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Characterization of bacteriophages active against lactic streptococci

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NOTE TO USERS

CHARACTERIZATION OF BACTERIOPHAGES ACTIVE AGAINST LACTIC STREPTOCOCCI

by

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INTRODUCTION

In the dairy industry rather widespread use is made of bacterial cultures known as "starters". These starters are used in buttermaking for the purpose of improving the flavor, odor and aroma of the butter. In cheese manufacture the starters are used for the development of acidity which is necessary, along with the rennet action, for the proper coagulation of the milk and proper expulsion of whey from the cheese curd. Acidity also helps retard growth of undesirable types of microbial contamination. Starters also are made by market milk dairies and sold as cultured buttermilk for human consumption. In these starters the most commonly occurring microorganisms are various strains of Streptococcus lactis or Streptococcus cremoris, these two species commonly being referred to as "lactic streptococci" because of their lactic acid-producing ability. These strains are used either as single strain cultures, as multiple strain cultures composed of several single strains, or as mixed cultures in which the so-called associate organisms, Streptococcus citrovorus and/or Streptococcus paracitrovorus, also are present.

It is well known that bacteriophages active against the lactic streptococci sometimes are present in starters.

Bacteriophage activity manifests itself by causing lysis of the susceptible bacteria with a resulting stoppage of the desired acidity development in the starters. Sometimes the rate of acid development merely is retarded rather than completely stopped, especially in the case of multiple-strain cultures. Correct maintenance of desirable fermentation in starters by protection of the S. lactis and S. cremoris organisms from bacteriophage action has become the concern of all who handle lactic cultures in the dairy industry. Slow or inactive starters result in inefficient manufacturing operations and in lower quality products, both of which result in monetary losses.

A completely satisfactory solution to the bacteriophage problem has not been found, even though work toward this end has been going on for more than twenty years. Several practical procedures have been suggested and tried with varying degrees of success. Usually the success has been only temporary and in due time an outbreak of bacteriophage would reoccur. If there were to be a solution to this problem it appeared that it would be one of more fundamental nature than the relatively empirical methods thus far employed. At the present time more basic avenues of approach are being followed and detailed experiments are being conducted in studying the relationship of the bacteriophages to the host microorganisms.

The purpose of this study was to characterize further, especially from the taxonomic standpoint, the bacteriophages active against the lactic streptococci. Those characteristics selected for further study were (1) cross-reactions of bacteriophages and host microorganisms, (2) plaque sizes, (3) serological typing, and (4) heat inactivation of the bacteriophage. The specific question for which an answer was desired was whether there exists in nature one homogeneous group of these bacteriophages distinguished principally on the basis of host strain specificity, or whether there are a number of heterologous groups which can be distinguished by some additional methods of identification. If the latter, using the characteristics studied in detail in this investigation, an attempt would be made to establish the basis for a systematic classification of the bacteriophages active against the lactic streptococci.

HISTORICAL

General Observations

The significance and applications of bacteriophage in bacteriological and virus research was reviewed by Craigie (1946), who pointed out that the bacteriophages (bacterial viruses) share several properties with animal and plant viruses. These similarities were similar range of size, reproduction only within living host cells, either wide host range or limited to a single species, destruction of host cells or existence within them in a latent state, extension or change of host specificity by adaptation or mutation, presence of one bacteriophage interfering with infection by another, and their similar antigenic properties.

The type of cell against which this activity is exhibited, the host specificity, has been the primary characteristic which identifies the various viruses. A voluminous amount of information on this general topic was available but a complete review of the entire field was not considered within the scope of this survey. Specific references are made only to those reports containing information directly applicable to the consideration of those characteristics by which bacteriophages are identified. A number of good review

articles are available, covering the various aspects of the viruses and bacteriophages and these may be consulted for further information.

Krueger (1936) comprehensively reviewed the available information on the nature of bacteriophage and its mode of action as understood up to that time. In considering the classification of bacteriophages he suggested that probably there were many different bacteriophages, each possessing individual characteristics under set conditions that are constant and differ widely between the various bacteriophages. The most important characteristics he listed were (1) strict serologic specificity independent of the bacterial substrate, (2) resistance to physical and chemical agents, (3) plaque characteristics, (4) particle size and (5) capacity to produce bacterial forms resistant to certain groups of phages.

In a review primarily on the plant viruses, Pirie (1946) suggested that in any attempt to classify the plant, bacterial and animal viruses, recognition of their ability to bring about defined physiological changes in the host and not their own intrinsic properties was of greatest importance. He added that the classification of the viruses was but one specialized branch of the more general theme of protein classification.

It is of historical interest to note briefly some of the earlier fundamental discoveries that lead to our present knowledge and interest in bacteriophages. In a brief review Rivers (1941) pointed out that in 1892, Iwanowski, working with tobacco mosaic, passed juice from an infected plant through a filter and noticed that the filtrate was capable of producing disease in healthy plants. For this work Iwanowski was credited with the discovery of the first virus.

Twort (1915), investigating the nature of ultramicroscopic viruses, noted the existence of a transmissible lytic agent active against staphylococcus organisms. This was the first investigation in which bacterial viruses or bacteriophages were encountered. Therapeutic use of bacteriophages for the treatment of infectious diseases because of their possible lytic effect on the invading organism was suggested, giving rise to a number of interesting investigations. One was that of Bordet and Ciuca (1921), who were the first to show that the blood sera of rabbits given injections of active bacteriophage filtrates contained antibodies (antiphage) which neutralized the activity of the lytic principle. In this manner they demonstrated the antigenicity of bacteriophage, the antigen being quite distinct from the normal bacterial antigen.

The first bacteriophage active against S. lactis was reported by Hadley and Dabney (1926), who found this bacteriophage present in a filtrate prepared from fecal material, the concentration being great enough to show activity after a 1:1,000 dilution. Bacteriophages active against other species of bacteria also were found in the sewage sample. It was not until some years later that the dairy industry became interested in the bacteriophage problem and only after it had definitely been established that much of the slow acid production in starters was due to bacteriophages active against the lactic streptococci.

General Characteristics of Bacteriophages

A comprehensive review of most of the earlier work on bacteriophages was compiled by d'Herelle (1926). At that time d'Herelle considered the bacteriophage as a single species capable of adaptation to activity against all species of bacteria known to be sensitive to the bacteriophage action. This view was supported by the fact that some strains of the lytic principle exhibited multiple virulence against several bacterial strains. No attempts at a systematic classification of bacteriophages were made up to that time, the work being directed primarily toward a better understanding of the nature and behavior of bacteriophage.

In view of newer knowledge, d'Herelle's single species concept is not generally accepted.

From the studies on the nature and behavior of bacteriophage emerge the few characteristics which form the bases for the present attempts to classify bacteriophages. d'Herelle reviewed the effects of heat on bacteriophage inactivation. In general, an exposure of 30 minutes at 65-75°C. was required to inactivate the bacteriophages known at that time.

Burnet and McKie (1933) proposed classification of the dysentery-coli bacteriophages on the basis of cross-resistance relationships. They concluded that major resistance groups could be established that were on the whole consistant with the serological groupings of the dysentery-coli bacteriophages that were classified systematically into twelve distinct serological groups by Burnet (1933). The specific antisera were titrated by a decrease in plaque counts. He found that the bacteriophages of a single serological group also were of the same plaque type, the same particle size and belonged to the same major resistance group. Within a serological group, minor antigenic differences were noted, but Burnet pointed out that there was practically no evidence of intermediates between the main groups.

Burnet and Freeman (1937) stated that the antigenic types of bacteriophages are as diverse as those of bacteria and that bacteriophages lysing the same bacteria may be antigenically distinct, while antigenically similar bacteriophages may lyse quite different bacterial species.

In a review of the immunological reactions of the filterable viruses, Burnet, Keogh and Lush (1937) emphasized the value of the antigen-antibody reaction in the classification of bacteriophages, but they admitted that no completely satisfactory explanation of the bacteriophage-antiphage reaction had yet been suggested.

Evans (1934) made a serological study of the hemolytic streptococcus bacteriophages and designated four types, A, B, C and D, based on their distinct behavior in cross-serological reactions. Two strains of bacteriophage were regarded as belonging to the same type when the antiserum prepared by treating rabbits with either one of them neutralized both. In the nascent state, type D bacteriophage was active against one strain of S. lacticus (S. lactis) of the six tested, but no further information was given, since the concern was primarily with the hemolytic streptococci. It was possible to group 421 strains of hemolytic streptococci into eight groups according to their sensitivities to the bacteriophages of the four serological types. The thermolability of the four bacteriophage types was reported to

be 60°C. for types A and B, 63°C. for type D, and 65°C. for type C, when heated for one hour at these temperatures. A more heat resistant type E was reported by Evans and Sockrider (1942) as being inactivated in 60 minutes at 75-76°C. at pH 7.4. The plaque diameter of type E was 0.5 to 0.75 mm. on 1.25 per cent agar medium containing 0.5 per cent glucose.

Andrewes and Elford (1933a) reported undiluted coli-bacteriophage heated for one hour at 65°C. was 99.8 per cent inactivated. The surviving 0.2 per cent showed normal resistance to antibodies. These investigators made an extensive study of the bacteriophage-antiphage reaction and proposed the "percentage law" which depended on a dishomogeniety of a virus population as regards susceptibility to inactivation by serum. This law stated that the percentage of virus inactivated in a given time, by a given concentration of serum, is constant over a wide range of virus concentrations.

Hershey (1943), in studying the bacteriophage-antiphage reaction, produced what he considered to be an immunologically distinct large-plaque bacteriophage from a coli-bacteriophage strain. Hershey et al. (1943) reported evidence supporting observations that serological classification of bacteriophage by means of neutralization tests separated strains of

bacteriophage into groups which were fairly homogeneous in other respects. They pointed out that distinguishable bacteriophages of the same general classification may stimulate the production of indistinguishable antibodies.

Delbrück (1945a) reported on some of the distinguishing characteristics of three bacteriophages, identified as alpha, gamma and delta, active against Escherichia coli (strain "B"). Their sizes, determined by electron micrographs, were 50 mμ for alpha and 65 x 80 mμ for gamma. The structures of alpha were round head, slender tail 150 mμ long; gamma's structure was oval head with discernable internal structure, straight tail 120 mμ long. A pour plate technic for plaque formation was used and the plaque sizes for alpha, gamma and delta were reported as medium, small and large, respectively. Delbrück (1945b) questioned the value of cross-resistance tests in the identification of viruses. He considered morphology (electron microscope) and cross-inactivation tests with specific antisera to be of more significance in bacteriophage classification.

Luria (1945), also working with E. coli bacteriophage, claimed to show mutations of bacterial viruses which affected their host range. He suggested that mutations of bacterial viruses enlarging their host range need not always be limited to activity upon closely related bacterial strains,

but may conceivably render a virus active on strains belonging to different species. He did not show whether these virus mutants were distinguishable by serological means. He also reported the existence of large and small plaque type bacteriophages.

In 1946, Delbrück comprehensively reviewed the advances of the preceding three years on the seven bacteriophages of the so-called "T"-system active against the non-motile strain "B" of E. coli. A classification based on the work of several investigators was presented in tabular form. The plaque sizes were reported simply as small, medium or large. The size ranged from 100 mμ down to 45 mμ in diameter, with tails 150 x 15 mμ, 120 x 12 mμ, or tail-less. Four serological groups and four cross-resistance groups were presented but these groups did not include the same virus strains. There was complete agreement of groupings of the seven coli viruses, when based on serological cross-reactions and on morphology.

Rountree (1948) reported the serological differentiation of 31 staphylococcal bacteriophages into six serological types, claiming that neutralization tests gave clear-cut differences between these types. These serological types also could be correlated with the origin of the bacteriophages and the strains of staphylococci which they attacked.

