugars. They also noted that secondary organisms were resistant to the bacteriophage type causing incomplete lysis of the parent culture but were sensitive to at least one other bacteriophage type.

Turner (1948), in a study of the dynamics of the populations of lactic organisms and bacteriophage on five organism-bacteriophage combinations, found that secondary growth became apparent immediately after completion of mass lysis of the susceptible cells with four of the combinations being tested. With one combination, secondary growth was not detectable, even after continued incubation for two weeks. Of those combinations showing secondary growth, the rate of growth of secondary cells was approximately the same as that of the sensitive culture before lysis occurred. The counts of the secondary growth of the five combinations at the end of mass lysis were <1, 250, 125, 300 and 1,000 per ml.

Nelson and Parmelee (1949), in studying organism-bacteriophage proliferation, found that different combinations used gave counts of secondary organisms varying from zero to 10,000 per ml. at the time of mass lysis.

EXPERIMENTAL METHODS

Preparation of Media

Litmus milk used for carrying S. lactis cultures and for the enumeration of bacteriophage was prepared by adding enough aqueous litmus solution to pasteurized skim milk to impart a bluish-purple color. To this milk was added 10 percent by volume of strained V-8 juice*. During the early part of the investigation, it was found that two of the organisms selected for study, 459 and 799, required longer than the usual period of time to produce normal coagulation of the litmus milk. This difficulty was remedied by adding 0.2 percent of glucose and 0.5 percent of trypticase** to the fortified litmus milk. After thorough mixing, the medium was dispensed in 8 ml. quantities into test tubes and sterilized under 15 pounds steam pressure for 15 minutes.

An early study of methods indicated that erratic results were obtained by the plate count method during the first few hours of the test runs when tomato juice agar or tryptone-glucose-extract-milk agar was used as the medium for the

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*A combination of vegetable juices prepared by the Campbell Soup Company, Camden, N. J.

**A pancreatic digest of casein.

enumeration of the bacteria selected for this investigation. Uniform counts were obtained after turning to the use of a relatively new medium, modified trypticase soy agar. This medium was the experimental modification of trypticase soy agar made from the formula suggested by the Baltimore Biological Laboratory, Inc., Baltimore, Md. The medium was prepared from the individual ingredients by dissolving with heating 15 g. trypticase, 5 g. phytone*, 0.2 g. L(-)cystine, 4 g. sodium chloride, 1 g. sodium citrate, 15 g. agar and 5 g. glucose in one liter of distilled water. Special care was taken to insure complete solution of the cystine. The mixture then was adjusted electrometrically to pH 7.0. Quantities of 100 ml. were measured into screw cap bottles and sterilized under 15 pounds steam pressure for 15 minutes. The pH after sterilization usually was about 6.8.

The substratum for test samples of bacteria plus bacteriophage and of control bacteria was prepared by dispensing 150 ml. quantities of pasteurized skim milk in screw cap bottles and sterilizing under 15 pounds steam pressure for 25 minutes.

Organism-Bacteriophage Combinations

The organism-bacteriophage combinations were selected from the Dairy Bacteriology Laboratory collection at Iowa State College. The sources and designations of the organisms are

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*A papaic digest of soybean.

listed in table 1, and the same information relative to the bacteriophages is shown in table 2.

To test the susceptibility of the streptococcus cultures to the homologous bacteriophages used, first the cultures were plated on trypticase soy agar, and after incubation at 32° C. for 48 hours, 25 well-separated colonies from each of six streptococcus strains were picked at random into sterile litmus milk. After incubation at 32° C. for 20 hours, each culture was tested for its susceptibility to its purified homologous bacteriophage. Two tubes of sterile litmus milk were inoculated with one drop of susceptible culture to each tube. To one of the pair, one drop of purified homologous bacteriophage filtrate was added. Pairs of tubes thus were set up with each culture isolated from the agar plate. Subsequent incubation at 32° C. overnight indicated the susceptibility of the selected culture to the purified bacteriophage. When the tube of litmus milk containing both bacteriophage filtrate and organism failed to coagulate at the same rate as the control tube to which no bacteriophage filtrate had been added, the culture was considered susceptible to the inoculated bacteriophage. All of the isolated colonies from each strain of culture used in this study were susceptible to each respective purified homologous bacteriophage. Therefore, the strain of streptococcus comprising a given culture was considered sufficiently pure for the purpose of this study.

Table 1.

Sources and designations of organisms

Organism designation	Source
H1-10	Iowa State College
H1-1	Iowa State College
122-1	Iowa State College
459	Dr. H. R. Whitehead, Dairy Research Institute, Palmerston North, New Zealand
ML1	
799	Mr. E. B. Anderson, United Dairies, Ltd., London W.12, England

Table 2.

Sources and designations of bacteriophages

Bacteriophage designation I. S. C.	Original	Date obtained	Source
PF11	PF11	Unknown	Iowa State College
F60	10	4-20-48	Dr. H. R. Whitehead, Dairy Research Institute, Palmerston North, New Zealand
F61	34	4-20-48	
F63	38	4-26-48	
F69	46	4-27-48	
F52	459	4-10-48	Mr. E. B. Anderson, United Dairies, Ltd., London W.12, England
F57	799	4-16-48	
F68	1p5	4-27-48	Miss Agnes A. Nichols, National Institute for Research in Dairying, Reading, England

One of the purified cultures of each strain was selected at random to be used as the test culture in the subsequent study.

To test the activity of the eight strains of bacteriophage against the corresponding purified culture susceptible to each, two isolated plaques for each bacteriophage were removed by means of a sterile loop from a plaque plate prepared according to the method of Turner (1948), and each was added to a tube of sterile litmus milk to which one drop of the corresponding susceptible culture previously had been added. A control for each was made by inoculating a tube of litmus milk with the corresponding susceptible strain of streptococcus, but with no added bacteriophage. After incubation for 20 hours, each of the control tubes showed normal coagulation. Each of the tubes to which bacteria and material from an isolated plaque had been added showed the bacteriophage was active against its corresponding culture. One of each of the pairs of tubes from each of the eight bacteriophage strains tested which showed activity then was used to prepare a large volume of filtrate. Each of eight screw cap bottles containing 150 ml. of sterile skim milk was inoculated with 1 ml. of the susceptible culture and five drops of the litmus milk showing bacteriophage activity for that particular culture. Whey filtrates were prepared after incubating the samples at 32° C. for 8 hours. The resulting filtrate from each of the eight purified bacteriophages again was tested for activity against its corresponding purified

streptococcus culture. In every test, the filtrate was active against its corresponding culture.

To further insure that each purified bacteriophage was active against the purified culture with which it later was to be used, each of the filtrates was tested for activity against each of the cultures. The results of these tests are shown in table 3. These results are in fairly close agreement with those of Wilkowske (1949), and they serve as a basis for assumption that the bacteriophage which had undergone the plaque purification procedure and the activity test was sufficiently pure for the purpose of this investigation.

Table 3.

Cross-reactions of six cultures of lactic streptococci and eight bacteriophage filtrates

Lactic streptococcus cultures	Bacteriophage filtrates							
	PF11	F52	F57	F60	F61	F63	F68	F69
H1-10	+	-	-	+	+	-	+	-
459	-	+	-	-	-	-	-	+
H1-1	-	+	-	-	-	-	-	+
ML1	-	+	-	-	-	-	-	+
799	-	-	+	-	-	+	-	-
122-1	+	-	-	+	+	-	+	-

+ = Sensitive
- = Resistant

Propagation of Cultures

The cultures were propagated in fortified litmus milk*. All cultures were subcultured daily and incubated at 32° C. for 12 to 15 hours, depending upon the firmness of the coagulum, throughout the course of this investigation. When properly coagulated, the cultures were held at 3 to 5° C. in a refrigerator.

Propagation and Storage of Bacteriophage

The propagation of a bacteriophage was accomplished by allowing it to proliferate on a susceptible host culture. The propagation procedure consisted of inoculating 150 ml. of sterile skim milk with 1 ml. of the susceptible culture and 5 drops of an active strain of bacteriophage. After incubating for 8 to 10 hours, the mixture was acidified with 5.5 ml. of sterile 10 percent lactic acid. The acidified material then was filtered aseptically through sterile coarse filter paper to remove the coagulated casein. The resulting filtrate then was filtered aseptically through a sterile Selas microporous porcelain filter (porosity #03) to obtain a cell-free whey filtrate containing the bacteriophage. The filtrates were stored at 3 to 5° C. during the period of use.

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*Fortified with 10 percent V-8 juice, 0.2 percent glucose and 0.5 percent trypticase.

Determination of Bacterial Populations

Plate counts of the bacteria were made following the procedure as outlined in Standard Methods (American Public Health Association, 1948), except that trypticase soy agar was used as the plating medium. The plates were incubated at 32° C. for 48 hours.

Determination of Bacteriophage Populations

Quantitative enumeration of bacteriophage may be accomplished by two methods. Either the two-layer agar plaque plate method of Delbrück (1945), later modified by Turner (1948), or the limiting dilution technique used by Harriman (1934) and by Nelson (1936) may be used. Krueger (1930) stated that accuracy of the limiting dilution method as applied to staphylococcal bacteriophage enumeration under carefully controlled conditions was ±5 percent. Turner (1948) found that satisfactory results could be obtained by either of two methods. A preliminary study of methods indicated that the limiting tube dilution method could be used advantageously in this work; therefore, this method was adopted and used in all trials reported herein.

This method consisted of making 100-fold dilutions, in sterile distilled water, of the medium being tested for an unknown number of bacteriophage particles and measuring 1.0 and 0.1 ml. quantities of the diluted medium into triplicate

tubes of litmus milk each containing 8 ml. quantities. The actual successive dilutions therefore were ten-fold. These tubes containing the diluted bacteriophage-containing material and one control tube containing no diluted medium then were inoculated with 1:10 dilution of the susceptible organism (Collins, 1949) and incubated at 32° C. for 12 to 16 hours. At the end of the incubation period, the last tube which did not show the typical acid, coagulation and reduction of the control tube was recorded. The bacteriophage titer of the original medium then was determined by referring to McCrady's tables of most probable numbers, as quoted by Buchanan and Fulmer (1928).

Procedure for a Representative Trial

A stepwise description of the methods used in carrying out a representative trial is presented at this point in order to give the reader a clearer understanding of the procedure. Two 150 ml. bottles of sterile skim milk were used for test purposes. Prior to beginning the trial, the calculated amount of bacteriophage material was added to one of the bottles of test medium upon which a bacteriophage titer then was made in order to insure that approximately the desired number of bacteriophage particles was present in the sample. Both bottles then were tempered in a constant-temperature water bath thermostatically controlled at 32 $\pm$0.1° C. To each of the bottles then was added the

the proper dilution of the test organism to give approximately the desired number of bacterial cells. The test material therefore consisted of two samples, the control sample which contained only the test organism and the test sample which contained the test organism-bacteriophage mixture.

Immediately following the addition of the test organism, 1 ml. aliquots were withdrawn from each of the bottles to determine the number of cells in the control sample and the number of non-lysed cells and the number of bacteriophage particles in the test sample. The enumerations made at this time, following procedures previously described, were those of zero hour. Subsequent determinations were made on the hour at intervals calculated to include the 2 hours immediately preceding and the 2 hours immediately following mass lysis.

Additional determinations were made at 16 and at 20 hours to determine the number of resistant organisms, if any, which had survived action of the bacteriophage.

Determination and Adjustment of pH

All pH determinations were made electrometrically with a Beckman pH meter, employing the glass electrode. The values obtained served merely as a guide to indicate the progress of the trial being carried out and were not included in the data presented.

It was necessary to adjust the pH in two instances. When approximately 10 million and 100 million cells were added to the test medium at zero hour, the pH was not allowed to drop below 6.0 in order that mass lysis might occur. Adjustment of pH was made by the addition of 1 N NaOH.

EXPERIMENTAL RESULTS

Effect of Varying Organism-Bacteriophage Ratio Upon the Counts of the Organism and of the Bacteriophage

The counts per ml. of organisms and bacteriophage used in this study were varied in three ways: first, by varying the quantity of countable bacteriophage particles added to an approximately constant count per ml. of bacterial cells; second, by using an approximately constant count per ml. of bacteriophage particles and varying the count per ml. of bacterial cells; and third, by varying both the count per ml. of bacterial cells and the count per ml. of bacteriophage particles to maintain approximately the same ratio of organism to bacteriophage. One organism-bacteriophage combination (799,F57) was used in all trials under this heading and the data are shown in figures 1 through 14.

Figures 1 through 4 show the effect of varying the initial count of bacteriophage using approximately 25, 2,500, 95,000 and 15 million particles per ml., while holding the initial count of the bacteria at approximately 200,000 to 250,000 cells per ml.

For the initial trial, the organism count was approximately 250,000 cells per ml. and the bacteriophage count was

approximately 25 particles per ml. (figure 1). Mass lysis occurred between 5 and 6 hours. The count of resistant organisms in the organism-bacteriophage mixture was 217 per ml. at 6 hours, while the count of bacteriophage reached nearly to its maximum of 950 million per ml. at the same time. This result agrees with the findings of Turner (1948), who showed that bacteriophage proliferation usually ceased when cell division stopped. It required 14 hours after mass lysis for the resistant organisms to attain a count equal to that of the parent organisms at zero hour. This rate of increase was considered moderately rapid, although the rate of increase was somewhat less rapid than for the control bacteria during the earlier stages of growth. The multiplication of the control bacteria was considered normal for a culture growing actively under favorable conditions.

The effect of further increasing the initial count of bacteriophage particles per ml. of milk while using an approximately constant count of bacterial cells is shown in figures 2, 3 and 4. A close relationship existed between the counts of the bacteriophage particles added and the time required for mass lysis. When the bacteriophage numbers used were 2,500 and 95,000 particles per ml., the respective times required for mass lysis were 4 and 3 hours. A probable explanation for the earlier occurrence of mass lysis when the count of bacteriophage particles was increased was that less time

was required for the number of bacteriophage particles to reach the same level as that of the bacterial cells. When the initial count of the bacteriophage particles was increased to 15 million per ml. (figure 4), only a slight increase in the count of bacteriophage particles was noted, and mass lysis began to occur during the first hour. In this instance, with a high initial inoculation of bacteriophage particles, adsorption of the bacteriophage particles probably occurred rapidly on essentially every cell and mass lysis apparently began before counts were made at the end of the first hour and before appreciable proliferation of the bacteria had occurred.

Final bacteriophage counts were the greatest when the initial count of bacteriophage was lowest. This can be explained by pointing out that with a low initial number of bacteriophage particles, the length of time required for the bacteriophage particles to equal the number of bacterial cells was increased. In the meantime, the number of bacterial cells continued to increase during the bacteriophage "lag", thus providing more substratum for bacteriophage proliferation.

With the samples for counts being taken at hourly intervals in these trials, it must be assumed that the counts of the bacteria with added bacteriophage closely followed the counts of the control bacteria, and that in those trials where the counts of the bacteria with added bacteriophage were lower than those of the control bacteria at the apparent time of

initiation of mass lysis, there was indication that mass lysis actually had begun some time before the hourly determination was made.

Figures 5 through 9 show the effect upon population changes resulting from varying the initial count of the bacteria while holding the initial count of bacteriophage particles constant. In general, the patterns obtained for the counts of control bacteria show that the count at any given time up to the time of maximum count depended upon the count per ml. of the initial inoculum. When the initial count was as low as 300 per ml. (figure 5), the time required to reach the maximum count was quite prolonged. As the initial count per ml. was increased, the time required for reaching the maximum count was reduced (figure 8). One exception to this condition occurred when the initial inoculation was abnormally high, as is shown in figure 9. As the count approached 100 million cells per ml., rapid growth of the culture did not occur, only a slow and slight increase in population occurring before some decline set in. This occurrence was significant because mass lysis failed to occur and the count of the bacteriophage remained abnormally low, never exceeding 100,000 per ml., while in other trials where bacterial growth was normal, bacteriophage counts reached much higher levels. This situation is in accordance with the generally accepted view that active cell division is necessary for bacteriophage

multiplication. When this trial was repeated, the results obtained followed closely those shown in figure 9.

Apart from the exception just mentioned, the levels of bacteriophage population reached were practically identical in the other trials (figures 5 through 8). The data show that as the initial count of bacteria was increased, the rate of bacteriophage multiplication also increased, and the time required for the initiation of mass lysis was decreased. This result can be explained by pointing out that the increased rate of bacteriophage proliferation was due, in part at least, to the presence of large numbers of active cells upon which the bacteriophage could proliferate, made available by the successively greater initial inocula of bacteria. In turn, the increased rate of bacteriophage proliferation allowed the number of bacteriophage particles to equal that of the bacterial cells sooner, thus initiating mass lysis more quickly. Allowing for slight experimental variations, it appeared that at least 10 million bacteria per ml. were necessary before mass lysis could occur under the conditions of this experiment, irrespective of the initial number of bacteria. Neither increasing the initial count of bacteriophage nor increasing the initial count of bacteria seemed to influence appreciably the count of secondary growth organisms at the completion of mass lysis, as shown by figures 1 through 8. In each trial, the number of survivors appeared to have been a relatively constant

segment of the number of cells present at the initiation of mass lysis; in no case did the number of survivors exceed 0.002 percent of the number of bacteria present at the initiation of mass lysis.

The effect of varying both the count of organisms per ml. and the count of bacteriophage particles per ml. in approximately the same ratio is shown in figure 6 and figures 10 through 14. The bacteriophage counts were 0.9, 25, 950, 2,500, 25,000 and 450,000 particles per ml. The corresponding counts of bacteria were 100, 15,000, 150,000, 800,000, 13 million and 100 million per ml. The ratio was approximately one bacteriophage particle to 1000 bacteria in most cases.

The data show that the time required for the initiation of mass lysis was influenced by the numbers of bacteria and bacteriophage. Progressively increasing the initial number from a count of 10 bacteriophage particles per ml. and 10,000 bacteria per ml. to a count of 10,000 bacteriophage particles per ml. and 10 million bacteria per ml. resulted in mass lysis occurring nearly 5 hours earlier with the higher initial inocula. In nearly every trial, the maximum bacteriophage count occurred at the same time that the end of mass lysis was reached. In general, the number of secondary growth organisms following mass lysis was relatively constant, considering the limits of the method used for their enumeration, except in that trial in which the initial counts of bacteria and bacteriophage were 10,000 and 10 million, respectively (figure 13). The count

of survivors in this trial was only 12 per ml. Since this trial was not repeated, one can not attach great importance to this unexpected variation. The data in figure 14 show that mass lysis failed to occur when the initial counts of bacteriophage and bacteria were increased to 450,000 and 100 million per ml., respectively. At this high level of organisms, apparently the rate of cell division was insufficient to sustain bacteriophage proliferation, as only a small and unsustained increase in bacteriophage count was observed. A parallel situation in which the initial count of the bacteria was 100 million per ml. in the test medium was pointed out earlier (figure 9). Here again, it appeared that in order for bacteriophage to multiply rapidly, the susceptible cells must be in a state of active division.

Throughout the course of these trials, two important observations were made: first, under the conditions of this experiment, no matter what the initial count of the bacteria might have been at the beginning of the trial, the proliferation of the bacteria apparently must reach a certain minimum level, usually about 10 million per ml., before mass lysis can occur, except when the initial bacteriophage count is approximately equal to, or greater than the initial organism count; second, considering the limitations of the method used for enumeration, the number of secondary organisms immediately following mass lysis appeared to have been a fairly constant segment of the organisms present at the onset of mass lysis.

