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Dynamics of the Streptococcus lactis-bacteriophage relationship

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NOTE TO USERS

DYNAMICS OF THE *STREPTOCOCCUS LACTIS*-BACTERIOPHAGE RELATIONSHIP

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INTRODUCTION

Bacteriophage problems pertaining to the dairy industry have been approached to date primarily with a view of preventing losses of revenue resulting from the failure of the lactic streptococci in starter cultures to perform normally. This condition is exemplified in cheesemaking. Mother cultures, bulk cultures or cheese milk contaminated with bacteriophage fail to show the desired changes in acidity during the normal time, while with cheddar cheese the resultant product does not ripen satisfactorily and may become unsaleable.

These conditions have been remedied largely by the establishment of practices in commercial plants which attempt to reduce to a minimum the concentration of bacteriophage that may gain entrance to the product. With such measures, the acid development required during the various stages of the cheese manufacturing process is completed before the bacteriophage reaches sufficient concentration to cause lysis of the lactic acid streptococci and consequent curtailment of acid production. The established practices used to combat bacteriophage consist of selection and rotation of starter cultures, maintainance of strict sanitation of plant equipment and surroundings, and special precautions for the preparation of mother and bulk starters.

In general, such procedures, where rigidly followed, have been quite successful in combating bacteriophage, even to the point where serious losses of revenue due to bacteriophage outbreaks in cheese manufacture have

become surprisingly few. Nevertheless, bacteriophage expresses itself sporadically even with strict adherence to the recommended measures of combat, while slight laxity in sanitation may result in heavy financial losses.

It appeared that the most fruitful approach to be taken in future studies of the bacteriophage active against Streptococcus lactis would be consideration of the more fundamental factors concerned with the bacteriophage-organism relationship. Therefore, a study of the dynamics of the bacteriophage-organism association was initiated. It was hoped this would result in partial elucidation of some factors affecting bacteria and bacteriophage growing together in milk.

STATEMENT OF PROBLEM

The purposes of the work reported herein were (a) to develop a method suitable for enumerating particles of bacteriophage active against Streptococcus lactis by a plaque method which would give reasonable precision and (b) to apply the resultant method to the detailed study of the proliferation of streptococcus bacteriophage when both bacteriophage and sensitive S. lactis bacteria were inoculated in sterilized milk.

REVIEW OF LITERATURE

Since the first report by Twort (1915) of the discovery of a transmissible lytic agent active against staphylococcus organisms isolated from calf vaccines, much evidence has accumulated to show that bacteriophages active against a wide variety of bacteria are demonstrable.

A tremendous amount of research work has been done on the problem of the nature of bacteriophage, and the amount of literature pertaining to the many aspects of bacteriophage is impossible to review in this type of presentation. However, many excellent reviews are available which attempt to summarize the known data and which theorize on the debatable aspects of the subject. These include those of d'Herelle (1926), Hadley (1928), Bronfenbrenner (1928), Burnet (1934), Kreuger (1936), Delbrück (1942) and Delbrück (1946).

The review of literature included in this thesis has been limited to those publications concerned with, first, a survey of the reports indicating the importance of bacteriophage active against S. lactis; second, methods of demonstrating bacteriophage activity; third, the mechanisms of bacteriophage proliferation; and, lastly, the effect of temperature on the rate of bacteriophage proliferation.

Bacteriophage Active Against Streptococcus lactis

The first reported appearance of bacteriophage probably responsible for "slowness" of acid production in cheesemaking was that of Whitehead

and Wards (1933) in New Zealand. A starter culture, originating at the Dairy Institute laboratory, suddenly failed to produce acid in bulk starters after a period of several months of successful use in a cheese factory. The agent responsible for the failure was found to be carried in the bulk starter itself, rather than in the mother culture. A year later, Whitehead and Cox (1934) while studying an outbreak of "slowness" in acid production, this time caused by an apparently normal mother culture, found that the commercial pasteurization of cheesemilk as practised in New Zealand was insufficient to destroy bacteriophage. Considerable work has been done on bacteriophage in New Zealand since the early reports, and this has been reviewed by Whitehead and Hunter (1945). The extent to which bacteriophage problems have been attacked and the methods used to overcome its effects are developed in that review. Considerable success in combating bacteriophage outbreaks has resulted from the long time study of this problem by the New Zealand group.

The Iowa Agricultural Experiment Station (1933) reported that certain butter cultures contained a transmissible agent capable of delaying the coagulation of milk when it was added together with fresh normal starter. The inhibiting agent could be demonstrated in whey filtrates freed from bacterial cells. Harriman (1934) reported that "addition of a slow butter culture to a normal culture markedly restrained the growth of the normal culture," thus indicating the transmissibility of the inhibitory principle. Nelson (1936) and Nelson et al. (1939) were the first in the United States to report that the filtered transmissible lytic inhibitory principle active against S. lactis cells was probably bacteriophage, as it possessed the characteristics ascribed to the bacteriophages. Babel

(1946) definitely implicated bacteriophage as the cause of "cessation of acid production" in the manufacture of cheddar and cottage cheeses. He was able to demonstrate bacteriophage in bacteria-free filtrates by addition of a small amount of the filtrate to S. lactis cells used in making the cheese, failure of subsequent acid production resulting, or by the appearance of lysed areas on solidified agar medium, over the surface of which was spread an even layer of susceptible S. lactis cells and suspected filtrate.

Mazé (1937) in France, Johns (1941) in Canada, Anderson and Meanwell (1942) and Nichols and Wolf (1944) in England, Mosimann (1946) in Switzerland, and Pette (1946) in Holland are among those who have studied certain aspects of the problem of bacteriophages in cultures of lactic streptococci and have demonstrated the widespread distribution of this bacteriophage in the dairy industry.

Bacteriophage Enumeration Studies

D'Herelle (1926) demonstrated the ability of the intestinal bacteriophage to bring about lysis of susceptible cells by using "clearing" of broth containing both bacteriophage and susceptible organism or by the appearance of lysed areas or "plaques" obtained by distributing a highly diluted bacteriophage filtrate over the hardened surface of a nutrient medium in a petri plate, the surface being seeded with a uniform layer of susceptible cells. Such procedures were used quantitatively to estimate the number of particles of bacteriophage in a given suspension. By a limiting dilution method using broth, a dilution eventually was reached, beyond which bacteriophage particles would be absent and not cause lysis of added

susceptible cells. Furthermore, if similar broth dilutions were spread over the surface of a hardened layer of agar medium smeared with susceptible cells, the plate corresponding to the limiting dilution produced one plaque, but none appeared from dilutions made beyond the limiting point.

The limiting dilution method was used by Harriman (1934) and Nelson (1936) in their work on determining the activity of a lytic principle active against S. lactis, but these investigators used absence of reduction and failure of acid development in litmus milk rather than "broth clearing" as an index of activity.

These procedures are the bases of methods used for enumeration of bacteriophage particles in the various technics in use at the present time.

According to d'Herelle (1926) a plaque was a colony of bacteriophage particles which originated from a single particle. Furthermore, the number of plaques that developed on an agar plate was in direct proportion to the quantity of bacteriophage contained in the filtrate. Hershey and Bronfenbrenner (1941), while investigating the influence of host resistance on virus activity by the plaque plate method, indicated that one bacteriophage particle per susceptible cell was sufficient to initiate the appearance of a plaque. Delbrück (1942, 1946), as a result of his studies on plaque formation, arrived at the conclusion that a plaque represented a locus of bacteriophage infection. Any given plaque may be the result of a single free bacteriophage particle or a bacteriophage infected cell containing one or more particles of bacteriophage. Regardless of the numbers of particles that form the locus of infection, only one plaque is formed once that locus is fixed.

The plaque plate method was modified by Yen (1935), who used a method

similar to that employed for plating bacteria. He considered that the bacteriophage and organisms would be better distributed within a single layer of solid medium as would not be the case with spreading a similar mixture on the surface of a solid medium. Difficulty was experienced in obtaining clearly defined plaques at different levels within the agar layer and those developing below the surface were difficult to count. Gratia (1936) overcame this by using a single layer of sterile solidified medium with a thin overlay of agar medium which contained the bacteriophage and susceptible organism. He considered that a more homogeneous mixture of bacteriophage and organism was obtained and as a result greater precision was possible. Hershey and Bronfenbrenner (1941) and Hershey et al. (1943) used this method in quantitative studies of bacteriophage and indicated that the method provided an essentially rich nutrient base and at the same time assured the necessary conditions for plaque formation.

Several workers, (Bronfenbrenner and Korb, 1923, 1925; Dreyer and Campbell-Renton, 1933; Yen, 1935; Hershey et al., 1943; Kleczkowska 1945; and Anderson, 1948) have reported that both numbers of plaques and their size may be varied widely by changing given conditions, regardless of the method used for plaque enumeration.

Bronfenbrenner and Korb (1923) found that increasing the agar concentration decreased the number and size of plaques. They also reported (1925) that increasing the agar concentration from 0.5 per cent to 1.0 per cent resulted in a decrease of 10 per cent in the plaque count and when the concentration reached 4.5 per cent the count was reduced to zero. By varying the agar concentration, these workers were able to reduce considerably the discrepancy between the broth clearing and the plaque plate methods for enumeration of bacteriophage activity. Hershey and

Bronfenbrenner (1941) demonstrated that an 8 ml. volume of agar reduced the plaque count when compared to using a 4 ml. volume of agar, both having the same agar concentration. The same workers pointed out in 1943 that while a lower concentration of agar favoured diffusion of the bacteriophage particles, the "critical factor" was agar volume rather than agar concentration, indicating that air supply was probably related to plaque formation. Dreyer and Campbell-Renton (1933) agreed with other workers regarding the importance of agar concentration on the numbers and size of plaques, but differed with respect to the effects of the concentration of cells added to plaque plates on the numbers of plaques obtainable. They referred to the work of Bronfenbrenner and Korb (1925), who found that cell concentration had no effect on the number of plaques, and to Burnet (1925), who reported that plaque numbers increased to a maximum and then decreased as the cell concentration was progressively increased. Instead, they indicated that greater numbers of plaques were secured on the more heavily seeded plates. Yen (1935) presented evidence that reducing the cell concentration to 50 million per ml. caused the resultant plaques to be smaller in size and fewer in numbers and that best results were obtained when the cell concentration was maintained between 1,000 and 5,000 million cells per ml. Kleczkowska (1945) found that as the agar concentration in plaque plates was increased from 0.5 per cent to 2.25 per cent there was a reduction in count from 180 to no plaques from 1 ml. of a 10^{-5} dilution. With the introduction of temperature as a variable, he secured results ranging from plaques too numerous to count with 0.5 per cent agar concentration at the incubation temperature of 15°C. down to 81 plaques at an incubation temperature of 30°C. using the same concentration of agar. This investigator also reported that wide variation in the thickness of the

bacterial suspension added to the plate was without effect on plaque size. Anderson (1948) indicated 100-fold reduction in the numbers of plaques obtainable, when using a synthetic nutrient medium, with coliform bacteriophage incubated at 15°C. as compared with the count obtained at 37°C. This difference was not apparent under similar conditions of incubation when an organic medium was substituted. The difference was attributed to a symbiotic nutritional factor, capable of production by the cell at 37°C. but not at 15°C. on the synthetic medium. When this substance was produced, it was utilized by the bacteriophage to effect adsorption to the cell and subsequent plaque formation.

While the reviewed literature definitely reveals the shortcomings of the plaque plate to demonstrate absolute numbers of bacteriophage particles in a given suspension, it is possible, by strictly following a given procedure, to secure by plaque formation a consistent, reproduceable proportion of the absolute quantity of bacteriophage plated out. The work reported by Hershey and Bronfenbrenner (1941) emphasized that the plaque count obtained from a given bacteriophage suspension was "strictly proportional to the aliquot of bacteriophage plated" and also indicated "the probability that a given particle will form a plaque varies with the conditions imposed, but under given conditions the probability is constant and independent of any association between the particles." This view was supported by statistical treatment of plaque formation. Kleczkowska (1945) also presented a statistical analysis of plaque formation which supported the views of Hershey and Bronfenbrenner (1941) that by strictly following a set procedure excellent duplication of results was possible. In addition, this worker pointed out that large variations in results were inevitable when plating conditions were not kept uniform.

Mechanism of Bacteriophage Proliferation

In the original researches of d'Herelle, cited in his review (d'Herelle, 1926), the statement was made that both bacteriophage and susceptible organisms proliferated in logarithmic fashion when in association, the rate of bacteriophage increase exceeding that of the bacterial cell to such an overwhelming extent that lysis of the cells subsequently followed. Furthermore, the observed facts showed that whereas the bacterial cells increased at a relatively uniform rate, bacteriophage increased by "steps." Most workers who have studied bacteriophage are in general agreement regarding the overall method of bacteriophage proliferation as explained by d'Herelle, although differences of opinion exist on the mechanism of the proliferation. As a result of the earlier work of Burnet (1925, 1929) and Kreuger and Northrup (1930), together with the more recent investigations of Kreuger and Fong (1937), Ellis and Delbrück (1939), Delbrück (1940a, 1945) and others, a much clearer picture can be obtained as to the probable mechanism of bacteriophage proliferation.

Burnet (1929), Kreuger and Northrup (1930), Clifton and Morrow (1936), Delbrück (1942), Delbrück and Luria (1942), Hershey et al. (1943) and Kreuger et al. (1946) all agree that bacteriophage proliferation occurs in three distinct phases. They are, in order, adsorption of the particle to the cell, proliferation of the bacteriophage on or within the cell, and the subsequent release of the newly formed particles into the surrounding medium. Under optimum conditions of growth, the stepwise nature of increase is hardly detectable and the use of special techniques is necessary to demonstrate the phenomenon (Kreuger et al., 1946). The technique most

generally employed is that of Burnet (1929). A bacteriophage-organism suspension is diluted to the extent that one bacterial cell, infected with a bacteriophage particle, is allowed to develop in nutrient medium and the number of newly formed particles is determined. By this "one step growth" technique, Burnet (1929), Hershey et al. (1943) and Delbrück (1945) were able to confirm d'Herelles' observations regarding the stepwise nature of bacteriophage proliferation.

Controversy exists, however, on the question of cell lysis and bacteriophage proliferation. While all workers agree that mass lysis of the susceptible cells takes place when a critical concentration of bacteriophage to cells is attained, Kreuger and Northrup (1930) do not accept the view that each cell is lysed when the newly formed particles are released, a view which is held by Delbrück (1945). The former workers concluded that individual cell lysis was not evident and that no cells were lysed until there was an excess of bacteriophage to cells, at which time mass lysis of all the susceptible cells took place over a short period of time. With such a viewpoint, bacteriophage increase took place without lysis of the individual cell, which instead, continued to divide normally. Hershey and Bronfenbrenner (1943) were unable to accept the Kreuger and Northrup contention of "continuous bacteriophage secretion" and indicated "that the possibility of stepwise liberation of bacteriophage without lysis seemed rather remote." However, Hershey and Bronfenbrenner (1943), theorized from the work of Delbrück (1940b) that if the bacteriophage particles are held to the bacterial cell by chemical forces of varying intensities, it would be possible for particles to be liberated from the cell almost as fast as they were formed. Thus, as stated by Hershey and Bronfenbrenner (1943),

under these conditions "bacteriophage could be secreted by a living cell which would undergo lysis only after the external concentration of bacteriophage had become high." The evidence that might lend support to this viewpoint of Kreuger and his workers is suggested by the investigations of Clifton and Morrow (1936) who found that "the rate of growth of cells in the presence of bacteriophage was not any different from that of the bacteriophage-free controls until the time of lysis." Kreuger (1936) presented arguments in support of the work of Kreuger and Northrup (1930) and that of Andrewes and Elford (1932) who found the slopes of the bacterial growth curves identical, with or without added bacteriophage, until mass lysis of the cells in combination with the bacteriophage occured. He reiterated that if lysis of individual cells took place, the presence of bacteriophage in the bacteriophage-organism mixture would of necessity need to stimulate the growth of the surviving cells in order to simulate the same rate of growth as the normal cells. Delbrück and Luria (1942), who have worked considerably on "one step growth curves" to demonstrate the stepwise nature of bacteriophage proliferation, and who have supported the premise of simultaneous lysis and bacteriophage liberation, state in this connection "most observers have consequently pictured lysis as the immediate cause of virus liberation." These investigators cited Cordtes (Delbrück and Luria, 1942) who reported a "case in which virus liberation occured in a form of a sudden burst but was not accompanied by lysis of the cell" . . . "the infected cell survived and proceeded to divide" in a nutrient medium containing 0.5 per cent NaCl. Therefore, Delbrück and Luria (1942) concluded that virus liberation of the burst type was not the result of the lysis of the cell but rather an accessory phenomenon which may or may not accompany virus liberation.

Another controversy relative to the bacteriophage-organism mechanism is that concerned with whether or not bacteriophage proliferation and bacterial multiplication are inseparable. Kreuger and Northrup (1930) indicated "bacterial growth as an essential conditioning agent for bacteriophage formation," a similar observation having been made by Burnet (1929) a year earlier. Spizizen (1943) used bacteriophage to study the mechanism of virus multiplication and reported that under normal conditions "cell growth occurred concomittent with virus multiplication" but that by special techniques "it was possible to separate growth of cells and growth of virus." For example, glycine and glycine anhydride in low concentration stimulated virus multiplication but was not enough to cause an increase in cell division. Hunter (1943) on the other hand, reported that under normal conditions bacteriophages active against S. lactis strains "are distinct entities whose growth conditions are similar to, but not necessarily identical with those of the bacteria upon which they develop." Clifton and Morrow (1936), while agreeing that bacterial cell multiplication was essential for bacteriophage production, pointed out that such was only true during the logarithmic growth of bacteriophage-organism association. The relationship was not apparent during the lag period, as the bacteriophage particle was being adsorbed to the cell during this time. By the use of penicillin to inhibit cell growth, Price (1947) was able to secure a 100,000-fold increase of staphylococcus bacteriophage at the expense of the inhibited substrate. He thus indicated that bacteriophage growth was not necessarily dependent upon bacterial growth. Kreuger and Fong (1937) presented material in which they were able to separate the mechanism into its two component parts. They indicated that whereas the optimum temperature for

staphylococcus bacteriophage was 35°C., that of the associated organism was approximately 40°C. Furthermore, by adjustment of the medium to pH 6.0 and to a temperature of 28°C. bacterial growth was inhibited, while that of the bacteriophage proceeded at a rapid rate. In addition to the consideration of the overall treatment of bacteriophage proliferation, it is desirable to review briefly the mechanics of each phase of the proliferation cycle.

The first phase, that of adsorption, includes the time necessary for the bacteriophage particles to become adsorbed to susceptible cells. Delbrück (1942) and Delbrück and Luria (1942) pointed out that, experimentally, the factors of physiological state of the cells, temperature, type of medium and the concentration of cells in relation to bacteriophage, all affect the rate of adsorption. For instance, actively growing cells adsorbed bacteriophage particles much faster than did cells in the lag period of growth (Delbrück, 1942), while reduction in temperature increased adsorption time (Delbrück and Luria, 1942).

The second phase represents that time from adsorption until actual release of the newly formed bacteriophage particles into the surrounding medium (Delbrück and Luria, 1942). During this interval no external activity is apparent and the bacteriophage is considered to be increasing in quantity within the cell during this time. The infected cell retains its normal appearance during this interval, but suddenly new particles of bacteriophage are liberated and in most cases lysis of the individual cells occurs at this time, (Delbrück and Luria, 1942).

The third phase, termed the "rise period" by Delbrück and Luria (1942), represents the step or burst by which the bacteriophage increase from a

single particle to many particles depending on the type of bacteriophage. Ellis and Delbrück (1939) estimated the average at 70 particles per burst, and Delbrück (1945) found the average to be 180 particles per burst for the coliform bacteriophage, while d'Herelle (1926) and Burnet (1929) reported 40 particles per burst for the dysentery bacteriophage.

After release of the newly formed particles, they are, under normal conditions, immediately adsorbed to other cells in the medium and thus the cycle is repeated. In the "one step growth" experiments, however, adsorption of the newly formed particle by other cells is prevented by dilution of the mixture and the numbers of particles released by a single cell may be enumerated by plaque plate technique. Under normal conditions the cycle of adsorption, growth of bacteriophage intracellularly and release of the new particles is repeated until a critical ratio of bacteriophage particles to cell numbers is attained, at which point mass lysis of the cells follows. At this time the bacteriophage particles remain free in the medium as no further adsorption is possible for lack of cell substrate (Clifton and Morrow, 1936).

Most workers have reported, since the observations of d'Herelle (1926), that sooner or later, after lysis of susceptible cells, there appeared in the lysate secondary growth of bacteria which possessed variable resistance to the bacteriophage within the existing culture. Gratia (1922), for instance, found that some resistant secondary growth cells obtained from a coliform lysate were just able to grow in the presence of the lytic agent, whereas other cells had the ability to withstand stronger concentrations of the lytic principle. Delbrück (1946) considered resistant bacterial cells the result of spontaneous mutation and after several transfers the

cells could regain their former sensitiveness to the parent bacteriophage. Nelson (1936) indicated that, with few exceptions, cells obtained from secondary growth S. lactis in combination with the homologous bacteriophage were resistant to the parent bacteriophage, but might be sensitive to other strains of bacteriophage. Similar results were reported by Whitehead and Hunter (1937) in that secondary growth S. lactis cells were variable in their sensitivity toward the parent bacteriophage; those resistant to the bacteriophage with which they were tested first were sensitive to other bacteriophage making its appearance later.

Effect of Temperature on the Rate of Bacteriophage Multiplication

Important factors known to affect proliferation rates of bacteriophage-organism mixtures include physiological condition of the susceptible cell, the pH of the culture medium and the temperature at which the reaction is permitted to take place.

Delbrück (1940a) emphasized the importance of the first factor. He found that the coliform cell in an active physiological state was larger than one not in an active state and proved experimentally that the probability of adsorption of the bacteriophage particle by the susceptible cell, therefore, was much greater due to the increased cell surface. This meant a shorter adsorption time. Kreuger and Northrup (1930) indicated the importance of temperature as a factor influencing the rate of growth of cells which in turn conditioned bacteriophage increase. A medium containing 1×10^7 staphylococcus bacteriophage particles per ml. and $1 \times 10^{7.4}$ susceptible cells per ml. incubated at 10°C. showed no growth of cells or bacteriophage for 22 hours. However, when cell growth commenced at that

time, bacteriophage increase soon followed and eventually lysis of the cells was accomplished. Thus it appeared that bacterial growth was essential to initiate bacteriophage increase. Kreuger et al. (1946) found, while working with staphylococcus bacteriophage, that increased temperature of the incubation mixture resulted in a shortened adsorption time which they attributed to the production of a cell precursor requisite for adsorption of the bacteriophage to the cell. The higher incubation temperature permitted a faster production of the cell precursor which in turn shortened the adsorption time. A similar idea was reported by Anderson (1948), who discussed the influence of temperature as a "conditioning factor" for the production, by coliform bacteria, of a co-factor necessary to effect adsorption of coliform bacteriophage. A temperature of 15°C. was too low for the cell to metabolize the necessary co-factor, but raising the temperature to 37°C. allowed production of the co-factor and adsorption was effected. Kreuger and Fong (1937) indicated from their work that manipulation of temperature markedly influenced bacteriophage production. At a temperature of 28°C., with certain adjustments in pH of the medium, staphylococcus bacteriophage production proceeded unhindered, while that of the susceptible cells was inhibited completely. By raising the temperature to 45°C. the opposite condition was realized, bacteriophage reproduction being decreased while cell multiplication remained unimpaired.

Bloch (1942) discussed the effect of temperature on bacteriophage increase in relation to cell lysis and reported that coliform bacteriophage and cell lysis were confined between temperature limits of 20°C. and 42°C., and at 34°C. the two processes proceeded at their optimum rates. It was further pointed out that within the temperature range of 20° to 34°C.

bacteriophage reproduction rate increased logarithmically with increase in temperature. This worker was unable to show a direct relationship between bacterial growth and bacteriophage proliferation. Hunter (1943) also indicated that the two processes were separate and distinct. He made a rather complete study of five strains of lactic streptococci together with their homologous bacteriophages with respect to the effect of temperature on proliferation rates and time of cell lysis. The optimum temperature for the growth of the susceptible cells for the different combinations was 30°C. and the time of cell lysis by the bacteriophage varied from 4 to 6 hours at that temperature. Reduction of the incubation temperature to 22°C. caused a considerable slowing of bacterial growth accompanied by an increased time required for lysis of the cells in all cases. However, with one combination, Hunter (1943) observed that the growth rate of the cells was slightly less at 37°C. than at 22°C., but lysis of the cells by the bacteriophage occured sooner at 37°C. than at 22°C. This was one case where slower rate of cell growth did not result in slower rate of bacteriophage multiplication. The conclusion arrived at by this worker was that this particular bacteriophage possessed a different optimum temperature for maximum growth than that for the corresponding susceptible organism.

EXPERIMENTAL METHODS

Preparation of Media

Litmus milk was prepared by adding sufficient aqueous solution of litmus to commercially pasteurized skim milk to impart a light mauve color. This milk then was dispensed in 8 ml. amounts into test tubes and sterilized at 15 pounds steam pressure for 25 minutes.

Tomato juice-peptonized milk medium containing both 1 and 1.5 per cent agar were used. Each was prepared identically except for the concentration of agar. For each liter of medium, 10 g. peptonized milk, 10 g. proteose peptone and 10 or 15 g. agar were dissolved in distilled water and the volume was made up to 600 ml. with distilled water. To this was added 400 ml. of clear tomato juice, previously adjusted colorimetrically to pH 6.4. The mixture then was adjusted electrometrically to exactly pH 6.4. The prepared agar medium was dispensed in 100 ml. amounts in screw cap bottles and sterilized at 15 pounds steam pressure for 20 minutes. After sterilization and while the medium was still hot, one bottle of medium was cooled to about 45°C. and the pH was determined electrometrically. Usually the reaction of the medium at this time was between pH 5.8 and 6.2. If the pH was not within this range, the required adjustment was made on the medium in the same bottle, noting the amount of reagent necessary to bring the pH of the medium to within the desired range. Then the required amount of sterile reagent was added aseptically to each bottle of medium.

Clear tomato juice was obtained by filtering canned whole tomatoes

through cheese cloth, absorbent cotton and coarse filter paper.

Sources of Bacteriophage-Organism Combinations

The bacteriophage-organism combinations used for study were obtained from the collection maintained in the Dairy Bacteriology Laboratories at Iowa State College. The sources and designations are presented in table 1.

Table 1.

Sources and designation of bacteriophage-organism combinations

Original bacteriophage designation	I. S. C. bacteriophage designation	*S. lactis* designation	Source of combination
Filtrate ip5	F68	IP5	Miss A. Nichols, Reading, England.
Filtrate 46	F69	ML1	H. R. Whitehead, Palmerston North, New Zealand.
Filtrate 1	F70	SH5*	St. Hyacinth, Quebec, Canada.
PF 2	PF2	H1-2	Iowa State College.
PF 11	PF11	H1-10	Iowa State College.

*Designation "SH" affixed by the writer: cultures otherwise designated as received.

To test susceptibility of the streptococcus cultures toward the homologous bacteriophages used, approximately thirty well-separated colonies from each of the five *S. lactis* strains were picked at random from plates of tomato juice-peptonized milk agar into sterile litmus milk. After incubation at 30°C. for approximately 18 to 20 hours, each culture was tested for its susceptibility toward its homologous bacteriophage. Two tubes of sterile litmus milk were inoculated with one drop of susceptible culture. To one of the pair, one drop of homologous bacteriophage filtrate was

added. Pairs of tubes containing litmus milk thus were set up with each culture developed from the agar plate colony. Subsequent incubation at 30°C. overnight indicated the susceptibility of the selected culture to the parent bacteriophage. The tube of litmus milk containing both bacteriophage and organism which failed to show growth of S. lactis at approximately the same rate as the control without added bacteriophage, indicated that the particular culture was susceptible to the inoculated bacteriophage. All the isolated colonies from each strain of S. lactis culture used in the study were susceptible to each respective homologous bacteriophage. Therefore, the strain of S. lactis comprising a given culture was considered sufficiently pure for the purpose of the study.

To test the activity of each of the five strains of bacteriophage against each parent susceptible culture, two isolated plaques for each bacteriophage were removed from a plaque plate with a sterile loop and each was added to a tube of sterile litmus milk containing one loopful of the susceptible strain of S. lactis cells. A control was made consisting of litmus milk inoculated with the corresponding susceptible strain of S. lactis but containing no added bacteriophage. After incubation for approximately 18 to 20 hours, cell-free bacteriophage whey filtrates were prepared from the litmus milk showing bacteriophage activity. The filtrates so obtained were tested for activity against the corresponding parent S. lactis cultures. In all cases, both plaques isolated from each bacteriophage strain lysed the organisms contained in the parent S. lactis culture. A single time through this plaque isolation procedure was considered sufficient purification for the purposes of this problem, because the bacteriophage strains had been propagated several times on single pure strains of S. lactis and thus

purified to some degree.

Preparation and Carrying of S. lactis Cultures

The S. lactis cultures used for the investigation were carried in litmus milk and were subcultured daily during actual use. After the required incubation time had elapsed the cultures were cooled in ice water and were stored in a mechanical refrigerator maintained at 2° to 3°C. Those cultures not in daily use were subcultured every third day and were kept under refrigeration after the required incubation time. When needed, such cultures were transferred daily for three successive days before actual use in order to have the organisms in a physiologically active state.

Because time of incubation influences the physiological condition of cells in cultures, it was necessary to establish a method with respect to length of time of incubation such that the physiological state of the different S. lactis strains would be somewhat the same. To control the variable of the physiological state of the S. lactis cultures and to ensure at the same time an easy method to quantitatively estimate the numbers of cells per ml. of culture, 0.1 ml. of 10^{-1} dilution of a given S. lactis culture was added to 8 ml. of sterile litmus milk and incubated at 30°C. until coagulation could be observed. At that time, the length of the incubation period being recorded, the number of organisms per ml. of culture was determined by plate count, using tomato juice-peptonized milk agar. The seeded plates were incubated at 30°C. for 48 hours before counting. Thus a record was obtained on each culture used as to the number of hours taken for coagulation, together with a plate count on the numbers of cells at the time of coagulation. By following this procedure for all the

cultures used in this study, it was possible to dispense with a daily direct count determination of cell numbers and every culture was considered to be in a similar state physiologically.