Later, Rountree (1949) pointed out the importance of adequate serological characterization of bacteriophages to be used for typing or for investigations of the bacteriophage-organism relationships and studies concerning apparent mutations. Heat treatment was of no value in freeing the bacteriophages from the organisms, since the bacteriophages were inactivated at lower temperatures, generally about 49°C. for one hour. Plaque sizes and stability to heat served only as additional information without any definite correlation.

Working with the bacteriophages of Pseudomonas pyocyanea, Dickinson (1948) reported inactivation of the bacteriophages at 65°C. for 10 minutes at approximately neutral reaction. This heat stability was characteristic of all strains studied. She further pointed out that this formed the basis for a convenient method of obtaining active bacteriophage from lysogenic strains, since the host cells were less heat resistant and were killed at 60°C. for 30 minutes.

In their textbook, Jordon and Burrows (1945) discussed the antigenicity of bacteriophage and pointed out the apparently homogeneous response of different immunological groups. They added that as yet there has been no formal classification of the bacteriophages, but if such is to be made, it would appear that immunological grouping would provide a rational basis.

In the sixth edition of Bergey's Manual of Determinative Bacteriology (1948), a supplementary classification of the filterable viruses has been included as the order Virales. Three constituent groups are recognized and tentatively classified as separate suborders showing no taxonomic overlapping of groups. The bacteriophages (viruses infecting bacteria) are classified in the suborder I Phagineae, viruses causing disease in seed plants in suborder II Phytophagineae and viruses causing diseases in animals (insects and mammals) in the suborder III Zoophagineae.

The family Phagaceae as proposed by Homes (1939) consisted of one genus Phagus containing 46 species of bacteriophages that have been characterized to varying extents, the classification being based primarily on their lytic activity against nine separate groups (genera) of microorganisms. In addition to their host specificity, the bacteriophages were characterized on the basis of size and type of plaques, particle size, thermal inactivation and serological and immunological relationships.

A variation from the attempts to classify bacteriophage has been to use bacteriophage as an agent for the classification and typing of bacteria. Craigie (1946) stated that many endeavors have been made, with varying success, to use bacteriophage as an aid in the classification of closely related groups or species of bacteria, or for the

differentiation of strains of a given species. Craigie and Yen (1938) first demonstrated various types of B. typhosus in simultaneous epidemics by means of bacteriophage. The classification of groups of related bacteria obtained by this method was found to coincide with the antigenic classification of the bacteria. Fisk (1942) reported that through the use of bacteriophage it sometimes is possible to demonstrate minor differences between bacterial strains which are not detectable by the usual serological procedures. Working with staphylococci, he presented evidence that the susceptibility of the organisms to the bacteriophages was not altered readily by changes in environment, verifying the dependability of the method. However, widespread use of this method of classification is limited, since it requires the maintenance of a bacteriophage collection.

Several other successful attempts to classify bacteria with bacteriophage have been made. Significantly, this type of classification is merely a practical application of the cross-resistance testing of bacteriophage, using the information obtained to identify the host bacteria rather than the bacteriophage.

Characteristics of Lactic Streptococcus Bacteriophages

The literature on bacteriophages active against both S. lactis and S. cremoris is reviewed here. These two microorganisms are recognized in Bergey's Manual (1948) as two distinct bacterial species of lactic acid-producing streptococci, based on Sherman's (1937) review of the streptococci. The two species are very closely related, the differences being the ability of S. lactis to ferment maltose and dextrin, produce ammonia from peptone, grow at 40°C., at pH 9.2 and in four per cent sodium chloride, while S. cremoris shows negative reactions for these differential tests.

Recently Whitehead and Hunter (1949) have reported that S. lactis could be differentiated from S. cremoris on the basis of growth at higher temperatures, formation of shorter chains, smaller cell size, difference in bacteriophage sensitivity patterns and fermentation of maltose.

Nichols and Hoyle (1948) identified 277 strains of lactic streptococci isolated from commercial starters and 72 "wild" strains from sour milk. They found that all starter strains were S. cremoris, while those from sour milk were S. lactis (with two possible exceptions). These observations have been confirmed by Collins (1949) who showed that 22 strains of lactic streptococci propagated

in the laboratory for considerable lengths of time all were classified as S. cremoris, based on absence of ammonia formation from arginine and no growth at 40°C., while 29 strains recently isolated from milk or cream all were found to be S. lactis, having shown positive tests for these two characteristics. Other studies of essential growth factors indicated that the cremoris type was more fastidious than the lactis type.

The work of Shattock and Mattick (1943) has indicated that both S. lactis and S. cremoris fall into the same serological group (Lancefield N).

These two species are very closely related and the evidence suggests that perhaps S. cremoris may be a variant form of S. lactis. Therefore, in this work it was considered desirable that all available bacteriophages active against both the S. lactis and S. cremoris species be investigated simultaneously.

In 1943, Hunter pointed out that the three factors available to characterize the lactic streptococcal bacteriophage races in order of their usefulness were (a) range of streptococcal strains on which the bacteriophage acts, (b) reaction of the bacteriophage to changes in incubation temperatures and (c) thermal deathpoint of the bacteriophage. He mentioned that the last criterion helps only rarely because most of the streptococcal bacteriophage

races are destroyed within a very narrow range of temperature. The second mentioned characteristic (b) recently has been studied and reported by Turner (1948), while the other two are considered herein. The specific characteristics with which this present investigation deals are the bacteriophage activity, plaque sizes, serological activity and heat inactivation; the literature on each of these particular characteristics is reviewed in the following sections.

Bacteriophage activity

In the United States, the first evidence of S. lactis bacteriophage was at the Iowa Agricultural Experiment Station (1933). It was mentioned in the annual report that bacteria-free filtrates from slow butter cultures had been shown to cause inhibition of the acid production of normal cultures. Harriman (1934) continued the studies of the causes of slow acid production in butter cultures, noting the transmissibility of the inhibitory principle present in the whey filtrates. He further indicated that the principle might come from the air.

A more detailed study of the inhibitory principle derived from slow cultures was reported by Nelson et al. (1939), at which time the general characteristics of the inhibitory principle were noted to be very similar to the general characteristics attributed to bacteriophage.

In New Zealand a considerable amount of work on bacteriophage has been done by Whitehead and various co-workers. Whitehead and Wards (1933) first noted the rapid loss of vitality (failure to clot) in dairy starters under commercial conditions; this usually occurred in the second successive propagation from the mother culture. Later, Whitehead and Cox (1934, 1935b) reported the isolation from a slow butter culture of a bacteriophage active against S. cremoris; they considered this bacteriophage the cause of the slow acid production.

Whitehead and Hunter (1939) reported in studies on the bacteriophage-organism relationships the isolation and purification of nine apparently separate bacteriophage strains by using various S. cremoris and one S. lactis strain as test organisms. They pointed out the difficulties and unreliability of differentiation of bacteriophage races solely on the basis of cross-reactions.

In England, Anderson and Meanwell (1942) reviewed some of the attempts of the United Dairies Research Laboratory to obtain a solution of the bacteriophage problem. They pointed out that most bacteriophages were strain-specific but commercial mixed-starter cultures sometimes are attacked by more than one bacteriophage strain. The use of mixed strains was recommended for cheese making, since they usually

showed slowness rather than complete stoppage of acid production when infected with bacteriophage.

Also in England, Nichols and Wolf (1945b) carried out cross-reaction tests with 64 culture strains and 72 bacteriophage types. Some bacteriophages were active against only their homologous organism strains, while others were active against as many as ten strains. On this basis they were able to arrive at the relationship and in some cases the identity of some of the culture strains circulating in commerce. All tests were carried out at 30°C. in yeast-dextrose broth, although they later noted that milk was a more sensitive medium for demonstration of bacteriophage activity.

Hunter (1946) used various bacteriophages to differentiate S. cremoris and S. lactis strains. He noted a tendency toward strain specificity in the bacteriophage races that attack the S. cremoris types and a general lack of specificity of the bacteriophage action on the S. lactis types.

Hunter (1947) carried out cross-resistance tests of bacteriophage-resistant and bacteriophage-carrying strains of lactic streptococci to ascertain whether the bacteriophage strains could be grouped into types or shown to be similar in any degree. He did not establish any clearly defined groups of bacteriophages or organisms because of

the varying degrees of activity by the same bacteriophage on various susceptible strains of culture tested. On the basis of cross-resistance tests he was able to group five bacteriophage strains into three "general types", but the members of these groups did not exhibit identical activity patterns.

Babel (1946) pointed out the influence of the bacteriophage in slowing down the rate of acid production during the manufacture of cheddar and cottage cheese.

Later, Babel (1947) isolated fifteen single strains of S. lactis from a multiple-strain cheese culture and used these cultures to determine the activity patterns exhibited by several bacteriophages isolated from various cheese factories experiencing difficulties due to slow acid production. Ten distinct bacteriophage types which were active against one or more of the fifteen cultures were reported, the differentiation being based solely on these cross-reaction tests.

Plaque sizes

Early in their work on bacteriophages active against S. cremoris, Whitehead and Cox (1935a) reported that the bacteriophage gave the classical appearance of plaques when spread on solid medium layered with susceptible streptococci. Later, Whitehead and Cox (1936), using a smearing

technique, reported the plaques to be about 0.5 mm. in diameter. Whitehead and Hunter (1937) reported the plaque diameters of the streptococcal bacteriophages to be in the range 0.25 to 0.6 mm. when placed on a plate spread with the sensitive organism, but added that the small size made plaque counts difficult and probably inaccurate due to the conditions used to bring about the plaque formation.

Hunter (1946) stated the diameter of the plaques produced by different bacteriophages varied from 0.25 to 1.0 mm., but no correlation of plaque size with other general characteristics of the bacteriophages was evident.

Turner (1948) made an extensive study of various methods and technics used for the demonstration and enumeration of plaques and pointed out the need for accurately controlled conditions to obtain maximum, reproducible plaque counts. He showed that the volume and agar concentration of the overlay, the concentration of susceptible cells, presence of skimmilk, kind of diluent, length of sterilization time and pH of the medium all accounted for variations in plaque counts. The technics found to give the highest counts were used in the determination of plaque sizes. Using a pour-plate method with a 3 ml. overlay volume and a 0.5 per cent final agar concentration, average plaque diameters of five bacteriophages were found to range from 1.0 to 2.0 mm.

Serological activity

In Bergey's Manual (1948) under the genus Phagus the bacteriophage attacking the streptococci comprise six species, four of which are active against the hemolytic streptococci and represent the four serological types proposed by Evans (1934). The other two, Phagus ineptus and Phagus streptococci (and also Phagus streptococci var. virilis), are reported to be active against two strains of S. cremoris, the classification being based primarily on the studies of Whitehead and Hunter (1937). These studies apparently are the only ones reported in which lactic streptococci bacteriophage preparations have been used for the preparation of antisera. The details concerned with the preparation of the immune sera were not given. One bacteriophage (RW), active against a parent culture strain, and a secondary bacteriophage (RW1), active against the secondary culture strain, were used to prepare the antisera. These two antisera were tested against their homologous filtrates, others in the RW group, and a few unrelated bacteriophage types (the R series) by preparing serial dilutions of the sera, mixing each with equal portions of bacteriophage filtrate, incubating for 1 hour at 30°C., placing one loop on a plate spread with sensitive organisms and noting the amount of lysis in the nearby zone after 18

hours incubation at 30°C. By reading the various degrees of lysis in relation to the various serum dilutions a semi-quantitative measure of the antiphage activity was obtained. Serum dilutions of 10^{-2} and 10^{-3} for RW1 and RW antisera, respectively, were antigenically active against their homologous bacteriophages, while practically no activity was shown against an unrelated bacteriophage group. This showed that the two bacteriophages used for injection into rabbits were closely related, if not identical, and serologically unrelated to the R series of the bacteriophage. No antisera was prepared for the other type or types (R series) of bacteriophages, so no cross-neutralization tests were possible with groups unrelated to the one RW-RW1 serological group.