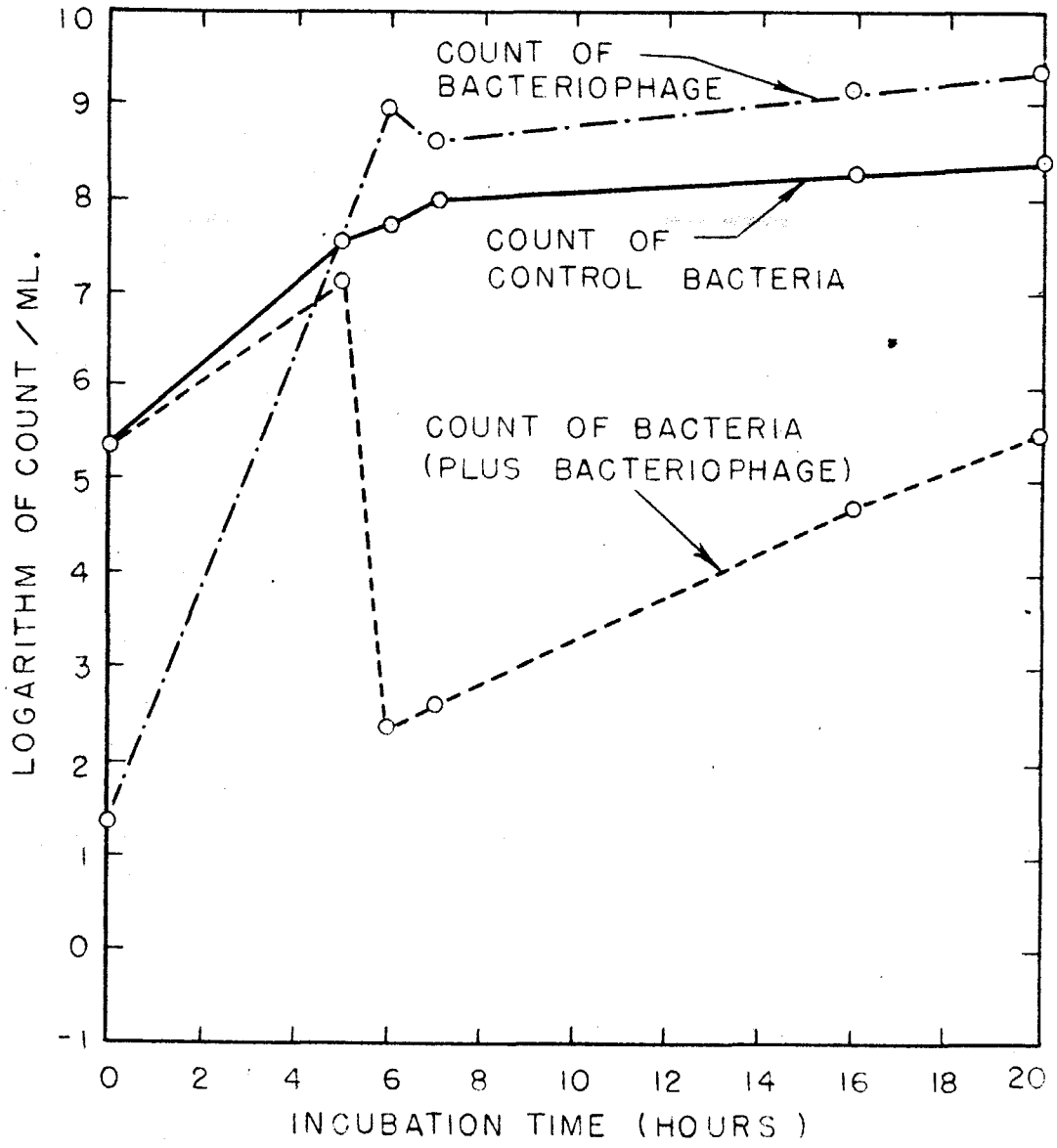

Fig. 1. Population changes of combination 799-F57 with initial bacteriophage count of 25 per ml. and initial bacteria count of 250,000 per ml.

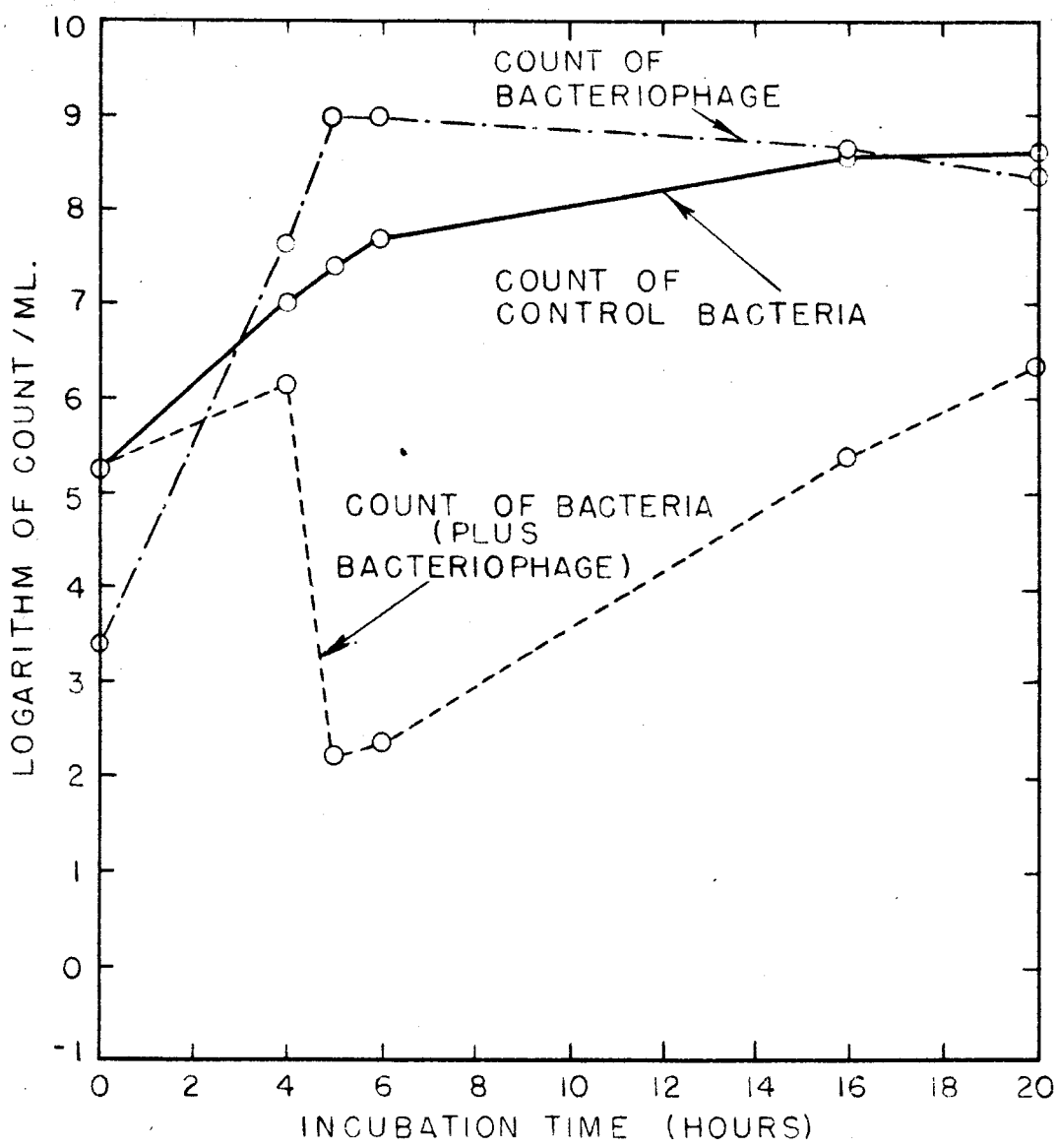

Fig. 2. Population changes of combination 799-F57 with initial bacteriophage count of 2,500 per ml. and initial bacteria count of 190,000 per ml.

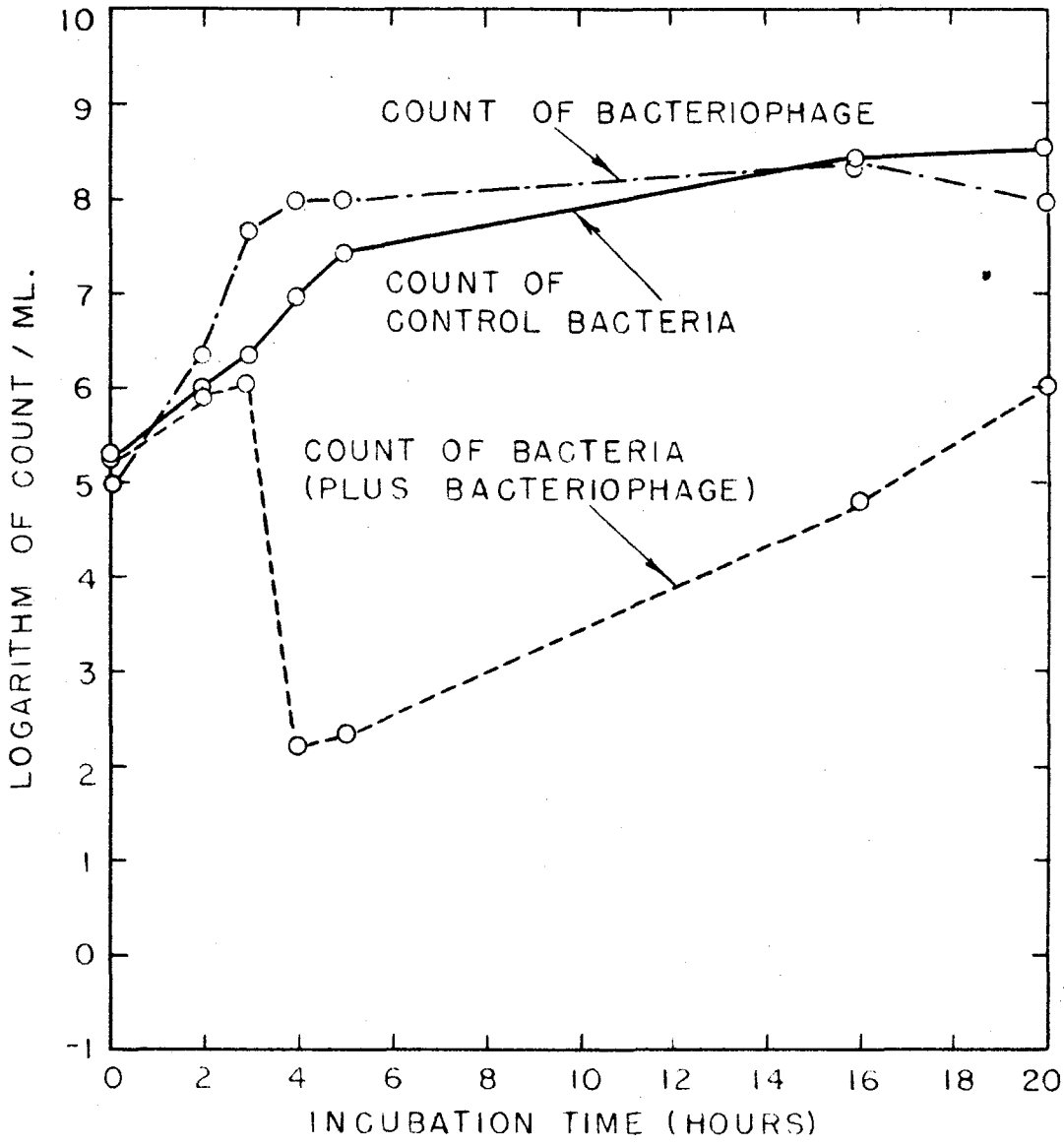

Fig. 3. Population changes of combination 799-F57 with initial bacteriophage count of 95,000 per ml. and initial bacteria count of 160,000 per ml.

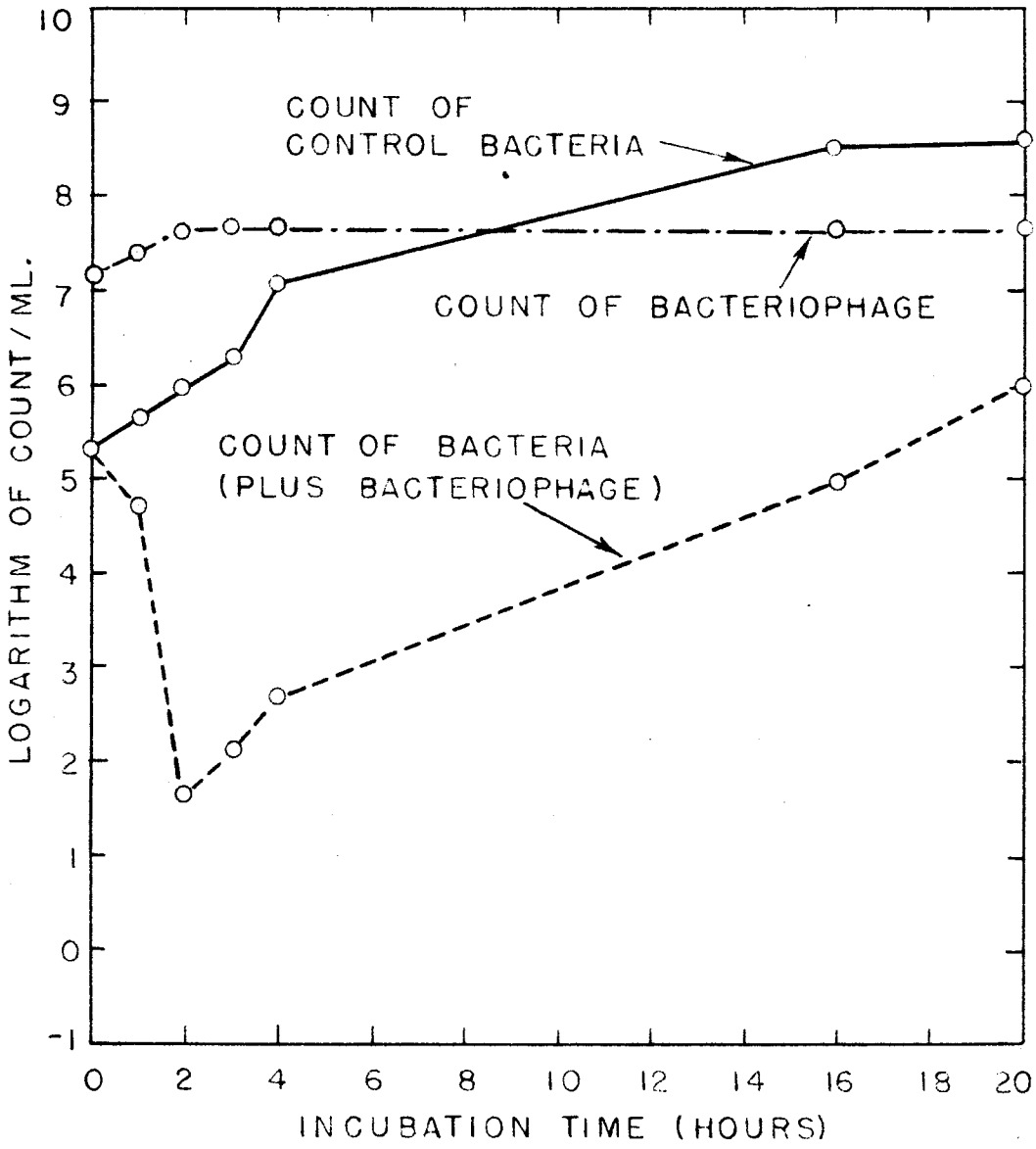

Fig. 4. Population changes of combination 799-F57 with initial bacteriophage count of 15 million per ml. and initial bacteria count of 200,000 per ml.

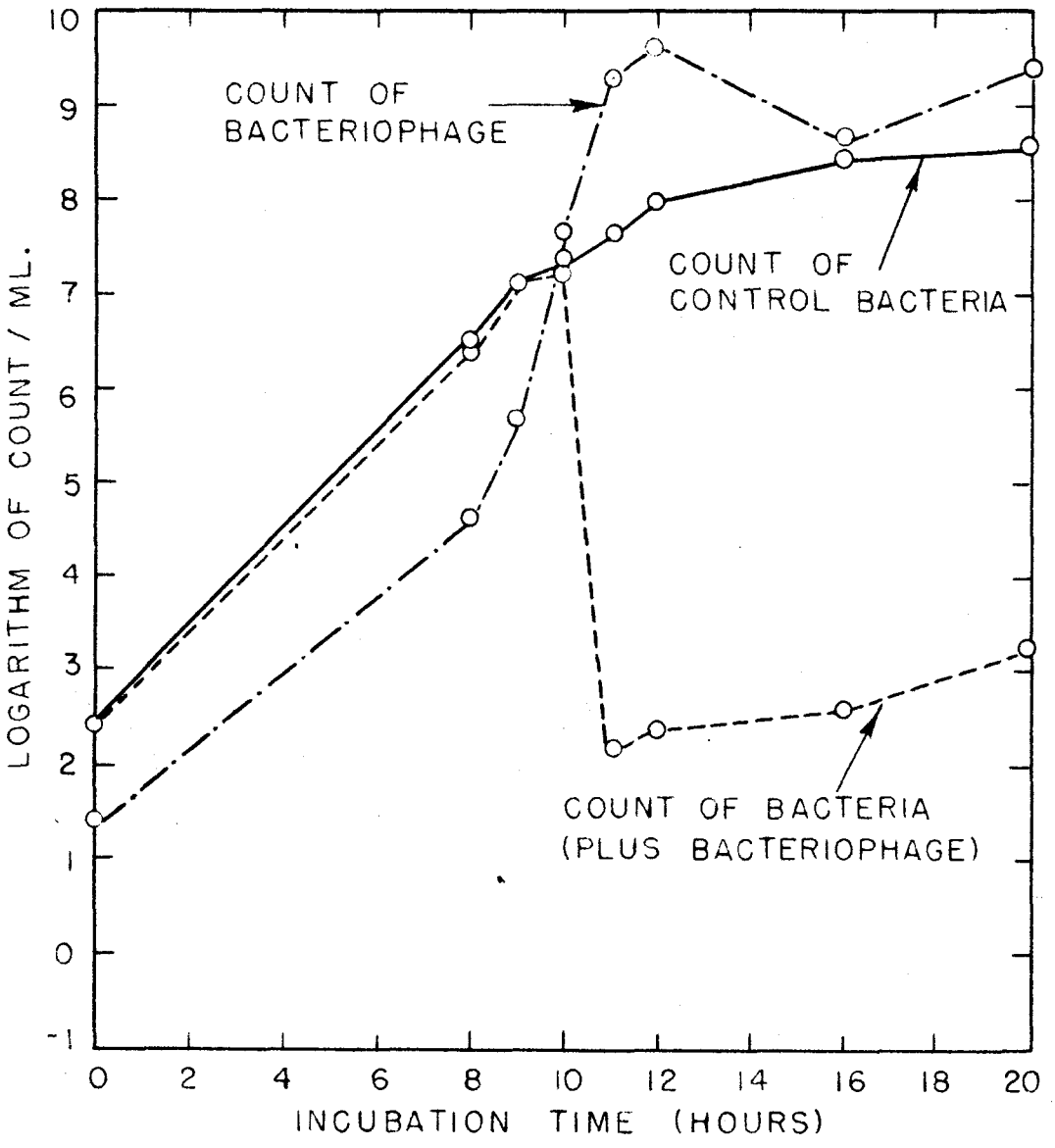

Fig. 5. Population changes of combination 799-F57 with initial bacteriophage count of 25 per ml. and initial bacteria count of 300 per ml.