Preparation and Carrying of Bacteriophage Filtrates

The streptococcus bacteriophage strains used in this study were carried in whey filtrates and it was necessary to repropagate the bacteriophage strains quite often to maintain a reasonably high level of activity. This was particularly necessary when the bacteriophage was used daily for comparative research work. Increasing the activity of a bacteriophage filtrate was accomplished in the following manner. Approximately two drops of an active culture of susceptible S. lactis cells used for propagation of the particular strain of bacteriophage were added to 100 ml. of sterile skim milk. This was incubated at 30°C. for 20 to 30 minutes, after which one drop of homologous bacteriophage whey filtrate was added. After gentle mixing, followed by incubation at 30°C. for 10 to 12 hours, the milk mixture was coagulated by addition of approximately 3.6 ml. of sterile 10 per cent lactic acid. The coagulum was caught in a sterile glass funnel containing sterilized coarse filter paper and the filtrate passed into a sterile bottle. The filtrate was immediately passed through a sterile Selas microporous porcelain filter (porosity #03) attached to a sterile suction flask, the whole assembly being maintained under a slight negative pressure. The bacteria-free whey filtrate containing the bacteriophage finally was placed in a sterile screw cap bottle and stored under refrigeration of 2° to 3°C. at all times.

Measurement of Bacteriophage Activity

The quantitative estimation of bacteriophage activity was made either by the limiting dilution technique used by Harriman (1934) and by Nelson (1936), or the two layer plaque plate method used by Delbrück (1945), the choice depending on the conditions of the particular study.

Limiting dilution method

This consisted of making successive ten-fold dilutions, usually in sterile distilled water, of the medium containing the unknown quantity of bacteriophage. One ml. of each dilution was added to 8 ml. of sterile litmus milk containing 0.1 ml. of active susceptible S. lactis culture. A control tube containing 8 ml. of litmus milk to which was added 0.1 ml. of culture as described above but with no added bacteriophage also was prepared. After incubation at 30°C. for 18 to 20 hours the control tubes of litmus milk were coagulated, while the extent of coagulation of the litmus milk containing both bacteriophage and organism gave a semi-quantitative measure of the bacteriophage activity. The highest dilution of the bacteriophage-containing medium which prevented normal coagulation of litmus milk was considered the bacteriophage titer for the particular preparation. Thus if the tube of litmus milk containing the 10^{-6} dilution of the original bacteriophage was not as coagulated as the control, and the litmus milk containing the 10^{-7} dilution was coagulated, the bacteriophage titer was considered as 10^{6}. This meant that 0.000001 ml. of the original bacteriophage preparation would prevent normal acid development by 0.1 ml. of active S. lactis culture added. The limiting dilution method described above was used for comparison with the plaque plate method of determining

bacteriophage activity. When greater accuracy was desired five tubes of each dilution were prepared and the "most probable number" of bacteriophage particles determined from the probability tables in Standard Methods (American Public Health Association, 1948).

Plaque plate method

The two layer agar plaque plate method adapted from that by Delbrück (1945) consisted of allowing 12 ml. of tomato juice-peptonized milk medium of 1 per cent agar strength to harden in a petri plate. The upper layer consisted of 1 ml. of 1:25 water dilution of susceptible cells from a culture which had just begun to coagulate, 1 ml. of milk containing the diluted bacteriophage and 1 ml. of the agar medium containing 1.5 per cent agar, the different materials being combined in the order mentioned. The final agar concentration of the agar overlay was 0.5 per cent. Immediately on addition of the 1 ml. of agar to the other materials comprising the overlay, the plate was rotated sufficiently to assure thorough mixing of the contents and the plate was placed in a level position until the agar overlay was hardened. The plates were incubated at 30°C. for approximately 10 hours, in an upright position, after which the plaques formed were enumerated.

The amount of the base layer of agar was not measured, but the amount of agar for the overlay was measured accurately because the concentration of agar in the overlay was found to affect the number of plaques appearing on the plates. The agar medium used for the overlay was held in a 55°C. incubator during the period of each trial. Experience indicated this as a necessary precaution, otherwise excessive cooling of the medium occured when 1 ml. of it was pipetted into the 2 ml. of material already in the plate, and this sometimes would result in a granular appearance of the

agar on hardening. Plaques developing on such plates were difficult to count accurately because the plaques were defined poorly. Other factors in addition to those mentioned in the review of literature that effect plaque formation are presented under experimental results in this thesis.

Considerable difficulty was experienced with regard to a suitable source of light for counting plaques on agar plates. This difficulty has not been overcome satisfactorily yet. However, reasonable success was obtained with a 15 watt daylight fluorescent tube using dull tin foil as a background. The plates containing the plaques were held approximately 12 to 14 inches distant from the light source and were counted against the tin foil background. Further work on a more suitable light source would be desirable.

Demonstration of Burst Time and Burst Size of *S. lactis* Bacteriophage

To demonstrate the stepwise proliferation of bacteriophage the procedure used by Delbrück (1945) was modified. One ml. of sterile skim milk containing approximately 40 million bacteriophage particles was mixed with 1 ml. of sterile skim milk containing approximately 80 million susceptible *S. lactis* cells. After adsorption for 5 minutes, 2 ml. were added aseptically to 98 ml. of sterile skim milk. After shaking, the milk suspension was further diluted 10,000 times, using two 99 ml. milk dilutions. Each ml. of milk in the last dilution, therefore, contained approximately 80 cells, 40 of which were infected with bacteriophage. Finally, 1 ml. of this last dilution was added to 39 ml. of sterile skim milk and, after thorough shaking, the entire 40 ml. were distributed evenly among 40 petri plates

containing solidified sterile tomato juice-peptonized milk medium of 1 per cent agar strength. The 40 petri dishes then were grouped into 4 lots of 10 plates each, and at stated intervals of time at room temperature each lot was completed by addition of the agar overlay already described. The plates were incubated for 10 to 12 hours and the numbers of plaques enumerated. In trials where temperatures of 37 and 45°C. were used all the 1 ml. amounts of diluted bacteriophage milk preparation were placed in serological test tubes for holding during the incubation period, after which the tube contents were emptied onto the surface of the solidified agar in the petri plates, and susceptible cells and 1.5 per cent agar were added. By this procedure, drying of the inoculum during incubation at higher temperatures and also shifts in pH where that of the medium was different from that of the milk were avoided.

Methods Used to Study Rates of Proliferation of S. lactis Bacteriophage

Commercially pasteurized skim milk was dispensed in 300 ml. amounts into one-pint screw cap bottles and was sterilized at 15 pounds steam preassure for 15 minutes. For each temperature under study, two such bottles of sterile skim milk were adjusted to the required temperature by immersion up to their necks in a constant temperature water bath thermostatically controlled to within 0.2°C. of the desired temperature. On attainment of the required temperature, both bottles of sterile milk were inoculated with sufficient susceptible S. lactis cells to give a plate count of approximately 100,000 per ml. of test material. After shaking, an aliquot was plated out from each bottle to establish the

actual count of S. lactis per ml. After 15 minutes in the water bath one bottle was inoculated with sufficient homologous bacteriophage particles to give a count of approximately 10 particles per ml. This bottle was shaken and the actual count of particles per ml. determined by the plaque plate. An adsorption time of 5 minutes was allowed, after which 1 ml. aliquots were removed from both bottles to determine the number of cells in the normal sample containing no bacteriophage and the number of non-lysed cells and the number of bacteriophage particles in the bacteriophage-organism mixture. The enumerations made at this time were those for zero time. Removal of 1 ml. aliquots with subsequent plating were carried out thereafter at hourly intervals for a period of 8 hours or more. The procedure as outlined in Standard Methods (American Public Health Association, 1948) was followed for enumerating the normal cells and the non-lysed cells in the bacteriophage-organism combination except that tomato juice-peptonized milk medium of 1 per cent agar strength was used as the plating medium. To enumerate the bacteriophage particles contained in the 1 ml. aliquot of the bacteriophage-organism mixture, the procedure as given for the plaque plate method for determination of bacteriophage activity was followed. Following these procedures, five bacteriophage-organism combinations were studied at temperatures of 21, 24, 27, 32, and 35°C. In two combinations a temperature of 37°C. was tried and for one combination data were obtained at 38.5°C. Two trials at each temperature for each of the combinations were made.

Method for pH Determinations

pH measurements using a quinhydrone potentiometric system were made

at hourly intervals on the sample containing normal S. lactis cells and on that containing the bacteriophage-organism combination. Approximately 3 ml. of test material were removed from each bottle at the same time as sampling was performed for enumeration studies. The designated amount was transferred to a cotton-stoppered sterile test tube and cooled in ice water and was stored in the refrigerator at 2° to 3°C. until the following day. Experience indicated this method of handling the test material prior to pH determinations had no appreciable influence on the result.

EXPERIMENTAL RESULTS

Development of a Plaque Enumeration Technic Applicable to S. lactis Bacteriophage

Preliminary work on the use of the two layer agar plate to determine numbers of S. lactis bacteriophage particles by plaque counts was patterned after the method of Delbrück (1945) who used this procedure to demonstrate the stepwise nature of the proliferation process of Escherichia coli bacteriophage. In the early studies approximately 12 ml. of sterile solidified tomato juice-peptonized milk medium of 1 per cent agar strength were used in the lower layer with an agar overlay containing 1 ml. of 10^{-2} dilution of susceptible cells, 1 ml. of bacteriophage suspension and 2 ml. of tomato juice agar as used for the basal layer. The total volume of the overlay was 4 ml. and its final agar concentration was 0.5 per cent.

Influence of agar volume and agar concentration on plaque formation

Although reasonably good results were obtained by the procedure outlined above, the effect of variations in agar thickness and agar concentration in the overlay on the plaque count was investigated as a potential cause of variation in counts. The plates were prepared with a bottom layer of agar described above and an increasing thickness of the agar overlay was obtained by using 1 ml. each of bacteriophage and susceptible cells as indicated above and 2, 3, or 4 ml. of 1 per cent agar-strength medium, giving total volumes of 4, 5, and 6 ml., respectively. The agar

concentrations in the overlay were 0.5, 0.6, and 0.67 per cent, respectively. Three trials were made with bacteriophage PF11 using 10^{-5} dilution. The results given in table 2 indicated that simultaneous increase in agar concentration and volume of the overlay reduced the plaque count to approximately 57 per cent of the maximum when 3 ml. of agar were used and to 46 per cent when 4 ml. were used, as compared to the maximum count obtained with 2 ml. of agar in the overlay.

An additional study was made on the five different bacteriophage-organism combinations, in which the final agar concentration of the overlay was fixed at 0.5 per cent and the thickness of the overlay was varied by using total volumes of 2, 3, or 4 ml., respectively for the second layer. The three volumes each contained 1 ml. of bacteriophage suspension and 1 ml. of susceptible organism, but in that containing 2 ml. total volume, the organisms were diluted with 1 per cent agar medium and 1 ml. of this mixture used. One ml. of 1.5 per cent agar was used to make up the total volume of the 3 ml. volume overlay and the 4 ml. volume was obtained with 2 ml. of 1 per cent agar. The preparation of material for plating and the plating procedure have been described earlier. The resulting plaque counts given in table 3 were totals of two plates for each overlay volume. The average plaque size at the time of enumeration also was determined. Using 3 ml. as a total volume of the agar overlay resulted in the greatest number of plaques obtained for all the bacteriophage strains studied with the exception of PF2, and the difference in the results between 3 and 4 ml. for PF2 undoubtedly was not significant. Increasing the volume of the overlay to 4 ml., or decreasing the volume to 2 ml. resulted in a drop in plaque numbers. Changes in overlay volume had a slightly greater depressing effect

Table 2.

The influence of simultaneous increase in volume and agar concentration of overlay on plaque enumeration of PF11 bacteriophage

Ml. 1% agar in overlay	Final % agar in overlay	Total plaque count Trial 1 1 plate	Trial 2 10 plates	Trial 3 2 plates	Ave. % of maximum
2	0.50	39	139	81	100
3	0.60	21	—	48	57
4	0.67	17	64	38	46

Table 3.

The influence of increase in volume of agar overlay on number and size of plaques of PF11 bacteriophage

Bacterio-phage Combination *	Bacterio-phage Diln. used	Total plaque count on 2 plates with an agar overlay volume of: 4 ml.	Av. plaque size	3ml.	Av. plaque size	2 ml.	Av. plaque size
F69/ML1.	10^{-5}	154	1.25 mm.	170	1.75 mm.	96	1.75 mm.
F68/IP5	10^{-6}	136	1.25 mm.	156	1.50 mm.	110	1.50 mm.
F70/SH5	10^{-4}	92	1.50 mm.	158	2.00 mm.	139	1.25 mm.
PF2/H1-2	10^{-7}	123	1.50 mm.	120	1.50 mm.	40	0.75 mm.
PF11/H1-10	10^{-7}	71	1.50 mm.	106	1.00 mm.	22	1.00 mm.

* The first designation refers to the bacteriophage strain and the second to the S.lactis strain.

on plaque numbers than did the small variation in agar concentration. The plaque sizes obtained varied with changes in the volume of the agar layer, but no definite trends appeared, except that the plaques tended to be somewhat smaller for three of the combinations when the 2 ml. volume was used.

As a result of these studies, the volume of the overlay finally adopted was 3 ml., composed of 1 ml. of susceptible cells prepared as described under "methods," 1 ml. of bacteriophage suspended in milk and 1 ml. of tomato juice-peptonized milk medium containing 1.5 per cent agar. This was superimposed upon the layer formed by about 12 ml. of tomato juice medium with an agar concentration of 1 per cent.

Influence of the concentration of susceptible cells added to the agar overlay on plaque formation

During the first trials for demonstrating plaques by the two-layer agar plate technique, 1 ml. of 10^{-2} water suspended cells prepared as described under "methods," equivalent to approximately 12 million, was used. Preliminary studies, with combination PF11/H1-10, showed that reducing the numbers of susceptible cells added to the agar overlay caused a decrease in plaque numbers. When 12 million, 6 million, and 1.2 million cells were added, plaque counts of 348, 287, and 161, respectively, were obtained. Further work followed, using all the five combinations under study. The varying concentrations of cells were prepared by taking 0.01, 0.1, 1, 2, 3, 4, and 5 ml. of undiluted susceptible culture, just at the time of coagulation, containing on the average, for the different combinations, approximately 700 million cells per ml. and making the volume to 100 ml. with water, thus giving approximately 0.07, 0.7, 7, 14, 21, 28, and 35 million cells, respectively, in the 1 ml. of the suspension

used for each plate. Triplicate plates were made for each cell concentration on each bacteriophage studied. The standardized methods were used for preparing dilutions and plaque plates. Data presented in table 4 show that the greatest numbers of plaques were obtained when the number of cells in the agar overlay was in the range from 21 to 35 million. Three combinations had highest plaque numbers with 28 million cells in the overlay. The slightly higher numbers of plaques obtained with PF2 and F70 using 21 million and 35 million cells, respectively, in the overlay was not considered significant.

As a result of these findings, the number of susceptible cells added to the agar overlay for further studies approximated 28 million. The method used was to add the equivalent of 4 ml. of a culture just at the point of coagulation to 100 ml. of sterile water, using 1 ml. of this suspension in the overlay of each plate.

Influence of milk added to the agar overlay on plaque numbers

During the early development of this part of the investigation, decimal dilutions of bacteriophage were at times made with water and at other times with milk. Greater numbers of plaques apparently were obtained from plates in which milk was used as the diluent. To establish the correctness of these observations, a comparative test was made using parallel dilutions of water and milk with bacteriophage-organism combination PF11/H1-10. The results gave a total of 63 plaques on four plates from the 10^{-6} water dilution and 645 plaques on four plates from the 10^{-6} milk dilution. This result indicated that the presence of milk stimulated plaque formation. The stimulatory action of milk could not be demonstrated with bacteriophage-organism combination PF2/H1-2. Therefore, a complete experiment was

Table 4.

The influence of varying concentrations of susceptible cells contained in agar overlay on plaque numbers.

Bacteriophage Combination	Bacteriophage diln. used	Total plaques on 3 replicate plates using the following numbers of cells in agar overlay						
		0.07M*	0.7M	7M	14M	21M	28M	35M
F69/ML2	10^{-5}	46	86	185	167	224	261	191
F68/IP5	10^{-6}	6	59	138	161	181	246	182
F70/SH5	10^{-7}	4	53	81	172	197	213	217
PF2/H1-2	10^{-7}	15	53	138	145	165	160	159
PF11/H2-10	10^{-7}	1	29	69	134	131	135	123

* M - Millions

Table 5.

The effect of the presence of milk in the agar overlay on the plaque numbers of five bacteriophage strains.

Bacteriophage Combination	Diln. used	No. plates in group	Total plaques on plate from diln's. of:			
			Milk	Water*	Water	Milk **
F69/ML2	10^{-7}	3	179	70	181	
F68/IP5	10^{-7}	2	92	99	102	
F70/SH5	10^{-7}	3	30	3	27	
PF2/H2-2	10^{-7}	2	69	75	72	
PF11/H1-10	10^{-7}	2	58	22	59	

* Both the bacteriophage and the susceptible cells were dispersed in water.

** The susceptible bacterial cells were added to sterile milk in place of the usual water.

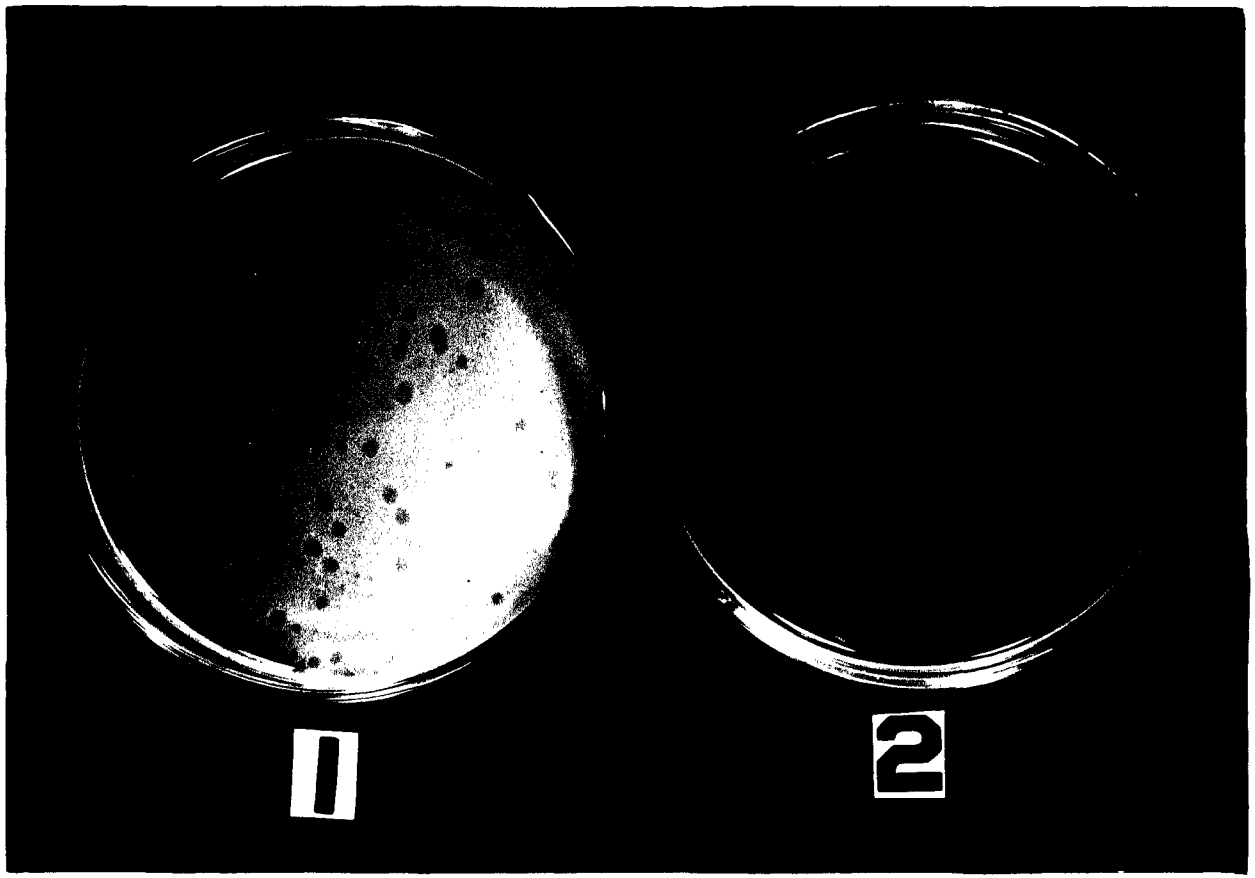


Figure 1. Effect of including milk in the agar overlay on plaque number obtained from 10^{-6} diln. of combination PF11/H1-10. 1- 1 ml. of milk in the agar overlay. 2- 1 ml. of water in the agar overlay.

set up to study the effect of milk on plaque enumeration for all the combinations under study. Bacteriophage was diluted out quantitatively in milk and similarly in water. Three dilutions were plated out to ensure securing sufficient plaques for counting. The diluted bacteriophage suspension in milk, in 1 ml. quantities, was added to each of two or three petri plates, after which 1 ml. of susceptible organisms suspended in water and 1 ml. of 1.5 per cent agar medium were added and mixed thoroughly before hardening of the agar could take place. The water suspension of bacteriophage was handled similarly. An additional two or three plates were prepared from the bacteriophage suspended in water and 1 ml. of milk was added to each petri plate. In order to maintain a constant volume it was necessary in this case to add the susceptible cells in milk rather than in water as was done for the two previous sets of plates. The results are given in table 5. The use of water as a bacteriophage diluent in place of milk caused drastic reduction in the plaque counts of bacteriophage strains F69, F70, and PF11. By adding milk to the water dilution of bacteriophage contained in the petri plate, the same plaque counts were obtained as were secured when the bacteriophage was suspended directly in milk. Plaque counts of bacteriophage F68 and PF 2 were not affected when diluted in water. Figure 1 shows the effect of including milk in the agar overlay on the number of plaques produced.

In all subsequent work, unless stated to the contrary, milk dilutions were used for plaque enumeration for all the combinations studied.

Influence of milk and other diluents for bacteriophage on the number of plaques

As a result of finding that milk enhanced the plaque count of some of

the bacteriophage strains under study, the question arose as to what effect removal of casein from milk might have on plaque numbers and also whether using diluents other than milk had any bearing on the plaque count. Two trials using PF11 bacteriophage were set up in which whey, water buffered at pH6.6 and milk were compared in their abilities to demonstrate plaques. The whey was prepared by acidulation of milk. Na_2HPO_4 and KH_2PO_4 were used to prepare the buffered water (Clark, 1933). The preparation of material and the procedure followed have been outlined. It is evident from table 6 that using whey as a bacteriophage diluent results in lower plaque counts than when milk is used. However, the 57 plaques obtained with whey are considerably higher than 22 plaques obtained with buffered water, which in turn gave a higher plaque count than when water was used alone. In a single trial using PF11 in which tomato juice-peptonized milk broth, milk and unbuffered water were compared as bacteriophage diluents, the total plaques obtained from four plates used for each type of diluent were 92, 323, and 38, respectively. The evidence, therefore, indicated that for bacteriophage PF11, milk was the best medium of those tested to use as a diluent for bacteriophage.

Influence of the length of the milk sterilization period on plaque formation

With the established routine of including milk as part of the plating procedure for demonstrating plaque formation, the effect of the length of time of milk sterilization at 15 pounds steam pressure on plaque production was considered. Commercially pasteurized skim milk was dispensed in 99 ml. amounts into screw cap bottles and was sterilized at 15 pounds pressure for different designated times. The steam flowed through the autoclave 5 minutes before the pressure valve was closed. The machine reached the pressure of

Table 6.

Comparison between milk and other types of diluents for bacteriophage suspensions on the resulant plaque count of bacteriophage PF11, using 10^{-6} dilution.

Type of diluent used	Total no. plaques on triplicate plates:	
	Trial 1	Trial 2
Water	7	6
Buffer water pH 6.6	22	13
Whey	57	48
Milk	138	152

Table 7.

Changes on plaque counts with different times of heat treatment at 15 lbs. steam pressure of milk used as bacteriophage diluent.

Type of diluent used	Time of treatment	Total plaques on 5 replicate plates in: Trial 1	Trial 2	Trial 3	Trial 4
Water	20 mins.	14	14	14	14
Skim milk	10 mins.	88	129	130	88
Skim milk	20 mins.	86	130	133	74
Skim milk	45 mins.	-	-	57	32

15 pounds in about 12 minutes and took approximately 20 minutes to return to atmospheric pressure after completion of sterilization. The standardized method for preparing the bacteriophage dilutions and for plating the prepared dilutions was used. As indicated in table 7, sterilization at 15 pounds pressure for either 10 or 20 minutes had no significant effect on plaque numbers. Increasing the time of sterilization to 45 minutes at 15 pounds pressure caused a significant drop in the plaque count in the two trials in which the longer heating time was used. Very slight caramelization of the milk took place at 15 pounds pressure for 20 minutes and serious browning was evident when milk was held for 45 minutes at the same pressure.

As a result of these findings, the milk used was sterilized at 15 pounds pressure for 15 minutes for all work in which milk was required to enumerate bacteriophage particles.

Influence of different pH levels of the plating medium on plaque formation

During the initial investigations on the application of the plaque plate to the quantitative estimation of bacteriophage particles, the agar medium was adjusted to pH 7.2 just prior to sterilization and no further pH check or adjustment was made. At one time, however, plaque formation with a particular batch of tomato juice-peptonized milk agar was not evident. Checking for causes indicated the agar medium dropped from pH 7.2 to pH 6.5 during the sterilization. While the actual cause eventually was traced to using very old dehydrated peptonized milk, the pH of the sterile agar medium was considered sufficiently acid that plaque formation might be affected. Several trials were performed on stepwise growth of bacteriophage with the medium maintained close to pH 7.2.

Consistently small burst sizes were evident when agar at this pH was used for enumeration. Later the pH of the plating medium was varied after sterilization and it was found that variation also occured in the numbers of plaques obtained from a given bacteriophage suspension. For instance, a 10^{-7} dilution of PF2 bacteriophage gave 143 plaques with the plating medium at pH 7.2, 512 plaques at pH 6.0, 282 plaques at pH 5.4, and 4 plaques at pH 5.2. No plaques were obtained when the medium was at pH 8.00 and above or pH 5.00 and below. As a result of the variations obtained, an experiment was designed to acertain what effect different levels of pH of the plating medium had on plaque numbers. Adjustment of the agar medium to the required pH was made electrometrically. After establishment of the required amounts of acid or base to effect the desired pH change, the necessary amount of sterile reagent was added aseptically to sterile melted agar medium. Eight different pH levels were chosen and 10 plates for each combination at each pH were prepared with a basal layer of solidified sterile agar and an agar overlay prepared by the standardized procedure but with the agar for both layers adjusted to the different pH levels. Plates for each pH level for each combination were completed before continuing with the next pH level of the same combination. The results are given in table 8. With all the combinations, a pH level of 5.8 to 6.0 gave the maximum number of plaques. As the pH was increased the plaque count decreased until at pH 7.2 the percentage recovery of plaques was reduced to approximately 57 per cent in one combination and to as low as 27 per cent in another. Decreasing the pH from 5.8-6.0 to 5.2 reduced the plaque count to extremely low values, to less than 7 per cent of the maximum in four cases out of five studied.

Table 8.

The influence of pH level of the agar plating medium on the plaque counts of five strains of bacteriophage.

Bacteriophage Comb.	Diln. used	Total plaques on 10 replicate plates at pH: 7.2	6.4	6.2	6.0	5.8	5.6	5.4	5.2
F69 MLI	10^{-7}	117 (40.2)*	232 (79.7)	254 (87.3)	290 (99.6)	291 (100)	283 (97.3)	237 (81.4)	18 (6.18)
F68 IP5	10^{-7}	462 (56.9)	661 (81.5)	666 (82.1)	811 (100)	721 (88.9)	717 (88.4)	573 (70.6)	364 (44.9)
F70 SH5	10^{-8}	96 (45.7)	149 (70.9)	177 (84.3)	178 (84.8)	210 (100)	149 (70.9)	47 (22.4)	7 (3.33)
FF2 H1-2	10^{-7}	143 (27.4)	210 (40.3)	429 (82.3)	521 (100)	470 (90.2)	482 (92.5)	282 (54.1)	16 (3.07)
FF11 H1-10	10^{-7}	168 (47.4)	262 (74.0)	286 (81.0)	354 (100)	291 (82.2)	298 (84.2)	228 (81.4)	11 (3.10)

* Figures in parenthesis indicate percent of maximum for that particular series.

As a result of these findings, the pH of the tomato juice-peptonized milk agar was maintained between pH 5.8 and pH 6.0. Each batch of agar medium was tested after sterilization and the pH was adjusted aseptically. The agar medium used for the bottom layer also was maintained between pH 5.8 and pH 6.0.

It is interesting to note that the pH level required to secure maximum plaque numbers under the conditions of this study is somewhat below that generally desired in a plating medium. The tomato juice-peptonized milk agar used to study rates of growth of S. lactis in combination with and not in combination with bacteriophage also was maintained between pH 5.8 to pH 6.0. The S. lactis cells developed countable colonies in 24 hours, while in 48 hours the colonies were well developed and readily enumerated when an incubation temperature of 30°C. was used.

Comparison between measuring bacteriophage activity by the plaque plate method and the limiting dilution method

In order to compare the plaque plate method with the limiting dilution method, the latter being more commonly used for the determination of titers of S. lactis bacteriophage preparations, all the bacteriophages under study were tested for activity under identical conditions by both plaque plate and limiting dilution methods. Filtrates of the five bacteriophages used in the work were prepared according to methods described earlier and initial titers were determined in a preliminary way by the two methods. The day following, each combination again was tested for titer, this time, by using triplicate plaque plates parallel with five tubes of litmus milk for each bacteriophage dilution used. This procedure was

repeated 10 days and 25 days later. The whey filtrates containing the bacteriophages were stored at approximately 2° to 3°C. during the period of study. The standardized procedure as previously described was used for determination of activity by plaque numbers, and the most probable number (American Public Health Association, 1948), using five tubes of litmus milk on each of five dilutions was used to determine bacteriophage activity by the limiting dilution method. To determine the extent of variation between the two methods as time progressed, the ratio of plaque count to most probable number was calculated. The data obtained from this study are presented in table 9. The titers as determined by the plaque plate method were slightly higher in every comparison made, except in one case where the results were identical. In comparing the ratio of plaque count to most probable number, the ratio became narrower over the 25-day period for F68 and F69, it increased for PF2 and was variable for both F70 and PF11. The significance of these latter observations is questionable, since the experiment was not repeated.