Nichols (1949) has communicated with Dr. C. E. Parmelee (March 5, 1949) relative to the work she and Margery Hoyle had just completed and which is scheduled to appear in the Journal of Dairy Research. This material appears to be the same general kind considered in this present investigation. They have classified approximately 450 strains of lactic streptococci into 11 types, based upon sensitivity to 72 test bacteriophages. They also have started on bacteriophage classification and prepared antiphage sera for about 14 bacteriophages and used the sera against the test bacteriophages used for the bacteriophage types above. The results of this work are not yet available.

Heat inactivation

When the Iowa Agricultural Experiment Station first reported (1933) that bacteria-free filtrates caused inhibition of acid production in butter cultures, it was also reported that the restraining agent was destroyed by boiling a filtrate containing it. Harriman (1934) reported that heating to 50°C. for 30 minutes seemed to have some destructive effect on the restraining ability of the filtrates, while 60°C. for 5 minutes seemed to destroy the filtrate action. These studies were carried out on acid filtrates.

Whitehead and Cox (1934) reported that the commercial pasteurization of cheese milk as practiced in New Zealand was insufficient to destroy a bacteriophage active against S. cremoris. Later Whitehead and Cox (1936) reported that one strain was not resistant to 70°C. for 30 minutes while another was destroyed by 50°C. for 30 minutes. Holding at 4°C. for several months had little effect on the bacteriophage activity. Whitehead and Hunter (1937) reported that all the bacteriophages they had investigated had a thermal death point of 70-75°C. at pH 6.0 with the exception of one which was destroyed at 55-60°C.

Mazé (1937) showed that certain of the bacteriophages of lactic acid bacteria resisted heating at 80°C. for 5

minutes if the host culture had also been added to the milk medium and the tubes incubated for 2 to 4 hours at 21°C. before heating. Recently Mazé (1946) reported that heating for 5 minutes in a milk medium inactivated three groups of bacteriophage at 80-82°, 70° and 65°C., respectively.

The most heat resistant bacteriophage has been reported by Yakovlev (1939). A bacteriophage active against a strain of S. cremoris used in the preparation of margarine was reported to resist 80°C. for 10 minutes in small volumes and in large volumes of whey it survived heating at 90°C. for 5 minutes.

Nelson et al. (1939) reviewed the general characteristics of the S. lactis bacteriophages and noted that the heat resistance was greatest at approximately neutral reaction, acid conditions being conducive to more rapid inactivation. They reported differences in resistance to heat of various strains, 15 minutes at 70°C. being required to inactivate the most resistant strain tested, while the most heat labile were inactivated in less than 5 minutes at 65°C.

Johns (1943) described a bacteriophage outbreak in a Canadian cheese factory where a multiple strain culture was in use. He pointed out that the incoming milk was a source

of bacteriophage and that pasteurization did not always eliminate the contaminating bacteriophage.

Whitehead (1944) showed that heating cream containing 50 per cent fat to 170°F. for 30 minutes by standing in hot water failed to destroy bacteriophage, but heating to 200°F. with live steam and holding for 30 minutes was adequate for bacteriophage destruction.

Hunter (1944) noted that regenerative flash pasteurization of milk (150-160°F. for approximately 30 seconds), as practiced in New Zealand cheese factories, did not destroy completely the lytic agent which was protected to some extent by the milk proteins. He noted that because of the heat resistance of bacteriophage contamination of the farmers' milk was a potentially important source of bacteriophage entering the cheese factories.

Bacteriophage contamination from farm equipment was further considered by Whitehead and Hunter (1947), who found that pasteurization of the whey at 180-190°F. destroyed the bacteriophage; this treatment was considered impractical due to cost of steam and the fact that the whey proteins "burned on" the plates were difficult to remove.

Nichols and Wolf (1945a) reported that 26 active bacteriophages attacking strains of lactic streptococci isolated from cheese starters usually did not survive a temperature of 75°C. for 7.5 minutes. Many of the

bacteriophages were not destroyed by heating for 50-60 minutes at 65-67°C. and most of them survived heating at 70°C. for 10-15 minutes. All determinations were made in milk at pH 6.42 to 6.28 with the original bacteriophage concentration of 10^6 particles per milliliter. In attempts to obtain this exact concentration they noted that plaque titers generally were lower (by about 10^2) than titers shown by clearing of broth.

Prouty (1948) also noted that commercial pasteurization did not destroy bacteriophage but that a temperature of 70°C. and an exposure period of 15 to 30 minutes was required for inactivation.

Morphology

The study of bacteriophage morphology has been made possible by the introduction of the electron microscope. The morphology of the bacteriophages active against S. lactis has been reported by Parmelee et al. (1949). Their electron micrographs clearly showed the typical sperm-shaped bacteriophage particles averaging 220 mμ long with a head diameter of 70 mμ and a tail that was 30 mμ wide and 150 mμ long. Nine strains of bacteriophage (two from New Zealand, four from England, one from Canada and two from The Iowa Agricultural Experiment Station) were studied with the electron microscope and the various strains were so nearly

alike in shape and size that they apparently could not be differentiated on the basis of morphology.

Other characteristics

In addition to the characteristics with which this investigation was concerned, a few others recently have been studied and reported which in general have aided in obtaining a better understanding of the nature of the S. lactis and S. cremoris bacteriophages.

Turner (1948) reported that for various bacteriophages the optimum temperature for maximum proliferation was 32°C., and preliminary studies indicated a burst time of about 65 minutes and a burst size averaging about 90 particles; these characteristics were found to be relatively similar for the various strains studied and apparently would be of no value for purposes of differentiation. The one characteristic studied that was markedly different for individual bacteriophage-organism combinations was that of secondary growth. Four of the strains permitted secondary growth, whereas one strain characteristically completely lysed the susceptible culture, permitting no secondary growth to appear. Whether this difference was due to a variation of the host microorganism or the bacteriophage was not determined.

Nichols and Hoyle (1948) obtained bacteriophages active against numerous culture strains for which no bacteriophage previously had been isolated by introducing the culture into a cheese factory where bacteriophage attack normally was heavy, and using the culture as a starter until active bacteriophage appeared in the whey. They also were able to develop bacteriophages active against insusceptible cultures by "feeding" whey samples from a cheese factory or bulk-milk samples, after acidification and filtration, to the respective strains of organisms. Bacteriophages also were obtained for resistant cultures by addition of concentrated undiluted bacteriophage suspensions capable of lysing some other strain of lactic streptococci to the resistant strain growing on agar. When a "new" bacteriophage occurred, it was propagated further on the formerly bacteriophage-resistant culture. Using these methods, Nichols and Hoyle have been able to isolate bacteriophages active against a majority of their resistant strains.

MATERIALS

Bacteriophages and Cultures

All bacteriophages and cultures used in this work are maintained in the Iowa State College collection. A listing of them in numerical order is presented in Table 1. As each new bacteriophage filtrate was added to the collection, it was given a number preceded with the letter "F" (filtrate) by which it is always identified. Those filtrates listed that are labeled "PF" (purified filtrate) were at some time repropagated from a single plaque isolation (Babel, 1949) and came from a variety of sources; the exact dates and sources are not known.

The cultures on which the bacteriophages are repropagated are listed in the second column beside the homologous bacteriophage filtrate. The dates the bacteriophages were obtained and the source are given as recorded in the current cataloging in as complete form as was available.

In attempting a systematic classification of the S. lactis and S. cremoris bacteriophages, it seemed desirable to work with types from as divergent sources as possible. Anticipating the work on this problem, bacteriophages and homologous cultures were obtained from investigators in

Table 1

Origins of Bacteriophage Strains Studied

Bacteriophage*	Culture**	Date Obtained	Source
F1	146-1	12-11-44	Slow vat of cheddar cheese, I.S.C.
F2	H1-1	1-3-45	Slow vat of cheddar cheese, I.S.C.
F3	H1-1	2-26-45	Slow vat of cheddar cheese, I.S.C.
F4	H1-1	3-2-45	Slow vat of cheddar cheese, I.S.C.
F5	H1-3	?	?
F6	H1-3	?	?
F7	H1-2	3-18-45	Slow vat of cheddar cheese, I.S.C.
F8	H1-7	?	?
F9	H1-11	?	?
F10	122-1	4-15-49	Slow culture in Market Milk Lab., I.S.C.
F11	146-1	3-28-45	Milk exposed in I.S.C. Cheese Lab.
F12	H1-1	5-29-49	Anderson-Erickson Dairy, Des Moines, Iowa, from slow cottage cheese vat.
F20	W8	7-6-45	Slow cottage cheese vat, I.S.C.

*Designation as of November 1, 1949.
**Culture used for bacteriophage propagation.

Table 1 (Continued)

Bacteriophage	Culture	Date Obtained	Source
F21	W4	7-6-45	Slow cottage cheese vat, I.S.C.
F22	H1-4	7-9-45	Slow cottage cheese vat, I.S.C.
F24	W2	7-25-45	Slow cottage cheese vat, I.S.C.
F25	M1	8-15-45	Slow culture in I.S.C. Cheese Lab.
F26	H1-2	9-20-45	Slow vat of cheddar cheese, I.S.C.
F27	H1-2	?	?
F32	H1-5	Prior to 11-18-47	Slow vat of cottage cheese, I.S.C.
F34	H1-4	?	?
F35	H1-4	Prior to 11-12-47	Purdue Creamery (Lafayette, Ind.)?
F42	H1-4	?	?
F43	122-1	9-13-47	Slow culture in Market Milk Lab., I.S.C.
F44	146-1	10-27-47	Slow culture in I.S.C. culture collection.
F46	122-1	11-6-47	I.S.C. culture collection.
F47	122-1	11-7-47	Slow milk culture from E. W. Bird, I.S.C.

Table 1 (Continued)

Bacteriophage	Culture	Date Obtained	Source
F48	122-1	11-7-47	Slow ripening cream from E. W. Bird, I.S.C.
F49	H1-8	11-29-47	Whey from cheese one day after slow culture in cheddar cheese vat, I.S.C.
F50	20	3-22-48	I.S.C. slow cheese vat.
F51	DL	4-6-48	Whey from Cheese Lab., I.S.C.
F52	459	4-10-48	E. B. Anderson, United Dairies Ltd., Central Laboratory, Wood Lane, London, W. 12, England
F53	497	4-10-48	
F54	565	4-16-48	
F55	573	4-16-48	
F56	712	4-16-48	
F57	799	4-16-48	
F58 (1)*	HP**	4-20-48	H. R. Whitehead, Dairy Research Inst., Palmerston North, New Zealand
F59 (2)	HP	4-20-48	
F60 (10)	HP	4-20-48	
F61 (34)	HP	4-20-48	

*Numbers in parentheses are the designations on the New Zealand filtrates when obtained.

**HP from New Zealand, not I.S.C. culture HP.

Table 1 (Continued)

Bacteriophage	Culture	Date Obtained	Source
F62 (4)	K2*	4-26-48	New Zealand
F63 (38)	E8	4-26-48	New Zealand
F64 (8)	FH8	4-26-48	New Zealand
F65 (11)	FH8	4-26-48	New Zealand
F66 (21)	FH8	4-26-48	New Zealand
F67	144F	4-27-48	Agnes A. Nichols, National Institute for Research in Dairying, University of Reading, Reading, England
F68	1P5	4-27-48	
F69 (46)	ML1	4-27-48	New Zealand
F70	5	4-30-48	Robert Dumais, St. Hyacinthe, Quebec, Canada
F71 (50)	KH	4-30-48	New Zealand
F72 (7)	R1-1	4-30-48	New Zealand
F73	799/11	5-12-48	Reading, England
F74	318B/28	5-12-48	Reading, England
F75	6	5-29-48	Canada
F76	122-1	8-2-48	I.S.C. Market Milk Lab.