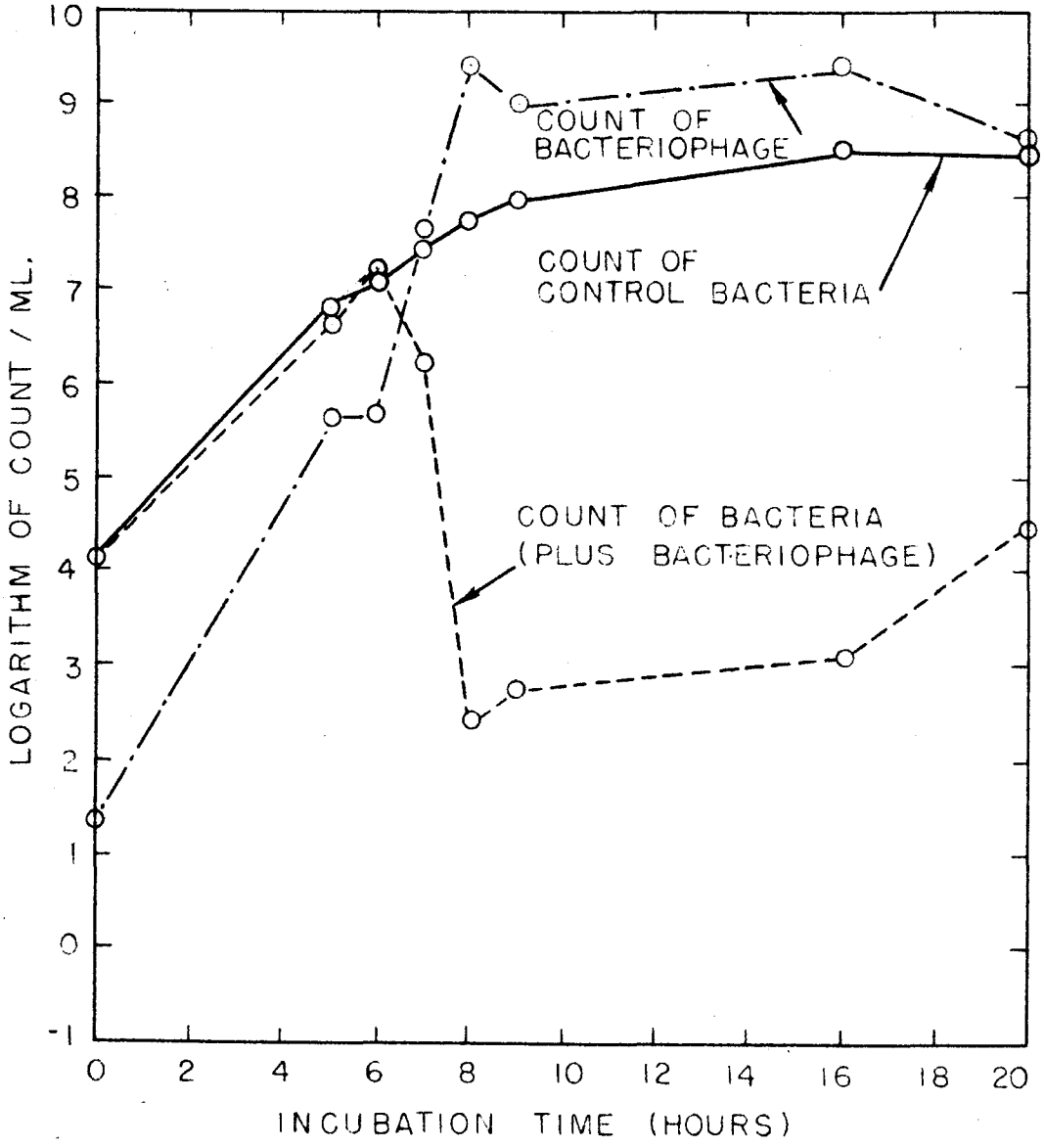

Fig. 6. Population changes of combination 799-P57 with initial bacteriophage count of 25 per ml. and initial bacteria count of 15,000 per ml.

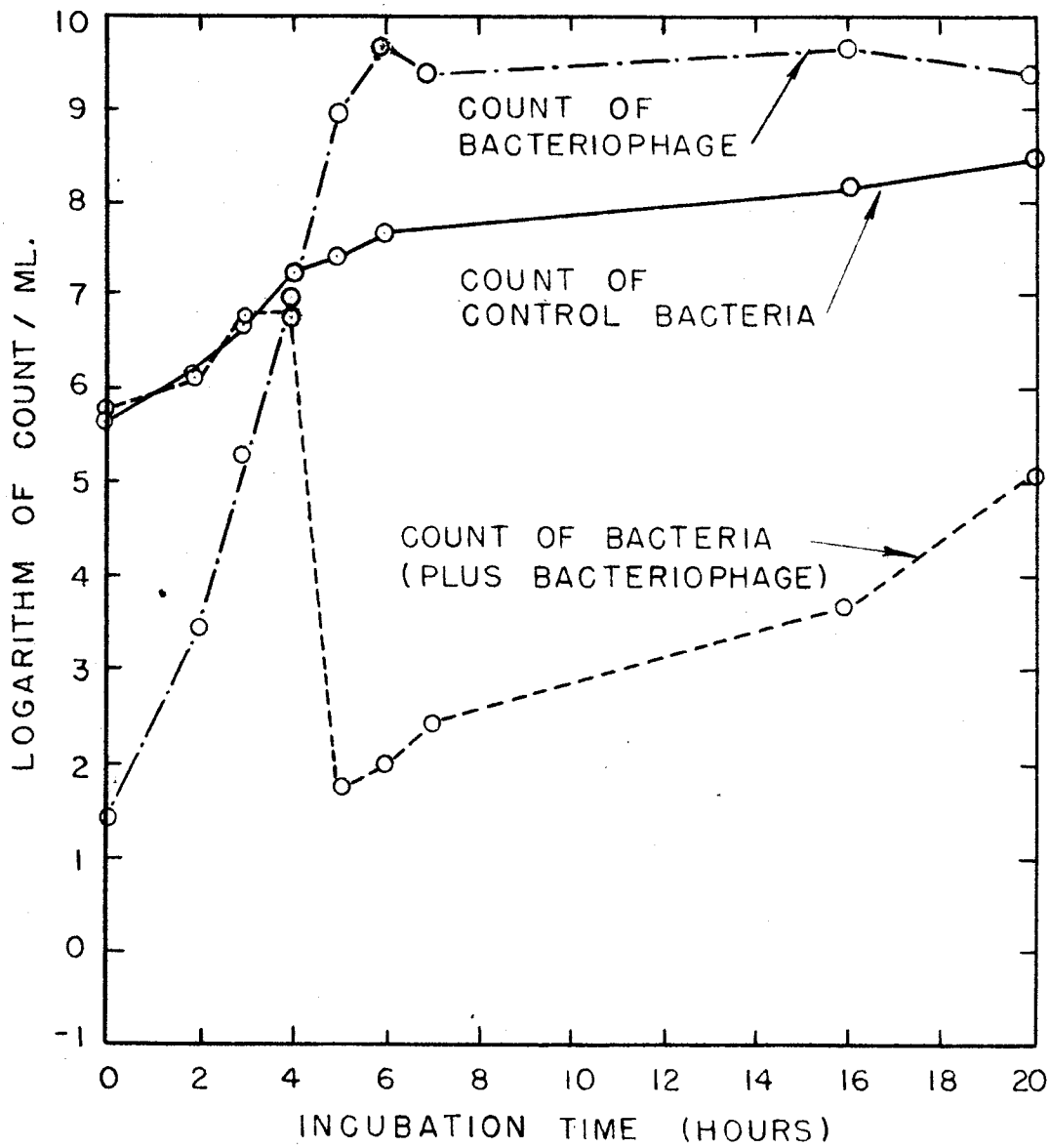

Fig. 7. Population changes of combination 799-F57 with initial bacteriophage count of 25 per ml. and initial bacteria count of 500,000 per ml.

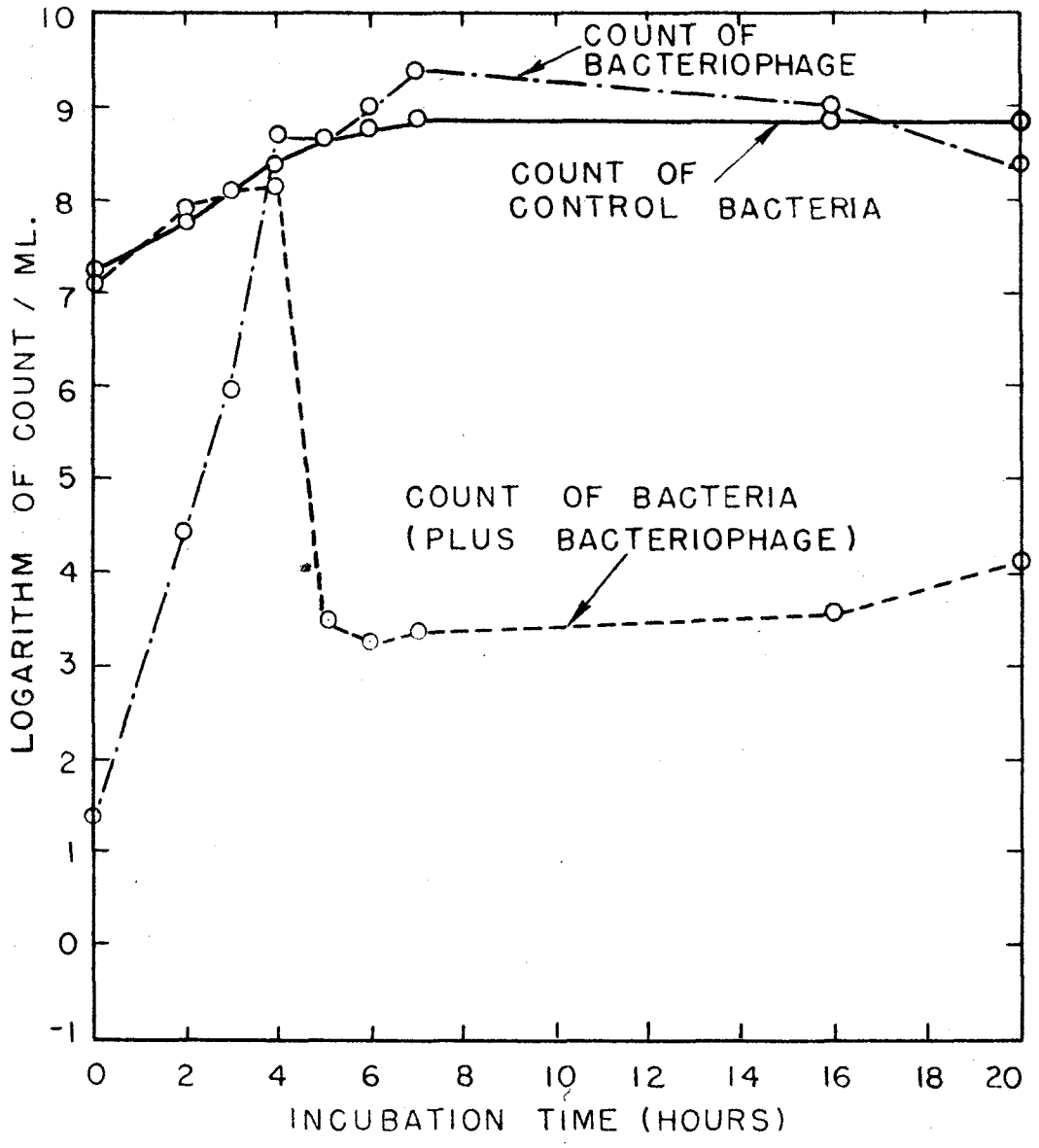

Fig. 8. Population changes of combination 799-F57 with initial bacteriophage count of 25 per ml. and initial bacteria count of 13 million per ml.

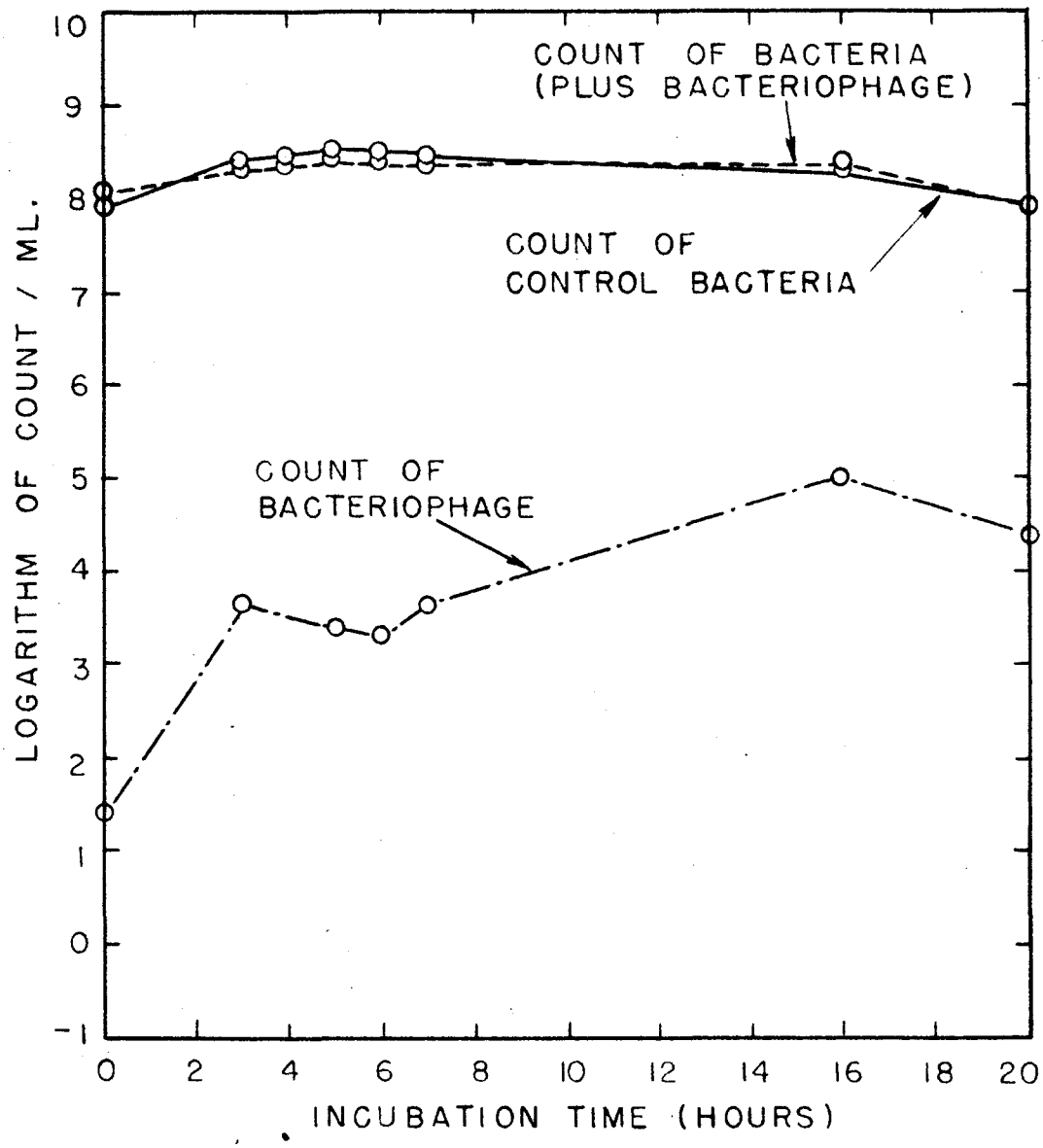

Fig. 9. **Population changes of combination 799-F57 with initial bacteriophage count of 25 per ml. and initial bacteria count of 100 million per ml.**

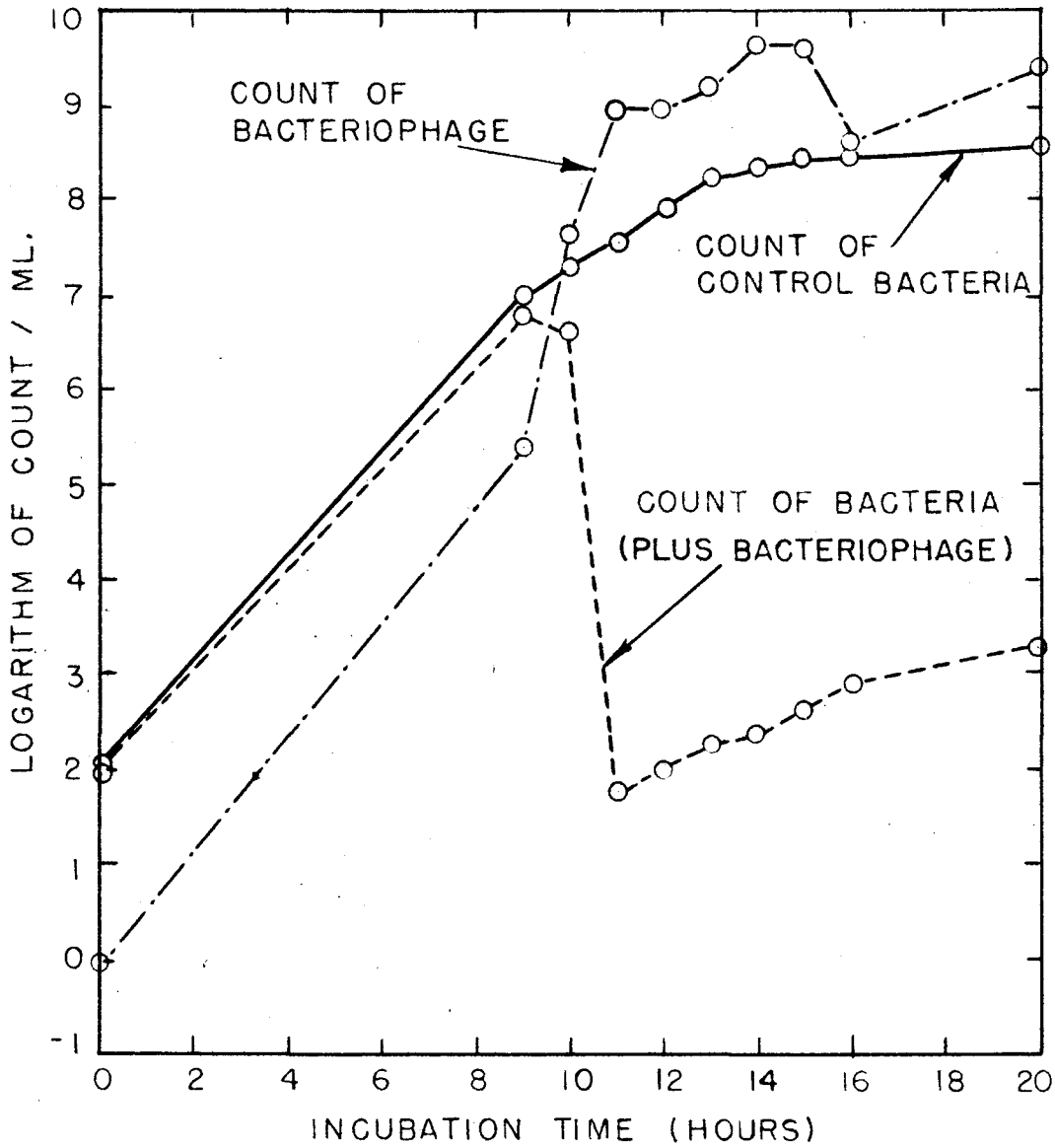

Fig. 10. Population changes of combination 799-F57 with initial bacteriophage count of 1 per ml. and initial bacteria count of 100 per ml.