For determining bacteriophage titers either the plaque plate or the limiting dilution method would give satisfactory results. However, when more exact counts of bacteriophage are desired, the plaque plate method of determining or following bacteriophage activity probably offers slight advantage because an actual count is made, rather than a determination of activity or absence thereof in a defined amount of material.

Discussion

These studies indicate that variations in thickness of the agar overlay and of the agar concentration of the overlay effect the number of plaques

Table 9.

Comparison of bacteriophage titer by plaque count and limiting dilution technics for five S.lactis bacteriophage strains.

Bacteriophage Comb.	Titer per ml* at: 0 days		1 day		10 days		25 days		Ratio Plaque count / Limiting Diln.(MPN).		
	Plaque count (1plate)	Limiting Diln. (1 tube)	Plaque count	MPN	Plaque count	MPN.	Plaque count	MPN.	1 day	10 days	25 days
F69 / ML1	2.9×10^{8}	10^{7}	30×10^{7}	9.5×10^{7}	19×10^{6}	13×10^{6}	10×10^{5}	7.9×10^{5}	3.16	1.46	1.27
F68 / IP5	4.1×10^{8}	10^{7}	45×10^{7}	14×10^{7}	12×10^{7}	4.9×10^{7}	62×10^{6}	33×10^{6}	3.22	2.45	1.88
F70 / SH5	8.6×10^{7}	10^{7}	91×10^{6}	70×10^{6}	28×10^{6}	28×10^{6}	46×10^{5}	23×10^{5}	1.28	1.00	2.00
PF2 / H1-2	2.8×10^{9}	10^{9}	29×10^{8}	24×10^{8}	21×10^{8}	7.9×10^{8}	94×10^{7}	24×10^{7}	1.21	2.66	3.91
PF11 / H1-10	3.2×10^{9}	10^{9}	35×10^{8}	24×10^{8}	14×10^{8}	7.9×10^{8}	53×10^{7}	35×10^{7}	1.46	1.78	1.51

* Titer based upon plaque counts (av. of 3 plates) or on most probably number (MPN) based on five tubes per dilution, calculated from the probability tables of Standard Methods (American Public Health Association 1948).

demonstrable. To secure comparable results it is essential that a constant agar concentration and agar thickness be adopted and always used. The results obtained, in general, substantiate the claims of Bronfenbrenner and Korb (1923) and Kleczkowska (1945), that reduced plaque numbers result when higher concentrations of agar are used and those of Hershey and Bronfenbrenner (1941), that increasing the thickness of the agar layer causes a drop in demonstrable plaques from a given bacteriophage suspension.

An optimum cell concentration must be maintained to secure the maximum numbers of plaques. Presumably, with low concentrations of added cells, resulting in lower plaque number, there is an insufficient concentration of cells onto which bacteriophage particles may adsorb. With fewer cells present, the bacteriophage particle has to travel a greater distance to make contact with the cell. The forces of attraction between cell and bacteriophage particle apparently are not active over any appreciable distance. Increasing the cell concentration enhances the chances that the particle will adsorb to the cell by decreasing the distance between the bacterial cell and bacteriophage particle. Data to be presented in a later section support this view, since the lag phase of bacteriophage multiplication was shortened by increasing the concentration of cells in relation to bacteriophage concentration. On the other hand, the reduced numbers of plaques obtained with higher concentrations of added cells could be explained on the basis of variation in activity of individual bacteriophage particles. Strongly active particles would lyse enough cells in a given time to produce a visible plaque. On the contrary, the weaker particles would lyse a number of cells insufficient to produce a visible plaque. However, with a slightly lower concentration of cells

these weaker particles would develop visible plaques. This agrees with the observations of Dreyer and Campbell-Renton (1933), who reported that greater numbers of plaques were secured as the cell concentration was increased, and of Burnet (1925), who found the plaque count increased to a maximum and then declined with increased amounts of added cells. Bronfenbrenner and Korb (1925) on the other hand, found the numbers of plaques unaffected by variation in cell concentration. The results obtained in this study tend to support the work of Burnet (1925) with four combinations, while F70 possibly leans toward the view of Dreyer and Campbell-Renton (1933).

The presence of milk obviously is necessary for optimum plaque formation by some bacteriophage strains, as absence of milk prevents the demonstration of the presence of some bacteriophage particles. According to Aschaffenburg (1940) the casein of milk has a marked influence on reducing the surface tension of milk. Therefore, inclusion of milk into the agar overlay would tend to decrease surface forces within the medium and allow the particles to diffuse more readily through the layer and enable them to adhere more readily to the surface of the bacterial cell. The fact that lower numbers of plaques were obtained when using whey than when milk was used, tends to emphasize the possible role of casein. In this connection Whitehead and Cox (1936) indicated that they preferred milk to whey for testing streptococcus bacteriophage activity, while Nichols and Wolf (1945) stated that milk possessed enhanced properties over broth for detecting bacteriophaging strains of lactic acid streptococci.

Reasonable care must be exercised regarding the extent of the heat treatment given to milk to be used in the procedure for demonstrating plaques. Heating over 20 minutes at 15 pounds pressure results in

decreasing the numbers of plaques demonstrable.

Bronfenbrenner and Korb (1923) indicated that pH of the medium may affect the appearance of plaques and pointed out that their medium was maintained at pH 7.4 for their studies on plaque formation. The present work definitely points to the importance of this factor on plaque formation. For the conditions under which these tests were made, maximum plaque numbers were obtained with all the bacteriophage strains studied when the plating medium was maintained between pH 5.8 and 6.0, together with the observance of the other factors that may effect plaque formation.

Failure of certain bacteriophage strains to produce countable plaques even under the most nearly optimum conditions available was encountered. Selection of five strains of bacteriophage which would permit accurate enumeration by the plaque procedure was considered justifiable because of the greater possible accuracy and convenience of the plaque enumeration method. To study those strains of bacteriophage that produce poorly defined plaques, one has no alternative but to use the limiting dilution method for enumeration of bacteriophage particles.

Determination of Burst Time and Burst Size for *S. lactis* Bacteriophage

In the preliminary studies to demonstrate the possible stepwise nature of the process of proliferation of *S. lactis* bacteriophage, the two layer agar technique of Delbrück (1945) was adapted to the bacteriophage-organism combinations being studied. Bacteriophage filtrate PF2 and *S. lactis* H1-2 each were diluted separately in milk to give counts of approximately 40 bacteriophage particles and 80 bacteria per ml., respectively. The diluted suspensions then were mixed for 5 minutes to permit adsorption

of the bacteriophage particles by the cells. The mixture then was dispensed among 40 petri plates, to give an average of one bacteriophage-infected cell per plate. The agar overlay of one per cent agar concentration was added, as described under methods, to each of ten plates after 45, 60, 75, and 100 minutes. No bursts occured on any plates during the total interval of 100 minutes, although single plaques appeared on about half of the plates, indicating the particles in the suspension were demonstrable by the plaque plate method.

Consideration, therefore, was given to the proximity of the bacteriophage particles to the cells. With the above procedure, the relatively great distances existing between a cell and a particle could account for the failure of the particles to be adsorbed. In a second trial, approximately 40 million bacteriophage particles per ml. and 80 million bacteria per ml. were used in the mixture and adsorption was allowed to take place for 5 minutes before the mixture was diluted to give less than one bacteriophage particle per ml. The diluted mixture was dispensed as before and the agar overlay added to each of ten plates after 43, 66, 105, and 130 minute intervals had elapsed. In a third trial, using the same procedure, the time intervals were 66, 80, 101, and 139 minutes. The data obtained for these two latter trials are given in table 10. The range of burst size for the two trials was 29 to 165 and the median burst size was 96, this representing the number of newly formed bacteriophage particles released per bacterial cell. Some bursts occured within 65 minutes, while some bacteriophage particles had not reproduced after a lapse of 139 minutes, although there was a high probability that the particles all had had an opportunity to be adsorbed on a susceptible bacterial cell. During the

Table 10.

Time of occurrence and size of burst for S.lactis bacteriophage PF2, maintained at room temperature.*

Trial no.	Total no. plates used	No. of plates infected	Number of plaques on plates incubated at room temps. at burst times: 43 mins	65-66 mins	80 mins	101-105 mins	130-139 mins
2	40	16	No bursts (5)**	93,59 (4)	-	No bursts (1)	77,84,103 29,96,165
3	40	8	-	No bursts (2)	122 (2)	120 (3)	97 (1)

* Room temp. June 1947 approximately 25°C.
** Figure in parenthesis indicates numbers of infected plates among the 10 used for that interval.

Table 11.

The influence of temperature on size of bursts occuring within 65 minutes with bacteriophage PF2

Temp.	Trial 1 Total no. plates	Trial 1 No. plates infected*	Trial 1 Burst sizes (plaque counts	Trial 2 Total no. plates	Trial 2 No. plates infected	Trial 2 Burst sizes (plaque counts)
Room**	10	8	45,43,48	10	10	132,52, 5
32°C	10	8	12,40,28 45,51, 9	10	7	67,46,71 168
37°C	10	6	23,35,17	10	5	20
45°C	10	3	No bursts	10	2	No bursts
Totals	40	25	12 bursts	40	24	8 bursts

* Some plates showed the presence of 1 or 2 bacteriophage particles, although no burst had occurred.
** Approximately 33°C

interval from zero time to 66 minutes only two bursts occured from the four infected cells, whereas, when the time interval had reached 139 minutes, six bursts had occured from six infected cells.

In a similar trial carried out at room temperature during August, using the same bacteriophage-organism combination at a fixed burst time of 65 minutes, ten bursts were obtained from a total of 40 plates. From 8 to 68 plaques per burst were obtained with the median burst size of 36-47 plaques. This represented less than one half the number obtained for the burst size during the preliminary trials described above. To account for the decreased burst size obtained during this latter trial, variation in room temperature due to changing seasons was considered. The preliminary trials were performed during June and no difficulty was experienced with respect to plating and incubation conditions. During August the room temperature at times reached approximately 35°C. which did not permit the second layer of agar to solidify quickly and necessitated placing the finished plates in a refrigerator for 15 minutes in order to bring about solidification of the agar in a reasonable period of time. After the agar hardened, the plates then were incubated at room temperature for 18 hours.

To acertain what effect temperature of incubation had on burst time and burst size, 40 petri plates were prepared and a suitable dilution of combination PF2/H1-2 designed to give less than one bacteriophage particle and not more than two bacterial cells per plate, was dispensed among 40 plates as described previously. Four groups of 10 plates each were incubated, one group at room temperature of approximately 33°C., a second group at 32°C., a third group at 37°C. and a fourth group at 45°C. After a lapse of 65 minutes

all the plates were completed by addition of the second layer of agar medium. The finished plates were placed in the refrigerator for 15 minutes to allow the agar to harden and then were incubated at 30°C. for 12 hours, after which the plaques were enumerated. From the results presented in table 11, the burst size for both trials at room temperature varied from 5 to 132 plaques per burst and at 32°C. it ranged from 9 to 168. The median size of the bursts was 45 to 48 at room temperature and 45 to 46 at 32°C. Although the median burst size at room temperature and at 32°C. did not vary significantly, the percentage of infected plates producing bursts was increased from 33 per cent at room temperature to 66 per cent at 32°C. Raising the temperature of incubation to 37°C. caused only four bursts to occur from eleven infected plates. The burst size varied from 17 to 35 countable particles with a median burst size of 20 to 23 at this temperature. No bursts occured at an incubation temperature of 45°C., although there were five plates containing one or two plaques.

During the winter of 1947-1948, burst time and burst size experiments were made using combination PF11/H1-10. These investigations considered the effects of variations in temperature on both the time and size of the bacteriophage burst. The procedures followed for the seven trials recorded in table 12 were the same as for those made on combination PF2/H1-2, except that the selected dilution mixture was dispensed into serological test tubes rather than directly onto the solidified agar in the plates. At designated time intervals, the incubated tubes of bacteriophage-organism mixture were emptied onto the solidified agar in the petri plate and the agar overlay then was added as described previously. Of 70 plates used at a temperature of 22-24°C., 50 plates contained one or more plaques and 25 of these infected

Table 12.

The influence of time and temperature of incubation of bacteriophage-organism mixture PF11/H1-10 on burst size.

(Summary of 7 trials.)

Temp of incub.	Total no. plates used	No. plates infected*	Frequency of bursts** from infected plates during:					Range of burst sizes at:					Total no. of bursts	Median burst size
			40 min.	60 min.	80 min.	100 min.	120 min.	40 min.	60 min.	80 min.	100 min.	120 min.		
R.T.x	70	50	1	3	7	6	8	5	19-76 (32)***	5-56 (20)	17-82 (30-64)	13-108 (29-30)	25	29
30°C	70	53	4	5	6	7	9	10-48 (44-48)	13-72 (25)	8-71 (12-13)	14-83 (26)	9-28 (14)	31	17
37°C	70	35	1	4	3	2	1	6	5-15 (9-13	4-14 (6)	14 -	6 -	11	9
45°C	70	7	0	0	0	0	0	-	-	-	-	-	0	-

x-R.T. (22-24°C)
* number of plates out of a total of 70, showing 1 or 2 plaques.
** number of plates from 14, used at each time intervals, showing bursts.
*** median burst size indicated in parentheses.

plates showed burst sizes ranging from 5 to 108 plaques over the total 120 minute interval. The median burst size at this temperature was 29 plaques, while the size of bursts varied from 5 to 108 countable particles. The number of plates showing bursts increased from three of the fourteen during the 60 minute incubation interval to eight of the fourteen during the 120 minute interval when an incubation temperature of 22-24°C. was used. Raising the temperature of incubation to 30°C. raised the number of bursts to 31 occuring from 53 infected plates, whereas the median burst size was reduced to 17 countable particles. At an incubation temperature of 37°C., the number of bursts occuring from the infected plates was reduced to 11 bursts out of a possible 35 infected plates and the median burst size was decreased to 11 countable particles. While seven infected plates occured at 45°C., no bursts occured at this temperature.

In comparing the median burst sizes of PF2 and PF11 obtained at similar temperatures, the latter bacteriophage possessed a much lower median burst size than the former. The recorded lower burst size of PF11 could be attributed to an inherent property of the particular bacteriophage. However, no strictly parallel comparisons were made. The investigations on the effect of the pH level of the plating medium on plaque numbers indicated that pH differences of the plating medium may have been responsible for the observed differences in burst size. The agar used in the seven trials presented in table 12 was adjusted to pH 7.2 after sterilization whereas that used for plaque enumeration of PF2 (table 10) was approximately pH 6.5. The latter pH has been shown more suitable for production of a greater number of plaques than the former.

Discussion

From the data presented, it is concluded that S. lactis bacteriophage increases by stepwise proliferation and is similar in this respect to coliform bacteriophage, as reported by Delbrück (1945). The burst size obtained during this study ranged from 5 to 168 countable particles per burst for PF2 and from 5 to 108 countable particles for PF11. Delbrück secured burst sizes ranging from below 20 to over 1,000 particles for E. coli strain B bacteriophage and indicated that the wide ranges obtained could not be accounted for on the basis of variations in size of bacterial cell. To account for the wide variation obtained with coliform bacteriophage, Delbrück (1940b) indicated that the release of newly formed bacteriophage particles from an individual cell took place over a period of 16 minutes, starting suddenly and ending abruptly. This viewpoint, theoretically applied, could account for the several low burst values which occured at the commencement of the period of bacteriophage release from the individual cell. However, this viewpoint would mean that no low burst values should have occured on the plates held for sufficient time to insure that all the newly-formed particles would be released by the time the overlay was added. Data in table 12 indicate that low burst values of five to eight countable plaques occured in the 80 minute time interval at both 24 and 30°C., while a burst size of nine countable plaques occured at 30°C. at the 120 minute interval. Data from table 10 and table 12 indicated that as the time interval allowed for bursts to occur is lengthened a greater number of bursts occured from the infected plates. Raising the temperature of incubation to 32°C. (tables 11 and 12) had a similar effect, while further increases resulted in a decrease in the number of bursts occuring. The

temperature of incubation affects the size of burst, the median size decreasing as the temperature exceeds that of 22 to 32°C. The median burst size suggested as an average for the two combinations studied was 90 countable particles, at a temperature of approximately 25°C. and with a minimum burst time of approximately 65 minutes. The values for the burst size obtained in the later studies seem to be too low and may be ascribed to the unsuitable pH of the plating medium.

In view of the role that the pH level of the plating medium plays in plaque formation and that of temperature on burst size, further work on the burst time and burst size appears desirable. Such a project would entail consideration of the application of frequency distribution measurements to analyze the data obtained.

Influence of Temperature on the *S. lactis* Bacteriophage Relationship

The plaque plate method of bacteriophage enumeration was used to study the effect of varying temperatures of incubation on the bacteriophage proliferation rates of five selected strains of bacteriophage when in association with their respective *S. lactis* cultures. The incubation temperatures used in this study were chosen with a view of covering the range of those temperatures commonly employed at one time or another during the preparation of starter cultures or in the manufacture of cheese. The procedure employed for the preparation of the dilutions and of the plaque and bacterial plates have been outlined previously. The hourly sampling of the test material, explained under "methods," was arranged so that three different temperatures on the same bacteriophage-organism combination were

run simultaneously. This arrangement allowed 20 minutes for each temperature level in which to remove the aliquots for plating and for pH determinations, prepare the required dilutions and complete the plating for plaque counts, and for bacterial counts on both the control culture containing bacteria alone and the culture containing both bacteria and bacteriophage.

From the experimental results obtained regression equations were calculated for the logarithmic phase of each proliferation curve determined. The calculated regression equation for the curve obtained from the growth of bacteria in the bacteriophage-organism mixture includes only the logarithmic rate of growth up to the point of mass lysis of the cells. The rapid rate of destruction of cells after lysis commenced did not permit calculation of a regression equation of any significance for population changes during lysis. The regression equations shown separately on each graph are those obtained for a second trial made under similar conditions.

From the graphs presented (figs. 2-26), the five bacteriophage-organism combinations studied all showed a similar pattern in many respects. The bacteriophage curves were typical-looking growth curves. The steepness of the slopes of the bacteriophage curves appeared related to those for the bacteria at the corresponding temperatures. Increasing the temperature of incubation from 21°C. to 32°C. caused increases in the slope values of the curves of both bacteriophage and related organism. At 32°C. the slopes were at their maximum values. Further increases in the temperature of incubation caused the slopes of both the bacteriophage and bacterial multiplication curves to become less than they were at 32°C. As a relationship between the rates of multiplication of bacteriophage and susceptible cell appeared apparent, the average slopes for each two curves together with

the calculated ratio of the average slope of the bacteriophage proliferation curve to the average slope of the bacterial growth curve obtained for the two trials are presented in table 13.

Maximum increase in bacteriophage and organism coincided at 32°C. for each combination, with the exception of F70/SH5, which had the greatest slope for the bacteriophage curve (2.49) at 27°C., although that for the bacteria occured at 32°C. with a value of 0.645 for the slope. The slopes at 32°C. for all the bacteriophage curves studied were greater than a value of 2.00, with an exception of the curve for F69 which had a slope of 1.81. Reducing the temperature of incubation to 27, 24, and 21°C. caused corresponding reductions in the slopes of both bacteriophage and organism multiplication curves. The calculated ratios of slopes of bacteriophage and bacterial curves for combinations F68/IP5 and F70/SH5 tended to narrow as temperature of incubation increased, although with F70/SH5 the value at 27°C. was somewhat higher than expected. The ratios obtained for the other three combinations had an erratic tendency to widen rather than to narrow with increased temperature of incubation, PF2/H1-2 and PF11/H1-10 having a ratio higher than expected at 24°C. and F69/MLI a lower value than expected at this temperature. As these ratios were calculated from only two trials, one may question the validity of attributing any great degree of significance to the two opposing trends in the various ratios of the bacteriophage-organism combinations as influenced by changes in temperature.

The total amount of bacteriophage produced reached a maximum which was always approximately the same, regardless of the temperature of incubation. However, the time taken to reach the maximum bacteriophage

Table 13.

Slope values from regression equations of proliferation curves of five S.lactis bacteriophage combinations obtained at five different temperatures of incubation. (Average slope values of two trials at each temperature)

Bacteriophage Comb.	21 degrees C.			24 degrees C.			27 degrees C.			32 degrees C.			35 degrees C.		
	A*	B**	C***	A	B	C	A	B	C	A	B	C	A	B	C
F68 IP5	1.35	0.28	4.90	1.65	0.35	4.78	1.97	0.44	4.53	2.19	0.57	3.84	1.39	0.38	3.66
F70 SH5	1.53	0.35	4.43	1.85	0.48	3.93	2.49	0.57	4.40	2.25	0.65	3.49	1.94	0.62	3.13
PF11 H1-10	1.36	0.36	3.78	1.68	0.36	4.67	1.87	0.46	4.06	2.16	0.51	4.23	-	-	-
PF2 H1-2	1.07	0.28	3.89	1.57	0.34	4.68	1.92	0.40	4.80	2.10	0.48	4.37	1.75	0.37	4.79
F69 ML1	1.02	0.29	3.58	1.08	0.35	3.08	1.64	0.41	4.05	1.81	0.45	4.02	1.50	0.35	4.35

* A-Slope of bacteriophage curve
** B-Slope of bacteria control curve
*** C-Ratio bacteriophage/bacteria control

numbers was shortest at the optimum incubation temperature of 32°C. and increasingly longer as the temperature was increased or decreased from that of the optimum. The range for the five combinations studied at the various temperatures was from 5 to 14 hours. After the maximum amount of bacteriophage was reached the curve levelled off quite abruptly and decreased slightly in some cases.

The end of the logarithmic phase of bacteriophage proliferation or, in some cases, the point of maximum bacteriophage recovery coincided with the point of completion of mass lysis of the susceptible cells. This indicated that the stoppage of further bacteriophage proliferation probably was due to the absence of living substrate. The subsequent slight reduction in total numbers of bacteriophage possibly may be ascribed to adsorption of some particles by the secondary growth cells under some circumstances.

The characteristic pattern of bacteriophage proliferation and cell lysis was not evident in some bacteriophage-organism combinations maintained at temperatures of 35°C. and above. While four of the bacteriophage-organism combinations studied gave normal patterns at 35°C., that of PF11/H1-10 failed to show bacteriophage proliferation at this temperature, although bacterial multiplication occured. In another trial in which PF11 was tested at temperatures of 32, 35, and 37°C., a normal pattern was obtained at 32°C., but no bacteriophage increase took place at 35 or 37°C., although increase in cell numbers was evident at all three temperatures. Such a result was unanticipated, and consequently the use of high dilutions due to expected higher rates of increase made bacteriophage enumeration impossible for temperatures of 35 and 37°C. In another trial with the same combination care was taken to ensure a plaque count of bacteriophage and

the results obtained at a temperature of 35°C. are shown in figure 27. The slope of the curve representing bacterial increase at this temperature was approximately the same as that obtained at an incubation temperature of 27°C. for the same combination (fig. 25). However, the bacteriophage proliferated at 27°C. and produced lysis of the cells, whereas at a temperature of 35°C. the bacteriophage did not proliferate at all. The same bacteriophage-organism combination incubated at 37°C. failed to produce any bacteriophage, while cell growth increased ten-fold after 4 hours of incubation. It is interesting to note from figure 27 that the pH values obtained for the normal medium and the bacteriophage-containing medium ran parallel for the entire 10 hours at 35°C., the decrease from pH 6.4 to pH 5.3 indicating that the bacteriophage PF11 was without effect when in combination with susceptible cells held at a temperature of 35°C. for a period of 10 hours.

Combination F68/IP5 incubated at 37°C. provided another example of the inability of bacteriophage to proliferate even when in combination with susceptible cells when certain critical temperatures of incubation were chosen. This particular combination was incubated for 30 hours at 37°C., but the bacteriophage failed to proliferate while the susceptible cells reached a count of 3.5 million per ml. This result is presented graphically in figure 28. In combination F70/SH5 both bacteria and bacteriophage proliferated well at 37°C. and produced a normal pattern at this temperature. However, at 40°C. neither the organism nor the bacteriophage were able to multiply, but at 38.5°C. the organism numbers increased from 0.1 to 3.2 million per ml. during 10 hours of incubation, whereas the homologous bacteriophage failed to increase at all. These data indicate that by

selection of a critical temperature, bacterial growth was able to proceed, while the bacteriophage proliferation was completely inhibited in some bacteriophage-organism mixtures.

While further study would be desirable on the relationship between the lag phases of multiplication for corresponding bacteriophage strains and bacteria at given temperatures, it is pointed out from this limited study that the lag period of bacteriophage increase always was longer than that for the corresponding susceptible organisms at the different temperatures of incubation, (Figs. 2-26). It was noted that shorter lag periods for the bacteriophage centered around the optimum or near-optimum temperatures required for bacterial multiplication. At these temperatures a period of about 2 hours usually was required before the bacteriophage entered the logarithmic rate of increase. As short a time as 1 hour was required for bacteriophage F70 at both temperatures of 32 and 35°C. An increasingly longer lag period amounting to 3 hours was evident for four of the bacteriophage strains studied at 24°C., but F70 at this temperature had only a 2 hours lag period. When a temperature of 21°C. was employed, the lag periods for the bacteriophage strains were lengthened to 3 hours for PF11, to 4 hours for F70, F68, and PF2 and to 8 hours for F69. These data indicate a possible partial explanation of why bacteriophage may not be apparent in the culture incubated at 21°C. but may show up quickly at the higher temperatures commonly encountered in the cheese vat.

While both the bacteriophage and the susceptible cells for each combination increased logarithmically, the rate of bacteriophage increase was always much greater than that of the corresponding bacteria and eventually caused mass lysis of the susceptible cells. The time required to bring about mass

lysis of susceptible bacterial cells appeared somewhat related to temperature and to the particular bacteriophage-organism combination. At a temperature of 27°C., 4 to 6 hours elapsed between inoculation and the start of mass lysis, at 32°C. 5 to 7 hours were required, at 35°C. 5 to 8 hours passed and at 24 and 21°C. lysis occurred after 6 to 11 hours. At the lower temperatures, the""break" in the curve for bacteria in the presence of bacteriophage was not as abrupt as it generally was at a temperature nearer to optimum. In most cases, the time elapsing from the "break point" to completion of cell lysis between the temperatures of 21 and 35°C. was 1 to 2 hours, although combination F69/ML1 took 3 hours at 21°C. and 3 to 4 hours at 35°C. The onset of mass lysis of cells appeared abrupt and the destruction of all susceptible cells apparently occurred within 1 hour in most cases, as shown in figures 2 to 26. In a series of single trials the aliquots used for the bacteria count in the bacteriophage-organism mixture were removed every 12 minutes rather than hourly during the period of mass lysis of each combination. The results shown in figures 29 to 33 indicate that at a temperature of 32°C. mass lysis usually occurred within a period of 36 to 40 minutes after the first detectable break in the bacterial population. For combination PF2/H1-2, this time was extended to 48 minutes. While these data indicate the reactions for all the five bacteriophage strains at a temperature of 32°C. only, presumably similar changes would be found for the same strains at the other temperatures of incubation at which mass lysis occurred.

For additional information regarding mass lysis of cells, bacteriophage F68 and S. lactis IP5 were mixed in milk in the ratio of 10 countable bacteriophage particles to one million countable bacteria per ml., and the

rate of proliferation of S. lactis at a temperature of 32°C. was followed by direct microscopic observation until mass lysis of the cells was visibly evident. Aliquots were removed hourly for 5 hours and stained smears were prepared and examined. A control consisting of the same inoculum of S. lactis IP5 but with no bacteriophage added was treated identically. Photomicrographs were prepared from the stained smears made hourly. The results for the period from 3 to 5 hours are depicted in figure 34. The populations of the control S. lactis and S. lactis plus bacteriophage appeared identical up to 3 hours incubation time, but during the following hour lysis of most of the cells in the bacteriophage-organism mixture took place and by 5 hours only an occasional single cell appeared in several fields examined. The control culture, on the other hand, continued to multiply at the normal rate.

At the completion of mass lysis of susceptible cells, secondary growth commenced immediately in all cases except in combination F69/ML1 in which secondary growth was not detectable even after continued incubation for 2 weeks. The numbers of secondary growth organisms surviving destruction by bacteriophage was characteristic for each bacteriophage-organism combination, regardless of the temperature, and varied from no measurable number in 5 ml. aliquots of the lysed material for combination F69/ML1 to 1000 per ml. for PF11/H1-10. The counts at the end of mass lysis for PF2/H1-2, F70/SH5, and F68/IP5 were 250, 125, and 300 per ml., respectively.

During some of the early trials on the effect of temperature on proliferation rates of S. lactis bacteriophage it was noted that some plates used to determine the bacteria count of the bacteriophage-organism

mixture in the region at which lysis of susceptible cells was expected looked peculiar. The plate from a 10^0 dilution contained but very few colonies, while that from a 10^{-1} dilution was covered with bacterial growth but the growth had a "moth-eaten" appearance. Plates from dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} appeared normal. Such a series is shown in figure 35. Choosing the plate from 10^0 dilution of this series for the bacterial count would result in an inaccurate estimate of the point of true lysis. Actually, the true lysis point in the system from which this material was taken occurred 1 hour later, at which time the appearance of the plate from 10^0 dilution was about the same as in the series pictured while those plates representing the corresponding increasing decimal dilutions showed no colonies at all. The absence of growth of bacteria in the plate from 10^0 dilution shown in figure 35 was due to the high concentration of bacteriophage in the 1 ml. aliquot plated; this caused lysis of the cells in the plate during the incubation. The plate from 10^{-1} dilution contained one-tenth the amount of bacteriophage found in the previous plate and was unable to bring about as complete lysis of the cells. As a result of this finding, plates from all dilutions were prepared during the expected time of lysis to prevent an inaccurate estimate of the time of mass lysis.

One of the main characteristics of bacteriophage action on S. lactis cells is the distinct retardation of acid development in the milk medium. A comparison of the rates of acid production was made between S. lactis cells with and without added bacteriophage in conjunction with the study on proliferation rates. The results obtained are presented together with the graphs on multiplication rates of the bacteriophage-organism combinations at the various temperatures (figs. 2-26). The pH curves for both

the control culture and the bacteriophage-organism mixture ran approximately parellel to each other until the concentration of the bacteriophage was sufficient to initiate lysis of the susceptible cells. Acid production continued in the control culture but stopped at once in the culture containing bacteriophage and remained at the same level for the balance of the experimental time. At no time did the pH level of the culture containing both bacteriophage and S. lactis cells drop below pH 6.2.