*Culture K from New Zealand redesignated K2 upon arrival to avoid confusion with I.S.C. culture K.

Table 1 (Continued)

Bacteriophage	Culture	Date Obtained	Source
PF2*	H1-2		
PF4	H1-4		
PF6	H1-1		
PF7	H1-1		
PF8	H1-5		
PF10	H1-10		
PF11	H1-10		
PF12	H1-9		
PF13	H1-2		
PF14	H1-5		

*Date obtained, source and time of purification of purified filtrates (PF-series) were not available.

New Zealand, Canada and England. These have been assigned numbers and also are listed along with the other filtrates in Table 1. Those cultures received from New Zealand were labeled as S. cremoris, while those from other sources were not identified further other than as lactic streptococci.

Purity of Cultures and Filtrates

The host microorganisms all are presumed to be single strain S. lactis or S. cremoris cultures. Most of the cultures are known to have been single colony isolates, while those from other countries were not further purified in this laboratory but are maintained in the collection as received in respect to purity.

The bacteriophage filtrates labeled "PF" are known to have been isolated from single plaques. Whether they are isolates from other filtrates in the "F" series and are present as duplicates or as different bacteriophages is not known. Some of the "F" series are known to not have been passed through a single plaque isolation, although most of them have been propagated on single strains of host organisms for several serial passages. No information is available as to the extent of purification which had been employed with the bacteriophage preparations from foreign cultures. It has been suggested by Conn et al. (1945)

that repeated repropagation of bacteriophages on a single strain culture probably tends toward purification. Babel (1947) has shown that as many as four distinct types of bacteriophage differentiated by cross-reaction tests may be present in a sample of whey taken from a vat in which a multiple-strain culture had undergone bacteriophage attack. It was not demonstrated whether these types all would remain in the filtrate upon repropagation on a single-strain culture.

Double plaque repurification of all bacteriophages on twice repurified cultures from single colonies was made of all material used in the plaque studies, thermolability studies and preparation of antisera for serological studies. In the cross-reaction studies of the bacteriophage and culture collection and the testing of the bacteriophage collection with prepared antiphage sera, the bacteriophage filtrates and cultures were used as they are maintained in the collection.

GENERAL METHODS

Propagation of Cultures

The cultures used in this work routinely were grown and maintained in litmus milk which was prepared by addition of litmus to skimmilk which then was dispensed in 16 x 125 mm. test tubes in approximately eight ml. quantities, cotton stoppered and sterilized by autoclaving at 15 lb. for 20 minutes. The stock cultures generally were transferred twice each week and were transferred daily when being used experimentally. After transfer of one loopful, or one drop in the case of slow cultures, the cultures were incubated at 32°C. until the typically acid, reduced and coagulated appearance was noted, usually within 16 hours. Very active cultures will present this typical reaction in 8-10 hours and slow cultures sometimes take up to 36 hours to reach the desired end-point of incubation. After incubation the cultures were stored in the refrigerator at 3-5°C.

Freshly prepared litmus milk or plain skimmilk cultures were used for inoculating purposes. When preparing bacteriophage filtrates, the undiluted cultures were used as inoculum. When used as inocula in the determination of bacteriophage

titers, a 1:10 skimmilk dilution of the litmus milk culture gave the most satisfactory results. The mixture was shaken vigorously to obtain homogeneous inoculum which was used at the rate of one drop per tube of litmus milk.

Propagation of Bacteriophages

The propagation of the bacteriophages was done by bringing about their proliferation on a susceptible host. One ml. of culture was added to 100 ml. of sterile skimmilk to which also was added a sufficient quantity of bacteriophage to bring about mass lysis of the cells. Usually one drop of a high-titer filtrate or one ml. of a low-titer filtrate was used. The culture-bacteriophage mixture was incubated for 6-8 hours at 32°C. The skimmilk then was coagulated by the addition of 3.5 ml. of a 10 per cent lactic acid solution, filtered through coarse filter paper, and finally through a Selas microporous porcelain filter (porosity #03) to obtain a bacteria-free whey filtrate containing the bacteriophage. Aseptic procedures were used throughout to avoid possible airborne bacteriophage contamination of the filtrates. Filtrates containing up to about 10^{10} bacteriophage particles per ml. generally were obtained by this method of bacteriophage propagation.

The pH of these filtrates was in the range of 4.0 to 4.5, which the bacteriophage can tolerate for fairly long periods of time when stored at 3-5°C. However, over a period of several months there was a continuous decrease in the titers of most filtrates, apparently due to the acid reaction. To lessen this possibility the filtrates were diluted 1:10 in skimmilk (final pH about 6.3) and stored in this diluted condition at 3-5°C.

Table 2 presents results of a few preliminary trials showing the changes in activity of four filtrates diluted in skimmilk to a pH of about 6.3 and stored at 3-5°C. for a period of 3.5 months. There was a gradual decrease in the titers, and when the bacteriophage population dropped to as low as 10^2 or 10^3 per ml., the preparations were repropagated. Further experience gained from working with several other filtrates indicates that those shown in Table 2 are somewhat better than the average in maintaining their activity, as the activity decreases to the repropagation level in a matter of only a very few months with some filtrates. This method of storage appeared to meet satisfactorily the requirements necessary to continue with the other portions of this investigation, so no further studies were conducted in regard to storage.

Table 2

Changes in Titer of Bacteriophage Filtrates Stored at 3 to 5°C.*

Filtrate Number	Titers		
	Aug. 28	Sept. 21	Nov. 8
F69	80×10^7	50×10^6	1×10^7
F70	60×10^7	10×10^6	1×10^6
PF2	25×10^6	10×10^6	5×10^6
F62	30×10^7	10×10^6	4.9×10^6

*Filtrates diluted 1:10 in skimmilk.

Measurement of Bacteriophage Activity

One means of bacteriophage enumeration is the limiting tube dilution method adapted by d'Herelle (1926) for bacteriophage and successfully used by Krueger (1930), Harriman (1934), Nelson et al. (1939) and others for accurate quantitative measurement of bacteriophage activity. Krueger (1930) claimed an accuracy of ±5 per cent in the method, but pointed out the need for controlled numbers of susceptible cells. Essentially, the method used herein consisted of making successive 100-fold dilutions of the bacteriophage filtrate in sterile distilled water and dispensing 1 and 0.1 ml. quantities of the diluted material to triplicate litmus milk tubes containing eight ml. of sterile litmus milk, giving an over-all effect of 10-fold serial dilutions. To each tube was added one drop of a 1:10 skimmilk dilution of a fresh susceptible culture. Incubation was at 32°C. for 14-16 hours. Tubes not showing the typical normal coagulation were considered to contain at least one bacteriophage particle or lytic unit. Using the McCrady probability tables quoted by Buchanan and Fulmer (1928), the "most probable number" of active bacteriophage particles present was determined. The "titer" is considered a measure of the concentration of bacteriophage particles, although the absolute number of particles per lytic unit remains to be

established. Until the exact quantitative relationship is determined, the assumption is that one lytic unit represents one active bacteriophage particle.

It is customary to report titers, for example, as 2.0 x 10^7 particles per ml. In some sections herein this practice is followed, while in others they are reported as logarithms, or simply 7.3 for this particle example.

The plaque plate count and the limiting tube method are used almost universally for counting bacteriophage activity. Cherry and Watson (1949) have pointed out that the variation in the plaque count, based on duplicate platings from the same or different dilution series of a stock virus suspension, does not exceed 13 per cent of the mean value and the accuracy of the "activity method" under controlled conditions approaches the above figure. Turner (1948) compared the plaque plate method with the limiting method by presenting the ratios of the former to the latter on a total of 15 comparable determinations, all falling between 1.00 and 3.91, which showed that two methods give much the same results for bacteriophage enumeration.

When working with a large number of apparently different types of bacteriophages no standard plaque plating procedure could be used for all strains since some strains have been shown to produce very small, almost invisible plaques. These can be enlarged and made more visible by decreasing

the concentration of agar in the upper layer in the plaque plating procedure. It seemed desirable to use a method of enumeration which could be used for all determinations without modification to meet individual strain characteristics. The limiting tube dilution method met this requirement, while the plaque plating method of counting did not. For the major portion of this investigation the limiting tube dilution method was selected for determining the active bacteriophage concentration, while the plaque plating technic was used only for obtaining plaques for measurement in the plaque size studies.

Plaque Plating Procedure

Throughout this investigation the two-layer plaque plating method developed by Turner (1948) in this laboratory was used as standard procedure for obtaining plaques for measurement. The medium used contained one per cent peptonized milk, one per cent proteose-peptone, one per cent agar, 57 per cent water and 40 per cent tomato juice (from commercially canned tomatoes). The first four ingredients were mixed and heated until the agar was melted. The tomato juice, which was neutralized to approximately pH 6.5 by addition of 70 ml. of normal NaOH to the juice from one No. 10 can of tomatoes, then was added to the slightly

cooled ingredients. The pH then was adjusted (usually to about 6.4) so that a final pH of 6.0 (±0.1) was obtained after autoclaving 25 minutes at 15 lb. pressure.

In the two-layer pour-plating method about 10-12 ml. of one per cent agar medium was allowed to solidify as the bottom layer in petri plates. The upper layer consisted of (a) one ml. of a 1:25 aqueous dilution of susceptible cells just at the coagulation point (approx. 15 hr. culture), (b) one ml. of skimmilk containing the bacteriophage and (c) one ml. of 1.5 per cent agar medium (temp. 55°C.). These were placed on the lower layer in the order shown, quickly mixed by rotation of the plate and allowed to solidify. Incubation was at 32°C. and plaques became visible after about 10-12 hours when examined with indirect light.

Plaque Size Determination Procedure

In the studies on the plaque sizes the method of Turner (1948) was used for the formation of the plaques. Appropriate serial dilutions of the bacteriophage filtrates were made and the plates exhibiting from 10 to 100 plaques were used for measurement. Measurements of the plaque diameters were made by means of an eyepiece micrometer in conjunction with a low power dissecting microscope. The

measuring system employed was standardized using a standard stage micrometer.

Measurement of pH

The pH determinations were made with a Leeds and Northrup glass electrode system or by a quinhydrone-calomel potentiometric method.

Cross-reaction Determination Procedure

The testing of several bacteriophages for lytic activity against several organisms has at various times been referred to as cross-resistance, cross-testing, cross-sensitivity, cross-lysis and cross-reaction. The latter term will be used herein and should not be confused with the term cross-neutralization used in a later section with reference to the bacteriophage-antiphage reaction.

The specific method used for cross-reaction testing was to place in a tube of litmus milk one drop of a 1:10 skimmilk dilution of the culture to be tested, followed by the addition of one drop of an active bacteriophage preparation. When several bacteriophages were to be tested against more than one culture, the appropriate number of tubes was inoculated so that the reaction of every culture-bacteriophage combination could be tested individually. The tubes were

incubated at 32°C. for 16 hours and those cultures showing significantly less growth than the bacteriophage-free control cultures were considered susceptible to the respective bacteriophages. The titers of all bacteriophage preparations used for testing were maintained above 10^6 particles per ml. for cross-reaction determinations.

Type of medium

Although some workers have used various types of broth media for cross-reaction studies, the most widely used medium has been skimmilk with or without added litmus. The reason for this choice is that the dairy industry in general is concerned primarily with the bacteriophage activity against starters which are made from milk or skimmilk. It is well known that various milk products are excellent media for the lactic streptococci and in most instances are adequate for the proliferation of bacteriophages. However, when some of the bacteriophages were cross-reacted with several of the cultures some inconsistencies were noted.

In some cases the reactions were opposite those expected, based on previous experience with the particular bacteriophage-culture combination in question. Also, in some cases five sub-strains isolated from a single strain culture which originally had been picked from a single colony showed variations in their susceptibility to the same bacteriophage

strain. These conflicting results suggested that perhaps a slight nutritional deficiency in the litmus skimmilk might be responsible for erratic results. An experiment was designed to test this supposition.