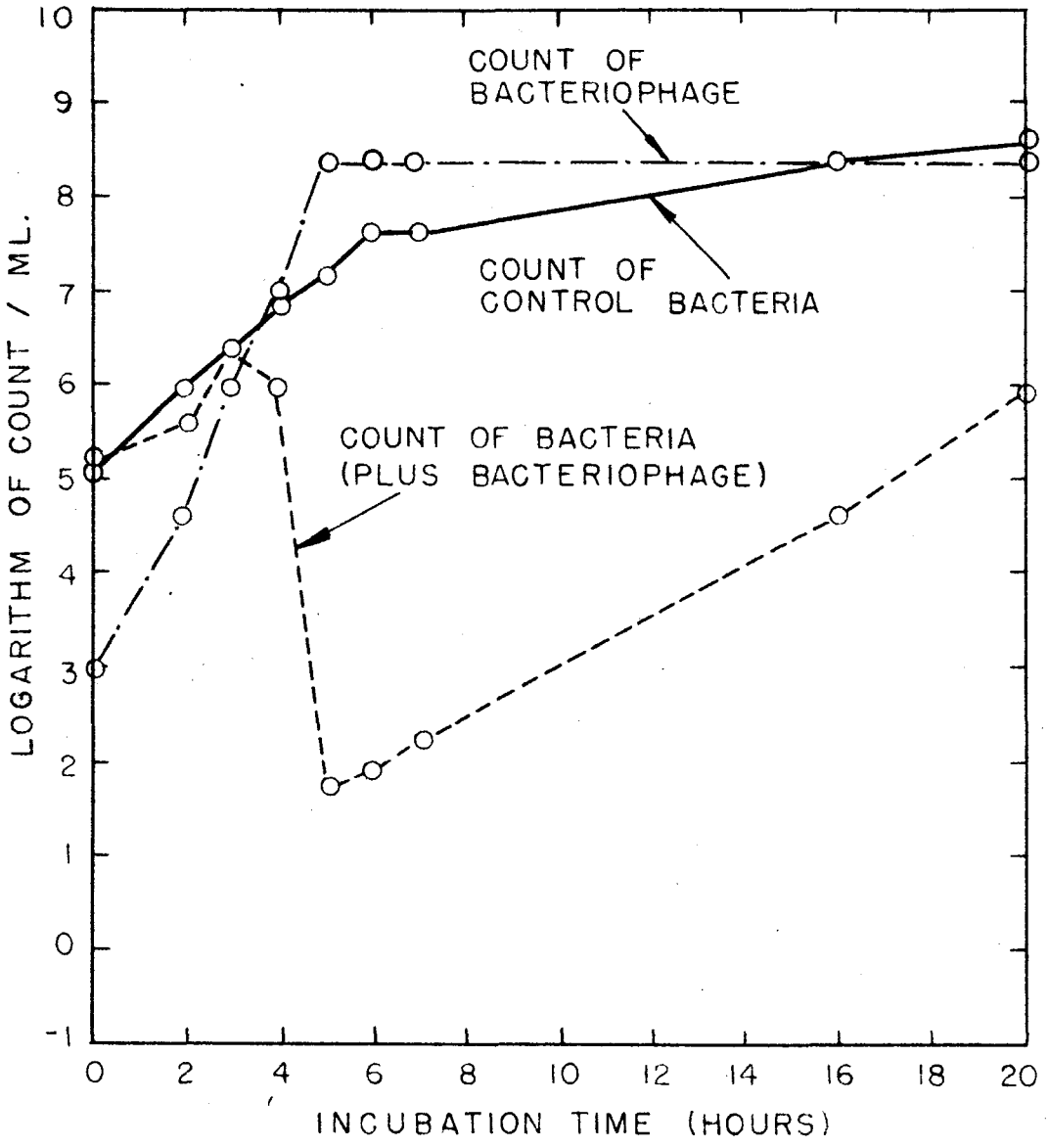

Fig. 11. Population changes of combination 799-F57 with initial bacteriophage count of 950 per ml. and initial bacteria count of 150,000 per ml.

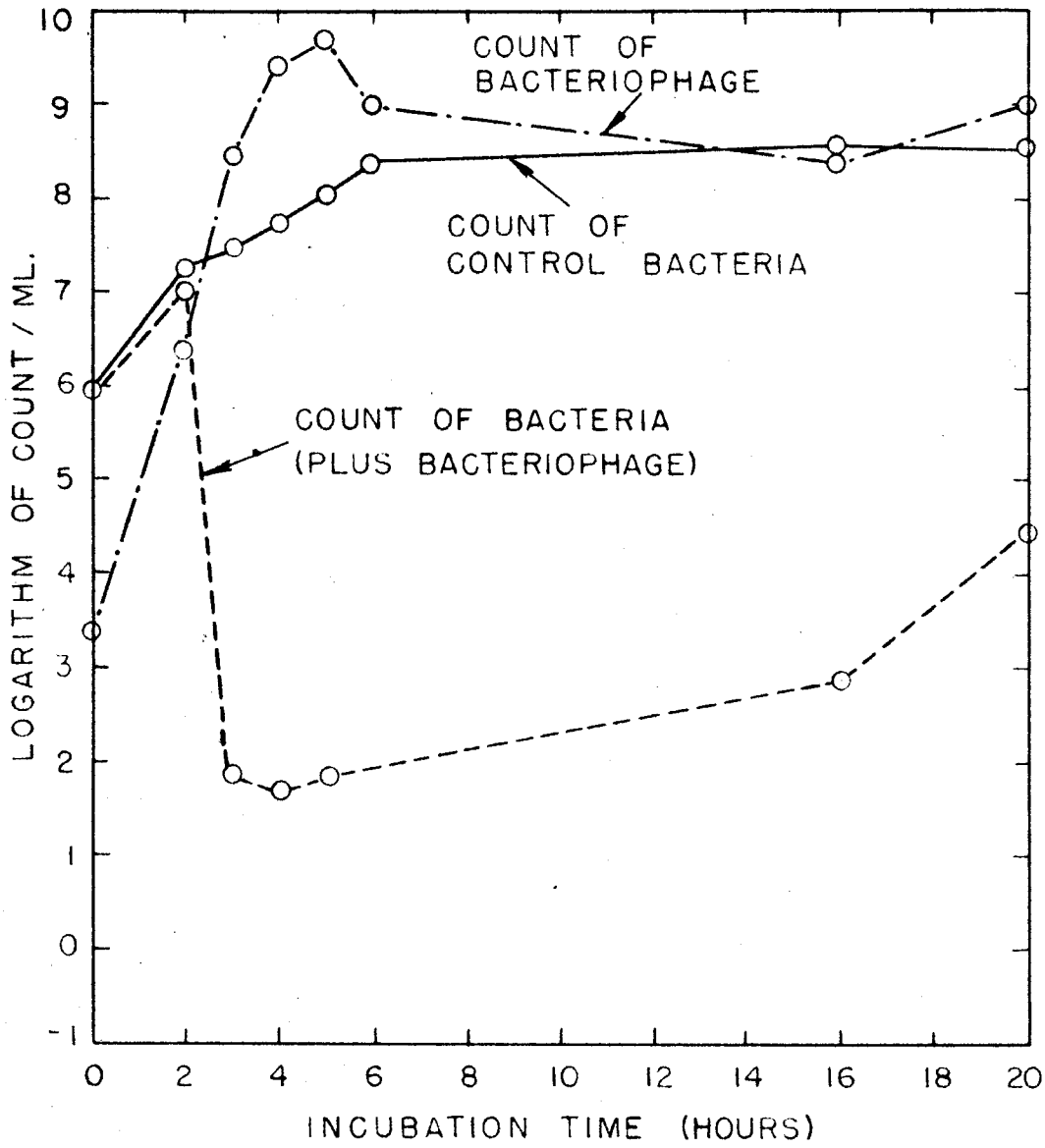

Fig. 12. Population changes of combination 799-P57 with initial bacteriophage count of 2,500 per ml. and initial bacteria count of 800,000 per ml.

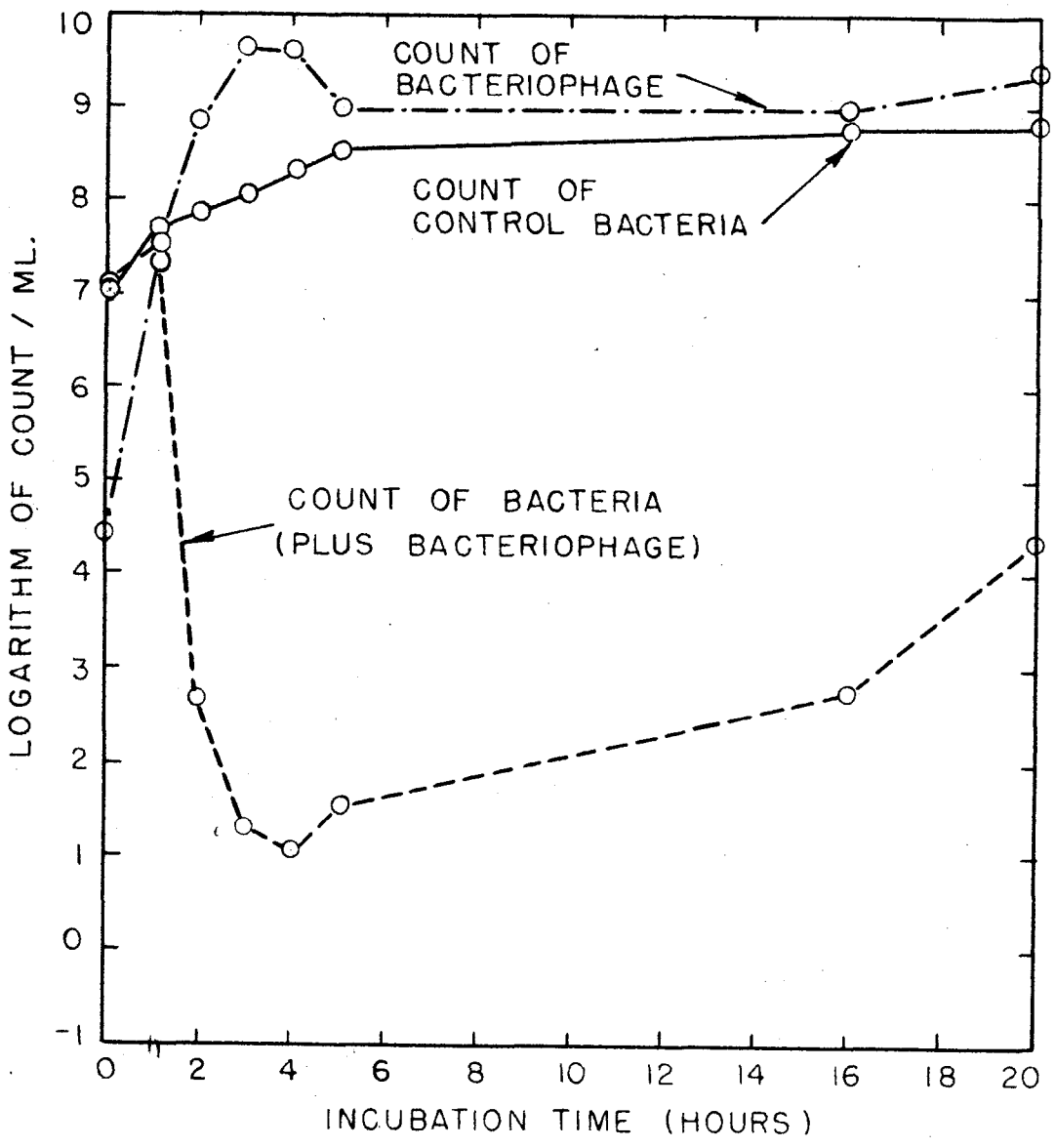

Fig. 13. **Population changes of combination 799-F57 with initial bacteriophage count of 25,000 per ml. and initial bacteria count of 13 million per ml.**

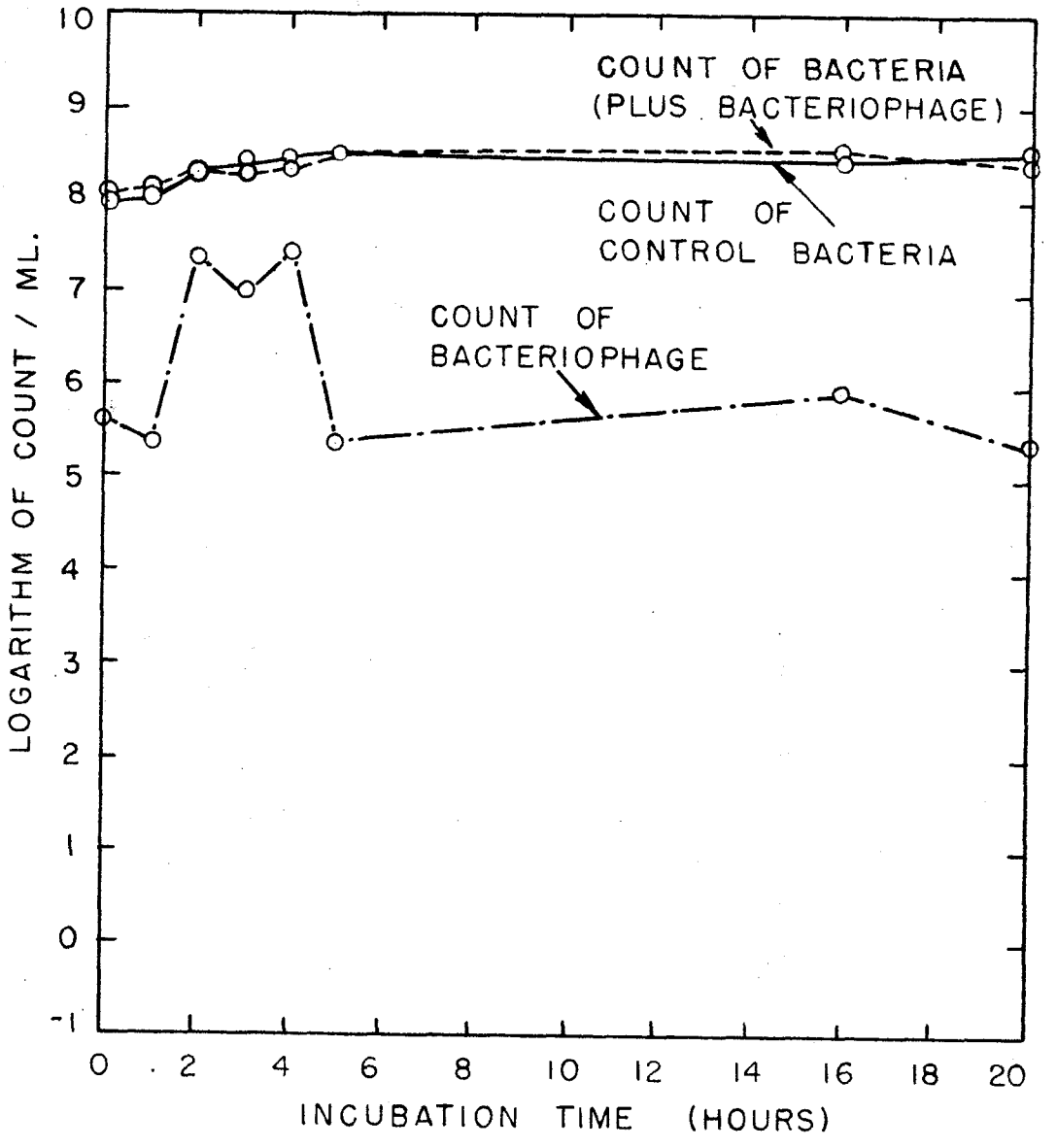

Fig. 14. Population changes of combination 799-F57 with initial bacteriophage count of 450,000 per ml. and initial bacteria count of 100 million per ml.

Population Studies of 13 Selected Organism-Bacteriophage Combinations

In this phase of the investigation six strains of lactic streptococci and eight strains of bacteriophage (table 1) were selected. When each of the organisms was combined with each of the bacteriophages to which it was sensitive, 13 different combinations resulted. The resulting data thus could be considered in three ways: as 13 individual combinations, as six comparisons of the activity of two or more bacteriophage strains on one culture and as three comparisons of the action of one bacteriophage strain on two or more cultures. Separate comparisons between each of the 13 individual combinations were not made except in a few instances where important differences between any two combinations existed.

The preceding study on varying initial counts served as a basis for determining the most desirable counts of bacteria and bacteriophage to use in this phase of the study. A bacteriophage count of 2,500 per ml. and a bacteria count of 250,000 per ml., as closely as they could be approximated in day-to-day runs, were selected as being most suitable. The initial bacteriophage counts ranged from approximately 950 to 4,500 per ml. and the initial bacteria counts ranged from approximately 110,000 to 350,000 per ml. Therefore, the ratio of the counts of bacteriophage to bacteria ranged from about 1:100 to about 1:350. This range was considered sufficiently

narrow to give comparable proliferation data. All of the other conditions and procedures were identical to those of previous trials, the only variable being the use of several organism-bacteriophage combinations instead of a single combination. The results of these trials are shown in figure 2 and figures 15 through 26.

The first data to be presented in this section will be that from several comparisons of the activities of two or more bacteriophage strains upon a single strain of organism.

Culture 799 was sensitive to bacteriophage strains F57 and F63. The population curves for combination 799,F57 are shown in figure 2. The bacteriophage appeared to have multiplied logarithmically following the initial inoculation and the increase continued at about the same rate until a maximum count of about 100 million per ml. was reached at 5 hours, after which a maximum stationary phase was reached. Mass lysis appeared to have begun before 4 hours, when the bacteria count had reached about 10 million per ml. Mass lysis appeared to have been complete at 5 hours, by which time the bacteria count was reduced to 170 per ml. The bacteriophage count reached its maximum of about 100 million per ml. at much the same time. The secondary growth organisms appeared to have multiplied at a fairly uniform rate following mass lysis, although at a somewhat slower rate than did the control bacteria. The count of secondary growth organisms at the end of the

20-hour trial was 2.2 million per ml.

In figure 15 are shown the population changes which occurred when organism 799 was acted upon by bacteriophage F63. At 3 hours following the initial inoculation the counts per milliliter of the control bacteria and the bacteria with added bacteriophage were practically the same. The count of the control bacteria continued to rise at a normal rate throughout the 20-hour period, but in the culture containing bacteriophage mass lysis began between 3 and 4 hours and continued for a portion of the next 2 hours. The count of resistant organisms was 450 per ml. when mass lysis was completed some time before 5 hours. The secondary growth bacteria in the bacteria-bacteriophage mixture reached a count of 3.5 million per ml. at the end of the 20-hour trial, and the recovery thus was considered rapid. A maximum bacteriophage count of about 250 million per ml. was reached at 5 hours, the same time at which the resistant organisms had reached the minimum level of 450 per ml.

In general, only minor differences in bacteria and bacteriophage populations existed when organism 799 was acted upon by bacteriophage strains F57 and F63. Counts of secondary growth organisms following mass lysis were 170 and 450 per ml., using strains F57 and F63, respectively. Considering the limitations of the enumeration method used, this difference probably has little or no significance. The

average generation time of the secondary growth organisms was slightly longer for combination 799,F63, being 70 minutes, and only 64 minutes for combination 799,F57.

Organism ML1 was acted upon by bacteriophage strains F52 and F69. Results with combination ML1,F52 (figure 16) differed from those obtained using combinations of 799 with bacteriophage strains F57 and F63 in that there was no evidence of the presence of secondary growth organisms following mass lysis. After incubation of the mixture containing bacteria and bacteriophage at 32° C. for 4 months, there still was no evidence that the sample contained resistant organisms as indicated by the plating procedure. The counts per milliliter of the control bacteria, the bacteriophage and the bacteria with added bacteriophage probably reached a common level of about 10 million at the time mass lysis started between 3 and 4 hours. The bacteriophage count reached its maximum between 4 and 5 hours, approximately the same time at which mass lysis was completed.

In figure 17 are shown the population changes for combination ML1,F69. Except for a slightly higher maximum bacteriophage level with this combination, the growth curves were almost identical with those for combination ML1,F52, with no secondary growth organisms resulting from either combination. Wilkowske (1949) showed that, with one exception the cross-reaction patterns for bacteriophage strains F52 and F69 were

the same, although according to his classification, they were placed in serological groups IA and VI, respectively. The results obtained with combinations ML1,F62 and ML1,F69, therefore, might have been expected to show some degree of similarity.

Organism H1-10 was sensitive to bacteriophage strains F61 and PF11. The data for the population changes of combination H1-10,F61 are presented in figure 18. Although there appeared to have been a slight lag in bacteriophage proliferation between the initial inoculation and second hourly interval, thereafter the bacteriophage increased logarithmically until a maximum count of about 2.5 billion per ml. was reached. Mass lysis began shortly after 3 hours and probably was complete a short time after 4 hours. The proliferation lines show that the maximum bacteriophage count and the minimum count of bacteria surviving mass lysis occurred at the same time. This result supports the belief of many workers that bacteriophage proliferation is dependent upon the presence of actively growing sensitive bacterial cells and that when almost all of the cells have been lysed, bacteriophage multiplication ceases.

Data on population changes occurring with combination H1-10,PF11 appear in figure 19. In general, quite similar results were obtained for both combinations H1-10,PF11 and H1-10,F61. One notable exception was in the rate of growth of secondary growth organisms. The count of these secondary

growth organisms from combination H1-10,F61 was 4 per ml. at the end of mass lysis, but at the end of the 20-hour test period the count had risen to only 15,000 per ml. Calculations show an average generation time of 75 minutes. With combination H1-10,PF11 the count of secondary growth organisms immediately following mass lysis was 19 per ml., but at the end of the 20-hour test period the count had risen to 660,000 per ml. The calculated average generation time for the secondary organisms of this combination was 64 minutes. From these results it is obvious that the proliferation rate of the secondary organisms from combination H1-10,PF11 was slightly greater than the proliferation rate of the secondary organisms from combination H1-10,F61, and the difference is clearly shown by the slopes of the population lines for these combinations (figures 18 and 19).