Discussion

From the results presented, it is evident that the five streptococcus bacteriophage strains obtained from various countries were practically identical in their main characteristics of concomittant multiplication of cells and bacteriophage, a more rapid rate of proliferation of the bacteriophage which eventually caused mass lysis of susceptible cells, and the subsequent appearance of secondary growth, except that combination F69/ML1 showed no secondary growth, even after incubation for 2 weeks. Each bacteriophage proliferation curve resembled the bacteria growth curve in possessing lag, logarithmic and resting periods. The length of the lag period appeared related to the temperature of incubation of the susceptible S. lactis cells, being shortest at the optimum temperature and longer as the temperature was increased or decreased from the optimum. These findings agree with the work of Kreuger and Northrup (1930), who found staphylococcus bacteriophage lag periods longer at lower temperatures of incubation and attributed the cause to the inability of the bacteriophage particle to adsorb to the susceptible cells at temperatures of 10°C. However, raising the temperature of incubation immediately promoted adsorption with subsequent multiplication of the organisms and the bacteriophage, followed by lysis

of the susceptible organisms. The rate of increase of the bacteriophage strains studied was found to be a function of the rate of growth of the susceptible cells, which in turn was definitely influenced by the temperature of incubation. Similar results were reported by Bloch (1942), in that coliform bacteriophage increased logarithmically together with susceptible cells incubated at temperatures between 20 and 34°C. Hunter (1943) also reported that reduction in the temperature of incubation from 30 to 22°C. caused a considerable delay in bacterial growth, which in turn increased the length of time required for lysis of the streptococcus cells.

With all the bacteriophage strains studied, proliferation of the bacteriophage eventually caused mass lysis of susceptible cells. As a result of this lysis, the acidity level of the bacteriophage-containing mixture remained at a level attained at the time of lysis for the balance of the time of the test run. Thus, the inability of a bacteriophage-containing streptococcus culture to develop acidity under the given conditions of these experiments is due primarily to lysis of the cells. If no proliferation of bacteriophage takes place, even though the bacteriophage may be present together with susceptible cells, acidity will develop normally, providing the cells multiply to a great enough extent. This point is well brought out in figures 27 and 28 in which the streptococcus cells grew sufficiently in the presence of homologous bacteriophage to effect changes in pH to the same extent as the organisms without bacteriophage.

The anomalies secured in these studies regarding growth of cells with no concomittant growth of bacteriophage at elevated temperatures of incubation also have been reported by Kreuger and Fong (1937) for staphylococcus bacteriophage. At an incubation temperature of 40°C., bacteriophage proliferation was inhibited, while cell multiplication

remained unimpaired. Work by Hunter (1943) indicated that with three New Zealand streptococcus bacteriophage-organism combinations, growth of the susceptible cultures proceeded well at 37°C., but the homologous bacteriophage strains failed to proliferate at this temperature.

From the review of literature and from results presented in this thesis on the effect of temperature on bacteriophage proliferation, it would seem that probably bacterial multiplication is necessary to initiate bacteriophage proliferation and the rates of increase for both processes proceed in logarithmic fashion once the bacteriophage has started to increase. Within certain temperature limits the rate of increase of bacteriophage appears to be related to cell multiplication rate. Outside these limits the relationship does not hold and the results under these conditions cannot be predicted. Thus it would appear that conditions other than multiplication of the sensitive host organism are necessary for multiplication of bacteriophage. The data available do not permit conclusions as to what this additional factor or factors may be.

Effect of Varying Concentration of *S. lactis* and Bacteriophage on Multiplication of the Organism and the Bacteriophage

The ratio of bacteriophage to cells commonly used in the studies of the effect of temperature on proliferation rates of *S. lactis* bacteriophage was maintained at 10 countable particles and 100,000 bacteria per ml. as an initial inoculum. Changing this ratio was contemplated to bring other factors into operation with respect to proliferation of the bacteriophage and of the susceptible *S. lactis* cells. The effect of changing this ratio,

first, by varying the concentration of S. lactis cells added to a fixed number of countable particles, and second, by varying the concentration of bacteriophage added to a fixed quantity of S. lactis cells, on the attendent changes in the multiplication of the organism and of the bacteriophage was studied.

Effect of varying the S. lactis concentration

Single trials using combination F68/IP5 were made with plate counts of 1,000, 10,000 and 1 million S. lactis cells per ml. as an initial inoculum in milk containing approximately 10 countable bacteriophage particles per ml. The procedure followed for this study of proliferation rate was identical to that already described. The results are indicated in figure 36. In general, the pattern obtained for the three different ratios was identical to that obtained for the ratio commonly used (fig. 10). The slopes obtained for the three ratios were for practical purposes of the same order as obtained for the commonly used ratio, although the data might suggest a tendency toward a slightly steeper slope as the concentration of the susceptible cells initially added was progressively increased. The most noticeable difference was the varying length of the bacteriophage lag. With 1,000, 10,000, and 1 million cells per ml., the lag periods were 5, 3, and 1 hours, respectively. The decreased lag probably was attributable to more rapid adsorption of bacteriophage due to the decreased distance the bacteriophage particles were required to travel when in more concentrated cell suspensions. The original postulation was that when the ratio was 10 bacteriophage to 1,000 cells per ml., multiplication would proceed for only a short time because the bacteriophage population, due to more rapid proliferation,

would quickly overtake the cells and cause their lysis. As the results indicated, this particular ratio took the longest of any to reach the point of mass lysis. It appeared that proximity of the bacteriophage particle to the cell was the dominating factor responsible for the observed differences in the bacteriophage lag periods. Another interesting point indicated by the graphs in figure 36 was the relatively short time required, regardless of the numbers of susceptible cells initially present, to effect mass lysis of susceptible cells, once the bacteriophage entered the logarithmic rate of proliferation. In one case mass lysis took place in 2 hours and in the other cases in 3 hours after the bacteriophage commenced logarithmic proliferation.

Effect of varying the concentration of bacteriophage

In a single trial using combination F68/IP5, the count of susceptible cells was held at approximately 100,000 per ml., while the counts of bacteriophage particles were approximately 1,000, 100,000, and 1 million per ml. at the time of the initial inoculation. The usual procedure was followed to enumerate both bacteriophage and bacteria, except that a determination was made every 12 minutes on two of the samples and once an hour for two hours, followed by 12 minute intervals for the third. From the results obtained (fig. 37) it appeared that successive increased additions of bacteriophage to the cells at the time of the initial inoculation had a distinct depressing effect on cell growth, while the bacteriophage increased rapidly in a logarithmic fashion. When the bacteriophage concentration was increased to 1000 particles per ml., bacterial growth continued for 204 minutes with an increase in cell numbers approximately ten-fold before lysis commenced. When the numbers of particles were increased to

100,000 per ml., the bacterial growth period lasted 108 minutes with a population increase of about four times, while with 1 million particles per ml., no increase of bacterial numbers took place; instead the numbers remained relatively at the same level for a period of 84 minutes, after which lysis of cells took place.

Discussion

Increasing the cell concentration in relation to bacteriophage concentration at the time of initial inoculation of the culture introduced an additional factor, apparently that of distance between any given cell and bacteriophage particle. Increasing the cell concentration caused progressive shortening of the bacteriophage lag periods in a system held at a temperature of 32°C. Still further increases in cell concentration possibly would overcome the effects of bacteriophage action, as the common practice in cheesemaking when bacteriophage is prevalent is the use of larger inocula of S. lactis cells in the cheese-milk to overcome the detrimental effect of bacteriophage. Specific data on this point are not available.

Increasing the concentration of bacteriophage initially added to a relatively constant number of S. lactis cells caused a marked decrease in the total population which could be attained by the time mass lysis of the susceptible cells occurred. Increasing the bacteriophage concentration to ten particles per bacterial cell at the time of the initial inoculation of the culture prevented measureable cell multiplication, although the bacteriophage proliferated several-fold under the same conditions. Kreuger and Northrup (1930) pointed out that 125 bacteriophage particles for each staphylococcus cell were necessary before mass lysis would take place.

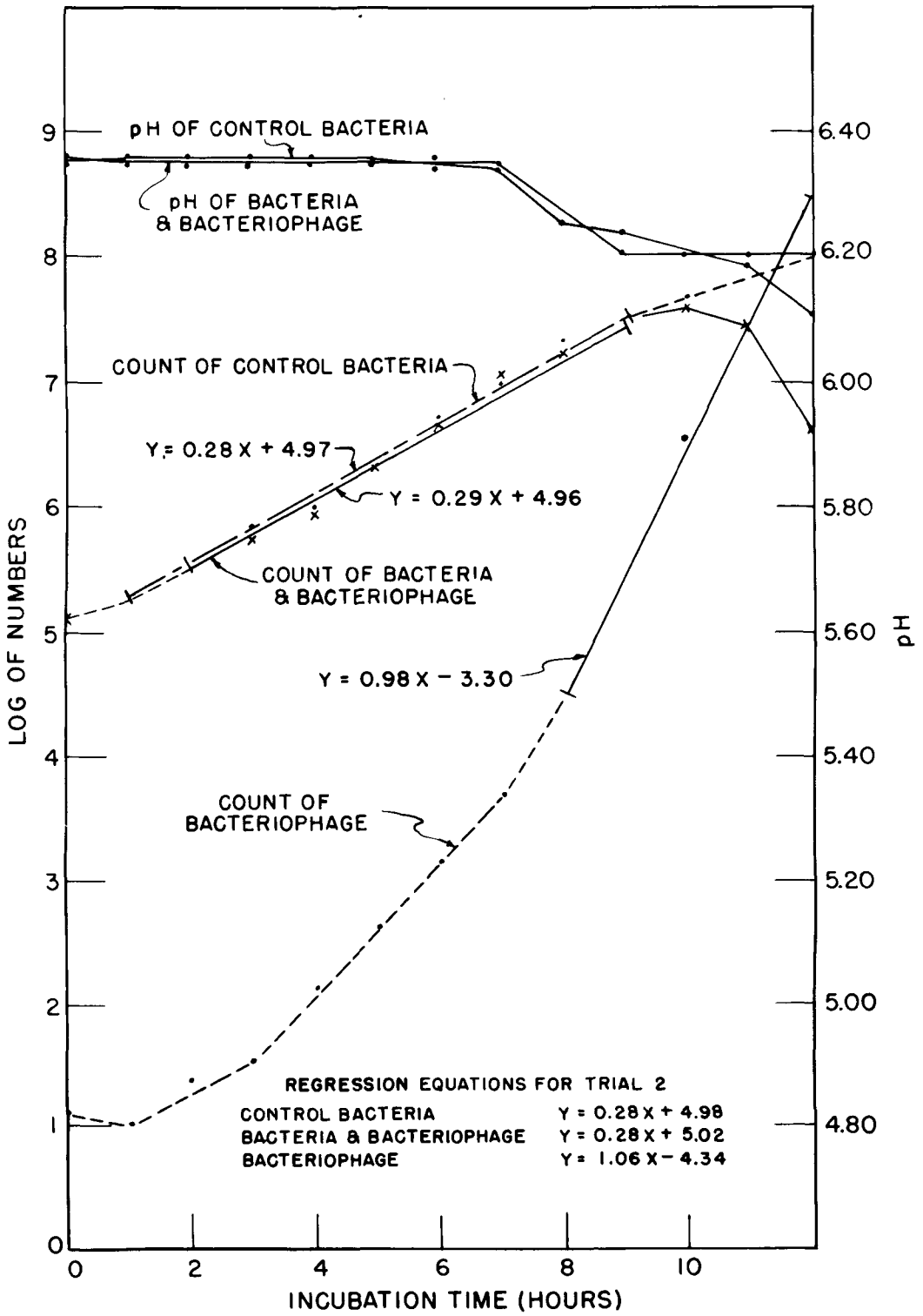


Figure 2. Influence of incubation temperature 21°C. on the proliferation rate and acid development of S. lactis bacteriophage combination F69/MLL.

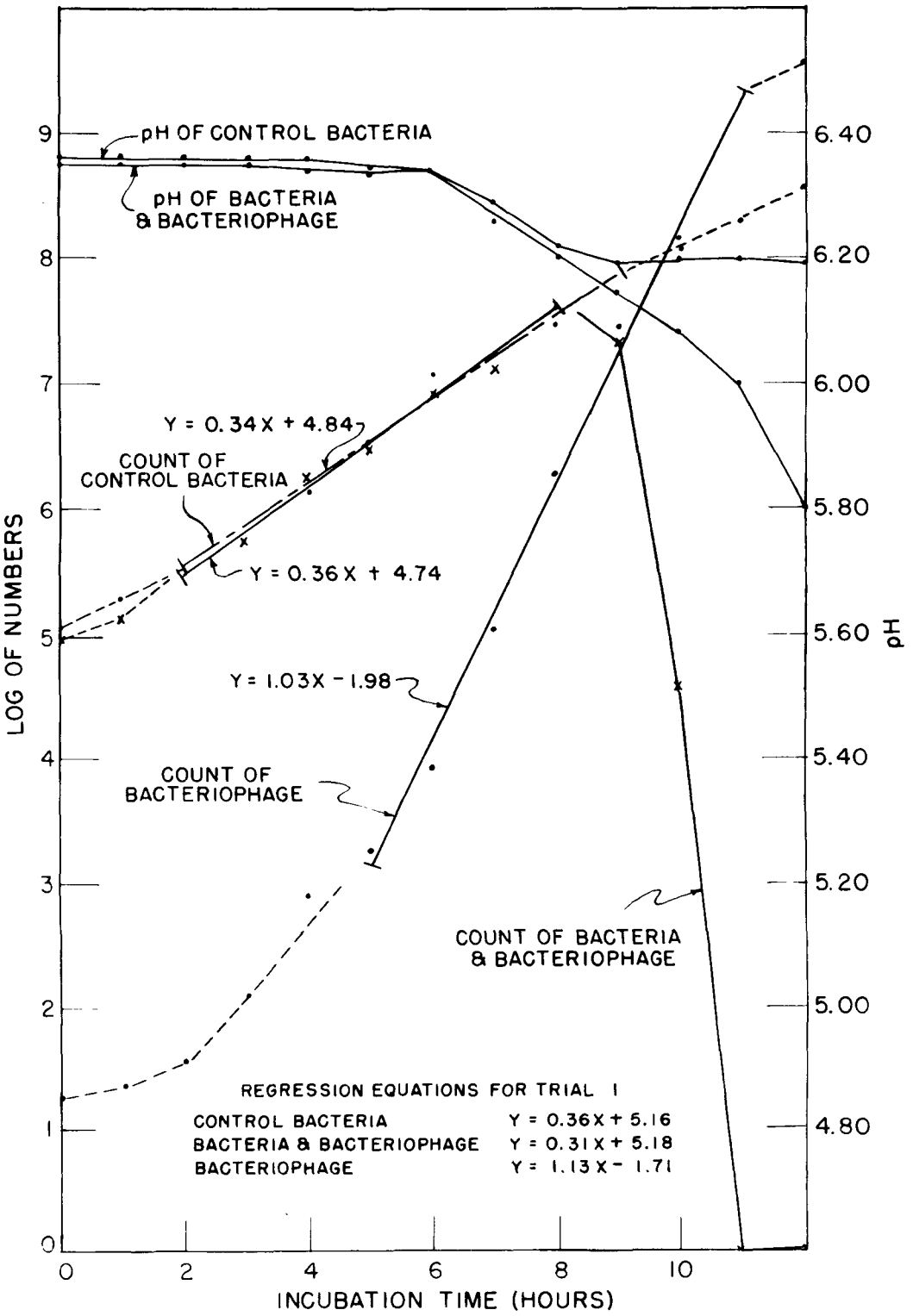


Figure 3. Influence of incubation temperature 24°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F69/ML1.

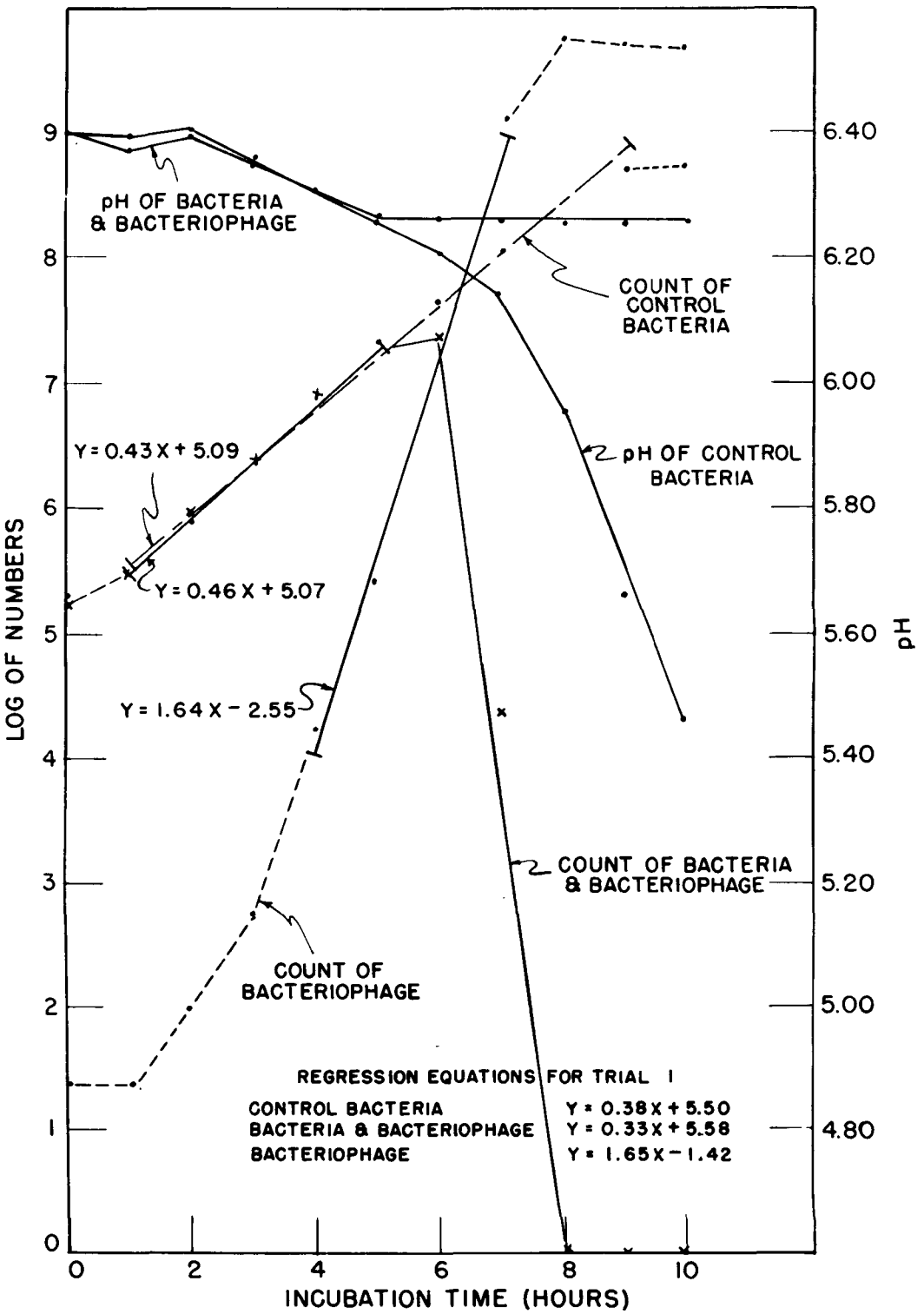


Figure 4. Influence of incubation temperature 27°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F69/MLl.

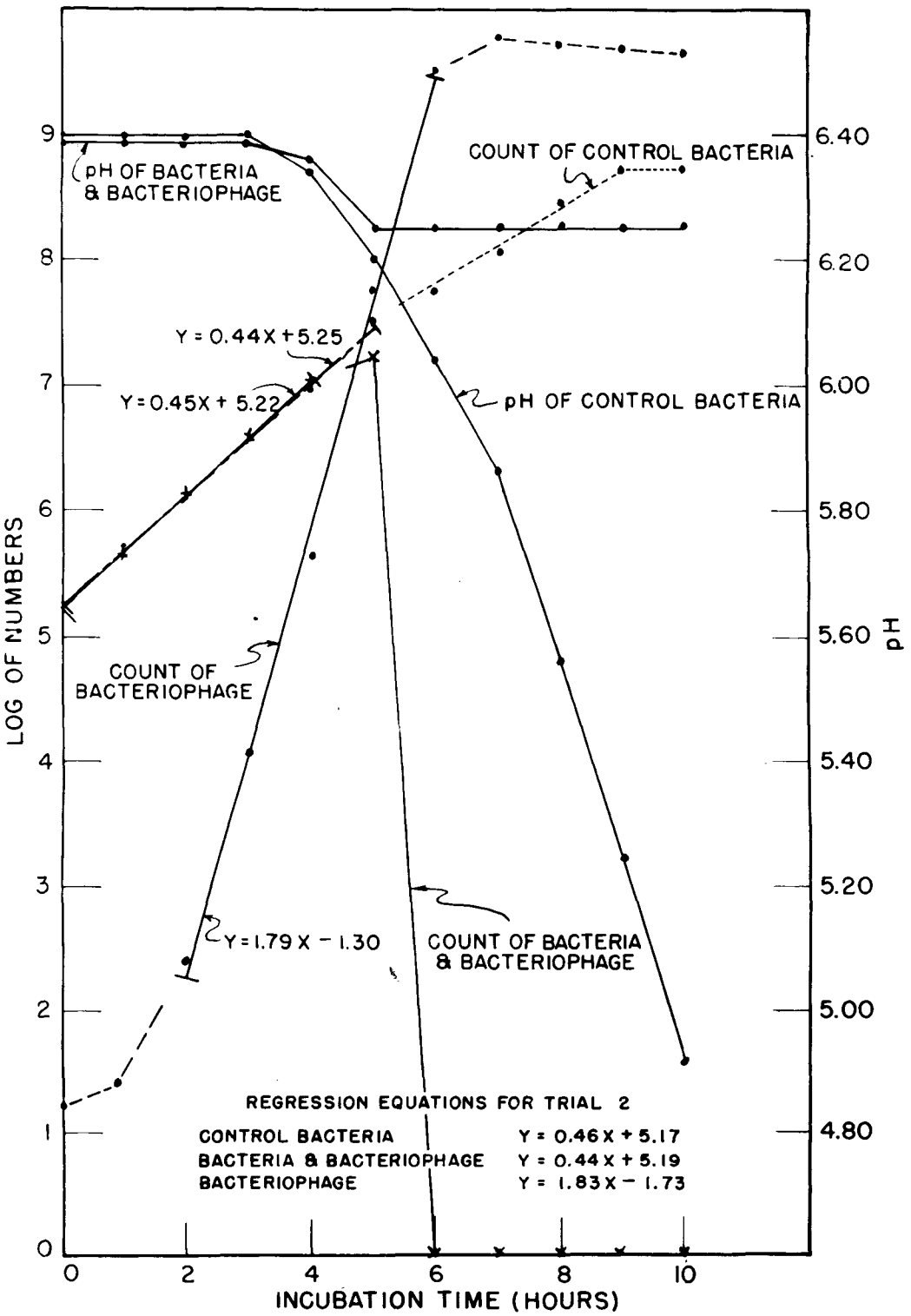


Figure 5. Influence of incubation temperature 32°C. on the proliferation rate and acid development of S.lactis bacteriophage combination P69/M11.

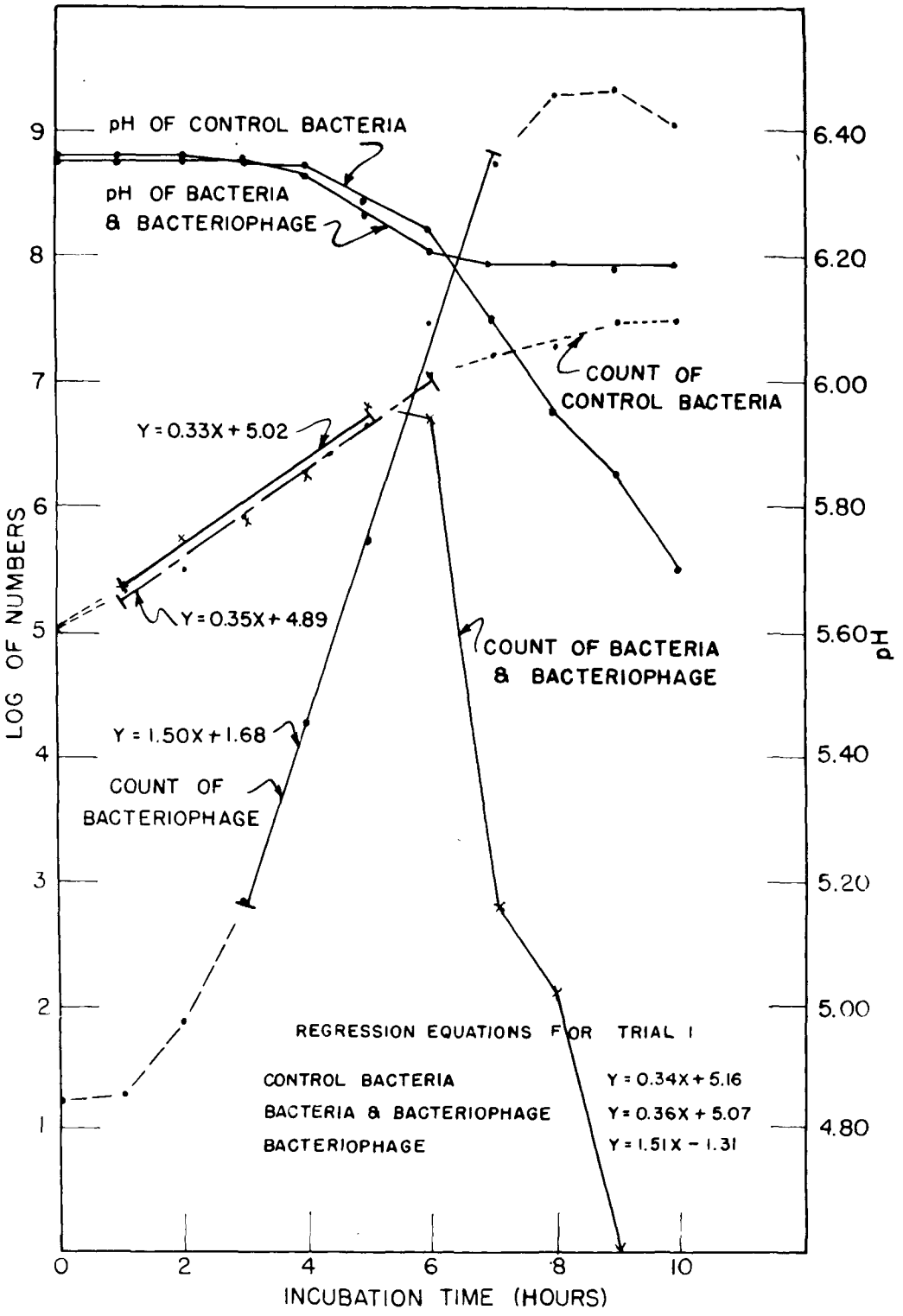


Figure 6. Influence of incubation temperature 35°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F69/ML1.

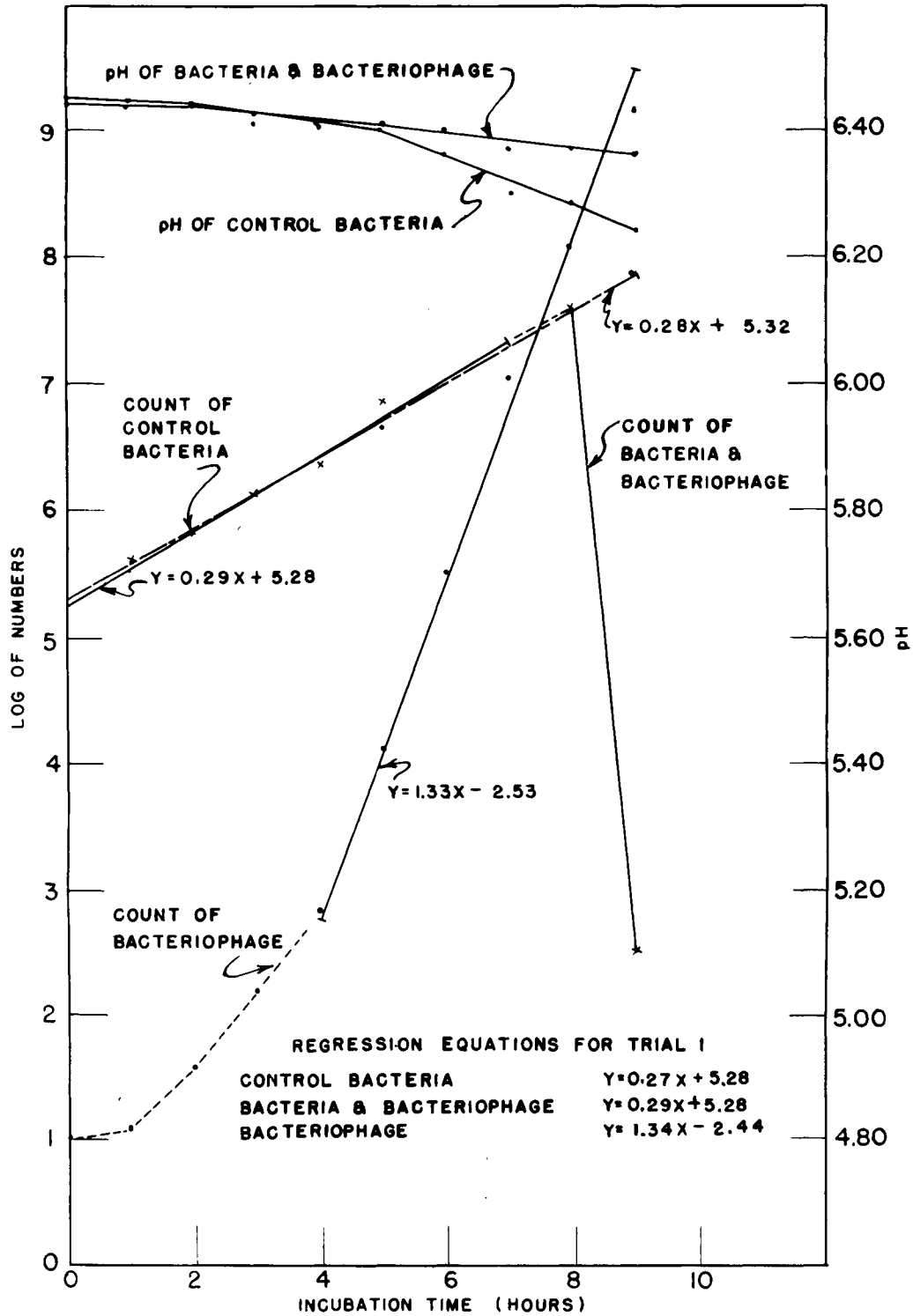


Figure 7. Influence of incubation temperature 21°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F68/IP5.