Enrichment of medium

To regular skimmilk used for preparing litmus milk 10 per cent strained vegetable juice* was added to fortify the medium nutritionally. The solids present were increased slightly by addition of two per cent of low-temperature spray-dried skimmilk solids** so that in cases of acid coagulation a more firm curd would result which would be more easily differentiated from a weak-bodied curd resulting from a "slowed" culture.

The reaction of the skimmilk was pH 6.6 and that of the strained vegetable juice was pH 4.3 before mixing. After the addition of two per cent skimmilk powder and litmus and autoclaving at 15 pounds pressure for 25 minutes, the final reaction of the enriched medium was pH 6.1.

Various bacteriophage and organism combinations were selected which were suspected of giving misleading results

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*Made by Campbell Soup Co., General Offices, Camden, N. J., U. S. A. and sold under the trade name "V-8 Cocktail Vegetable Juices".

**Manufactured by Sanna Dairies, Inc., Madison, Wisconsin.

and replicate cross-reaction tests were made to establish definitely whether the bacteriophage would show variations in activity which correlated with the test medium used. As expected, both the plain and fortified skim usually showed the same results but, in those cases where a discrepancy arose, the fortified litmus generally gave the fewer discrepancies, as can be noted in the summations shown in Table 3.

In the upper portion of the table the six combinations selected as exhibiting positive bacteriophage activity most of the time showed that in six out of 36 tests in regular skim the reaction was erroneous, while with fortified skim only in one of 24 trials was a discrepancy noted. This indicated that the fortified skim was somewhat superior to the regular skim in demonstrating activity of the bacteriophages. In the lower portion of the table several combinations are shown which generally did not exhibit any reaction. In regular skim 23 tubes were read as positive and 64 as negative, while in the fortified medium only six out of 59 tubes were found to exhibit reactions which were at variance with the majority. Just why there should be any activity exhibited at all cannot be explained satisfactorily.

In Table 4 the influence of vegetable juice on the growth rate of seven cultures is shown. When grown in fortified litmus milk medium these seven cultures showed

Table 3

Comparison of Regular and Fortified Litmus Milk in Selected Cross-reactions

(Figures represent number of trials)

Combination		Bacteriophage activity			
		Regular		Fortified*	
Organism	Bacteriophage	Positive	Negative	Positive	Negative
5	F75	11	1	8	0
146-2	F68	4	2	4	0
H1-1	PF2	2	1	2	0
W8	F56	6	0	3	1
W2	F56	2	1	2	0
1P5	F68	5	1	4	0
Totals		30	6	23	1
5	F24	1	4	0	4
6	F24	1	4	0	3
W4	F75	3	3	0	4
H1-4	F62	3	6	0	6
H1-4	F70	1	2	0	2
H1-4	F75	1	2	0	2
H1-4	PF2	2	4	2	2
H1-10	F68	2	4	2	2
146-2	F70	2	10	0	8
146-2	F24	1	3	0	3
W8	F68	2	4	1	3
1P5	F62	1	5	0	4
W2	F68	2	10	1	6
W8	F75	1	3	0	4
Totals		23	64	6	53

*Skimmilk fortified by addition of 10 per cent canned vegetable juice and 2 per cent skimmilk powder.

Table 4

Influence of Vegetable Juice on the Growth Rate of Selected Cultures

(Figures represent hours of time to coagulate)*

Culture no.	1st trial 4 tubes of each		2nd trial 10 tubes of each		3rd trial 6 tubes of each	
	Skim + vegetable juice	Skim	Skim + vegetable juice	Skim	Skim + vegetable juice	Skim
W4	12	16	11	16	12	15
W8	12	14	12	13	12	14
146-2	11	15	12	14	12	16
H1-1	11	14	12	15	12	14
H1-2	11	15	11	15	11	15
H1-3	12	15	12	16	12	14
H1-4	11	14	11	15	12	15

*Incubation at 32°C.

complete coagulation a number of hours sooner than when grown in ordinary skimmilk.

When the first cross-reaction studies were made regular litmus milk was used, but after the superiority of the fortified medium was established, the addition of vegetable juice to all litmus milk was adopted as standard procedure.

EXPERIMENTAL

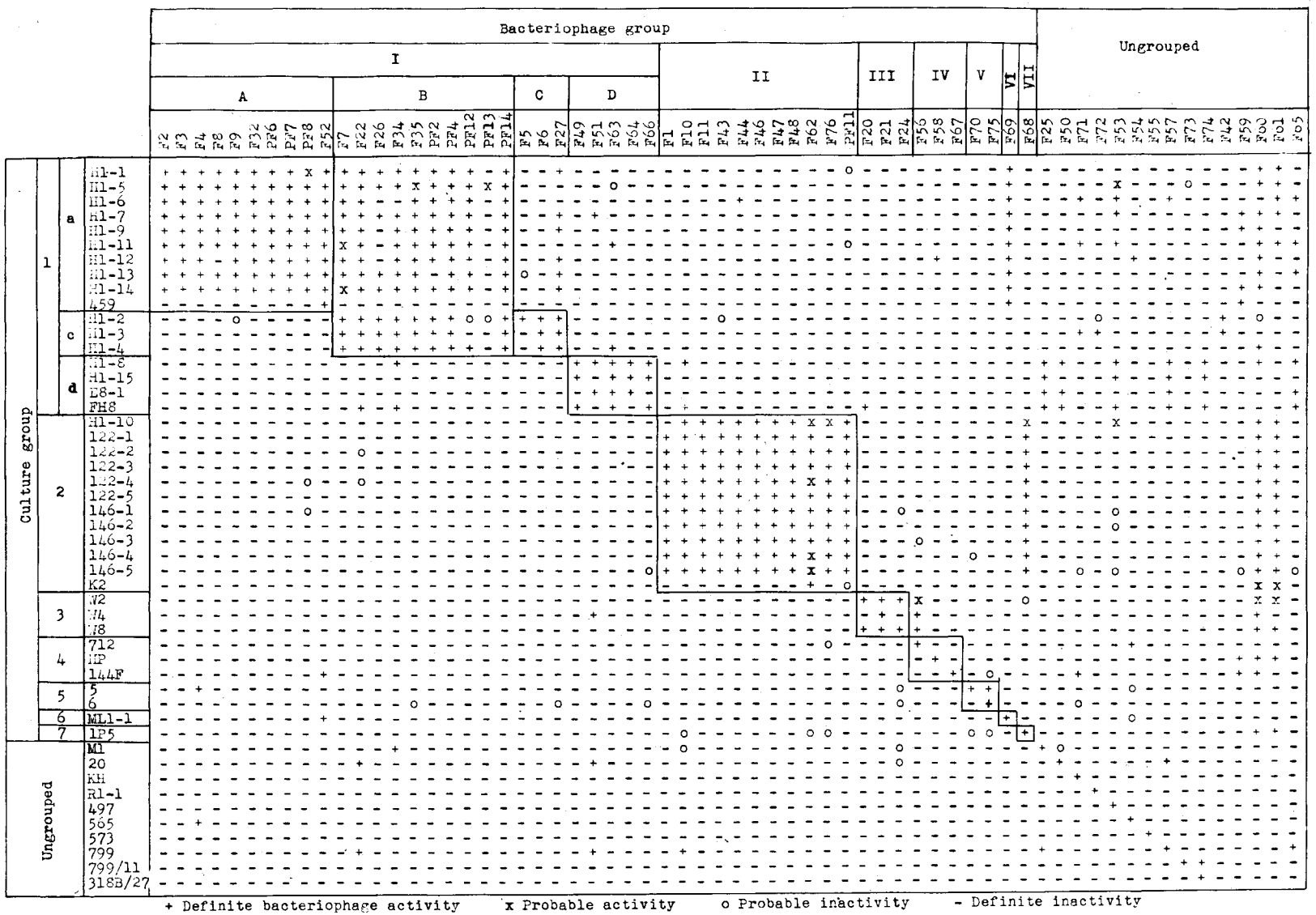
Cross-reaction Determinations

In the first cross-reaction determinations 49 strains of culture and 66 bacteriophage filtrates were used. Duplicates obtained by repurification also were included, bringing the actual totals used to 65 cultures and 77 filtrates. Since it was impossible to complete this large number of cross-reaction tests in one day, it was decided that for comparative purposes all cultures should be used at a given time and different bacteriophages used on successive days until all were tested. Thus, when a single titer determination was made of the bacteriophage preparation, it was known that this same activity was present when each of the different cultures was tested for susceptibility. In the first trial regular litmus milk was used as the test medium. The data obtained from this cross-reacting were recorded on cross-lined charts which are reproduced in part in Table 5, except the order of listing has been rearranged and duplicates omitted. Data for filtrates F12 and PF10 were not recorded, since active filtrates were not available at the time of testing. In some instances the duplicate reactions did not agree in all trials; these

Table 5

Cross-reactions of 64 Bacteriophage Filtrates and 49 Cultures of Lactic Streptococci

(In regular litmus milk)



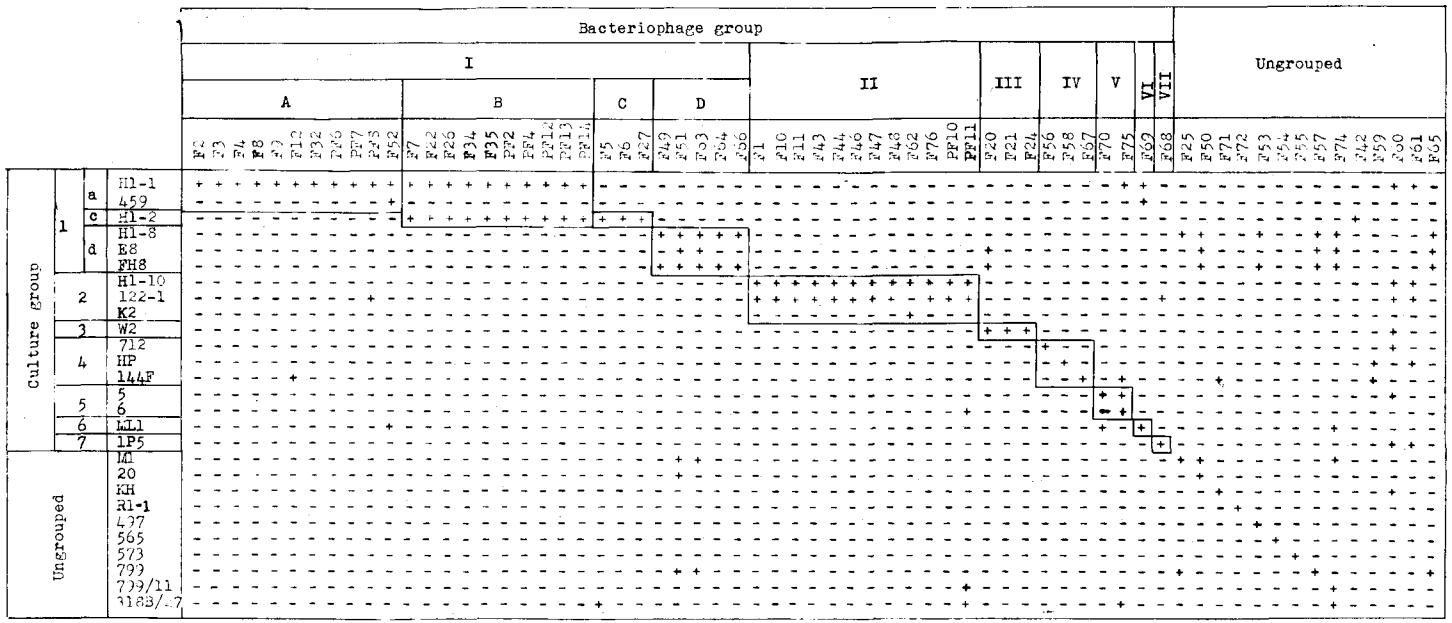
reactions are recorded as probable activity (x) or probable inactivity (o), based upon averages of all trials obtained for a given combination. This work was completed prior to the studies concerning the use of vegetable juice fortification of the litmus milk, so the use of regular litmus milk may account for some of the irregularities.