It could not be expected that the counts of secondary organisms immediately following mass lysis always would be exactly the same in repeated trials because of slight variations which usually exist when enumerations of bacteria are made by the method used in this investigation, although the counts probably would be at the same general level. The data show that the level of populations at the time of mass lysis of organism H1-10 by bacteriophages F61 and PF11 (figures 18 and 19) was slightly lower than that of organism ML1 acted upon by bacteriophages F52 and F69 (figures 16 and 17), but the

difference may not be significant. Data on repeatability are not available.

Bacteriophage strains F52 and F69 both were active on culture 459. The population curves obtained from bacteria-bacteriophage combination 459,F52 are shown in figure 20. Some differences were noted in the behavior of both the secondary growth organisms and the bacteriophage when compared to some of the other combinations studied. Ordinarily the bacteriophage count at the completion of mass lysis reached a level in the range of 400 million to 1 billion per ml., but with this combination, the bacteriophage count was only 9.5 million per ml. immediately following mass lysis, while a maximum count of 450 million per ml. finally was reached before the end of the 20-hour test period. The initial count of secondary organisms was 4 per ml., while the final count at the end of the 20-hour trial period reached only 400 per ml. When compared to the proliferation rate of the control bacteria, this rate of increase was extremely slow. The secondary organisms had an average generation time of nearly 5 hours, as compared to the usual average generation time of slightly more than 1 hour for the secondary organisms of other combinations used in these trials.

The results obtained for combination 459,F69 (figure 21) differed from the results for combination 459,F52 only in one important respect, that being the absence of secondary growth

organisms following mass lysis when F69 was used. The other population levels follow quite closely those obtained with combination 459,F52. As with combination 459,F52, the low bacteriophage count at the completion of mass lysis and the eventual increase before the end of the trial were apparent.

Although the control bacteria behaved normally and the bacteria in the presence of added bacteriophage reached nearly to the level at which mass lysis usually begins, some relationship existed between the bacteria and the bacteriophage, not readily explainable on the basis of the data available, which resulted in the incidence of a very low level of secondary organisms from combination 459,F52, and the complete absence of secondary growth organisms with combination 459,F69 and a relatively low level of bacteriophage proliferation for both combinations at the completion of mass lysis. These results were confirmed by repeating the trials.

Population changes occurring when organism H1-1 was acted upon by bacteriophages F52 and F69 (figures 22 and 23) are quite similar in many ways to population changes noted for organism 459 after the action of the same bacteriophages.

With combination H1-1,F52, mass lysis began when the bacteria and bacteriophage counts reached nearly 10 million per ml. The bacteriophage count had increased to 450 million per ml. and the bacteria count had decreased to 3 per ml. at the completion of mass lysis. Following mass lysis, the rate of growth

of the secondary growth organisms was comparatively slow, a count of 120 per ml. being reached at the end of the 20-hour trial period, with evidence that most of the growth occurred during the last 4 hours. In this trial, the average generation time of 168 minutes for the secondary growth organisms was considered as being quite long. The bacteriophage reached nearly to its maximum at about the same time as when the minimum bacteria count occurred.

Data showing the population changes of combination Hl-l, F69 (figure 23) were practically identical to those of combination Hl-l,F52, the one important difference being the absence of secondary growth organisms from combination Hl-l,F69 following mass lysis. The failure to obtain secondary growth organisms from this combination was confirmed in two additional trials. Since the population differences between combinations Hl-l,F52 and Hl-l,F69 parallel the differences between combinations 459,F52 and 459,F69 previously pointed out, no discussion of these data will be made here in order to avoid repetition.

S. cremoris 122-1 was sensitive to three bacteriophage strains, F60, F68 and PFll. Proliferation curves for organism-bacteriophage combination 122-1,F60 are presented in figure 24. Mass lysis probably began at 4 hours when the bacteria and bacteriophage apparently had reached about the same level. Although this level appeared to be slightly low, it was the

same as that at which mass lysis occurred when organism H1-10 was acted upon by bacteriophage strains F61 and PF11. The count of secondary growth organisms remaining after mass lysis was only 7 per ml., but an increase to 43,000 per ml. at the end of the 20-hour test period was noted. The rate of growth of the secondary organisms was slow at first, but later increased, most of the growth occurring during the last 4 hours. The average generation time for the secondary organisms during the entire period after mass lysis was 75 minutes, but the average generation time for the period from 5 to 16 hours was 110 minutes, while the average generation time from 16 to 20 hours decreased to 60 minutes. Thus, the proliferation rate of the secondary organisms was about twice as rapid during the last 4 hours. Additional runs indicated that this result could be closely duplicated. This phenomenon was observed in only one other trial, that in which combination H1-1,F52 was used, and in this case the rate of proliferation was considerably less rapid than observed with combination 122-1,F60. The data provide no explanation for this phenomenon.

The overall population levels for combination 122-1,F68 (figure 25) followed quite closely those of combination 122-1, PF11 (figure 26). With both combinations mass lysis began when the bacteria and bacteriophage reached a common level, probably a short time after 3 hours, and in both trials the number of secondary growth organisms at the end of mass lysis

was markedly higher than with combination 122-1,F60. The number of secondary growth organisms from combination 122-1, F68 immediately following mass lysis was 290 per ml., while that from combination 122-1,PF11 was 320 per ml. The average generation times for the secondary growth organisms of combinations 122-1,F68 and 122-1,PF11 were 70 minutes and 74 minutes, respectively.

The influence of the host organism upon the changes in populations could be studied in several series of combinations, because several of the bacteriophage strains chosen for study were active on two or more strains of bacteria selected. Bacteriophage F52 was active on cultures ML1, 459 and H1-1 (figures 16, 20 and 22), bacteriophage F69 on the same group of cultures (figures 17, 21 and 23), and bacteriophage PF11 on cultures H1-10 and 122-1 (figures 19 and 26).

When cultures ML1, 459 and H1-1 were used with bacteriophage strains F52 and F69, the counts of bacteria and bacteriophage prior to mass lysis were much the same for each combination, both counts being about 10 million per ml. in each case. The bacteriophage populations at the completion of mass lysis reached the same comparative level of about 1 billion per ml. in each of the trials except those of combinations 459,F52 and 459,F69. In these two trials the bacteriophage counts did not exceed 10 million per ml. when mass lysis was completed. Not only was this level of bacteriophage

proliferation low at the completion of mass lysis, but it remained considerably lower throughout the trials than in the case of the other combinations, although a level of 100 million per ml. finally was reached at the end of the 20-hour trial.

Secondary growth organisms did not result from combining any of the three organisms with bacteriophage F69; neither did they result from combination ML1,F52, but with combinations 459,F52 and H1-1,F52 secondary growth organisms were produced, the counts per milliliter immediately following mass lysis being 46 and 3, respectively. These population levels were considered as being quite low. These secondary growth organisms exhibited a slow rate of growth during the remainder of the 20-hour trial period, showing average generation times of 300 minutes for combination 459,F52 and 168 minutes for combination H1-1,F52.

Some similarities in behavior between combinations 459,F52 and H1-1,F52 and combinations 459,F69 and H1-1,F69 suggest that some relationship might have existed between organisms 459 and H1-1, as well as between bacteriophages F52 and F69. Evidence supporting this view is strengthened by the work of Wilkowske (1949), who placed organisms H1-1 and 459 in the same culture group, Ia, although culture 459 was somewhat atypical, and bacteriophage strains F52 and F69 in serological groups IA and VI, respectively, although their activity patterns were

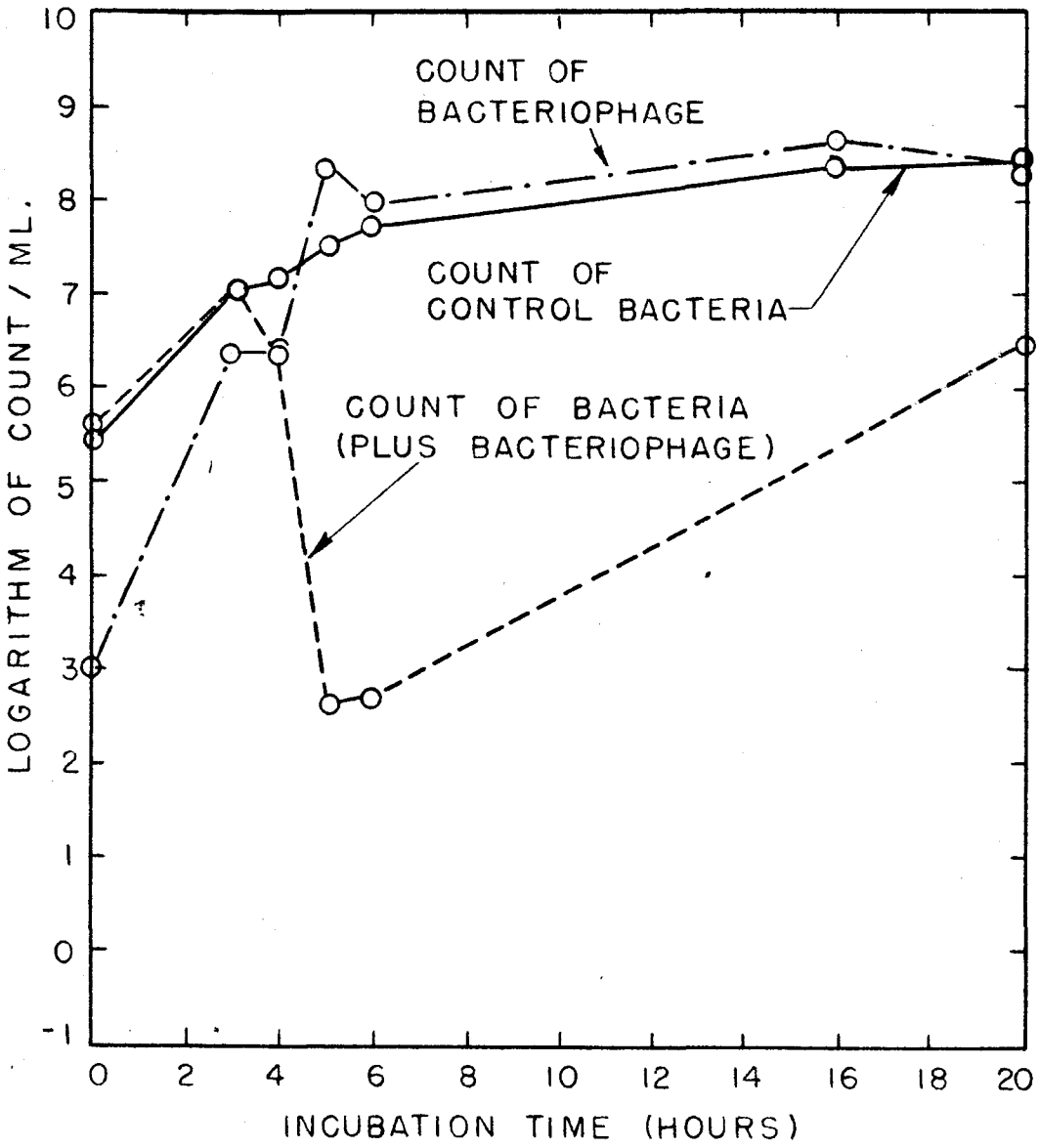

Fig. 15. **Population changes of combination 799-F63 with initial bacteriophage count of 950 per ml. and initial bacteria count of 350,000 per ml.**

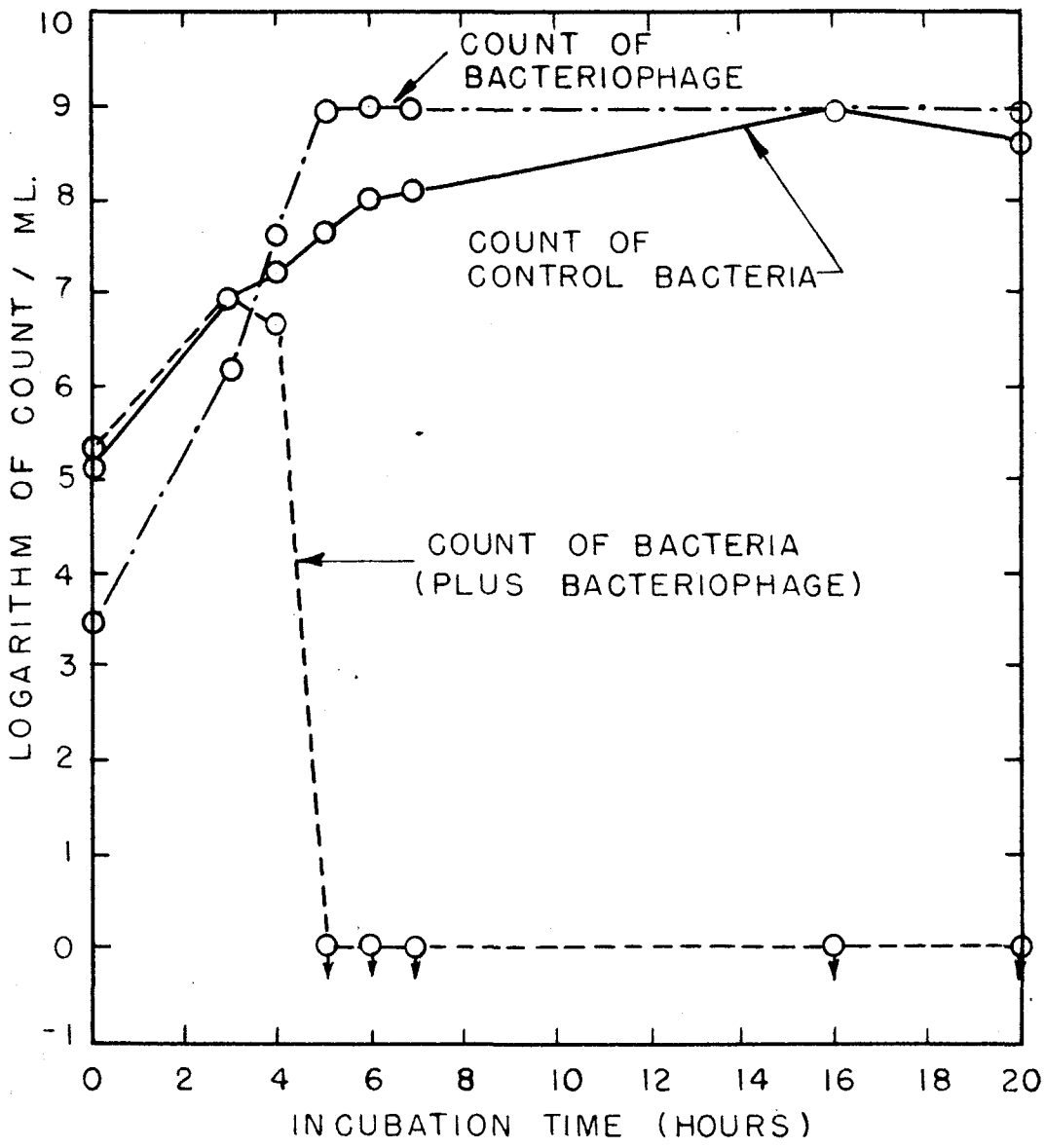

Fig. 16. Population changes of combination ML1-F52 with initial bacteriophage count of 2,500 per ml. and initial bacteria count of 180,000 per ml.

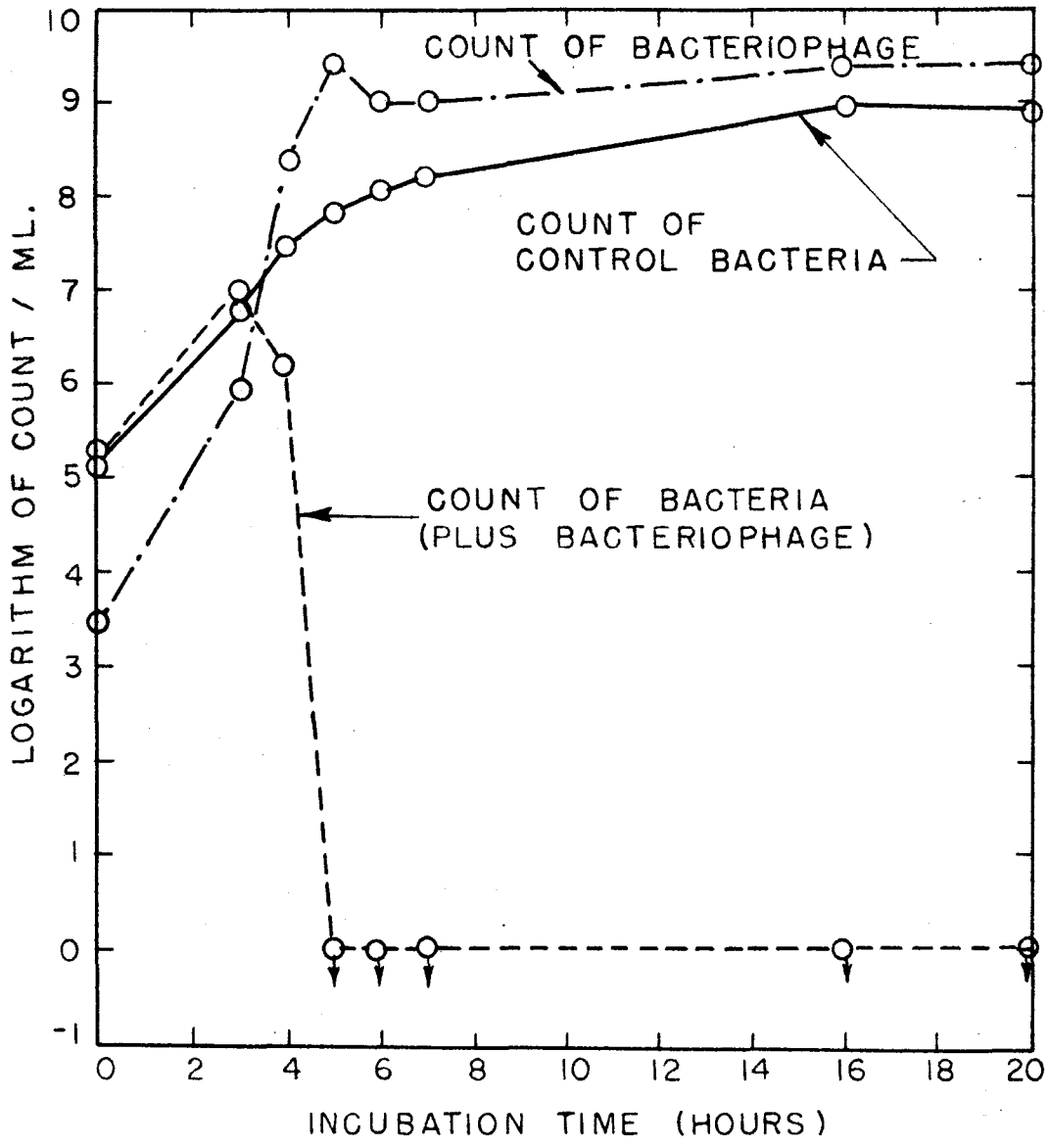

Fig. 17. Population changes of combination ML1-F69 with initial bacteriophage count of 2,500 per ml. and initial bacteria count of 150,000 per ml.