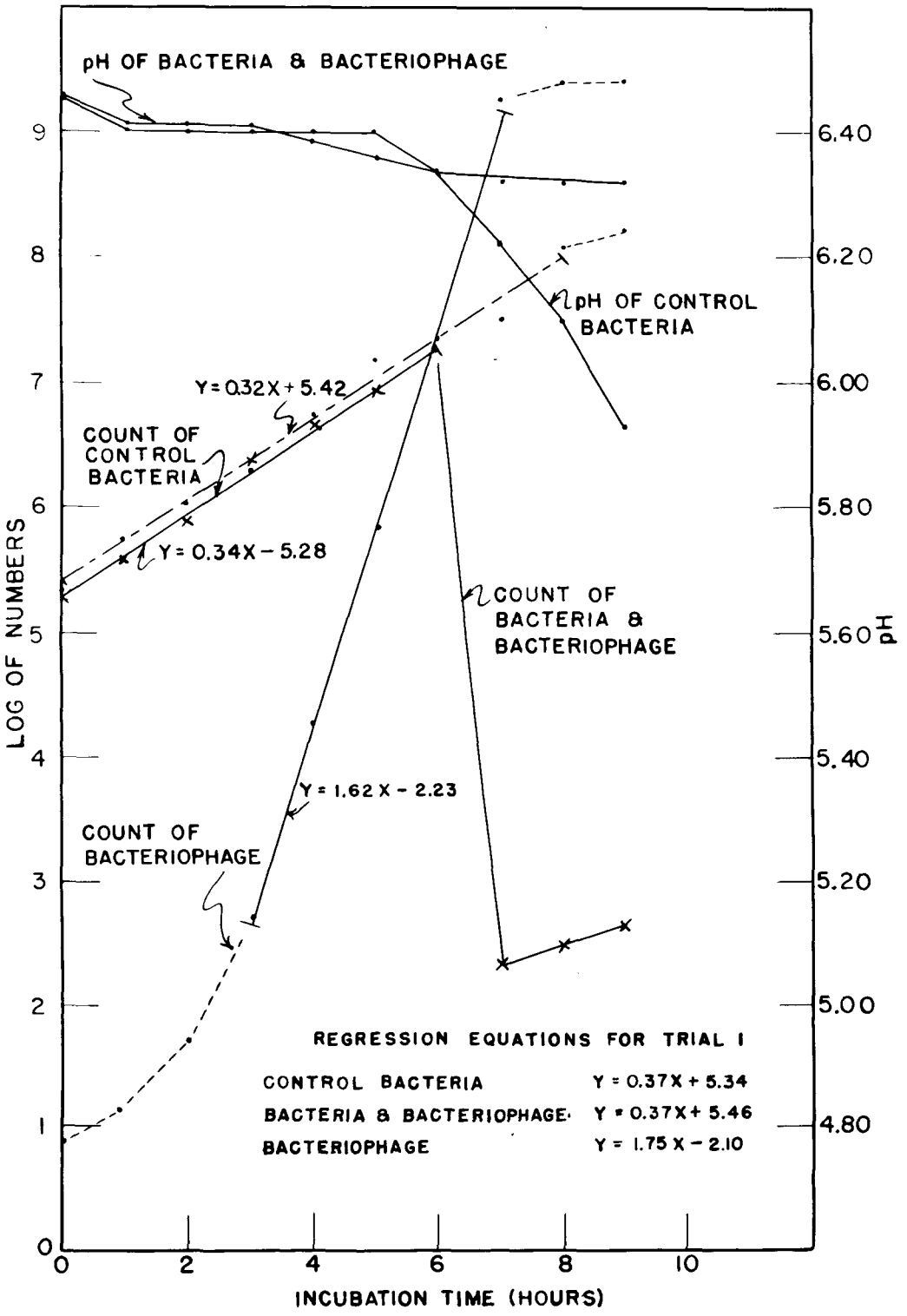


Figure 8. Influence of incubation temperature 24°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F68/IP5.

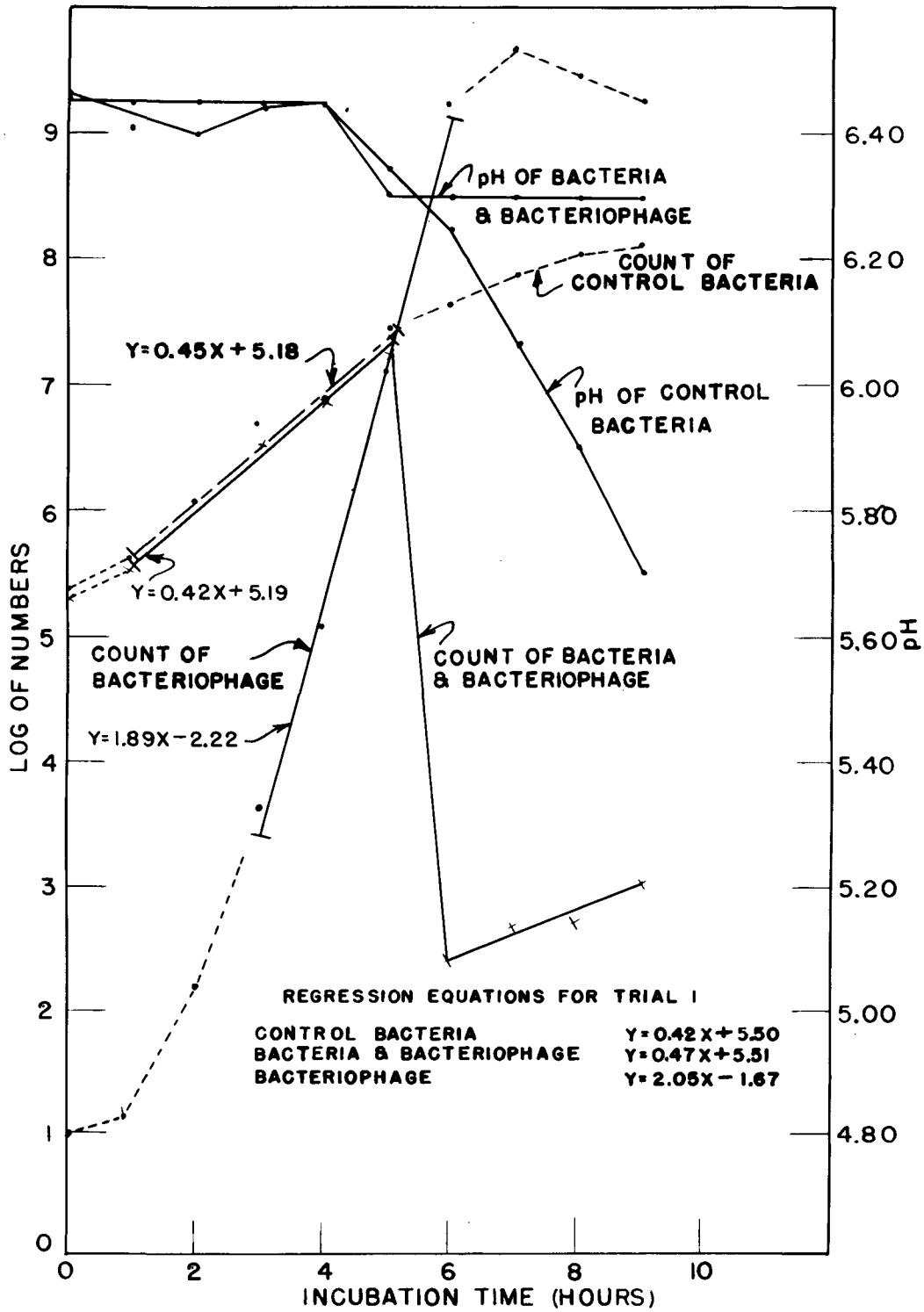


Figure 1. Influence of incubation temperature 27°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F68/TP5 .

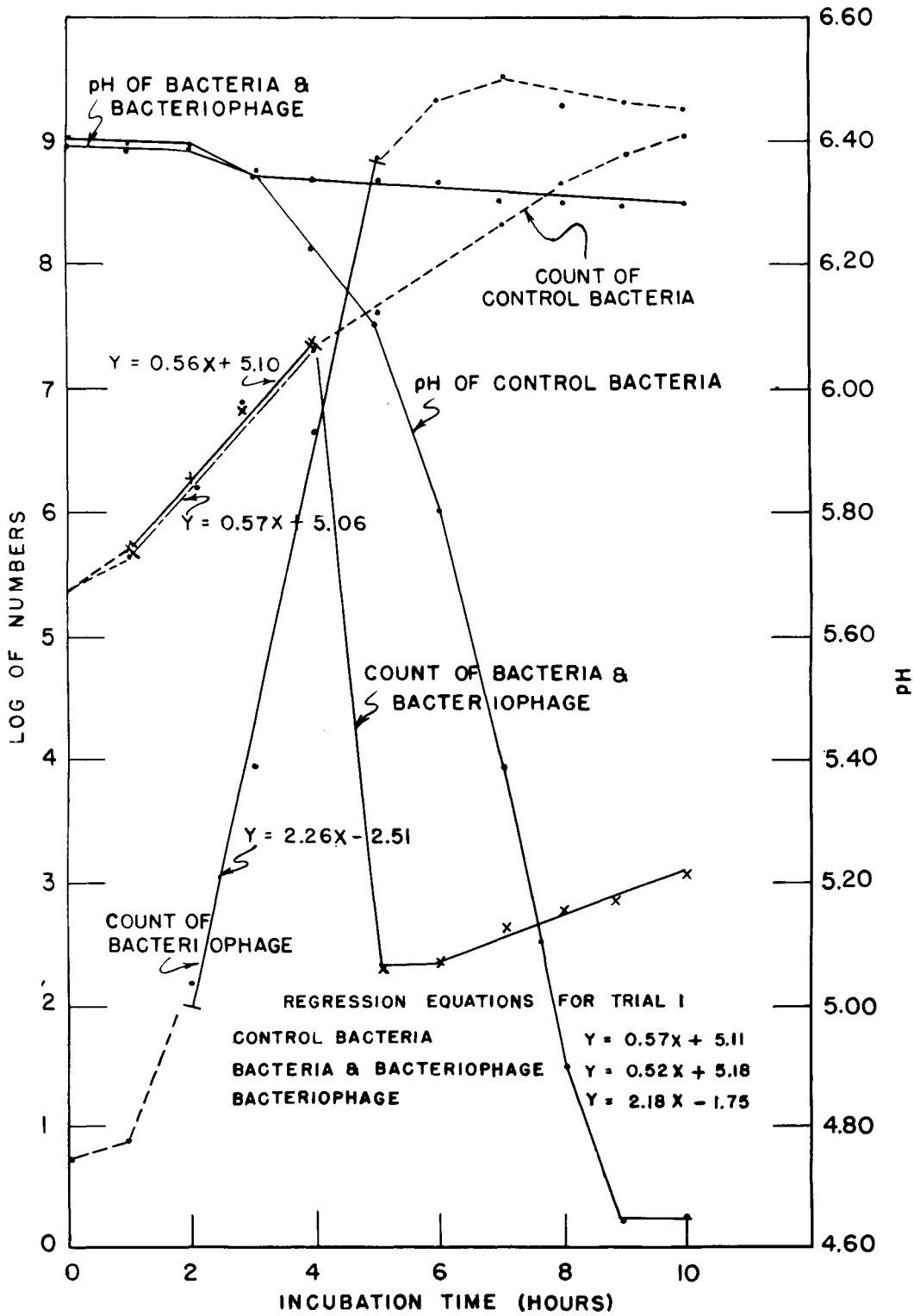


Figure 10. Influence of incubation temperature 32°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F68/IP5.

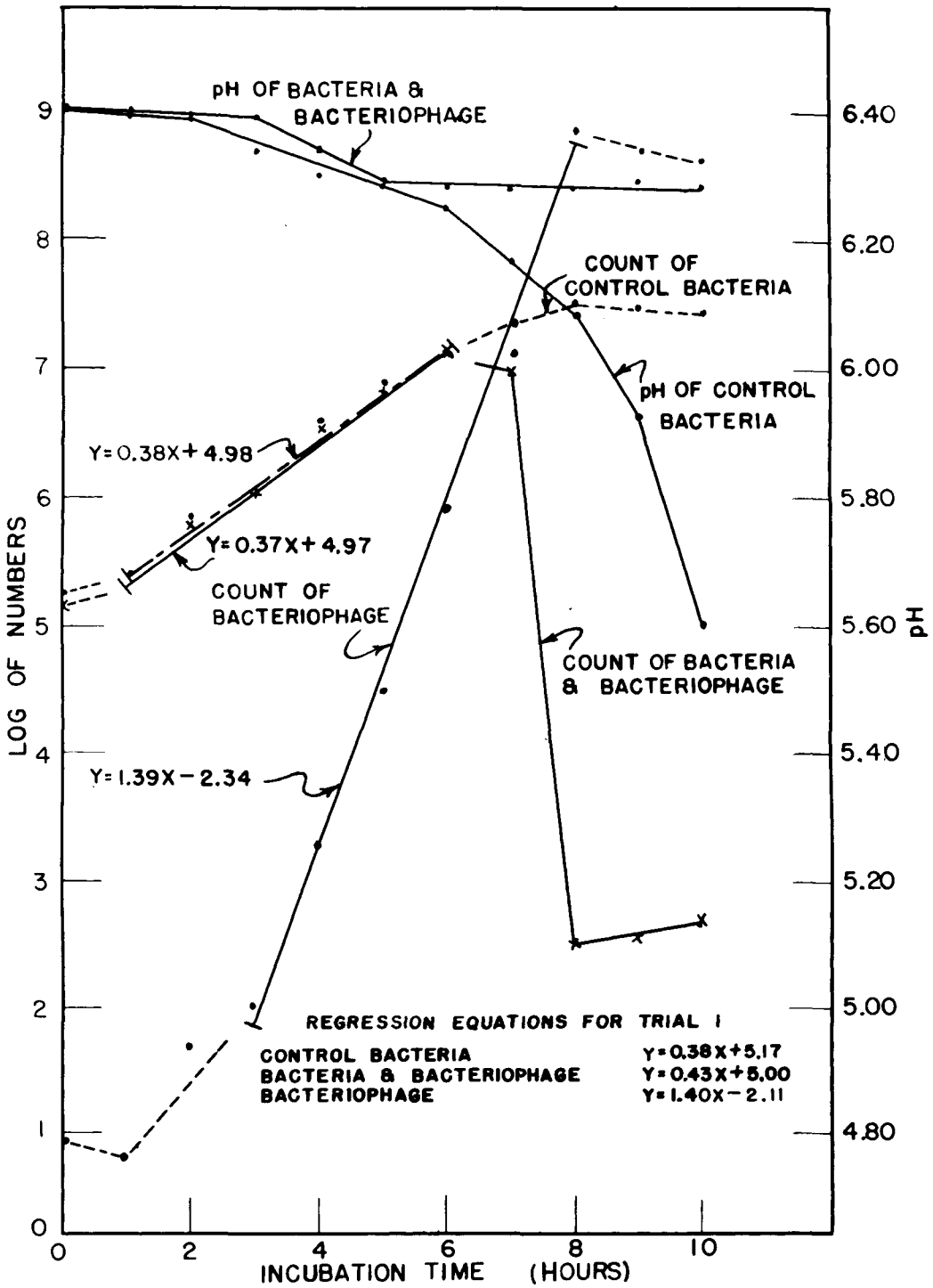


Figure 11. Influence of incubation temperature 35°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F63/IP5.

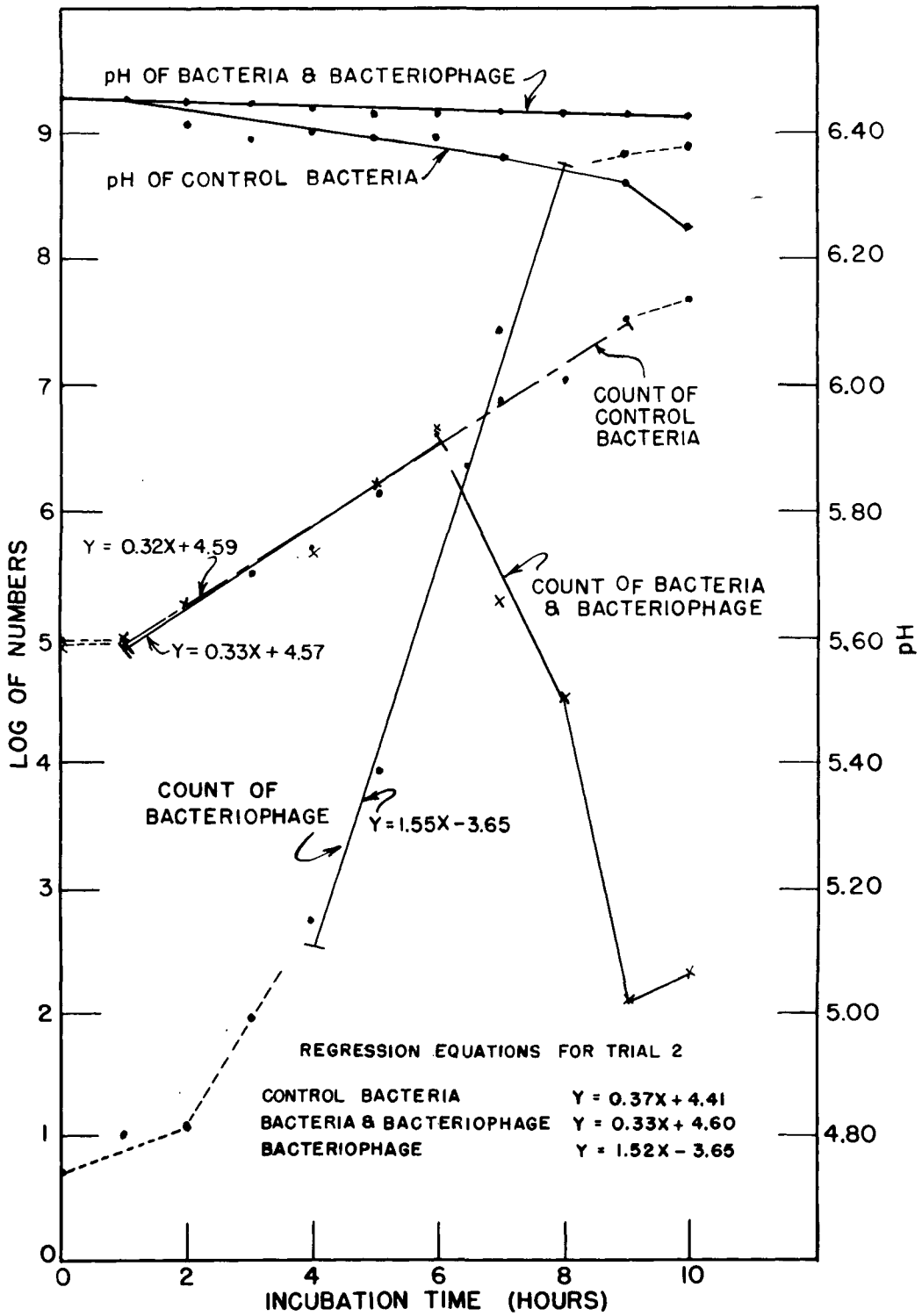


Figure 12. Influence of incubation temperature 21°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F70/SH5.

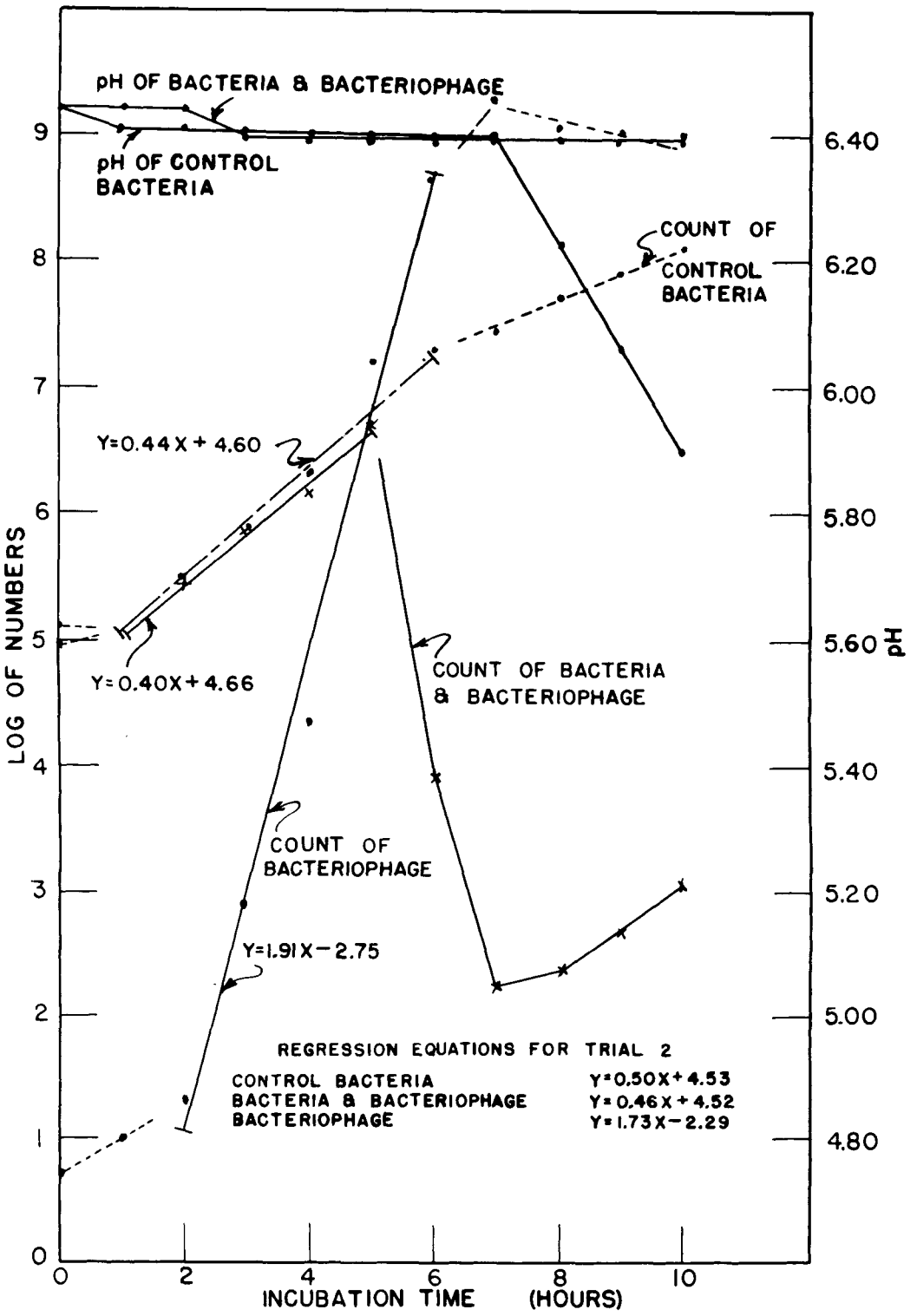


Figure 13. Influence of incubation temperature 24°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F70/SH5.

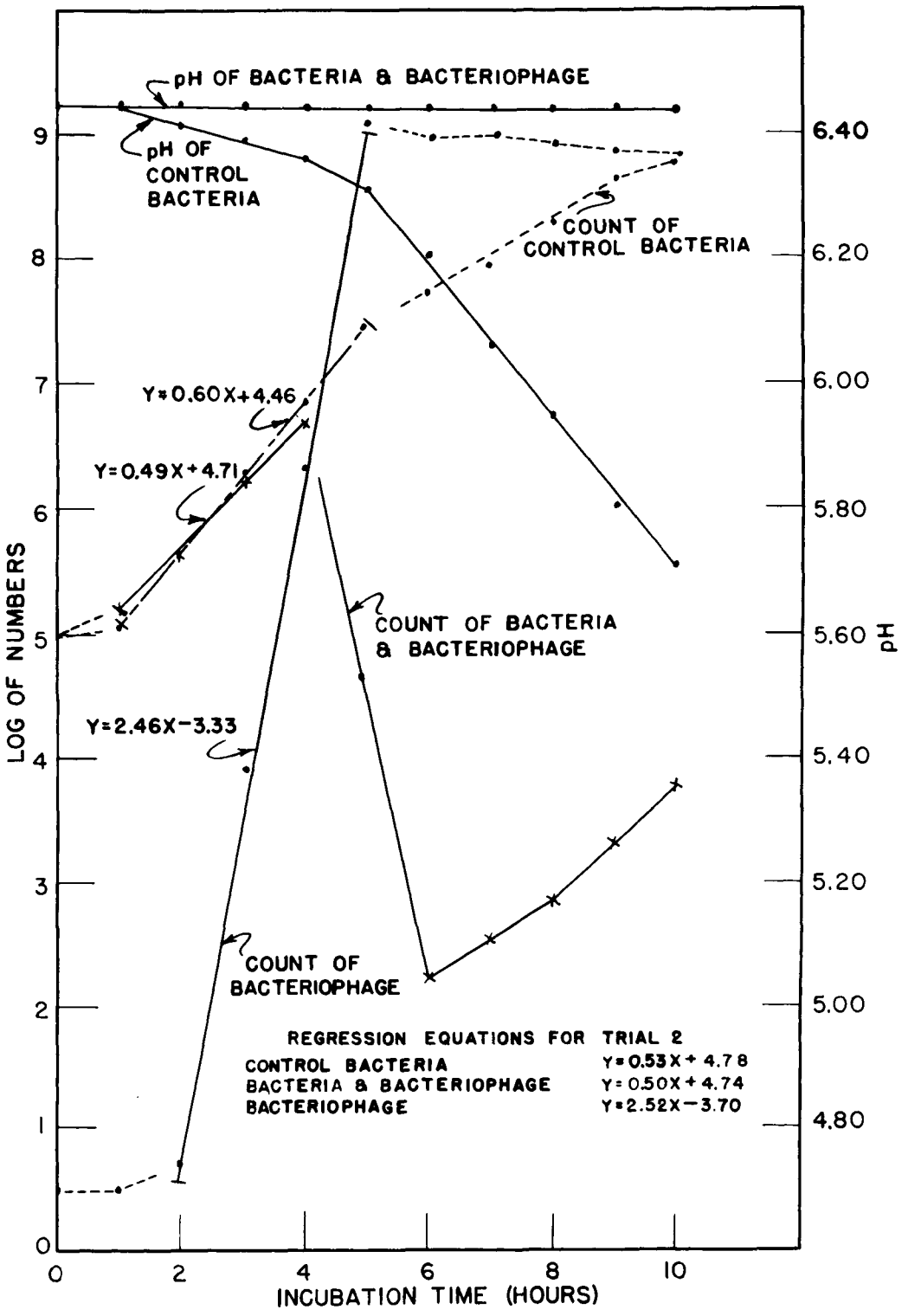


Figure 14. Influence of incubation temperature 27°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F70/SH5.

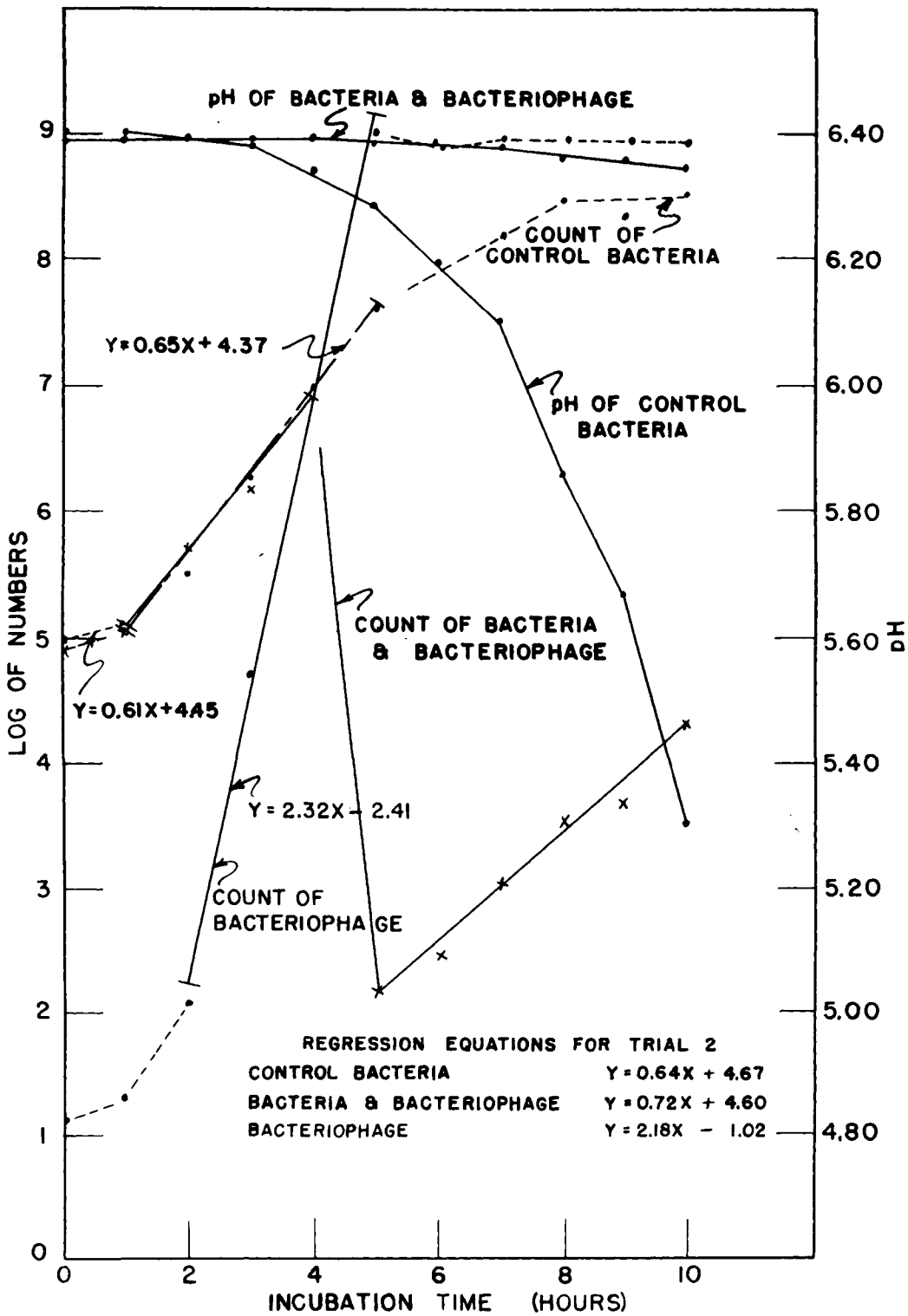


Figure 15. Influence of incubation temperature 32°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F70/SH5.