Following the work on the use of vegetable juice to fortify the skimmilk, another trial of 65 filtrates were cross-reacted with 27 cultures selected as representatives of the various culture groups. At this time the technics considered to be the most reliable, based upon previous experience, were used. These included use of vegetable juice-fortified litmus milk, dilution of inoculum, vigorous shaking for proper mixing prior to incubation and use of slightly larger (19 x 115 mm.) test tubes instead of the smaller (16 x 125 mm.) previously used. The purpose of this trial was to determine whether some of the irregularities noted in earlier trials were due to technics and whether the cross-reaction method of culture and bacteriophage classification was reliable. The results are recorded in Table 6. By comparing the data of Tables 5 and 6 it can be seen that slightly improved results were obtained by use of the improved technics.

Table 6

Cross-reactions of 65 Bacteriophage Filtrates and 27 Representative Cultures of Lactic Streptococci

(In vegetable juice fortified litmus milk)



\+ Definite bacteriophage activity - Definite inactivity

Discussion of cross-reaction studies

The areas set off by lines in Tables 5 and 6 circumscribe the reactions which, if good agreement were to be found, should show positive reactions, while outside these squares all reactions would have been negative. This ideal was not realized, as can be noted by the several inconsistencies.

By comparing the activity patterns exhibited by the various bacteriophages, it was possible to determine closely related bacteriophage groups based on their activities against the various cultures used as test organisms. Conversely, by comparing the sensitivity patterns shown by the various cultures, it was possible to determine closely related groups based on their susceptibility to the numerous bacteriophage strains used in the cross-reaction studies. It should be noted that the groupings and order of listing in the tables were made after considerably more work had been done, especially serological classification, so the arrangement in Tables 5 and 6 appears more orderly than was actually the case when first tested, at which time the relationships of the various cultures and the various bacteriophages were unknown.

The various bacteriophage groups have been assigned Roman numerals and their sub-groups capital letters. The

culture groups were assigned Arabic numbers with small letters to denote the subgroups. The numbering system employed was related as follows:

		Group number					
Bacteriophages:	I (A B C D)	II	III	IV	V	VI	VII
Cultures:	1 (a ac c d)	2	3	4	5	6	7

The cultures of group 1 appeared to fall into four subgroups in respect to sensitivity toward the action of the bacteriophages of group I. The subdivisions of the first group are based on cross-reactions only, the other groupings being based on both cross-reactions and the serological reactions which are reported in a later section. Bacteriophage groups IA, IC and ID were active against culture groups 1a, 1c and 1d, respectively, while bacteriophage group IB was active against both culture groups 1a and 1c.

Several inconsistencies in reactions may be noted. Culture 459 (group 1a) appears misplaced on the basis of sensitivity, but was so located because its homologous bacteriophage F52 belonged to serological group I. Since the primary basis for establishing the groups was the serological reactions, F52 was placed in group IA. F52 was active against the cultures of group 1a, including

its homologous strain 459, although the latter was not sensitive to the other filtrates of group IA. The same applies to culture K2 and filtrate F62. Although filtrates F56, F58 and F67 (group IV) showed serologically similar reactions, each was strain specific in activity against its homologous group 4 culture. The two bacteriophage strains F70 and F75 were of the same origin, yet culture 6 was susceptible only to its homologous filtrate F75 while culture 5 was susceptible to both F70 and F75.

Those cultures and bacteriophages for which no definite patterns were apparent are considered "ungrouped" pending further clarification of their taxonomic positions. Filtrates F60 and F61 have reactions which are too broad to conform to any one established group. This was in agreement with Hunter (1946) who placed F60 and F61 (his numbers 10 and 34, respectively) in the polyvalent intermediate group of bacteriophages because of their wide lytic capacities and ability to attack several cultures of both the S. lactis and S. cremoris species. It may be that these filtrates were mixtures, both having been used as received from New Zealand in respect to purity. Additional study of these ungrouped strains would be desirable.

It was difficult to establish clearly defined groups of bacteriophage or groups of organisms on the basis of cross-reactions alone. When used as a guide, and recognizing

that some inconsistencies exist, several workers, including Babel (1947), Nichols and Ineson (1947), Hunter (1946, 1947) and Whitehead and Hunter (1949), have found this method useful in bacteriophage identification.

The various bacteriophage filtrates belonging to the same groups are summarized in tabular form in Table 7. Using the cross-reaction tests to classify the cultures as well as the bacteriophages, the related culture groups similarly are summarized in Table 8.

The history of the various cultures further substantiates some of the suggested groupings. The H1-series of cultures are believed all to have been isolated from a commercial mixed culture. There appeared to be at least three different strain types in this series (1a, 1c and 1d), while culture H1-10 definitely appears to belong to culture group 2 of the 122-146 series. Since the 122 and 146 butter cultures have been used widely by the dairy industry ever since 1922, these strains may have found their way into commercial channels and have been used in the preparation of the H1-series commercial culture.

There is a certain amount of culture exchange between various investigators, which may account for the similarities of some of the foreign cultures supposedly from widely separated sources.

Table 7

Grouping of Related Bacteriophage Filtrates
Based on Activity Against Various Lactic Streptococcus Cultures

Filtrates in bacteriophage group number										
IA	IB	IC	ID	II	III	IV	V	VI	VII	Ungrouped
F2	F7	F5	F49	F1	F20	F56	F70	F69	F68	F25
F3	F22	F6	F51	F10	F21	F58	F75			F42
F4	F26	F27	F63	F11	F24	F67				F50
F8	F34		F64	F43						F53
F9	F35		F66	F44						F54
F12	PF2			F46						F55
F32	PF4			F47						F57
F52	PF12			F48						F59
PF6	PF13			F62						F60
PF7	PF14			F76						F61
PF8				PF10						F65
				PF11						F71
										F72
										F73
										F74
Sub totals:										
11	10	3	5	12	3	3	2	1	1	15
Total: 66 filtrates										

Table 8

Grouping of Related Strains of Lactic Streptococci Based on Sensitivity to Bacteriophages

Organisms in culture group number									
1a	1c	1d	2	3	4	5	6	7	Ungrouped
H1-1	H1-2	H1-8	H1-10	W2	712	5	ML1	1P5	M1
H1-5	H1-3	H1-15	122-1	W4	HP	6			20
H1-6	H1-4	E8-1	122-2	W8	144F				KH
H1-7		FH8	122-3						R1-1
H1-9			122-4						497
H1-11			122-5						565
H1-12			146-1						573
H1-13			146-2						799
H1-14			146-3						799/11
459			146-4						318B/27
			146-5						
			K2						
Sub totals:									
10	3	4	12	3	3	2	1	1	10
Total: 49 cultures									

In grouping the bacteriophage filtrates, knowledge of their history aided materially in establishing that some probably are substrains of the same bacteriophage group. Most of the filtrates in group I have been isolated at Iowa State College since 1945. Only F63, F64 and F66 from New Zealand also belonged to this group, but they also were active against cultures which were apparently related to the H1-series of culture in group 1d.

All members of group II, except F62 from New Zealand, have been isolated from 122 and 146 cultures at Iowa State College during the past four years. They have been re-propagated many times since isolation and still show similar patterns in respect to their cross reactions.

Although F20, F21 and F24 have all been placed in group III, their cross-reactions do not agree entirely, the serological neutralization reactions were weak and their range of thermal inactivation was relatively wide. This grouping should be considered tentative only.

Filtrate group IV consists of F56, F58 and F67 from London, New Zealand, and Reading, respectively. An exchange of filtrates or cultures in the past would explain their similar classification. Both F70 and F75 (group V) were received from Canada; they were similar serologically and probably were of the same origin. Although groups VI and VII each contain only one filtrate, they are considered "groups"

since their activity patterns each were different from the patterns of any other group. Their antiphage sera also have been prepared and shown not to react with other groups.

In view of the fact that several irregularities and inconsistencies have been shown when the bacteriophages and cultures are cross-tested, it appears that when used alone this method is of limited value in classification. When used in conjunction with other differential tests, especially serological grouping and heat resistance, these combined methods form a workable basis for both bacteriophage classification and culture differentiation.

Although the bacteriophages and cultures are placed in definite groups, it should be recognized that they are only suggested groupings based on the information now available. Their positions are considered only as a workable basis for classification. In fact, cross-reaction experiments as now known are of limited value as a means of classification, needing confirmation by other differential tests.

Plaque Size Determinations

Results and discussion

The plaque sizes of eleven different bacteriophages are tabulated in Table 9. The figures given for the smallest and largest sizes were those obtained after measuring

Table 9

Plaque Sizes of Various Bacteriophage Strains

Group	Bacteriophage	Millimeters diameter		
		Smallest	Largest	Median
I	PF2	0.77	1.65	1.32
	PF8	0.66	1.32	1.21
II	F43	0.55	1.43	1.32
	F62	0.44	1.21	1.10
	PF11	0.88	1.32	1.10
III	F24	--*	0.44	0.33
IV	F56	0.77	1.65	1.21
V	F70	0.88	1.54	1.43
	F75	0.66	1.65	1.43
VI	F69	0.66	1.76	1.54
VII	F68	0.77	1.76	1.54

*Too small to measure accurately.

several plaques on a given plate. The median does not represent the average of all the plaques appearing on the plate but rather the size characteristic of the largest number of plaques, as determined by observation and measurement. In general, most of the plaques on a plate were about the same size with a few larger and smaller ones occurring on almost every plate.

Plaques of ten of the eleven different bacteriophage strains measured usually were between 1.1 and 1.6 mm. in diameter and diameter appears to be of little value in differentiating these strains. One bacteriophage strain (F24) consistently produced very small plaques; this was a reproducible characteristic by which this strain could be distinguished from the others. The "median" of 0.33 mm. diameter was about as small as could be measured reliably, so the smallest were not recorded for this strain. When the F24 bacteriophage was purified by single plaque isolation, it was necessary to modify the plating method by using only 2/3 ml. of agar medium in the upper layer. Decreasing the agar concentration enlarged the plaque size to about 0.6 mm., which aided materially when isolating from plaques with a sterile needle.

The plaques from all bacteriophage types were clear with well-defined edges, of consistently round shape and presented no unusual appearances. The very small plaques

were somewhat opaque but when their size was increased by modification of the plating method the enlarged plaques were clear with sharp edges.

Serological Grouping

The precise usage of some of the terminology employed in the serological investigations involving bacteriophages will be explained briefly to avoid possible confusion. An antigen may be defined as a substance whose parenteral introduction into the tissue of an animal results in the formation of substances known as antibodies which may react specifically with the antigen in some observable manner. In this work the bacteriophages are considered the antigens. The antibodies are termed "antiphages" and the meaning of the term is entirely distinct from that used in the literature in reference to chemical and microbially-produced inhibitors known as "antiphage agents". When the bacteriophage antigen reacts specifically with the antiphage antibody the reaction is termed "neutralization" according to usage, although not analogous to chemical neutralization. This bacteriophage-antiphage neutralization reaction has been shown by several investigators to be temporary in that the bacteriophage activity can be restored under the proper conditions.

Selection of material

The various bacteriophage strains selected for use as antigens are listed in Table 10. Selection was based on attempts to use strains from widely separated sources. The first six were selected at random while the last six were chosen in an effort to obtain bacteriophages that were antigenically distinct from the others, except PF2 which was used for a second trial after repurification. The cultures on which the bacteriophages were propagated and the titers obtained also are given. All cultures and bacteriophages used in the preparation of antigens were repurified from the stock collection by single colony isolations of the organisms followed by plaque isolations of the bacteriophages.