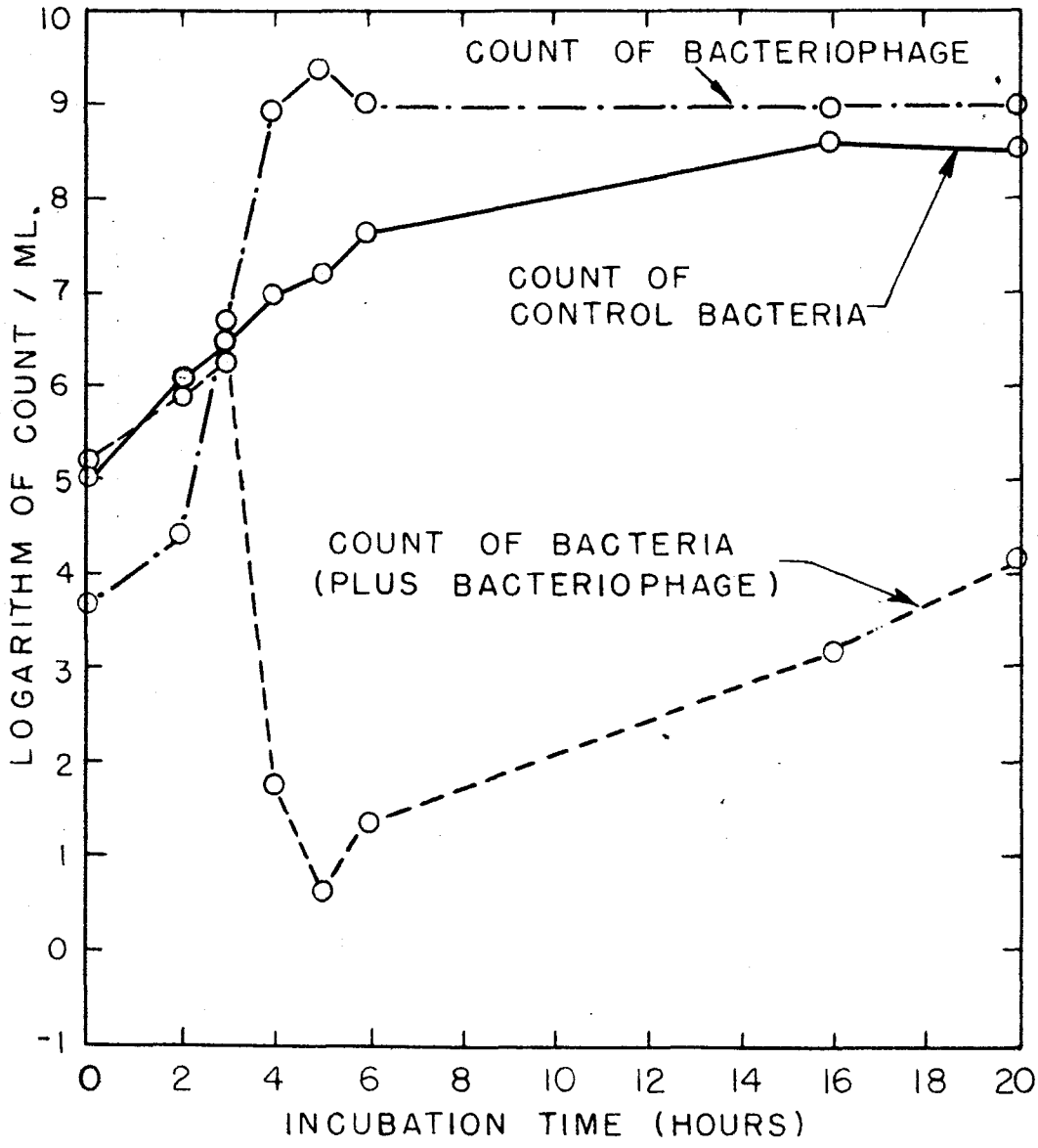

Fig. 18. Population changes of combination H1-10,F61 with initial bacteriophage count of 4,500 per ml. and initial bacteria count of 140,000 per ml.

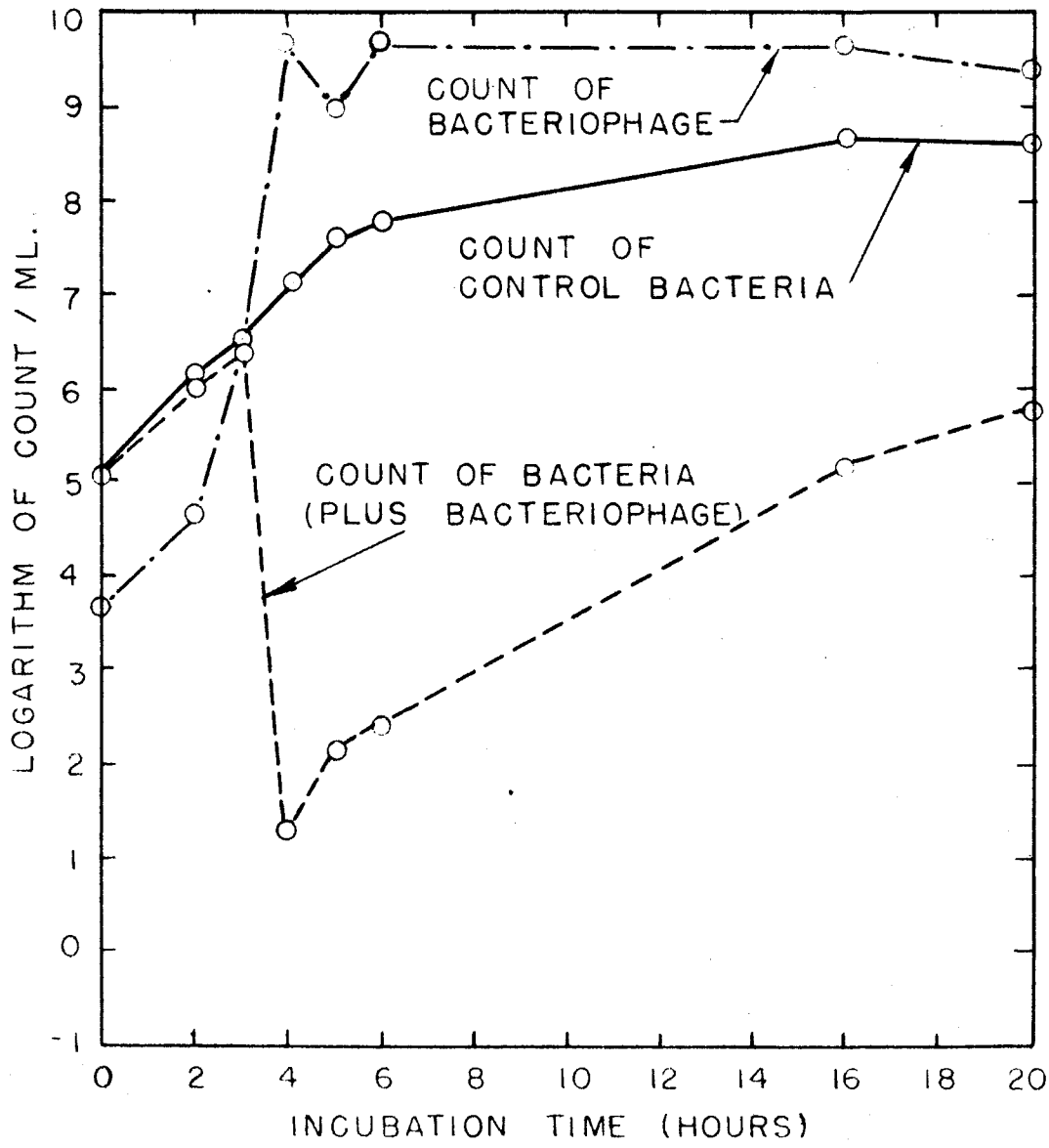

Fig. 19. Population changes of combination H1-10, PF11 with initial bacteriophage count of 4,500 per ml. and initial bacteria count of 130,000 per ml.

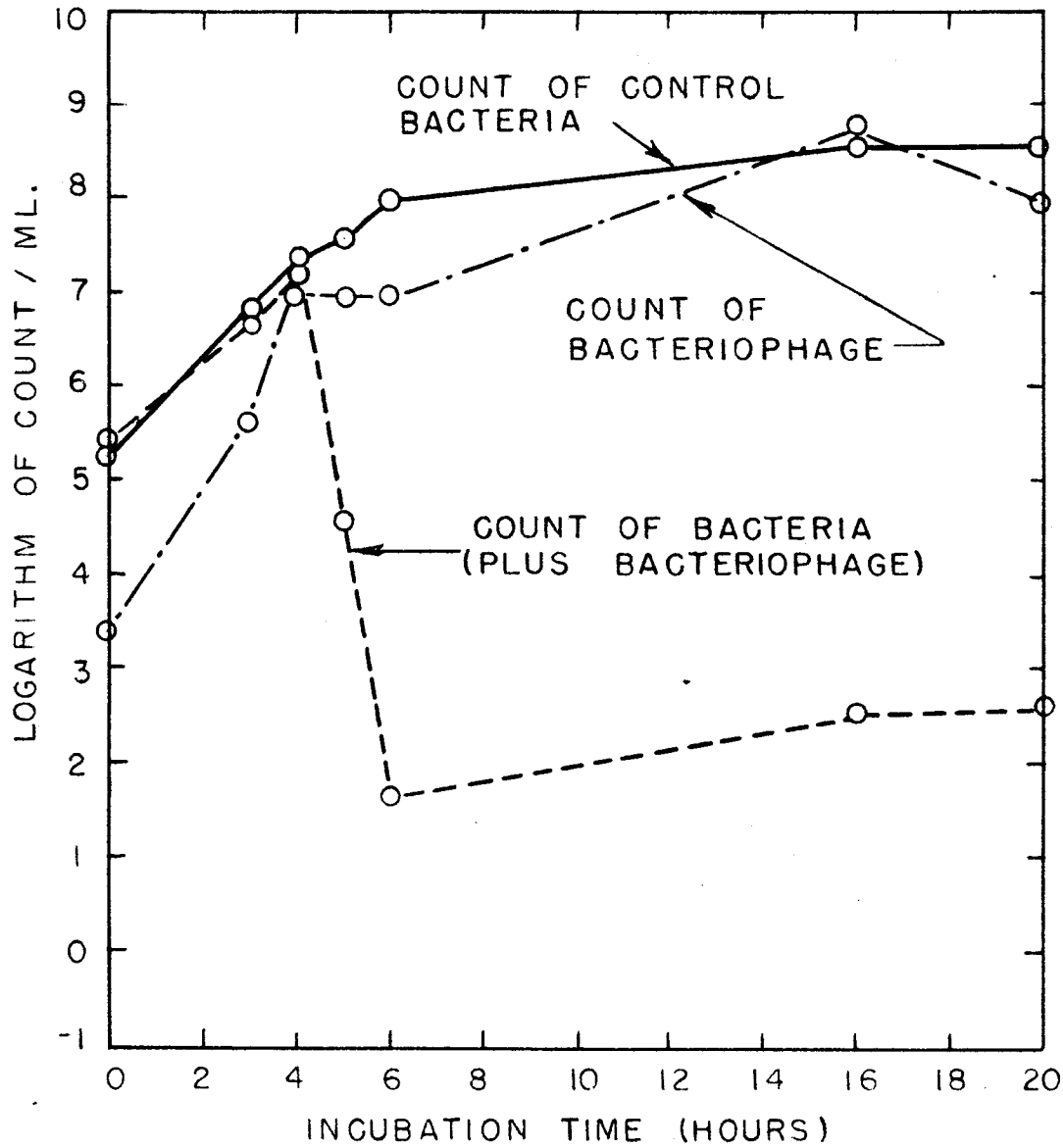

Fig. 20. Population changes of combination 459-F52 with initial bacteriophage count of 2,500 per ml. and initial bacteria count of 210,000 per ml.

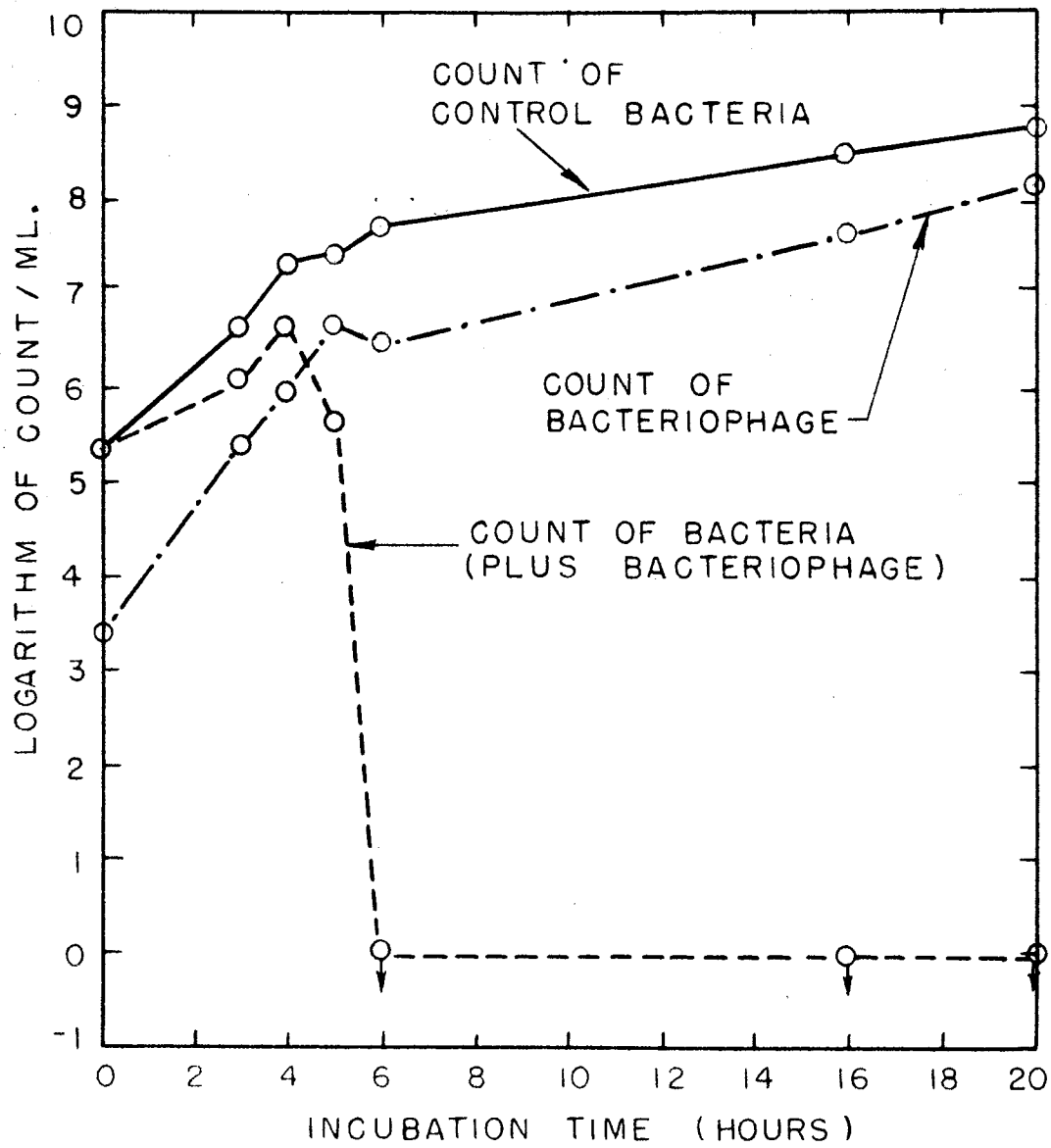

Fig. 21. Population changes of combination 459-F69 with initial bacteriophage count of 2,500 per ml. and initial bacteria count of 220,000 per ml.

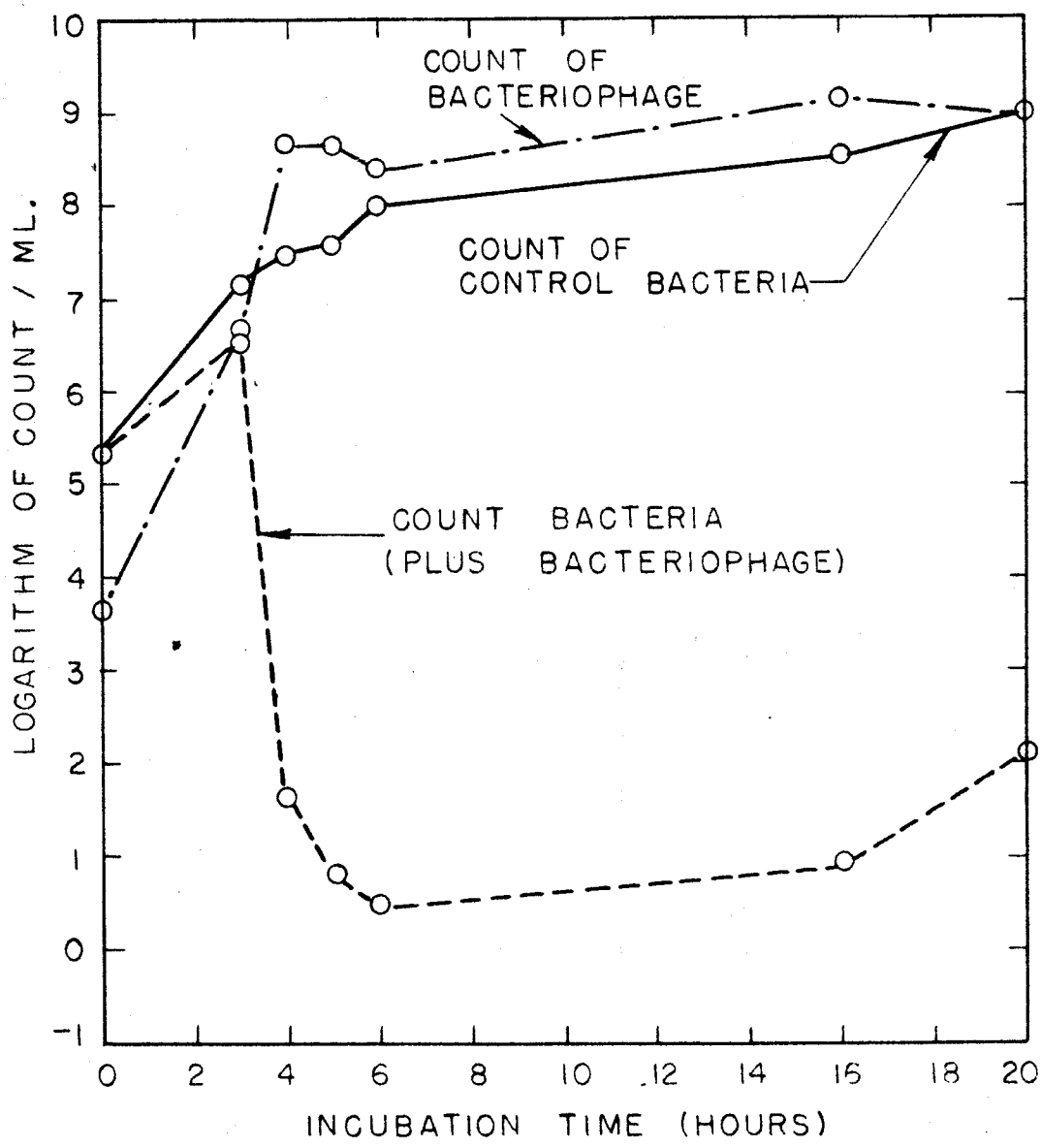

Fig. 22. Population changes of combination H1-1,F52 with initial bacteriophage count of 4,500 per ml. and initial bacteria count of 240,000 per ml.