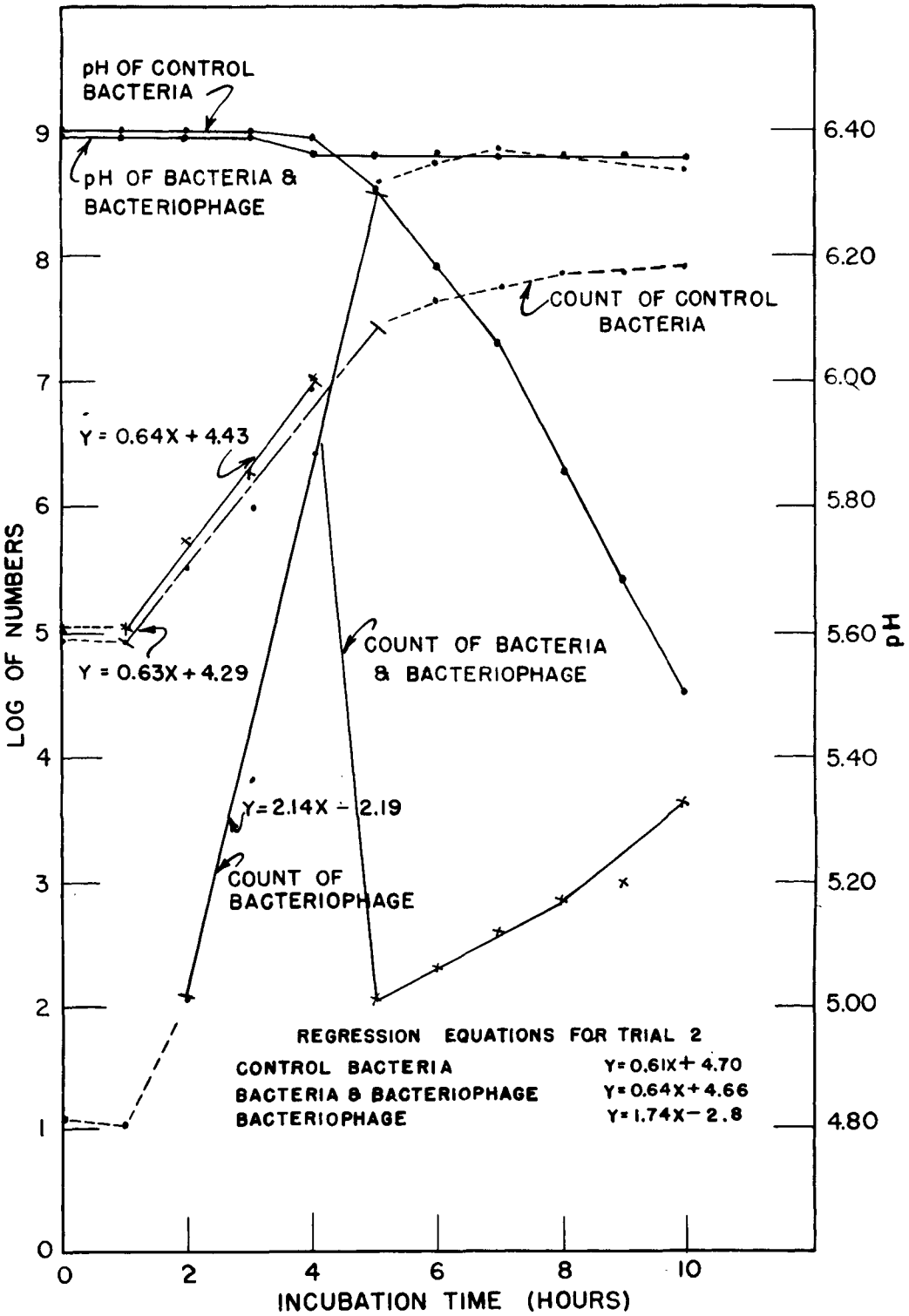


Figure 16. Influence of incubation temperature 35°C. on the proliferation rate and acid development of S. lactis bacteriophage combination F70/SH5.

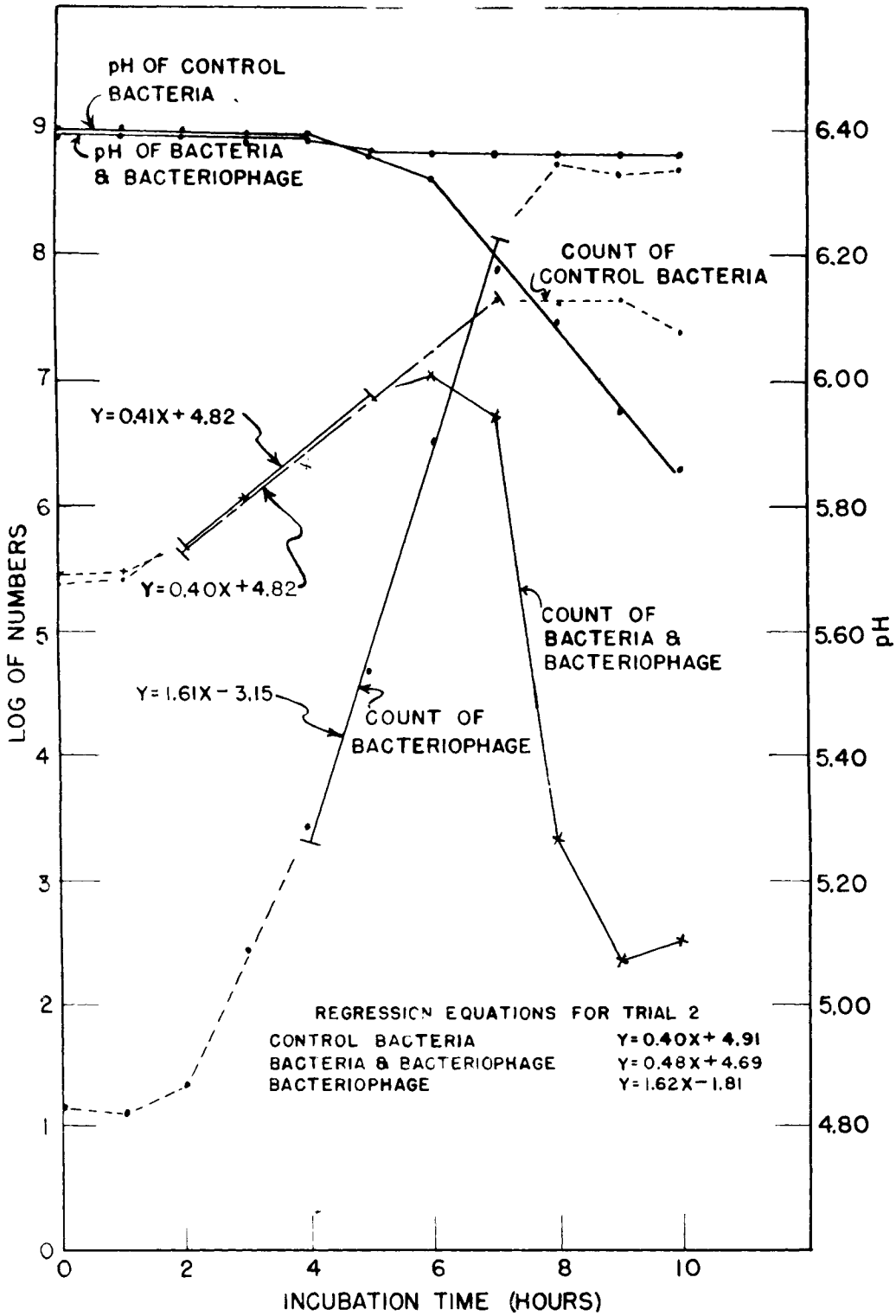


Figure 17. Influence of incubation temperature 37°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F70/SH5.

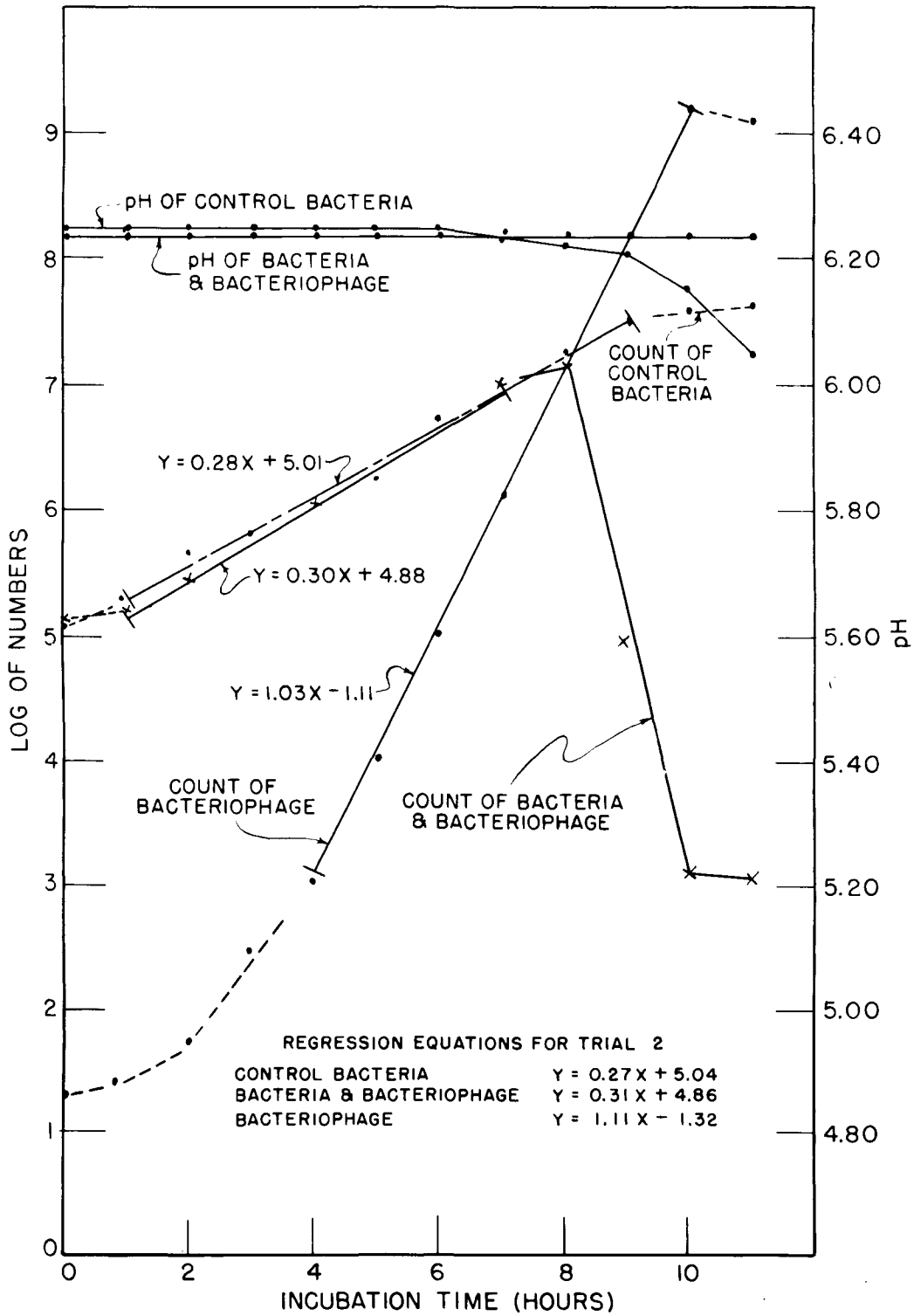


Figure 18. Influence of incubation temperature 21°C. on the proliferation rate and acid development of S.lactis bacteriophage combination PF2/H1-2.

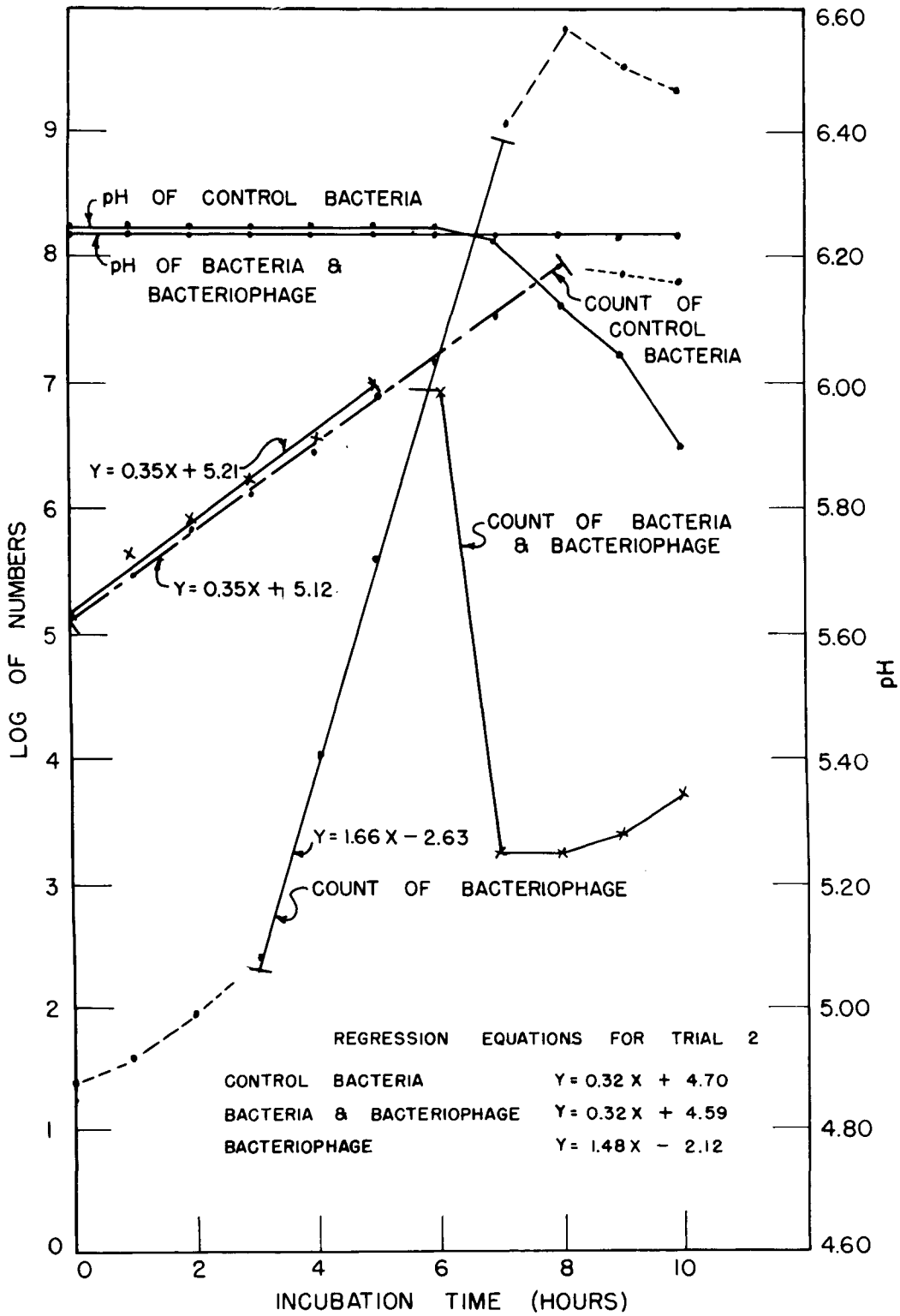


Figure 19. Influence of incubation temperature 24°C. on the proliferation rate and acid development of S.lactis bacteriophage combination PF2/H1-2.

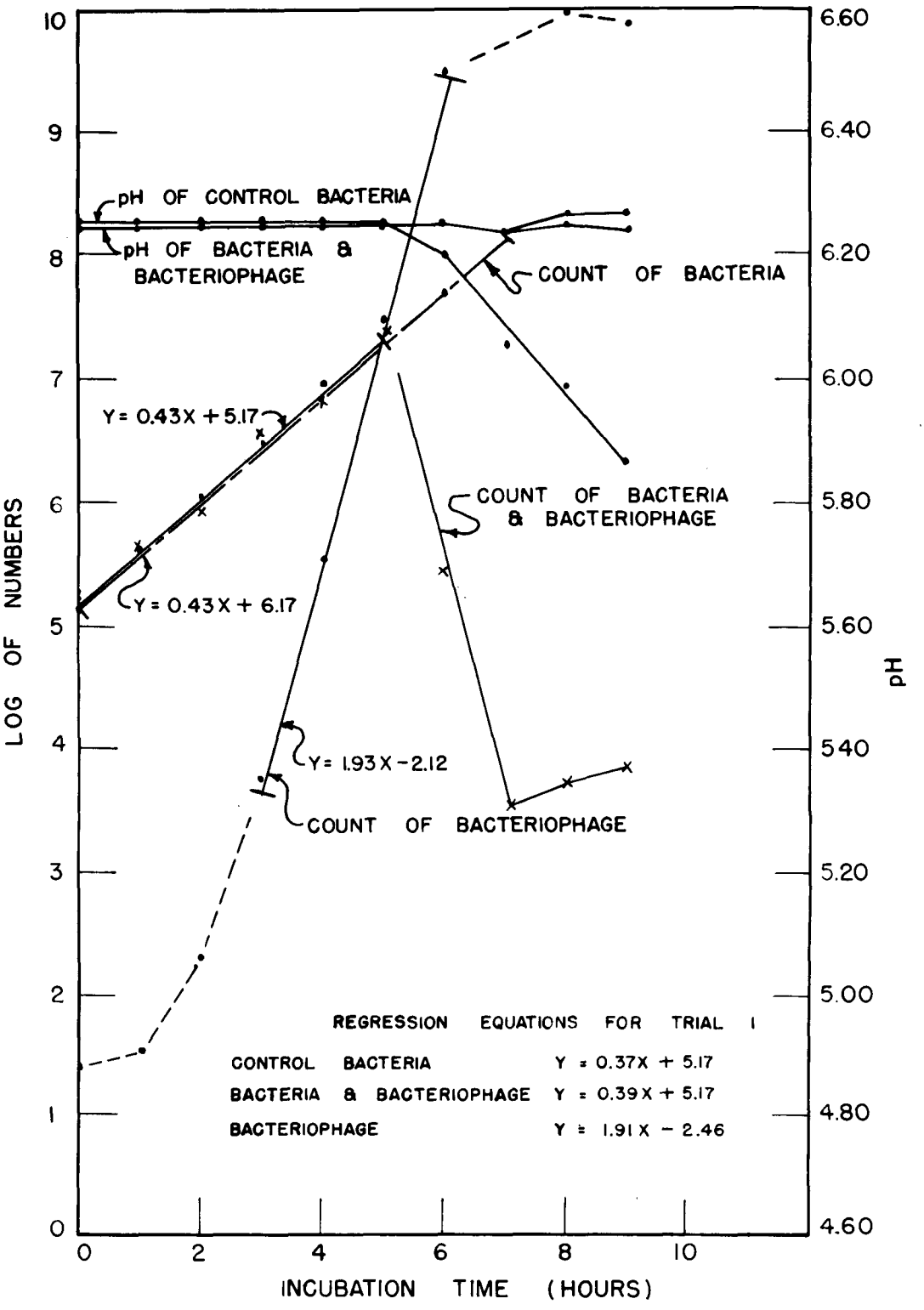


Figure 20. Influence of incubation temperature 27°C. on the proliferation rate and acid development of S.lactis bacteriophage combination PF2/H1-2.

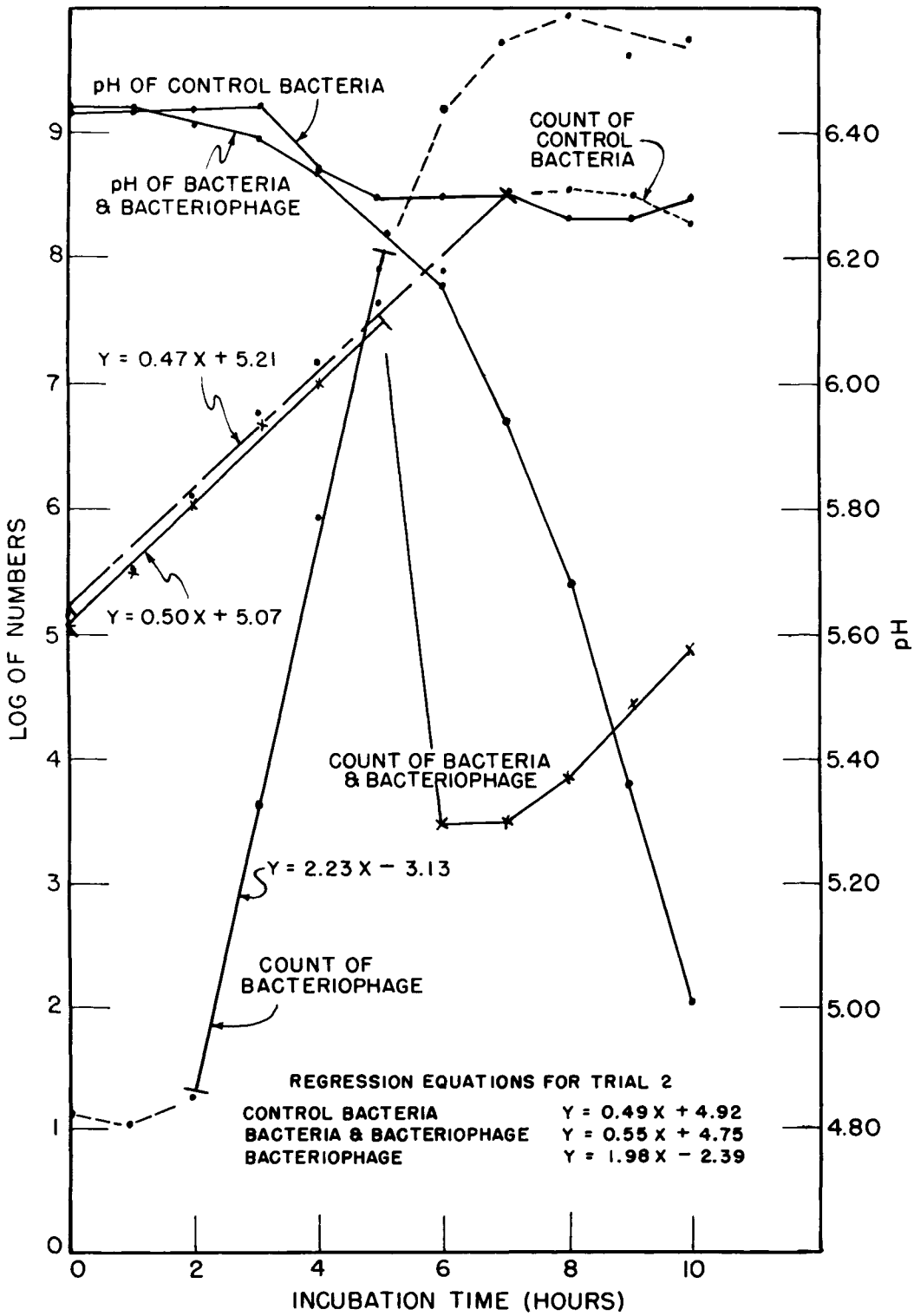


Figure 21. Influence of incubation temperature 32°C. on the proliferation rate and acid development of S.lactis bacteriophage combination PF2/H1-2.

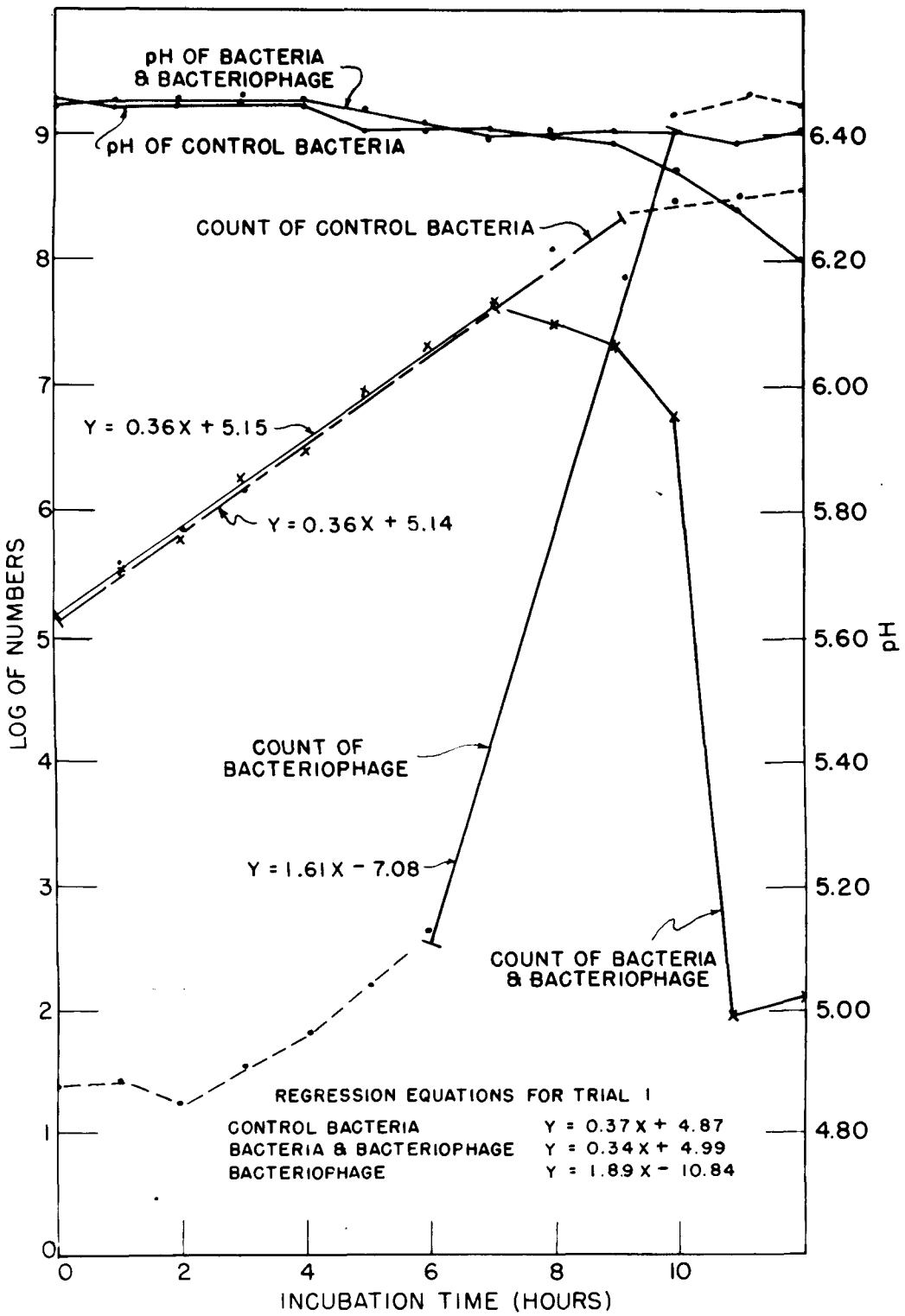


Figure 22. Influence of incubation temperature 35°C. on the proliferation rate and acid development of S.lactis bacteriophage combination PF2/H1-2.

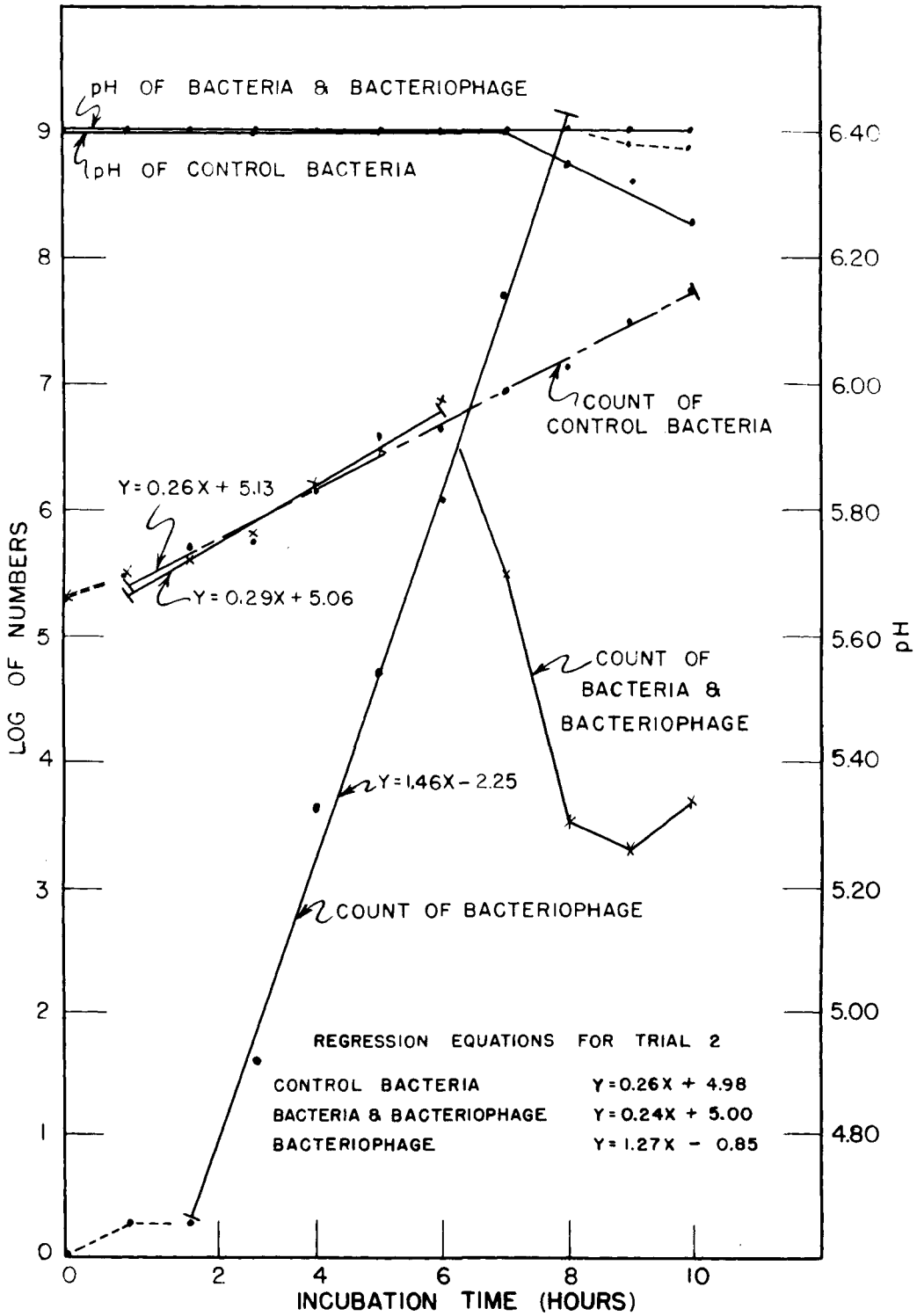


Figure 23. Influence of incubation temperature 21°C. on the proliferation rate and acid development of S.lactis bacteriophage combination PF11/H1-10.