In reviewing the work of Evans (1934), Burnet (1933), Hershey (1943) and Delbrück (1945c), considerable differences in procedures were noted in regard to preparation of antigen, course of injections and handling of immune sera. The following procedures are various modifications of their technics which were adapted to this particular investigation.

Preparation of antigen

The bacteriophage-containing whey filtrates that were prepared for injection into the animals had an initial

Table 10

Bacteriophage Filtrates Used for Preparation of Antisera

Rabbit no.	Bacteriophage	Grown on culture	Titer when frozen	Titer after 2 months	Antiserum designation	Original source
1	F69	ML1	9+	3.0×10^7	R1	New Zealand
2	F62	K2	9+	2.5×10^7	R2	New Zealand
3	F70	5	9	7.5×10^7	R3	Quebec, Canada
4	PF2	H1-2	9	2.3×10^8	R4	I.S.C.
5	PF11	H1-10	9	2.0×10^8	R5	I.S.C.
6	F56	712	8	7.5×10^6	R6	London, England
7	PF2	H1-2	9	1.5×10^8	R7	I.S.C.
8	PF8	H1-5	9	1.0×10^9	R8	I.S.C.
9	F75	6	8	4.5×10^7	R9	Quebec, Canada
10	F43	122-1	9	1.5×10^8	R10	I.S.C.
11	F68	1P5	9	2.5×10^8	R11	Reading, England
12	F24	W2	7	1.0×10^7	R12	I.S.C.

acidity of approximately pH 4.5. To lessen the possibility of a reaction in the animal, the filtrates were adjusted to pH 7.4 by the addition of N/10 NaOH. When the sodium hydroxide was added slowly to the clear, light yellow-colored filtrates a white, flocculent precipitation occurred, causing the filtrate to become quite opaque. This precipitate was presumed to be milk salts, but was not identified, since it was removed by centrifugation at 4500 rpm for 15 minutes and the relatively clear supernatant was decanted for use. A preliminary trial was made in which the supernatant was refiltered after centrifugation to remove the last traces of the precipitate but too great a decrease in the bacteriophage titer was noted.

The slightly opaque supernatant material was used as such as the antigen in the preparation of the immune sera. The antigenic material was dispensed into a sufficient number of screw-cap tubes in the appropriate quantities so that one tube of each strain could be thawed when needed. A single freezing and thawing did not affect the bacteriophage activity to any measurable degree. Storage at -20°C. for several months was found to be very satisfactory for maintaining a fairly high titer in the filtrates, as shown in Table 10. It is well known that many biological materials exhibit antigenic activity in the inactive or dead form as well as in the living state. In all cases reported herein

the bacteriophage was in its active state and its activity had not been lessened or modified in any demonstrable way up to the time it was injected. All of the above procedures were carried out aseptically and the antigens were bacteria-free when injected into the animals.

It was possible that bacterial debris resulting from the disruption of the bacterial cells was present in the material injected and thus might also act as an antigen. Some workers have been able to show the presence of low concentrations of anti-bacterial substances which could be removed from the sera by adsorption on homologous bacterial cells. Several attempts to demonstrate the presence of bacterial antibodies were unsuccessful; if such were present they did not seem to interfere with the bacteriophage-antiphage reaction as carried out in this work.

Injection of rabbits

New Zealand white rabbits were selected as the test animal, since they would furnish the desired quantity of blood for subsequent experiment work. The use of rabbits in the field of immunology has been well established and several workers have used rabbits successfully in the preparation of antiphage sera. The rabbits were 7-8 weeks old at the beginning of the course of injections. Rabbits number 6, 7 and 12 were males and the others were females; they all were caged separately.

Normal rabbit blood for use as controls was removed from the rabbits prior to the injection of the bacteriophages. Approximately 5 ml. quantities were aseptically drawn from the marginal vein of the right ear (26 gauge needle and 5 ml. syringe), transferred into sterile centrifuge tubes, allowed to clot at room temperature for 1 hour, loosened from the sides of the test tubes with a sterile needle and held in the refrigerator (40°F.) overnight. The tubes then were centrifuged for 15 minutes at 3500 rpm and the relatively clear supernatant sera decanted. The sera were dispensed in 1 ml. quantities into sterile screw cap test tubes and stored at -20°C.

Tables 11 and 12 show the course of injections given to the first and second series of six rabbits, respectively. All injections were made intraperitoneally, using sterile 22 gauge needles and 5 ml. syringes. All animals showed normal gains in weight and good health during the course of injections. In the first series some trial bleedings were made from the ears in order to determine whether the injections were being effective in stimulating the production of antiphage. When the course of injections was completed the blood was removed from the heart, using sterile 18 gauge needles and 30 ml. syringes. All rabbits were sacrificed after a single bleeding except rabbit number 5, which was given two more injections and re-bled six days

Table 11

Record of Bacteriophage Injections
(First series)

Date 1948	Rabbit Number 1	2	3	4	5	6
	ml.	ml.	ml.	ml.	ml.	ml.
10-7	0.5					
10-9	0.75	0.5	0.5			
10-11	1.0	0.75	0.75	0.5	0.5	0.5
10-13	1.5	1.0	1.0	0.75	0.75	0.75
10-15	1.75	1.25	1.25	1.0	0.75	1.0
10-17	2.0	1.25	1.75	1.25	1.5	2.0
10-19	3.0	2.25	2.5	2.0	2.0	3.25
10-21	4.0	3.0	3.0	3.0	3.0	4.0
10-23	4.75	4.0	4.0	4.0	3.5	4.5
10-25	5.0	5.0	5.0	5.0	5.0	5.0
10-27	5.0*	5.0	5.0	5.0	5.0	5.5*
10-29	5.0	5.0	5.0	4.5	5.0	5.25
10-31	5.0	5.25	4.5*	4.75*	5.5	5.0
11-2	4.5	4.5	4.75	5.0	5.25	5.0
11-4	5.0	5.0	5.0	5.0	5.0	5.0
11-6	5.0	5.0	5.0	5.5	5.0	-
11-8	Bled	4.5	4.5	5.0	5.0	5.0
11-10		5.0	5.0	5.0	5.0	5.0
11-12		5.0	5.0	5.0	5.0	Bled
11-14		5.0	5.0	5.0	5.0	
11-16		5.0	5.0	5.0	Bled	
11-18		Bled	Bled	-	5.0	
11-20				Bled	5.0	
11-22					Bled	
Totals						
Inoculum	53.75	73.25	73.5	72.25	77.75	56.75
Blood	70	40	70	30	50-45	70
Serum	33	15	33	14	19-27	24

*Preliminary trial bleeding from ear.

Table 12

Record of Bacteriophage Injections
(Second series)

Date 1949	Rabbit Number					
	7	8	9	10	11	12
	ml.	ml.	ml.	ml.	ml.	ml.
2-6	0.5	0.5	0.5	0.5	0.5	0.5
2-8	1.0	1.0	1.0	1.0	0.75	1.0
2-10	1.5	1.5	1.5	1.5	1.5	1.5
2-12	2.0	2.0	2.0	2.0	2.0	2.0
2-14	3.0	3.0	3.0	3.0	3.0	3.0
2-16	4.0	4.0	4.0	4.0	4.0	4.0
2-18	5.0	5.0	5.0	5.0	5.0	5.0
2-20	5.0	5.0	5.0	5.0	5.0	5.0
2-22	5.0	5.0	5.0	5.0	5.0	5.0
2-24	5.0	5.0	5.0	5.0	5.0	5.0
2-26	5.0	5.0	5.0	5.0	5.0	5.0
2-28	5.0	5.0	5.0	5.0	5.0	5.0
3-2	5.0	5.0	5.0	5.0	5.0	5.0
3-4	5.0	5.0	5.0	5.0	5.0	5.0
3-6	-	-	5.0	5.0	5.0	5.0
3-7	Bled	Bled	-	-	-	-
3-8			Bled	Bled	-	-
3-9					Bled	Bled
Totals						
Inoculum	52.0	52.0	57.0	57.0	56.75	57.0
Blood	25	30	45	40	40	40
Serum	13	14	23	17	18	19

later. All rabbits were bled 48 or more hours after the last injection. The sera were obtained from the blood in the same manner as described above. All sera were stored in a frozen condition without the addition of preservatives.

After the first series of rabbits had been injected, Latarjet (1948) reported, that in the case of bacteriophage active against E. coli, a larger number of small intravenous injections apparently was more effective in the production of strong antiphage sera than a smaller number of large intraperitoneal injections. However, since the amount and course of injections that had been used for the first series produced satisfactory results under these experimental conditions, it was used without modification for the second series of rabbit injections.

Bacteriophage-antiphage reaction

van Rooyen and Rhodes (1948) pointed out that there is no standard method for estimating accurately the potency of immune sera which contains virus-neutralizing antibodies. The method used in this work to bring about the neutralization of the bacteriophage with the antiphage sera was similar to that of Burnet, Keogh and Lush (1937). Essentially, the method consisted of mixing equal portions of high-titer bacteriophage and immune serum and allowing them to react at 35°C. for not less than 1 hr. Titers of the mixture and serum-free

bacteriophage controls then were determined by the limiting dilution method with litmus milk as the test medium. The difference between titers of original bacteriophage and bacteriophage plus antiserum represents a measure of the strength of the antiphage activity.

It is customary in immunological work to use the term "titer" to refer to the maximum dilution of the blood sera showing a demonstrable reaction. In this work the term "titer" is used as customary in bacteriophage work to refer to the concentration or activity of the bacteriophage. However, in order to determine the potency of the antiphage sera in terms of limiting dilution of the sera, two trials were made, results of which are presented in Table 13. Serial dilutions of the homologous sera were made and a portion of each dilution was used to carry out the bacteriophage-antiphage neutralization reaction. In these two cases it was found that the "immunological titer" would occur in the range of 1:1,000 to 1:10,000 serum dilution.

For purposes of this investigation it was desirable to maintain a constant serum concentration, so a 1:10 aqueous dilution was made of all sera used to carry out the bacteriophage neutralization reactions. This tenfold dilution slightly lessened the possibility of interference

Table 13

Influence of Antisera Dilution on Antiphage Activity

Antiserum dilution	Titers after antiphage action	
	F69 + R1	F56 + R6
1:10	7.9×10^2	3.3×10^1
1:100	1.3×10^4	3.3×10^2
1:1,000	1.3×10^5	1.3×10^5
1:10,000	1.3×10^7	4.9×10^5
1:100,000	1.0×10^7	4.9×10^6
1:1,000,000	3.3×10^7	4.9×10^6
Original bacteriophage titer	2.3×10^7	4.9×10^6

by naturally occurring blood constituents and yet retained a strong antiphage concentration.

To determine whether any naturally occurring constituents in the blood were present that might cause a decrease in the bacteriophage titer, the normal blood sera obtained from the rabbits prior to injection was used in tests designed as controls for later experimental studies. In these control tests normal blood serum which had been diluted tenfold with sterile distilled water was mixed with an equal portion of high-titer bacteriophage preparation in skimmilk. The mixture was incubated for one or two hours at 35°C., followed by bacteriophage enumeration using the limiting tube dilution method for determining the decrease in titer, if any, caused by the normal blood serum. In all cases tested there was no evidence of any decrease in titer caused by the normal blood. It was concluded that there usually were no naturally occurring blood constituents which would show bacteriophage-inhibition activity or antiphage activity under these experimental conditions.