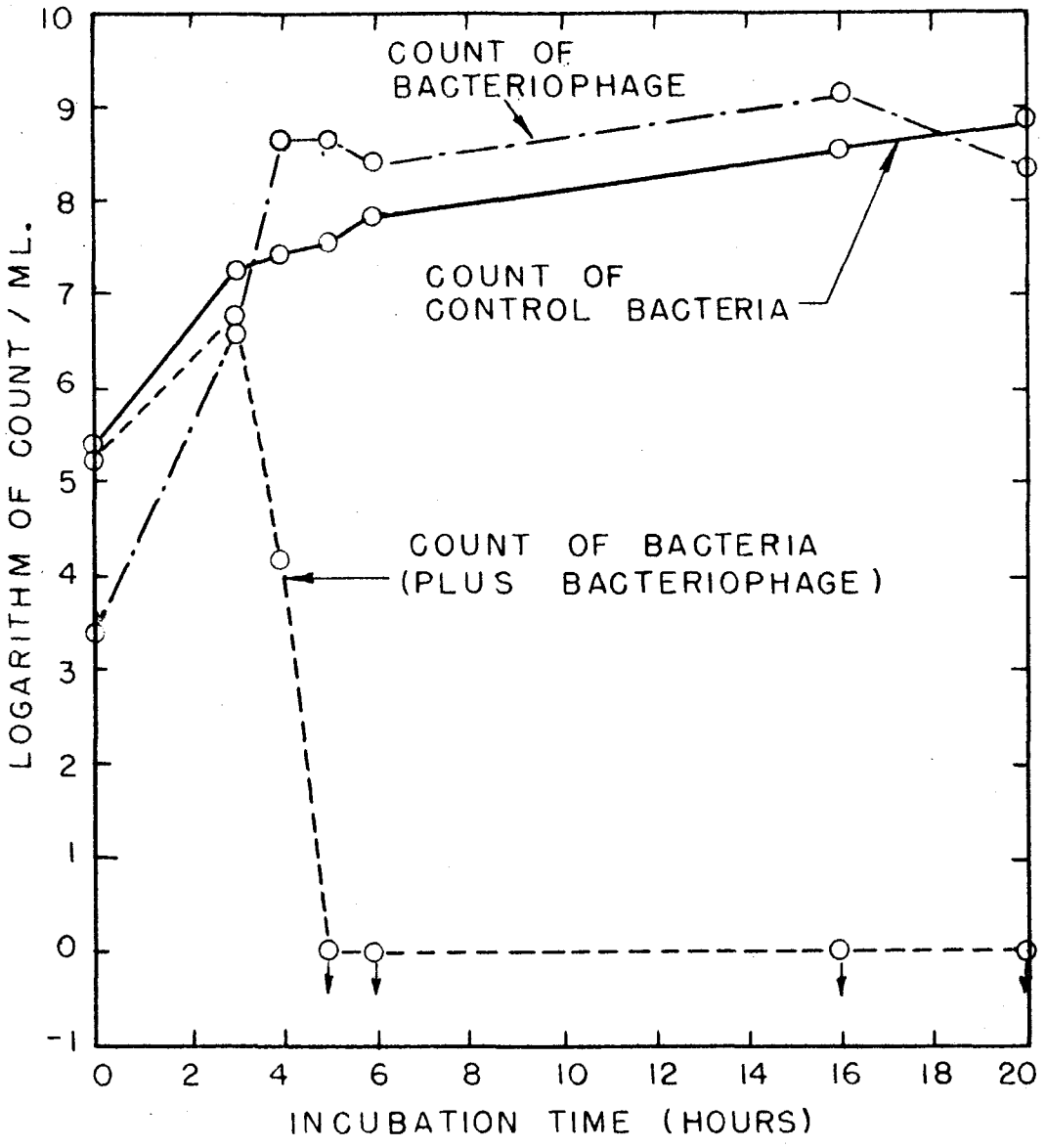

Fig. 23. Population changes of combination Hl-1,F69 with initial bacteriophage count of 2,500 per ml. and initial bacteria count of 180,000 per ml.

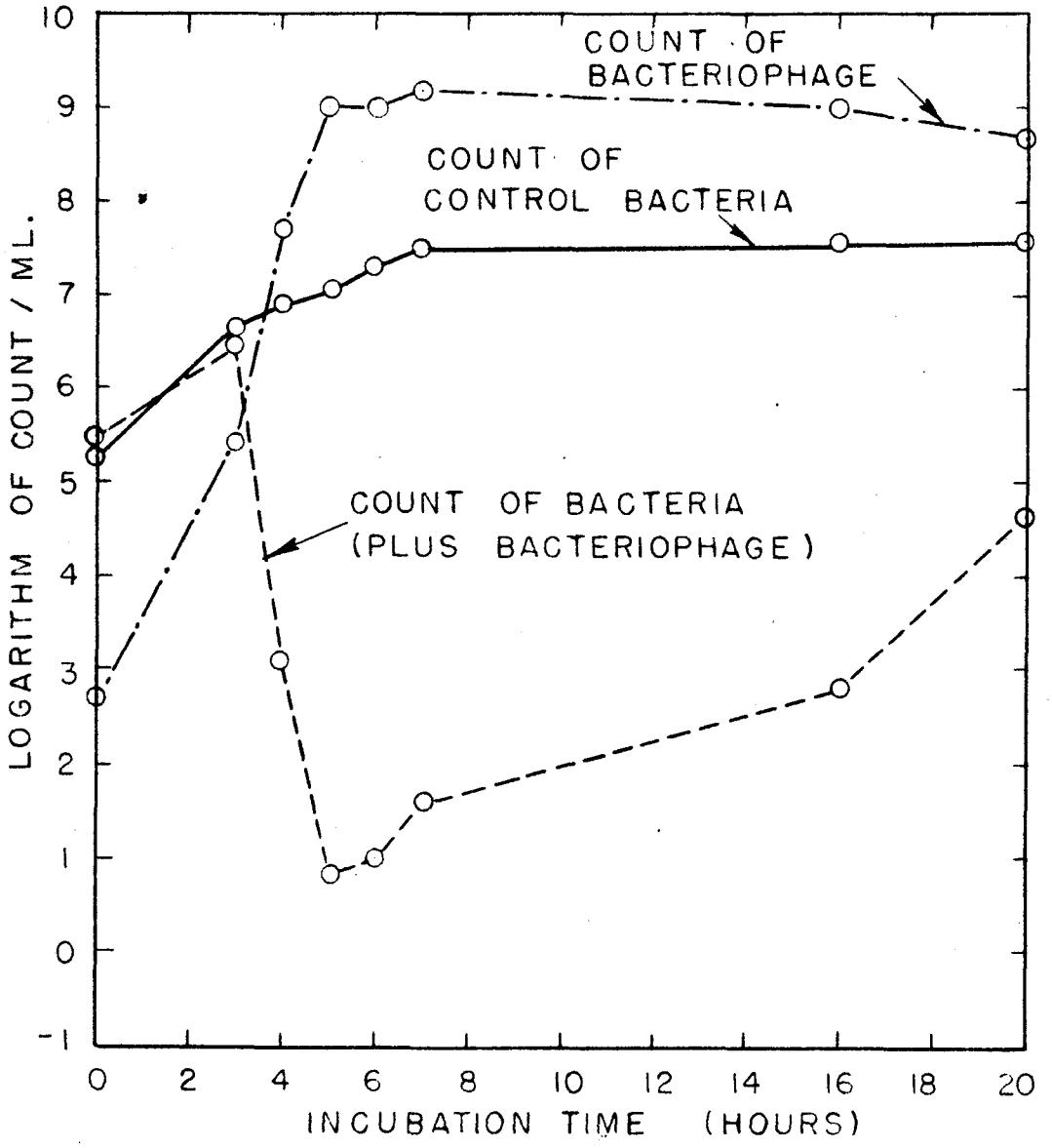

Fig. 24. Population changes of combination 122-1,F60 with initial bacteriophage count of 950 per ml. and initial bacteria count of 350,000 per ml.

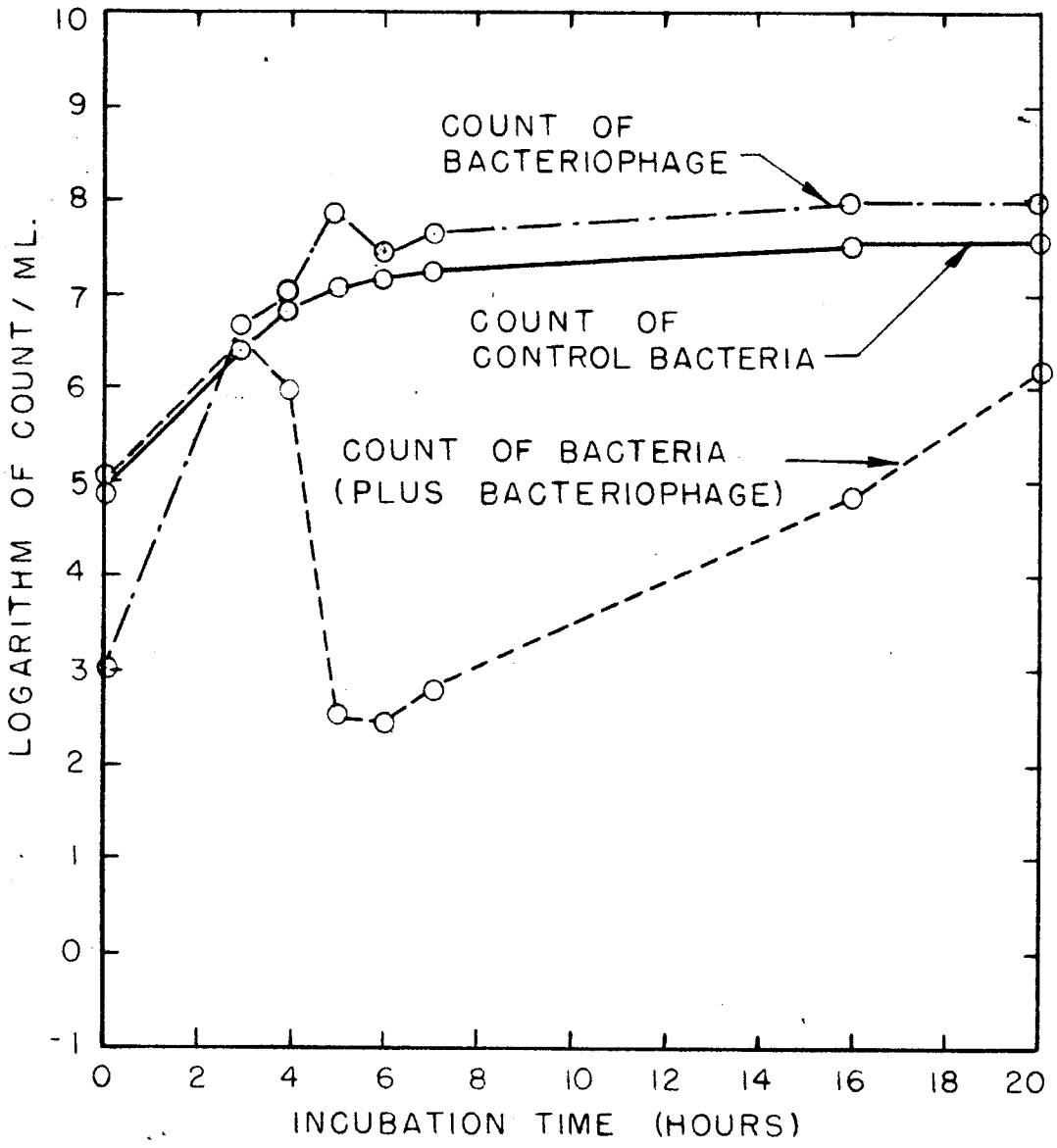

Fig. 25. Population changes of combination 122-1,F68 with initial bacteriophage count of 950 per ml. and initial bacteria count of 110,000 per ml.

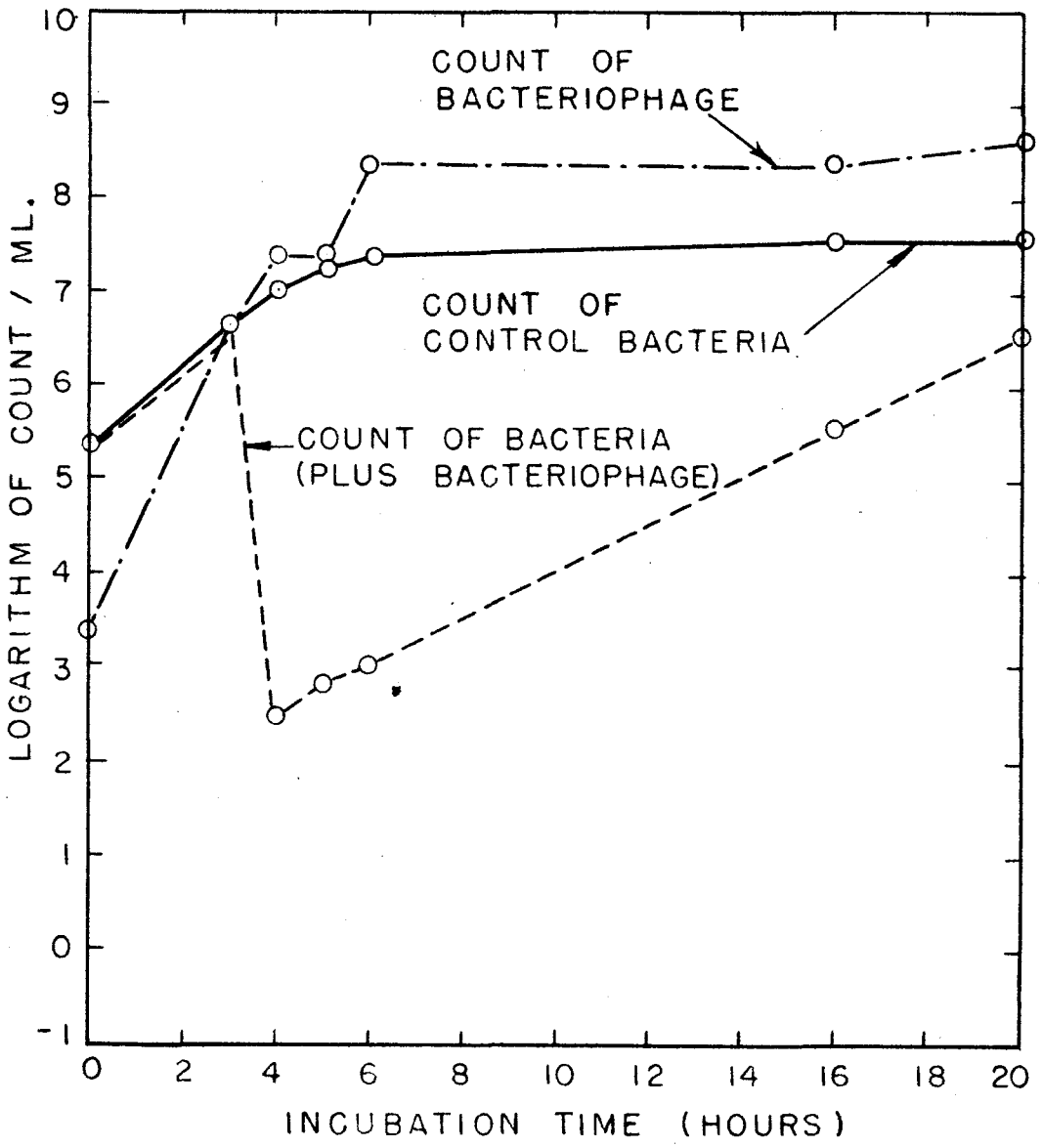

Fig. 26. Population changes of combination 122-1,PF11 with initial bacteriophage count of 2,500 per ml. and initial bacteria count of 220,000 per ml.

practically identical.

Data for the population changes involving organisms H1-10 and 122-1, when acted upon by bacteriophage strain PF11, appear in figures 19 and 26. Mass lysis appears to have been initiated at the same stage of the trial period for both combinations and the counts of bacteria and bacteriophage attained the same level at the same time, prior to the initiation of mass lysis. Two important differences between the combinations occurred. A maximum bacteriophage count of 4.5 billion per ml. from combination H1-10,PF11 was attained at the same time that mass lysis was completed, and the count of secondary organisms was only 19 per ml. Combination 122-1,PF11, on the other hand, showed a bacteriophage count of only 25 million per ml. at the end of mass lysis but the count of secondary organisms at the same time was 320 per ml. The data provide no explanation for these differences.

Sensitivity Characteristics of Secondary Growth Organisms

Organism 122-1 was selected for a study of the differences in sensitivity characteristics of the secondary growth organisms resulting from the action of a series of bacteriophages, PF11, F60 and F68 being used. The sensitivity patterns of these secondary growth organisms tested against the three filtrates are shown in table 4. Control cultures without added

Table 4

The Sensitivity of Secondary Growth Cultures to the Original Bacteriophage Filtrates

Culture	Origin of secondary growth cultures: 122-1,PF11 — Action of original bacteriophage filtrates: PF11	F60	F68	122-1,F60 — PF11	F60	F68	122-1,F68 — PF11	F60	F68
Trial 1									
1	-	+	-				+	+	+
2	-	+	-				+	+	+
3	-	+	-				+	+	+
4	-	-	-				+	+	+
5	-	-	-				+	+	+
6	-	-	-				+	+	+
7	-	+	-				+	+	+
8	-	+	-				+	+	+
9	-	+	-				+	+	+
10	-	+	-				+	+	+
11	-	-	-				+	+	+
12	-	+	-				+	+	+
13	-	-	-				+	+	+
14	-	-	-				+	+	+
Trial 2									
15	-	+	-	+	-	+	-	+	-
16	-	-	-	+	-	+	-	+	-
17	-	+	-	+	-	+	-	-	-
18	-	-	-	+	-	+	-	-	-
Trial 3									
19	-	-	-	+	-	+	-	+	-
20	-	-	-	+	-	+	-	-	-
21	-	-	-	+	-	+	-	-	-
22	-	+	-	+	-	+	-	-	-
23	-	-	-	+	-	+	-	-	-
24	-	+	-	+	-	+	-	-	-
25	-	-	-	+	-	+	-	-	-
26	-	-	-	+	-	+	-	-	-
27	-	+	-	+	-	+	-	+	-
28	-	-	-	+	-	+	-	+	-

\+ = Sensitive
\- = Resistant

bacteriophage were used for each of the secondary growth cultures of each combination in these trials and in every instance the control cultures showed normal coagulation. The secondary growth cultures were derived from plates prepared from test samples of bacteria and bacteriophage, the plates having been poured after the culture plus bacteriophage had been incubated for 20 hours. The method used for propagation and determination of sensitivities of the secondary growth organisms was the same as those described under experimental methods.

The secondary growth cultures from combination 122-1,PF11 were not sensitive to the original bacteriophages PF11 and F68 in any of the three trials. However, 30 to 60 percent of the secondary cultures from any one series of isolations from this combination were sensitive to bacteriophage F60.

The secondary growth organisms from combination 122-1,F60 in trial 1 showed abnormal fermentations, and therefore were discarded. In trials 2 and 3, all of the secondary cultures from combination 122-1,F60 were sensitive to bacteriophages PF11 and F68, while none of them was sensitive to bacteriophage F60.

Behavior of the secondary growth cultures of combination 122-1,F68 varied to some extent. All of the cultures obtained were sensitive to all three bacteriophages in trial 1. That none of these cultures was resistant to the original bacterio-

phage F68 would indicate that these cultures possibly might not have been true secondary organisms, or that they had rapidly reverted from the resistant state to the sensitive state. In trials 2 and 3 none of the secondary cultures were sensitive to bacteriophage strains PF11 and F68, but 30 to 60 percent were sensitive to bacteriophage strain F60.

On the basis of the results of these trials, it appeared that the sensitivity characteristics of the secondary growth organisms from a given culture-bacteriophage combination might be the same or they might be of at least two different types. In any given trial, the sensitivity of all of the cultures of a given combination was the same toward bacteriophage strains PF11 and F68. It was apparent, however, that different groups of secondary cultures may or may not all possess the same sensitivities in different trials.

The behavior of secondary growth cultures toward bacteriophage F60 showed some variation. As expected, none of the secondary cultures of combination 122-1,F60 was sensitive to strain F60. Only one demonstrable type of secondary culture from combination 122-1,F60 apparently existed, since all of the cultures were sensitive to bacteriophages PF11 and F68 and resistant to bacteriophage F60. On the other hand, there appeared to have been two types of mutants originating from both combinations 122-1,PF11 and 122-1,F68 -- those which were and those which were not sensitive to bacteriophage F60. From 30

to 60 percent of the secondary cultures of combination 122-1, PF11 in each of the three trials, and from 30 to 50 percent of the secondary cultures of combination 122-1,F68 in trials 2 and 3 were of the type sensitive to bacteriophage F60, the remainder having lost this sensitivity, as well as the sensitivity to the bacteriophages responsible for the selection of these mutants from the larger populations. Probably the two mutant types were the result of independent mutations occurring at much the same rate and thus forming essentially the same proportions of the populations.