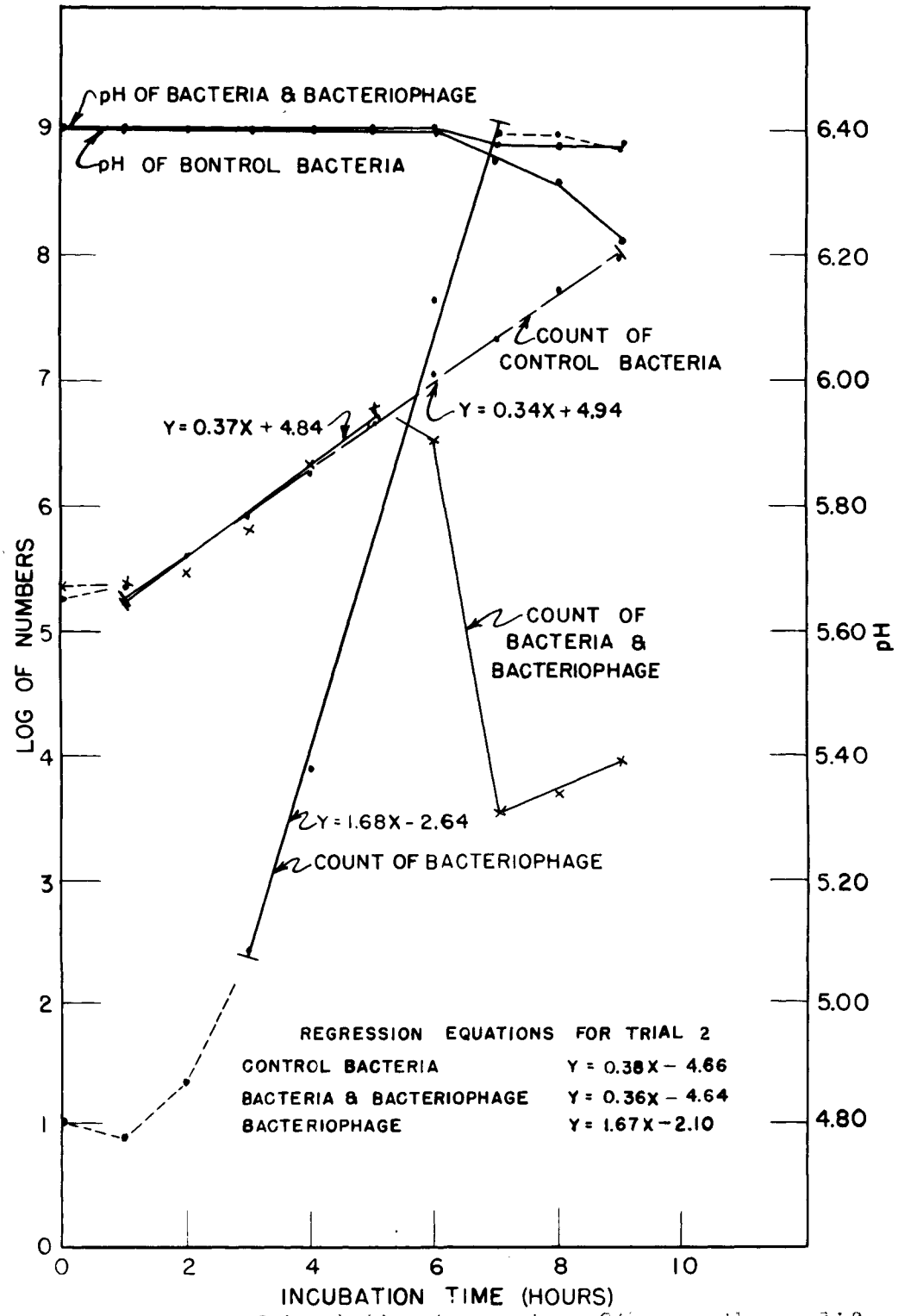


Figure 24. Influence of incubation temperature 24°C. on the proliferation rate and acid development of S. lactis bacteriophage combination PF11/HI-10.

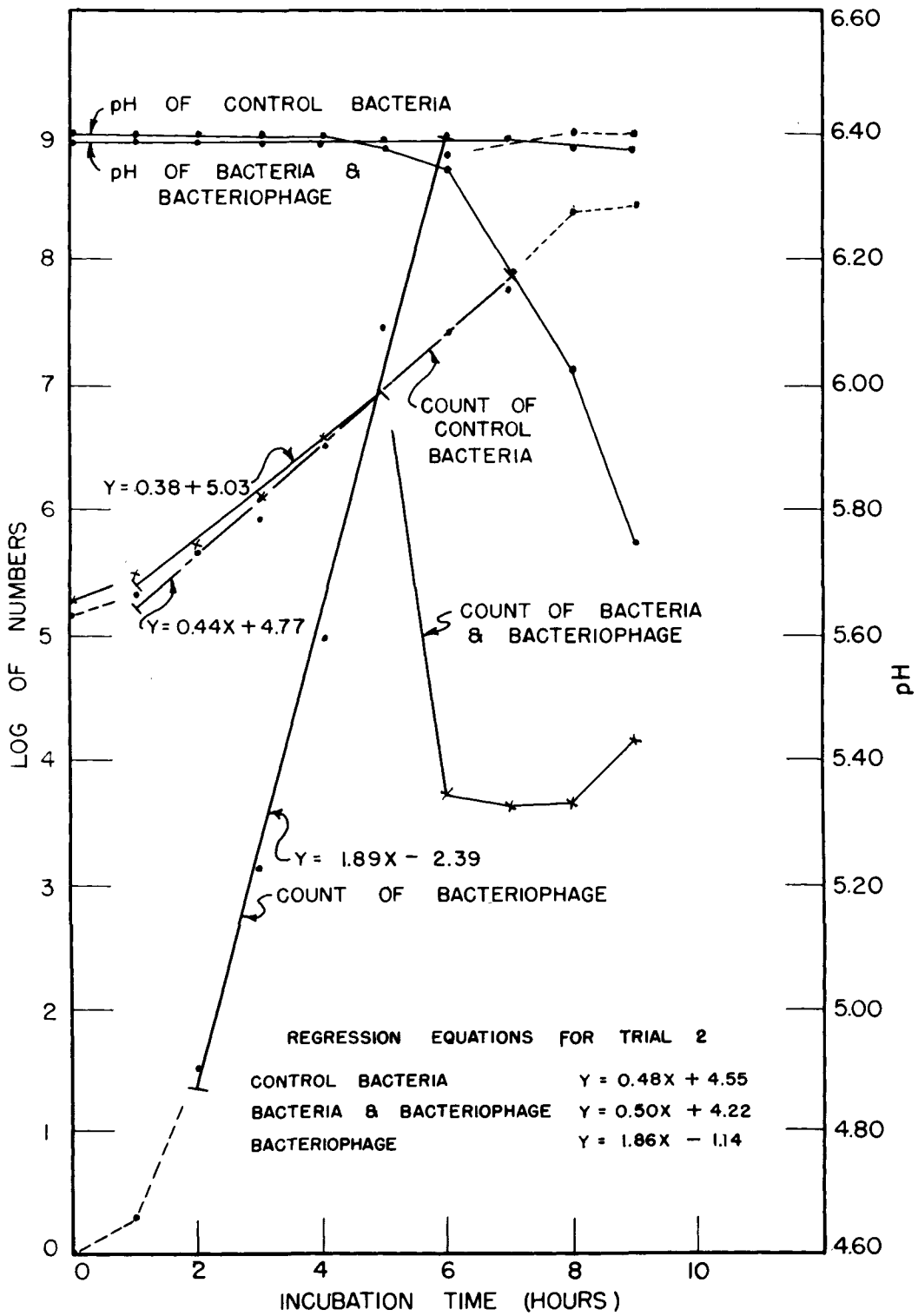
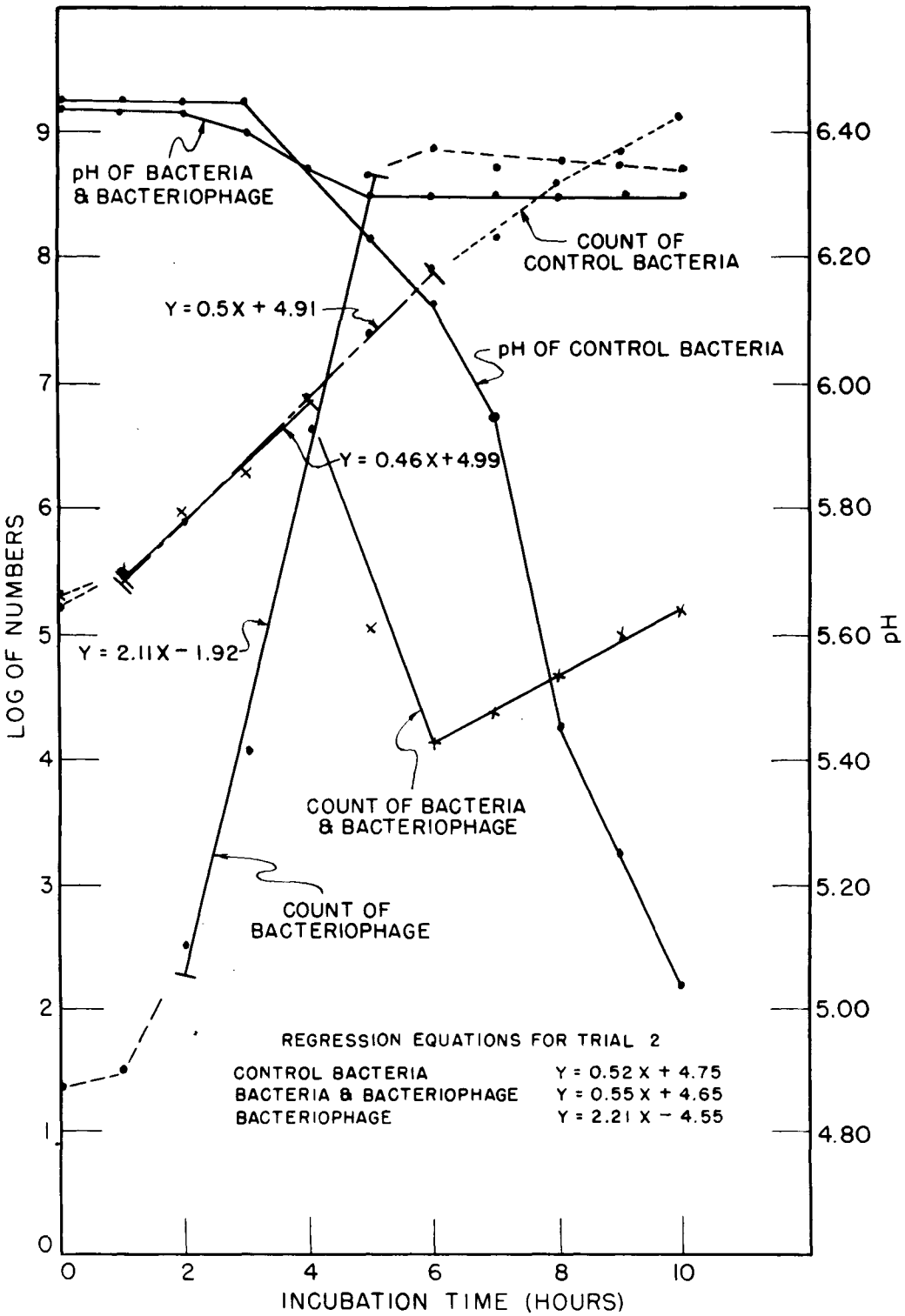


Figure 25. Influence of incubation temperature 27°C. on the proliferation rate and acid development of S.lactis bacteriophage combination PF11/H1-10.



[illegible] Influence of incubating temperature 32°C. on the proliferation [illegible] development of S. [illegible] bacteriophage combination PFil/H1-10.

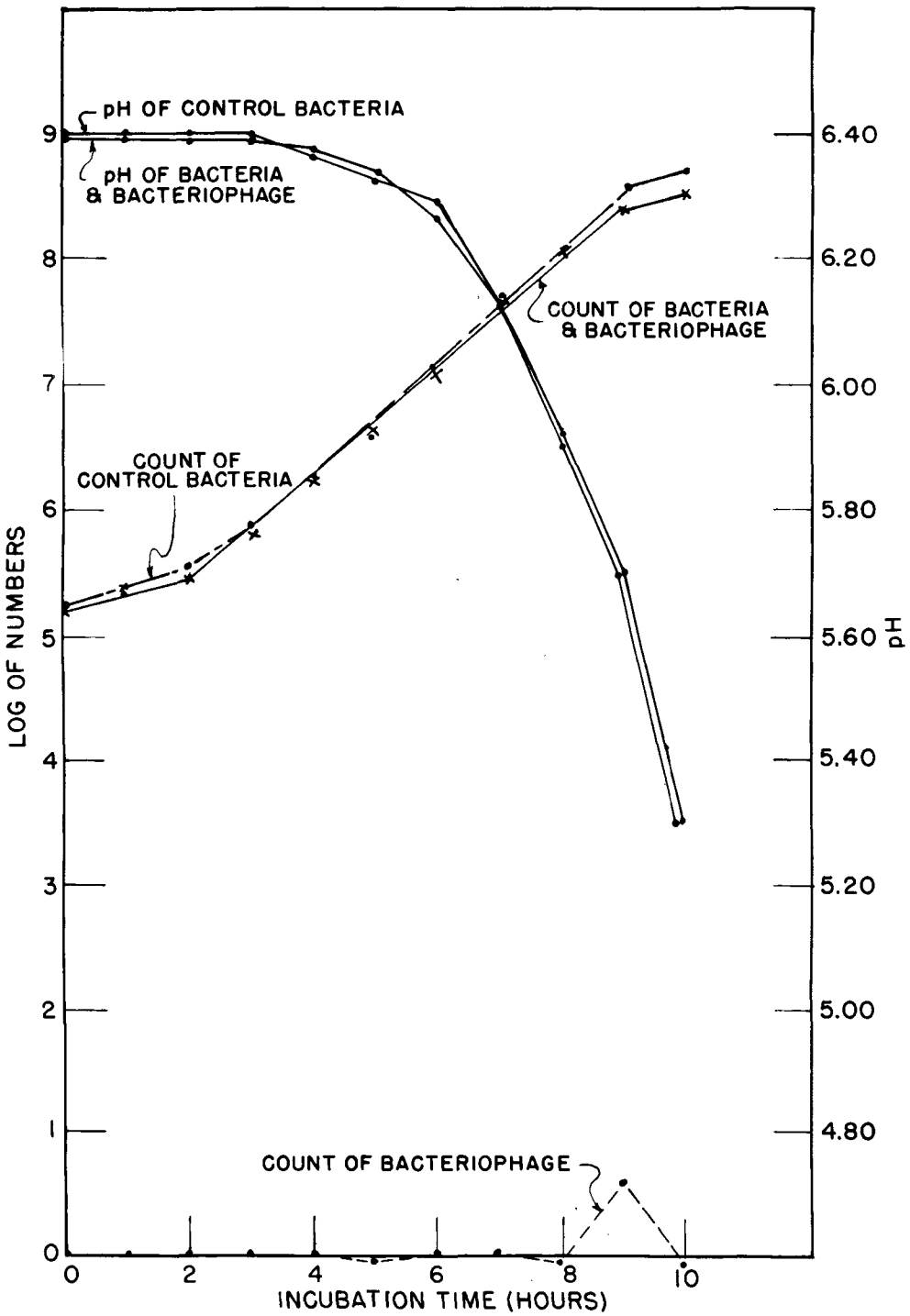


Figure 27. Influence of incubation temperature 35°C. on the proliferation rate and acid development of S.lactis bacteriophage combination PF11/H1-10.

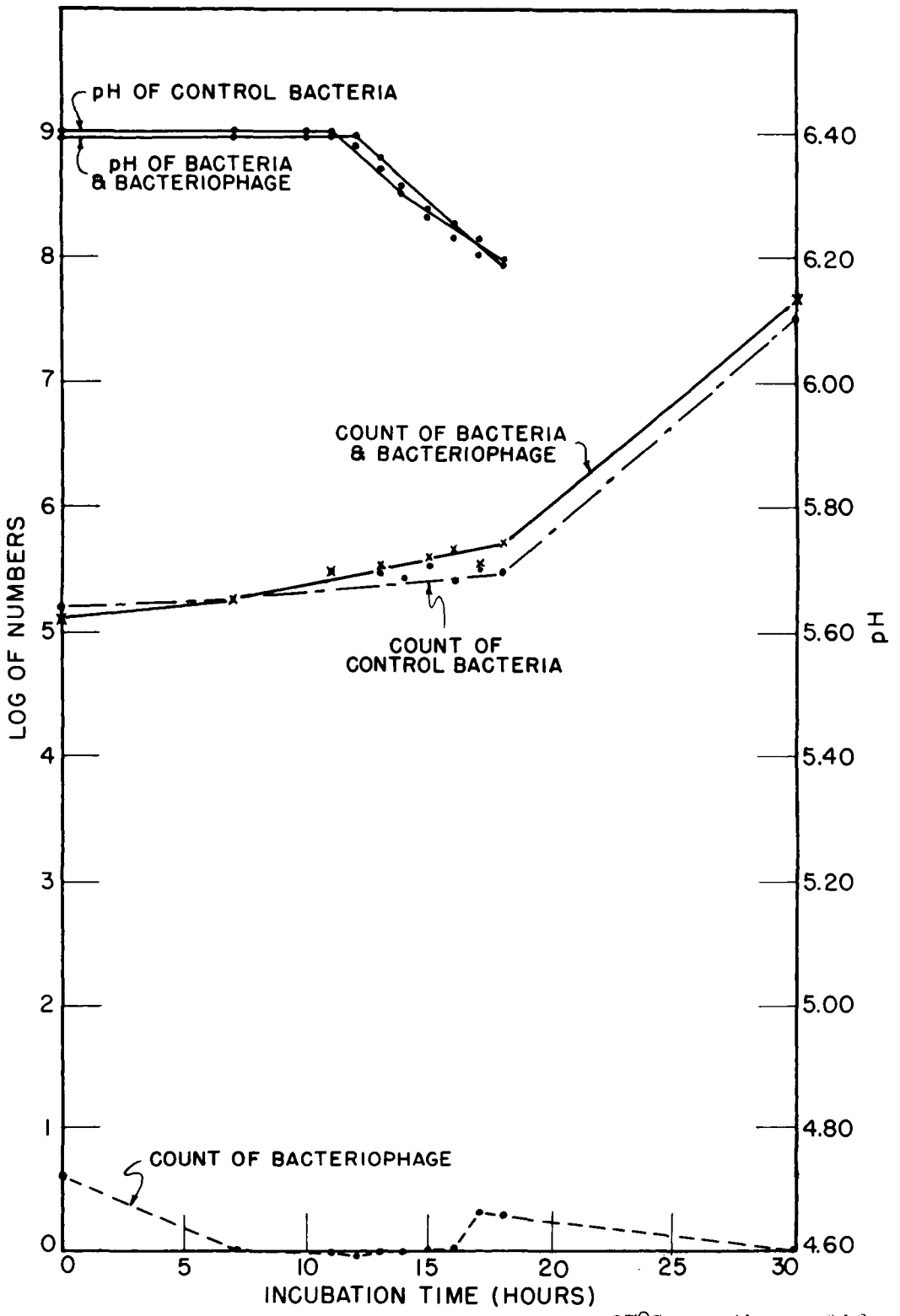


Figure 28. Influence of incubation temperature 37°C. on the proliferation rate and acid development of S.lactis bacteriophage combination F68/IP5.

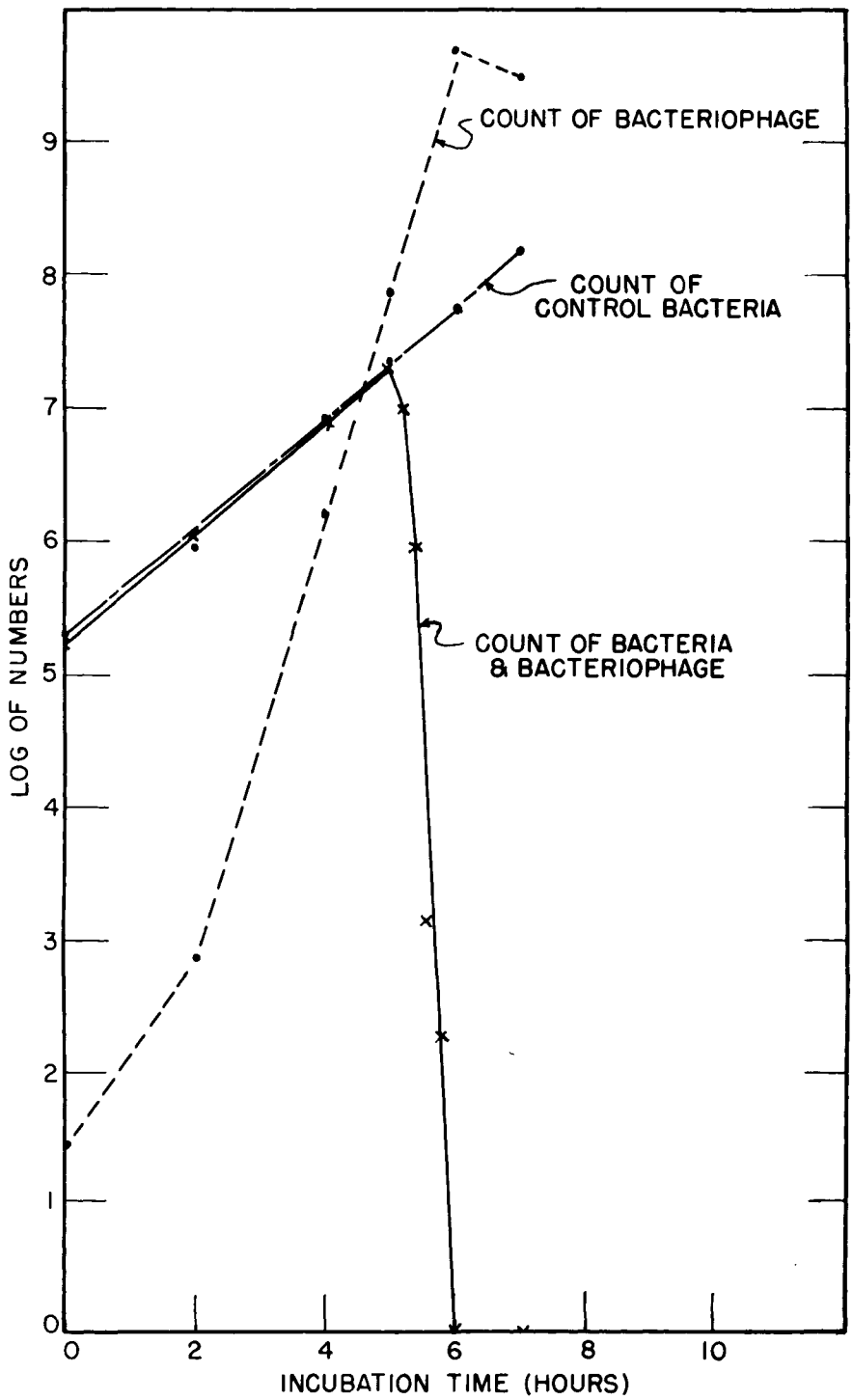


Figure 29. Determination of the length of time of the period of mass lysis of S.lactis ML1 by bacteriophage F69 at temperature of 32°C.

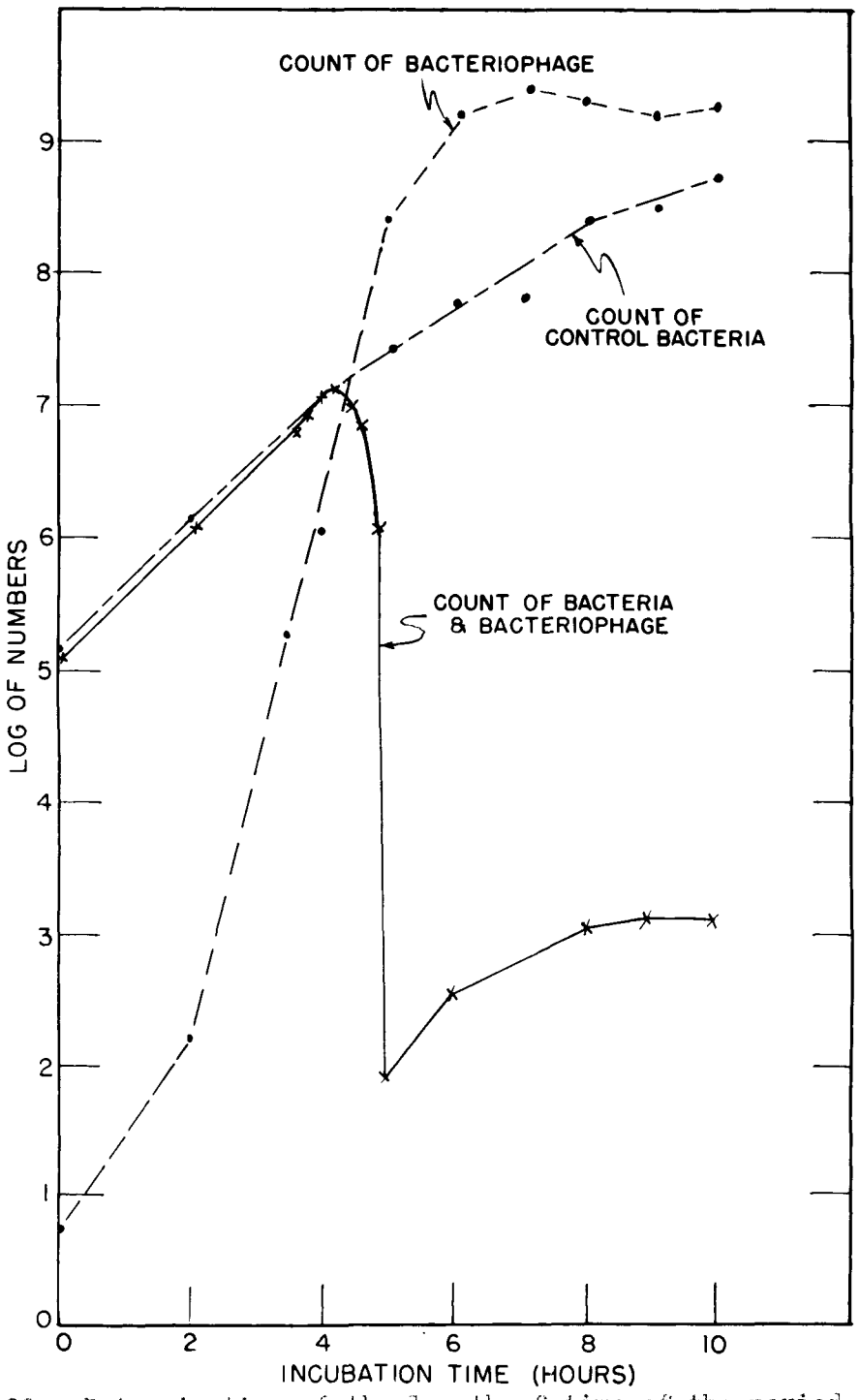


Figure 30. Determination of the length of time of the period of mass lysis of S.lactis IP5 by bacteriophage F68 at temperature of 32°C.

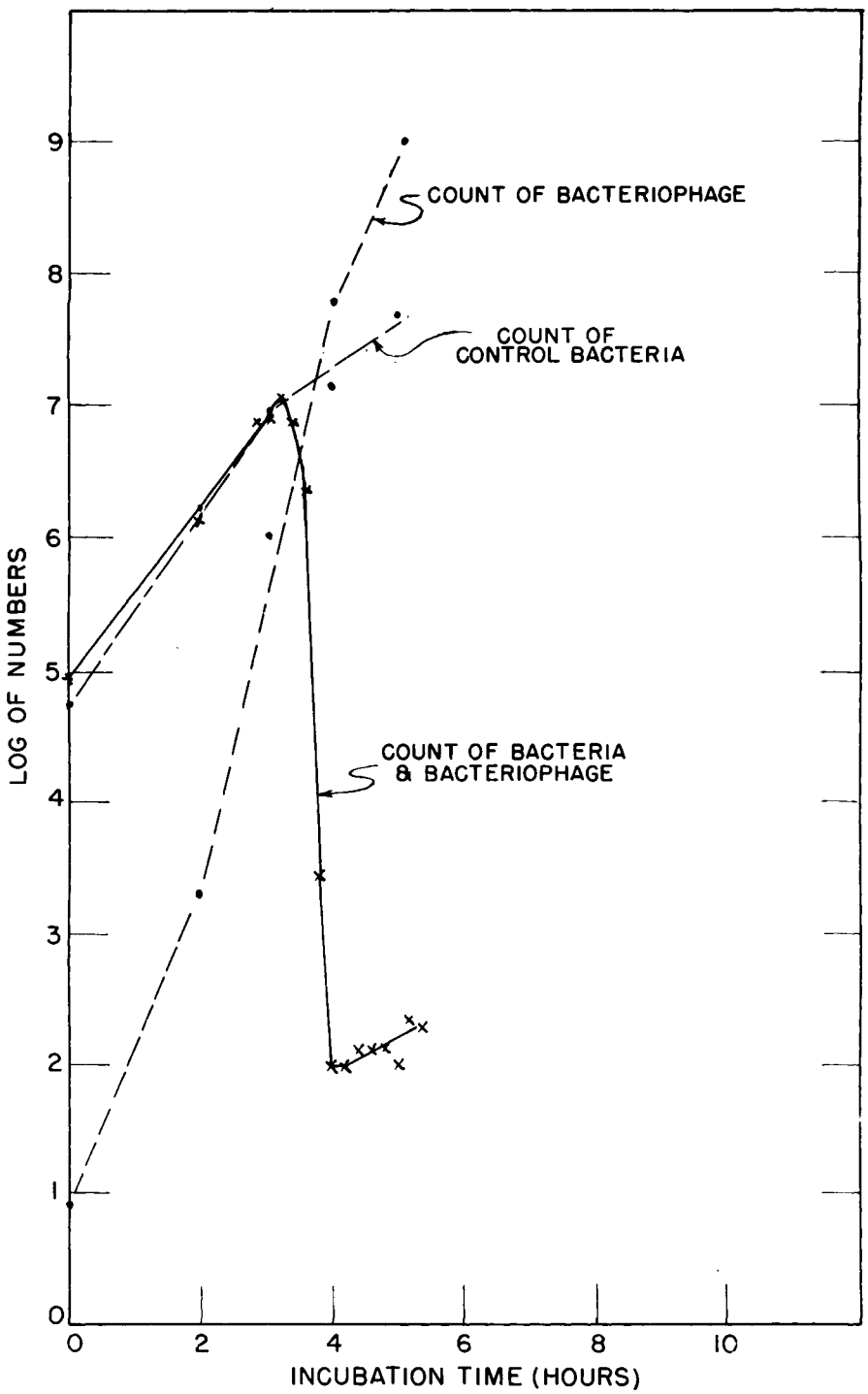


Figure 31. Determination of the length of time of the period of mass lysis of S.lactis SH5 by bacteriophage F70 at temperature of 32°C.

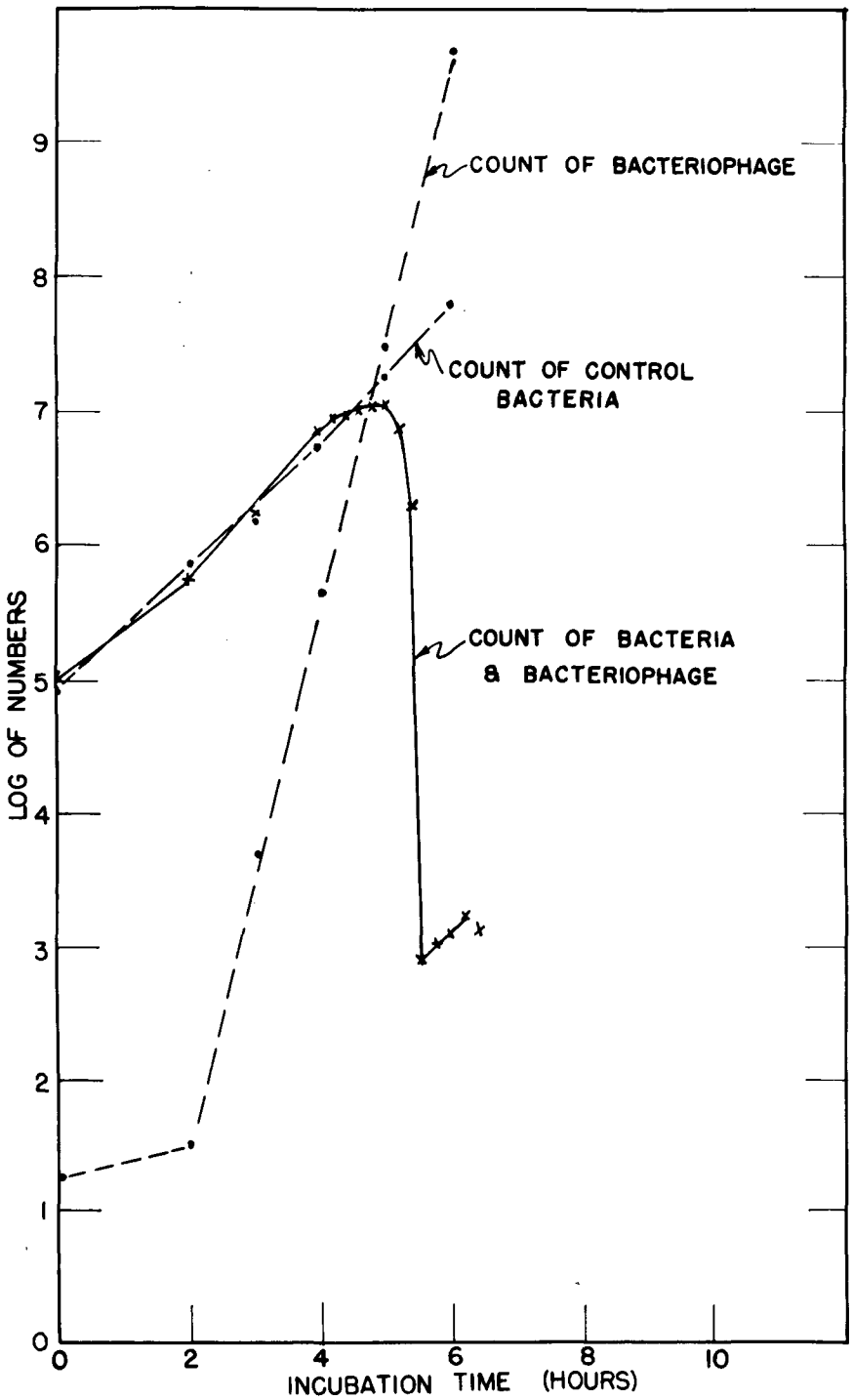


Figure 32. Determination of the length of time of the period of mass lysis of S.lactis H1-2 by bacteriophage PF2 at temperature of 32°C.

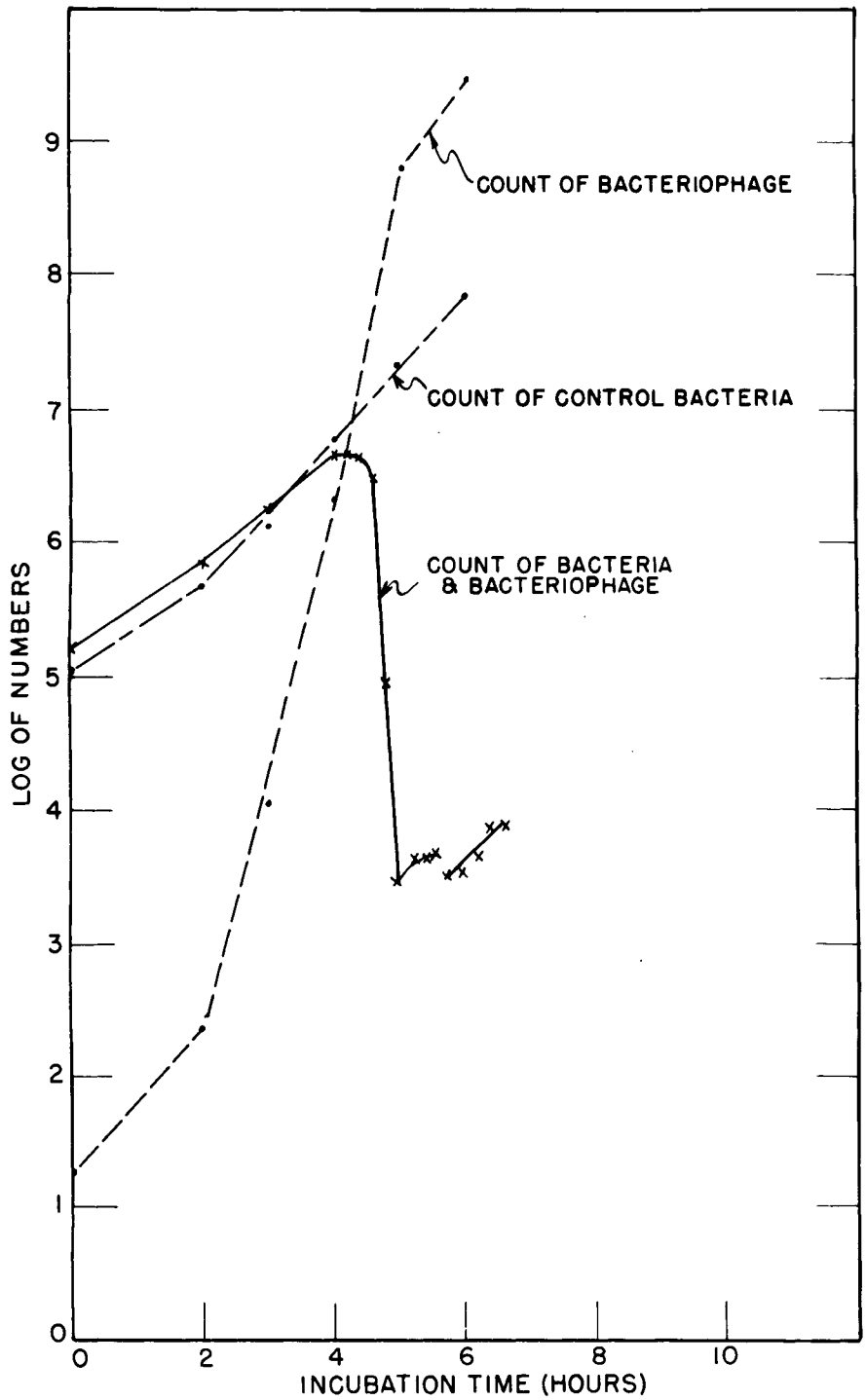


Figure 33. Determination of the length of time of the period of mass lysis of S.lactis H1-10 by bacteriophage PF11 at temperature of 32°C.

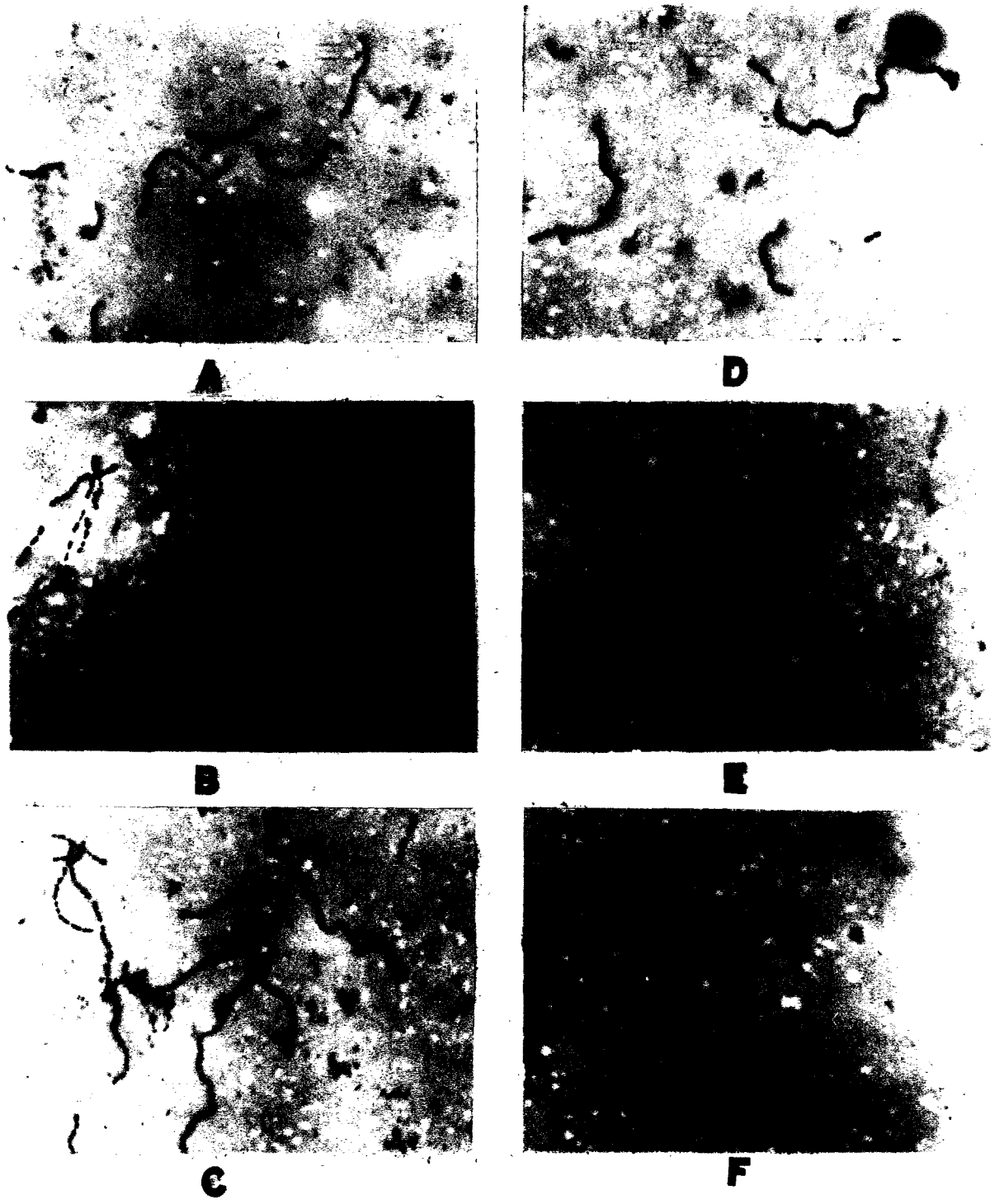


Figure 34. Appearance of S.lactis cells with & without added bacteriophage from 3 to 5 hours incubation at 32°C. (Combination F68/IP5). Bacteria controls A- 3 hrs. B- 4 hrs. C- 5 hrs. incubation. Bacteria & bacteriophage D- 3 hrs. E- 4 hrs. F- 5 hrs. incubation.

Figure 35. Appearance of plates from bacteria count of bacteriophage-organism mixture F68/IP5 made during the period of mass lysis of the susceptible S.lactis cells. 1- 10^{0} diln. 2- 10^{-1} diln. 3- 10^{-2} diln. 4- 10^{-3} diln. 5- 10^{-4} diln. 6- 10^{-5} diln.

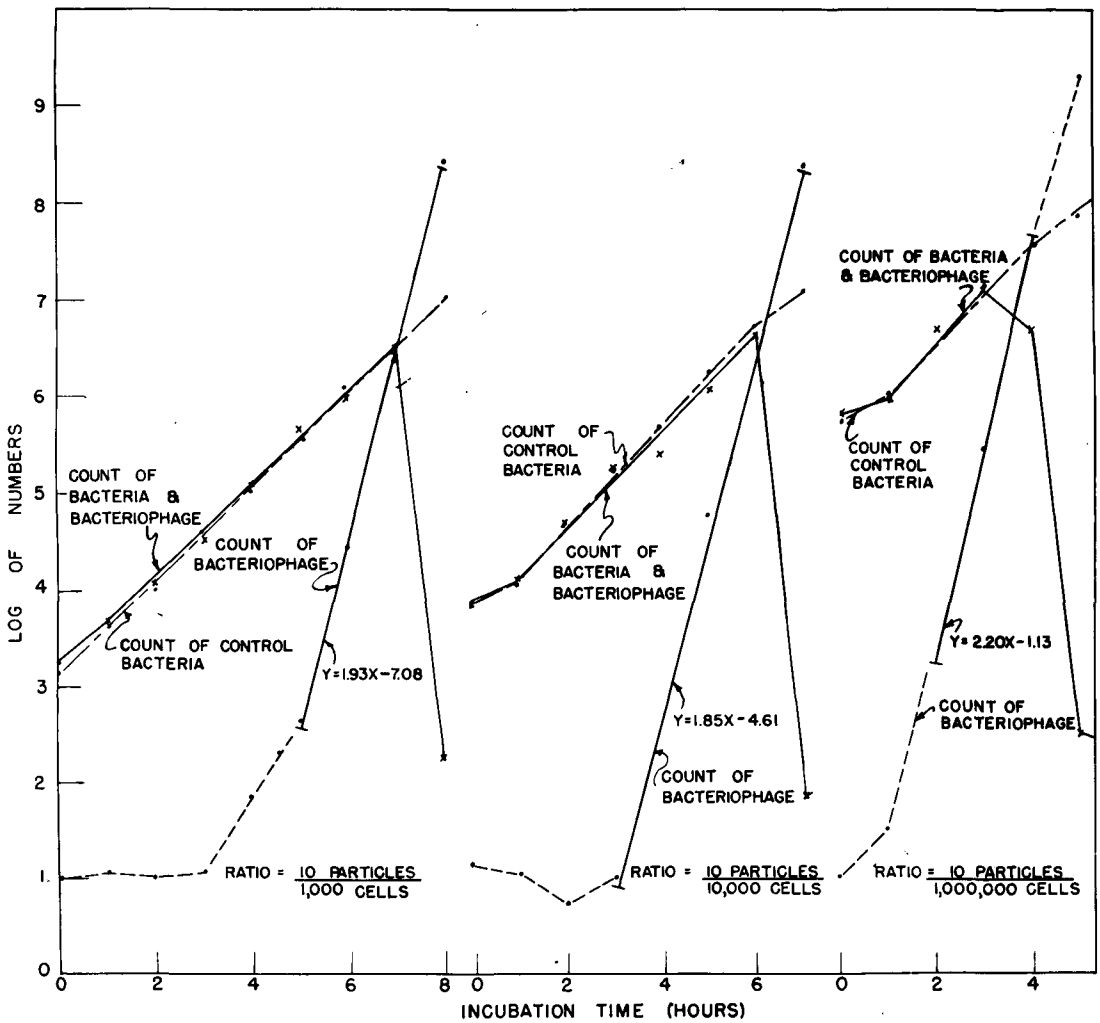


Figure 36. Effect of varying the S.lactis concentration to a given concentration of bacteriophage on the proliferation rate of combination F68/IP5 incubated at 32°C.

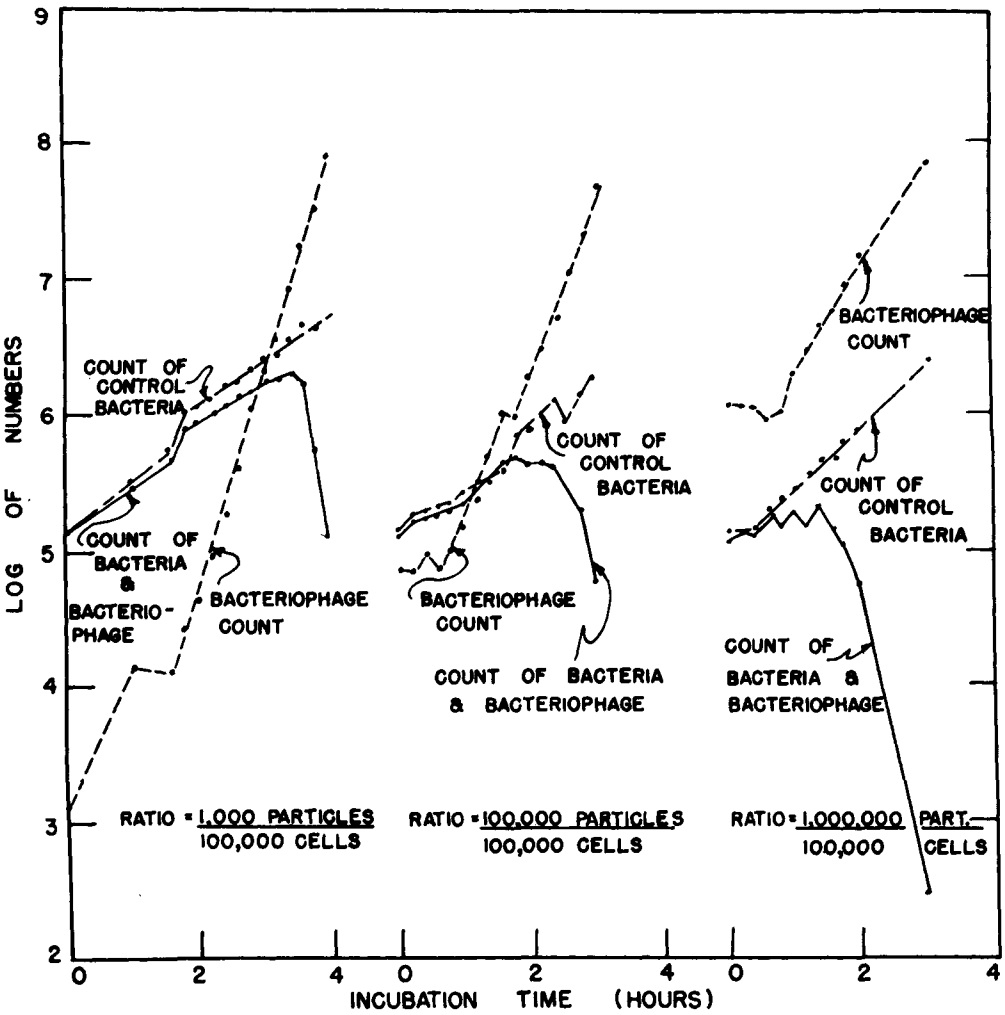


Figure 37. Effect of varying the bacteriophage concentration to a given concentration of S.lactis cells on the proliferation rates of combination F68/IP5 incubated at 32°C.

Delbrück (1940b) indicated that adsorption of bacteriophage above a threshold value was necessary to bring about mass lysis of susceptible cells. On reaching this value "lysis from without" took place, and the susceptible cells were destroyed in a very short period of time.

From the results presented in this phase of the study the effect of increasing the cell concentration resulted in shortening the bacteriophage lag but the slope of the logarithmic curve was not significantly changed with increases of bacterial numbers. Increasing the concentration of bacteriophage relative to cell concentration resulted in a decreased bacterial population which was eventually prevented from increasing with additional increase in concentration of bacteriophage.

Additional studies of bacteriophage proliferation, when the ratio of countable bacteriophage particles to bacterial population is varied over a wide range, are necessary before any very general conclusions on the effects of such variations can be drawn. This is particularly important with very low bacteriophage populations and high populations of susceptible cells at the temperatures encountered in carrying cultures and in the manufacture of cheese.

DISCUSSION

Obviously the different potential variables which may occur in the plating procedure for enumeration of bacteriophage particles by plaque formation must be controlled rigorously or this procedure will be quite unsatisfactory for experimental purposes. A wide range of variation may occur in bacteriophage strains with respect to their demonstration by the plaque method. Therefore, this procedure is limited to those strains whose characteristics are such that they produce plaques by following this particular method and thus may be enumerated quantitatively. However, the bacteriophage strains selected for this study have made possible a graphic presentation of some of the common observations encountered in commercial cheesemaking.

From the results presented it is evident that the temperatures commonly employed during the earlier stages of cheesemaking are conducive to maximum bacteriophage proliferation. In addition, the extremely rapid rate of proliferation permits the bacteriophage to reach a sufficient concentration in a period of 4 to 6 hours to cause mass destruction of susceptible cells. Thus in cheesemaking, one generally encounters no difficulty with the manufacturing process, when the cheese milk contains bacteriophage, until a lapse of 4 to 6 hours at which time acid production stops. This cessation of acid so commonly encountered at this time appears to coincide with the mass lysis of the susceptible bacteria graphically presented in this thesis. The low concentrations of bacteriophage mostly used in these studies, coupled with the ability

of bacteriophage to proliferate very rapidly indicates that cheesemilk containing one countable bacteriophage particle for every 10,000 susceptible cells maintained at temperatures between 24 and 32°C. may, at times, not readily be manufactured into cheese during the normal period of time. This finding also emphasizes the need for maintaining strict plant sanitation in order to keep the concentration of bacteriophage that may gain entrance to milk at lower levels than indicated above, otherwise, bacteriophage outbreaks may occur where slight laxity in sanitation is permitted.

Adsorption of the bacteriophage particle to the cell is an absolute requirement for demonstrating bacteriophage particles by plaques and for continued proliferation of the bacteriophage. Adsorption, in turn, appears dependent upon the factor of proximity between bacteriophage particle and susceptible cell and upon the presence of a component of milk which presumably may enhance adsorption of the particles in some strains of bacteriophage. This work indicates that by increasing the cell concentration in relation to the amount of bacteriophage present initially, one may shorten the bacteriophage lag at a given temperature, thus implying that adsorption is enhanced by bringing the particle and the cell closer together which permits more rapid adsorption and consequently a shortening of the lag period. Reducing the temperature of incubation also prolongs the bacteriophage lag, in one combination, to as long as 8 hours at 21°C., but raising the temperature toward the optimum for the particular bacteriophage immediately shortened the lag period. Such a result as presented may help to explain, in part, a possible cause of outbreaks of bacteriophage in commercial cheese plants. The lower temperatures of incubation maintained at times during the preparation of starter cultures may permit the

bacteriophage to remain in a somewhat dormant state until a more optimum temperature prevails in the cheese milk which promptly permits rapid adsorption of the bacteriophage.

The work presented on the bacteriophage burst time suggested 65 minutes as the probable burst time for S. lactis bacteriophage. The establishment of this time for the burst of streptococcus bacteriophage is somewhat substantiated in an indirect way from the studies made on the effect of incubation temperatures of 24 to 32°C. on bacteriophage proliferation rate in which a lag period of roughly 60 minutes was obtained when using hourly samplings of the test material. Similarily, it is noted from figure 37 that the "step" of the initial bacteriophage increase, after the logarithmic phase was reached, extended over a period of 60 minutes for two of the ratios presented.

The different bacteriophages studied varied considerably in the number of cells which resisted lysis by the respective homologous bacteriophage strains and from the results obtained one may possibly account for the varying rapidity of subsequent acid development due to growth of the secondary S. lactis cells which one, at times, encounters in commercial cheesemaking.

CONCLUSIONS

1. Maximum plaque counts of a reproducible nature were possible using a two-layer agar plaque plate containing 12 ml. of tomato juice-peptonized milk medium of 1 per cent agar concentration in the basal layer upon which was superimposed an agar overlay composed of 1 ml. of susceptible S. lactis cells, 1 ml. of bacteriophage suspension, and 1 ml. of the above agar medium but containing 1.5 per cent agar.

2. Simultaneous increases in agar volume and agar concentration of the overlay caused reduction of plaque counts to the extent of 46 per cent in the variations studied.

3. Varying the concentration of susceptible cells added to the agar overlay effected plaque numbers. Maximum plaques were obtained when approximately 28 million susceptible cells were added to the agar overlay. Less than and more than this cell concentration resulted in decreased numbers of plaques.

4. Inclusion of sterilized milk, heated for 15 minutes under 15 pounds steam pressure, in the agar overlay stimulated plaque formation with some strains of bacteriophage studied. Prolonged heating for 45 minutes or longer at this pressure caused reduction in plaque counts.

5. Maximum numbers of plaques were obtained for the several bacteriophage strains studied when the plating medium was maintained at pH 5.8 to 6.0. Lower plaque numbers were evident when the pH of the medium was not

within this range.

6. The two-layer agar plaque plate has a slight advantage over the limiting dilution method for determining bacteriophage activity. The former gives a count of particle foci in a sample volume containing a number of such foci, whereas the latter is a determination of the activity or absence thereof in a defined amount of material.

7. The streptococcus bacteriophage strains studied proliferate by stepwise increase, the suggested burst time is approximately 65 minutes and the average burst size is about 90 particles.

8. Maximum proliferation of each bacteriophage strain studied occurred at 32°C., this temperature also being the optimum necessary for maximum growth of the susceptible S. lactis culture. Varying the temperature of incubation produced corresponding changes in the proliferation rates of both the homologous bacteriophage and susceptible cell strains.

9. The total amount of bacteriophage produced under the conditions of this study was approximately 3 billion countable particles per ml. regardless of the incubation temperatures used. However, the time required to reach the maximum varied with the temperature of incubation, being shortest at the temperature of 32°C. and progressively longer as the temperature was varied from this optimum.

10. The proliferation curves of the S. lactis bacteriophage strains studied exhibited lag, logarithmic and resting phases.

11. Between an incubation temperature of 21 and 35°C. for some bacteriophage strains studied and at 37°C. for one strain, bacteriophage proliferation appeared dependent upon bacterial multiplication; however, when the temperature of incubation was slightly above this range this relationship was not evident. Instead, the bacteriophage failed to proliferate, whereas the bacteria multiplied quite well.

12. The bacteriophage strains held at specified temperatures of incubation proliferated at a much faster rate than the corresponding cells held at the same temperature, and eventually caused mass lysis of the susceptible cells. Mass lysis occurred in the shortest time at the optimum temperature of growth for the bacterial cells, while the time to effect lysis at temperatures on either side of the optimum was progressively longer.

13. Subsequent to mass lysis of susceptible cells, secondary growth appeared immediately in four of the selected bacteriophage strains, the rate of growth of the secondary cells approximating that existing in the culture before lysis occurred. The fifth bacteriophage strain failed to show evidence of secondary growth cells even after incubation for 2 weeks.

14. Increasing the cell concentration initially added to a given number of countable bacteriophage particles maintained at a temperature of 32°C. progressively shortened the bacteriophage lag, but the slopes of the curves showing bacteriophage population apparently are not affected significantly by the changes in initial cell population.

15. Increasing the concentration of bacteriophage initially added to a given number of S. lactis cells in a milk culture caused the amount of subsequent bacterial growth to be decreased to where no measureable increase was obtained although the bacteriophage proliferated under all the conditions imposed.

SUMMARY

By modification of a two-layer agar technic, a quantitative enumeration of bacteriophage particles by development of plaques in agar was used to study the burst time, the burst size, and the effect of varying temperatures of incubation on the proliferation rates of several selected strains of bacteriophage each added to milk cultures containing the corresponding susceptible Streptococcus lactis strains.

Several factors caused variation in the numbers of particles demonstrable by the two-layer agar plate technic, but by strictly following the optimum procedure a consistent quantitative proportion of the absolute numbers of bacteriophage particles was demonstrable and thus reproducible results were possible.

The application of the two-layer agar plaque plate to study the proliferation rates of five strains of S. lactis bacteriophage at different incubation temperatures indicated that a definite pattern was exhibited by all five strains within the range of incubation temperatures used, and that bacteriophage proliferation was dependent upon bacterial multiplication, both processes proceeding at their maximum at the optimum temperature for the multiplication of the organism but decreasing as the temperature varied either side of the optimum. Because of the extremely rapid proliferation rate of the bacteriophages below certain maximum temperatures, the bacterial cells soon were outnumbered and mass lysis of the susceptible cells occurred. In four of the five strains studied subsequent secondary growth became evident immediately after mass lysis of the susceptible cells. At higher

temperatures bacterial multiplication without bacteriophage proliferation was demonstrable; the exact temperature range in which this phenomenon occurred varied with different bacteriophage-bacteria combinations.

When the bacteriophage was active the S. lactis cultures failed to produce acid after mass lysis occurred.

Modification of the ratios of bacteriophage and susceptible cells added to milk were investigated and the attendent changes were studied and discussed.

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