In general, the secondary growth cultures of organism 122-1 showed fairly consistent sensitivity characteristics toward bacteriophages PF11 and F68, but they differed in their sensitivities to the original bacteriophage F60. This behavior suggests that bacteriophages PF11 and F68 may have been related to each other and that bacteriophage F60 possessed the same cross-reactions as bacteriophages PF11 and F68, as well as some additional cross-reactions. These observations are partially substantiated by the cross-reaction data of Wilkowske (1949), which show that bacteriophage PF11 and F68 of serological groups II and VII, respectively, have almost identical cross-reaction patterns and that bacteriophage F60 (ungrouped) possessed not only the same cross-reactions of bacteriophages PF11 and F68, but a fairly large number of other cross-reactions in addition.

Further studies relating to the sensitivities of secondary growth organisms appear to be necessary before any very general conclusions on this subject can be drawn.

DISCUSSION

With organism-bacteriophage combination 799,F57, variation of initial numbers, whether this variation was one of progressive increases of bacteriophage numbers while maintaining a relatively constant initial number of bacteria, or progressive increases of initial bacterial numbers with a relatively constant number of bacteriophage particles, or progressive increases in initial numbers of both bacteria and bacteriophage in a relatively constant ratio, resulted in somewhat similar population patterns of bacteria and bacteriophage. Only minor variations occurred with relation to the shape of the bacteriophage population curve, time of initiation of mass lysis, bacterial population at the initiation of mass lysis, secondary growth organism population at the completion of mass lysis, proliferation rate of secondary growth organisms and maximum bacteriophage population. These generalizations were not applicable when initial counts of bacteria reached 100 million per ml., or when initial bacteriophage counts reached 15 million per ml.

Most important in any of these trials was the critical stage referred to as mass lysis, which occurred when populations of bacteria and bacteriophage increased to such levels that their counts were about equal. At this point, destruction of most of the bacterial cells occurred, while the rapid rate

of increase of the bacteriophage population continued until the end of the period of mass lysis when the substratum of sensitive cells was exhausted. When other conditions were the same, the time at which mass lysis occurred was governed by the initial numbers of bacteria and bacteriophage. Within limits, increased initial numbers of bacteria and bacteriophage reduced the time required to reach the critical level necessary for the initiation of mass lysis.

Indirectly, the extent of bacteriophage proliferation was influenced somewhat by the initial concentration of bacteriophage. Under the condition used in this investigation, the primary prerequisites for the initiation of mass lysis were an actively growing bacterial population with a count approaching 10 million per ml. and an equal number of bacteriophage particles. In general, somewhat higher final bacteriophage populations resulted when small initial inocula of bacteriophage were used. Turner (1948) pointed out that the proximity of the bacteriophage particle to the cell appeared to be the dominating factor responsible for the observed differences in bacteriophage lag periods. In the present trials, a low initial bacteriophage count resulted in delay in the initiation of mass lysis, a longer time being required for the bacteriophage to reach the critical level. The additional time allowed the total bacterial population to become slightly greater, thereby providing more substratum upon which the

bacteriophage could proliferate and in turn allowed higher bacteriophage populations to result.

The relatively constant number of secondary growth organisms with combination 799,F57, even when there was considerable variation in quantity of initial inocula of bacteria and bacteriophage, indicated that the secondary growth organisms detected probably resulted from a selection of naturally occurring mutants. These mutants would be expected to form a fairly constant fraction of the reasonably constant bacterial population usually found at lysis under the standardized conditions employed for this study.

Trials were conducted involving 13 combinations of bacteria and bacteriophage in which the initial counts were held relatively constant at 2,500 and 250,000, respectively. With these counts, 3 to 4 hours were required in each trial for mass lysis to occur. In general, only slight differences in the counts of bacteria and bacteriophage at the time of mass lysis existed between the 13 combinations. Apparently a bacterial population of about 10 million per ml. was necessary before mass lysis could be initiated. Appreciable variations in numbers of secondary growth organisms and their respective proliferation rates existed among the 13 combinations. Some of the more extreme variations were exhibited by combinations ML1,F69, which was incapable of producing secondary growth organisms, H1-1,F52, which produced only a few secondary growth

organisms with a comparatively low growth rate, H1-10,PF11, which produced a small number of secondary growth organisms with a relatively high growth rate, and 799,F63, which produced a fairly large number of secondary growth organisms with a relatively high growth rate.

Culture 122-1 and bacteriophage strains PF11, F60 and F68 were selected as a representative group to use in determining sensitivity patterns of secondary growth organisms. Only one mutant type, sensitive to both strains PF11 and F68 but resistant to strain F60, resulted when culture 122-1 was acted upon by strain F60. When organism 122-1 was acted upon by bacteriophage strain PF11 or F68, relatively larger numbers of secondary growth organisms of two types resulted, one sensitive to strain F60 and one resistant to strain F60 but both resistant to strains PF11 and F68. The two types of secondary organisms resulting from action of bacteriophage strains PF11 or F68 on organism 122-1 occurred in about equal numbers and both grew rather rapidly. The secondary growth organisms from combination 122-1,F60 were present in small numbers, probably partially because their relative growth rate was much slower, but also possibly in part because of a lower rate of mutation to this secondary growth type.

Based upon the results obtained with the series of combinations with organism 122-1, it is probable that a similar set of factors was operating when two or more bacteriophages

acted upon the same sensitive organism in several of the other combinations. Organism MLl, for example, was incapable of producing, in numbers detected by the procedures used, a mutant strain resistant to either of the two bacteriophage strains F52 and F69, but organism strains 459 and Hl-l, both of which were placed in sensitivity group la by Wilkowske (1949), apparently produced a mutant type which was sensitive to bacteriophage strain F69 but resistant to bacteriophage strain F52. Neither of these strains of bacteria produced a mutant resistant to both strains F52 and F69. The mutant selected out in combination 459,F52 may have been different from the type arising from combination Hl-l,F52, basing this difference between the two combinations upon the comparative numbers of mutants and their proliferation rates, but this possibility was not explored. A difference in mutation rate to a single sensitivity type could be used to explain the variations in population which were observed, but possibly these differences were not associated in any way with differences in sensitivity.

The results when one bacteriophage strain acted upon organism strains MLl, 459 and Hl-l showed that no resistant mutants were obtained from any of the three organisms when acted upon by bacteriophage strain F69. Resistant mutants were obtained from combinations Hl-l,F52 and 459,F52, but not from combination MLl,F52. This result indicates that strain F69 apparently possessed a somewhat wider range of activity than

strain F52. The mutant type resulting when strain F52 acted upon organisms H1-1 and 459 indicated that these organisms were capable of producing mutants, but the mutant type(s) were not resistant to the action of strain F69; also strain F69 did not permit the selection of a mutant type which was resistant to strain F69 but sensitive to strain F52. No resistant mutant was obtained from organism ML1, which indicated that the organism differed from organisms H1-1 and 459 and was incapable, under the experimental conditions employed, of producing a mutant resistant to either bacteriophage F52 or F69.

It appeared that the production of a resistant mutant may have been the result of complementary actions of both the organism and the bacteriophage and that both members of the combination play an important role in mutant manifestation. Apparently the bacteriophage acts as a selective agent for mutants resistant to that bacteriophage, but the organism must have a demonstrable rate of mutation to resistant forms if the bacteriophage is to have a population from which it can select resistant forms.

A close relationship did not seem to exist between the mutation rate of an organism and the growth rate of the resultant mutant(s). The growth rate appears to be a characteristic which differs among the mutants arising from different organisms, some exhibiting a high growth rate, as shown by the mutants of combination 122-1,PF11, some showing a moderate

growth rate as was the case with mutants from combination H1-10,F61, and others showing a low growth rate as with the mutants of combination 459,F52.

Use of a heavy initial inoculum of starter bacteria conceivably could help prevent the occurrence of mass lysis during the cheesemaking operation by getting the population of bacteria at an extremely high and essentially static level early, but such a practice would be impractical because of the relatively large quantity of culture which would be required to prevent mass lysis, and also because of the possible undesirable effects upon the quality of the resulting cheese due to the disruption of the normal cheesemaking procedure.

In some instances, cheesemaking operations have been completed even after bacteriophage attack, a combination of secondary growth organisms and other bacteriophage-resistant strains being responsible for continued acid development, although at a low rate; however, the time required for the completion of the process was greatly prolonged. Placing dependence upon culture recovery or using secondary growth cultures to replace the normal cheese culture organisms is not a recommended practice.

Even though resistant secondary cultures might be less subject to bacteriophage attack, their use as cheese cultures usually would be undesirable because of their reduced rate of growth and their probable inability to produce acid at a

normal rate. Whitehead and Cox (1936) attempted to produce cheese cultures resistant to bacteriophage by isolating resistant strains of bacteria from cultures which had been attacked by bacteriophage. They found that the resistance of the secondary cultures obtained in this manner was not permanent, and that the cultures were not immune to attack by other bacteriophage strains. Meanwell (1941) advocated the use of mixed lactic streptococcus cultures for cheesemaking instead of relying upon the action of single strain cultures or secondary growth cultures, as resistant organisms then usually were present in sufficient numbers to complete the cheesemaking process after a bacteriophage attack. In the event of a bacteriophage attack, a culture consisting of two or more strains might afford less chance of all of the strains being lysed at the same time, and the unlysed strains could complete the process more satisfactorily than could the secondary growth organisms. The results obtained in this thesis indicate that the use of active original strains, rather than resistant secondary strains, probably would prove more acceptable because of more rapid growth and consequently more adequate acid production.

SUMMARY AND CONCLUSIONS

Six strains of lactic streptococci and eight strains of lactic streptococcus bacteriophage were studied to determine the influence upon populations of (1) varying the initial numbers of bacteria and bacteriophage, (2) combining a single susceptible organism with two or more active bacteriophages and (3) combining one bacteriophage with two or more susceptible organisms.

Using one organism-bacteriophage combination, 799,F57, progressively increasing the initial count of bacteriophage particles per milliliter, while holding the initial count of the bacteria relatively constant at 200,000 per ml., reduced the time required for the initiation of mass lysis, but had no appreciable effect upon either the number of secondary growth organisms per milliliter at the end of mass lysis, or the proliferation rate of the secondary growth organisms. Progressively increasing the initial number of bacterial cells per milliliter while holding the initial count of bacteriophage particles relatively constant at 25 particles per ml., reduced the time required for mass lysis, except that mass lysis failed to occur when the initial count of the bacteria reached 100 million per ml. Progressive proportionate increases in counts per milliliter of bacteria and bacteriophage, at and below the level of 13 million bacteria per ml. and 25,000 bacteriophage

particles per ml. decreased the time required for mass lysis. Mass lysis did not occur when the initial counts of bacteria and bacteriophage reached 100 million and 450,000 per ml., respectively.

In general, when organism-bacteriophage combination 799,F57 was used, mass lysis was not initiated until the counts of both the bacteria and the bacteriophage had reached a minimum level of about 10 million per ml., except when the initial count is approximately equal to, or greater than the initial organism count. Within the limitations of the method used for enumeration, the secondary organism population immediately following mass lysis appeared to have been a relatively constant segment of the bacteria at the beginning of mass lysis.

Population studies involving 13 organism-bacteriophage combinations, with the initial count of the bacteriophage held relatively constant at 2,500 particles per ml., and the initial count of the bacteria held relatively constant at 200,000 per ml., revealed that mass lysis usually occurred between 3 and 4 hours following the initiation of the experiment, after a relatively constant level of 10 million per ml. was reached by both the bacteria and the bacteriophage. The counts of the secondary growth organisms immediately following mass lysis varied from zero per ml. from four combinations to 450 per ml. for combination 799,F63. In no case did the proliferation rate of the secondary growth organisms equal the proliferation rate during the logarithmic phase of the control culture.

Upon the basis of phage sensitivity range, mutants of two different types were obtained when either bacteriophage PF11 or bacteriophage F68 acted on organism 122-1, while action of bacteriophage F60 on 122-1 permitted isolation of only one mutant type. These findings were employed as the basis for explanation of some of the patterns of secondary growth obtained from other organism-bacteriophage combinations studied in these investigations.

The data apparently indicate that secondary growth cultures arise as a result of selection by bacteriophage of relatively small numbers of naturally occurring bacteriophage resistant mutants which are present in many lactic cultures.

LITERATURE CITED

American Public Health Association. Standard methods for
1948 the examination of dairy products. 9th ed. Am. Pub. Health Assn., New York, N. Y.

Anderson, E. B. and Meanwell, L. J. The problem of bac-
1948 teriophage in cheesemaking. I. Observations and investigations on slow acid production. J. Dairy Research, 13:58-72.

Anderson, T. F. Morphological and chemical relations in
1946 viruses and bacteriophages. Cold Spring Harbor Symposia on Quant. Biol., 11:1-13.

Anderson, T. F. Bacteriophages. Ann. Rev. of Microbiol.,
1950 4:21-34.

Babel, F. J. Factors influencing acid production on cheese
1946 cultures. II. Influence of bacteriophage on acid production in the manufacture of cheddar and cottage cheese. J. Dairy Sci., 29:597-606.

Babel, F. J. Studies on the susceptibility of certain
1947 cheese cultures to the action of bacteriophage. J. Dairy Sci., 30:507-516.

Babel, F. J. Bacteriophage production by cultures of
1948 Streptococcus lactis. (Abstracts of Papers Presented at the Forty-third Annual Meeting) J. Dairy Sci., 29:708.

Bennett, F. W. Action of certain viruscidal agents on
1950 lactic streptococcus bacteriophage. Unpublished Ph. D. Thesis. Ames, Iowa. Iowa State College Library.

Bronfenbrenner, J. Virus diseases of bacteria-bacteriophagy.
1928 In Rivers, T. M. Filterable viruses. p. 373-415. Williams and Wilkins Co., Baltimore, Md.

Buchanan, R. E. and Fulmer, E. I. Physiology and chemistry
1928 of bacteria. Vol. I, p. 11. The Williams and Wilkins Co., Baltimore, Md.

Burnet, F. M. "Smooth-rough" variation in bacteria and its
1929 relation to bacteriophage. J. Path. Bact., 32:15-42.

Burnet, F. M. 1934 The bacteriophages. Biol. Rev. Cambridge Phil. Soc., 9:332-350.

Cohen, S. S. 1949 Growth requirements of bacterial viruses. Bact. Rev., 13:1-24.

Collins, E. B. 1949 Relationship of the nutrition of Streptococcus lactis to bacteriophage proliferation. Unpublished Ph. D. Thesis. Ames, Iowa. Iowa State College Library.

Craigie, J. 1946 The significance and applications of bacteriophage in bacteriological and virus research. Bact. Rev., 10:73-88.

Delbrück, M. 1942 Bacterial viruses (bacteriophages). Advances in Enzymol., 2:1-30.

Delbrück, M. 1945 The burst size in the growth of bacterial viruses (bacteriophages). J. Bact., 50:131-135.

Delbrück, M. 1946 Bacterial viruses or bacteriophages. Biol. Rev. Cambridge Phil. Soc., 21:30-40.

Evans, A. C. 1934 Streptococcus bacteriophage: a study of four serological types. U. S. Pub. Health Rep., 49:1386-1401.

Flu, P. C. 1946 The bacteriophage. Universitaire Pers. Leiden, Leiden.

Fong, J. 1949 Bacteriophage. Ann. Rev. Microbiol., 3:423-444.

Ford, H. F. and Babel, F. J. 1950 Effect of incubation temperature on the retention of bacteriophage by a culture of Streptococcus lactis. J. Dairy Sci., 33:466-472.

Gratia, A. 1921 Studies on the d'Herelle phenomenon. J. Exp. Med., 34:115-126.

Greene, G. I. and Babel, F. J. 1948 Effect of ultraviolet irradiation on bacteriophage active against Streptococcus lactis. J. Dairy Sci., 31:509-515.

Hadley, P. 1928 The Twort-d'Herelle phenomenon. A critical review and presentation of a new conception (homogamic theory) of bacteriophage action. J. Infectious Diseases, 42:263-434.

Harriman, L. A. 1934 Cause of slow acid production in butter cultures. Unpublished Ph. D. Thesis. Ames, Iowa. Iowa State College Library.

Herelle, F. d'. 1922 The bacteriophage, its role in immunity (English edition). Williams and Wilkins Co., Baltimore, Md.

Herelle, F. d'. 1926 The bacteriophage and its behavior. (English translation by G. H. Smith) The Williams and Wilkins Co., Baltimore, Md.

Johns, C. K. and Katznelson, H. 1941 Studies on bacteriophage in relation to cheddar cheesemaking. Can. J. Research, Sect. C, 19:49-58.

Krueger, A. P. 1930 A method for the quantitative determination of bacteriophage. J. Gen. Physiol., 13:557-564.

Krueger, A. P. 1936 The nature of bacteriophage and its mode of action. Physiol. Rev., 16:129-172.

Lauffer, M. A., Price, W. C. and Petre, A. W. 1949 The nature of viruses. Advances in Enzymol., 9:171-240.

Luria, S. E. and Delbrück, M. 1943 Mutations of bacteria from virus sensitivity to virus resistance. Genetics, 28:491-511.

Meanwell, L. J. 1941 The problem of bacteriophage in cheese starters. (Abstract) Proc. Soc. Agr. Bacteriologists, 1941:16-18.

Nelson, F. E. 1936 Study of a factor inhibiting the growth of Streptococcus lactis. Unpublished Ph. D. Thesis. Ames, Iowa. Iowa State College Library.

Nelson, F. E., Harriman, L. A. and Hammer, B. W. 1939 Slow acid production by butter cultures. Iowa Agr. Expt. Sta. Res. Bull. 256.

Nelson, F. E. and Parmelee, C. E. Proliferation of bacteriophage on Streptococcus lactis. Soc. Am. Bact., Abstracts of Papers Presented at 49th General Meeting. 22-23.
1949

Nichols, A. A. The control of slowness in cheesemaking. Dairy Ind., 10:250-256, 265.
1945

Nichols, A. A. and Hoyle, M. Bacteriophage in typing lactic streptococci. J. Dairy Research, 16:167-208.
1949

Nichols, A. A. and Ineson, P. J. Cheese starter "recovery" after attack by bacteriophage. (Strain dominance in multiple strain starters.) J. Dairy Research, 15:99-111.
1947

Overcast, W. W. Influence of pH on proliferation of Streptococcus lactis bacteriophage. Unpublished Ph. D. Thesis. Ames, Iowa. Iowa State College Library.
1950

Parmelee, C. E., Carr, P. H. and Nelson, F. E. Electron microscope studies of bacteriophage active against Streptococcus lactis. J. Bact., 57: 391-397.
1949

Price, W. H. Bacterial viruses (bacteriophages). Sci. Monthly, 67:124-127.
1948

Turner, G. E. Dynamics of the Streptococcus lactis-bacteriophage relationship. Unpublished Ph. D. Thesis. Ames, Iowa. Iowa State College Library.
1948

Whitehead, H. R. and Cox, G. A. The occurrence of bacteriophage in cultures of lactic streptococci. New Zealand J. Sci. Technol., 16:319-320.
1935

Whitehead, H. R. and Cox, G. A. Bacteriophage phenomena in cultures of lactic streptococci. J. Dairy Research, 7:55-62.
1936

Whitehead, H. R. and Hunter, G. J. E. Observations on the activity of bacteriophage in the group of lactic streptococci. J. Path. Bact., 44:337-347.
1937

Whitehead, H. R. and Hunter, G. J. E. The progress and present position of research on cheese starters in New Zealand. New Zealand J. Sci. Technol., 23:40A-46A.
1941

Whitehead, H. R. and Hunter, G. J. E. Bacteriophage infection in cheese manufacture. J. Dairy Research, 14:64-80.
1946

Whitehead, H. R. and Hunter, G. J. E. Bacteriophage in cheese manufacture. Contamination from farm equipment. J. Dairy Research, 15:112-120.
1947

Wilkowske, H. H. Characterization of bacteriophages active against lactic streptococci. Unpublished Ph. D. Thesis. Ames, Iowa. Iowa State College Library.
1949

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