However, an abnormal reaction was observed on several occasions which may have been caused by the presence of blood sera. The reaction could not be reproduced consistently. It was noted that occasionally in the three tubes representing the lowest dilution used (the 1:10 dilution of the

bacteriophage-antiphage mixture) the cultures developed in the normal manner, while in several of the higher serial dilutions active bacteriophage was demonstrated. Large amounts of bacteriophage had been present in the low dilution and yet did not cause inhibition of the culture. By calculation it can be shown that slightly over 1 per cent of blood serum was present in the final dilution. Nutritionally this would be enough to stimulate the rapid growth of the culture, which if it overgrew the bacteriophage would explain this zoning phenomenon. Another explanation would be that there may have been some material in the blood sera, which, at this concentration, inhibited the action of the bacteriophage. This type of reaction occurred only rarely and not sufficiently often to affect the results in the serological studies.

Influence of pH

When some of the antiphage sera were tested for activity against stock filtrates of acid reaction, the bacteriophage neutralizations were not in some cases the same as those obtained when the bacteriophage preparation used for injection of the rabbits were tested similarly. It was considered unlikely that there should be any sensitivity differences in the original bottle filtrate and purified filtrates prepared from them by plaque isolations. A difference in

the hydrogen ion concentration of the reacting mixtures was suspected. The original bottle filtrates had been prepared by addition of sufficient amounts of lactic acid to coagulate the milk proteins prior to filtration. The reactions of the filtrates were within the pH range of 4.2 to 4.5. The diluted antiphage sera was pH 7.1 to 7.3. When equal portions of acidified filtrate and diluted antiphage sera were mixed, the final reaction in a number of trials always was below pH 5.2.

At pH 4.8, filtrate F69 and homologous antiserum did not show any appreciable reaction, while a tenfold dilution of the filtrate in skimmilk showed a strong reaction at pH 6.3. The reaction of PF2 with R4 and R7 antisera behaved in a similar manner. When F56 was tested against all antisera from R1 to R11, neutralization was demonstrated with R5 and R10, but with the other nine antisera the expected neutralization was not shown. The pH of the mixture of this bacteriophage and its homologous antiserum was 5.2. The others were not determined but would be very close to this value, since the normal rabbit sera all were of approximately the same pH. The above considerations suggested that either pH 5.2 was very near the critical point of the bacteriophage-antiphage reaction or a concentration of hydrogen ions sufficient to impair the bacteriophage-antiphage reaction was dependent upon the particular combination of bacteriophage

and serum under investigation. In order to further clarify the question of pH, a more quantitative study was made, the data obtained being plotted in Figure 1. Three different bacteriophages were selected and allowed to react with their homologous sera at various known pH levels. The decreases in titers of the reacting mixtures were determined for all combinations and plotted against the various pH values. Below pH 5.0 there was a marked inhibition of the bacteriophage neutralization reaction while between pH 5.5 and 8.5 the reaction was not influenced to an appreciable degree by the hydrogen ion concentration. The upper limiting pH for the neutralization reaction was not determined. Further data on the retardation of the reaction at the lower pH values are presented in Figure 2. Since the plot showed the range between pH 6.0 and 7.0 to be satisfactory, all further reactions were carried out within these limits.

Influence of time

Although a complete investigation of the bacteriophage-antiphage reaction was not considered within the scope of this investigation, it was considered necessary that the reaction be carried out under standardized conditions known to be reliable so that the data obtained could be evaluated properly. An incubation temperature of 35°C. selected for

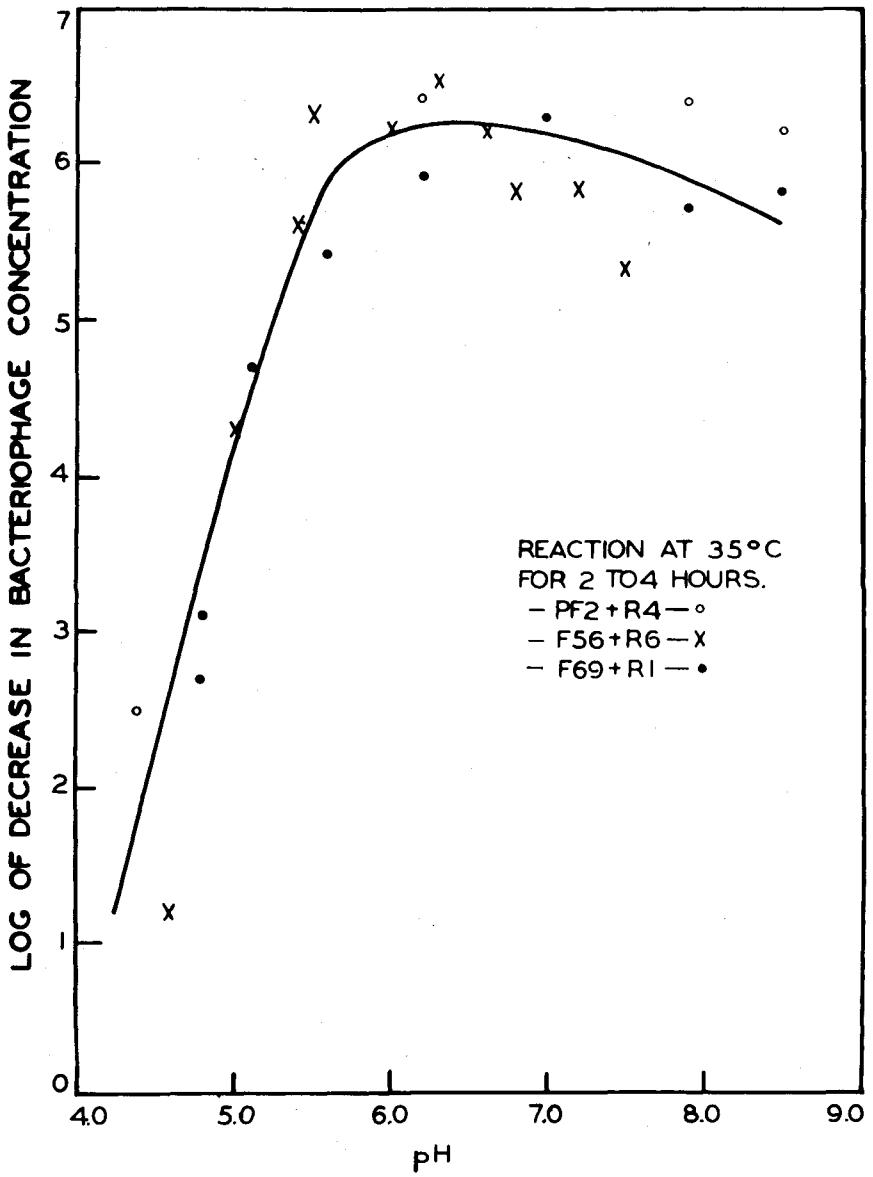


Fig. 1. Influence of pH on the Neutralization of Bacteriophage by Homologous Antiphage Serum.

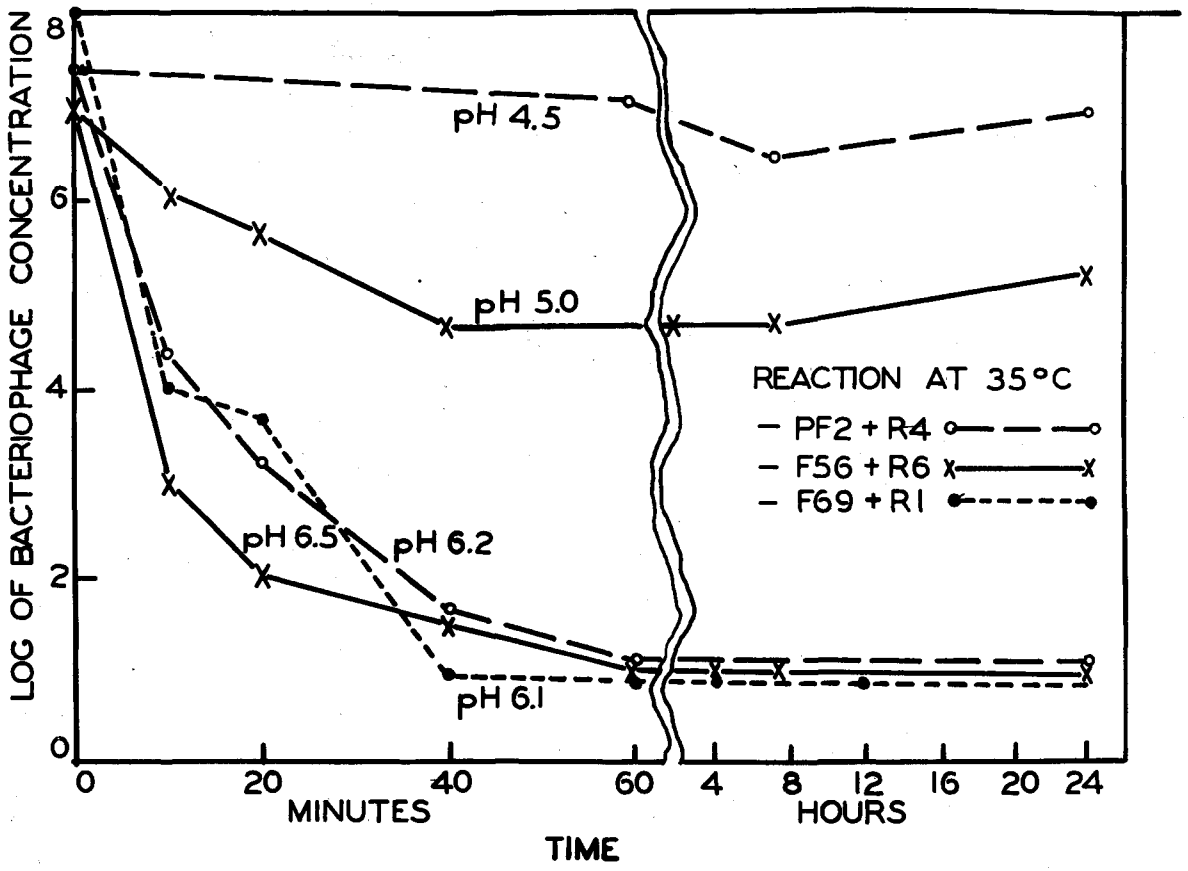


Fig. 2. Influence of pH on Rate of Bacteriophage Inactivation by Homologous Antiphage Serum.

the reaction was found to give satisfactory results and was used for all reactions.

A study of the time required for the completion of the bacteriophage-antiphage reaction was made. The reactions for three combinations of bacteriophage and homologous antisera were allowed to proceed at 35°C. and the titers were determined at various time intervals. These have been plotted in Figure 2 to obtain a better understanding of the dynamics of this reaction. Before one hour had elapsed all three reactions when carried out in the pH range from 6.1 to 6.5 had dropped to a titer of 1.0 or less. These curves showed that the antiphage serum was relatively potent as an inhibitor of bacteriophage activity and that the one hour minimum incubation period selected would allow sufficient time for the reaction to take place.

Cross-neutralization reactions

Cross-neutralization reactions were carried out using the 12 antisera against the 65 bacteriophages available for this investigation. One ml. of 1:10 aqueous dilution of serum was mixed with 1 ml. of bacteriophage in skimmilk by shaking in a screw-cap test tube. The mixture then was incubated for at least 1 hour and never more than 4 hours, at 35°C. Serial dilutions of the mixture then were made to determine the bacteriophage titer and this value compared

to the titer of the control, which was another 1 ml. of the same bacteriophage handled in a similar manner except that 1 ml. of sterile distilled water was added in lieu of the diluted antiserum. Table 14 shows the titers obtained by the cross-neutralization reactions of the 12 different sera and the 12 bacteriophages that had been used for the preparation of the antisera. The titers presented are averages of two or more individual determinations. Due to the large amount of work involved, these determinations were made over a period of several months and thus a slight experimental error is introduced due to small day to day variations in the controls. Titers differing by 1.0 or less should not be considered significant when comparisons are made. In the 12 homologous reactions, strong inactivation reactions were noted. The exact titer decrease for a given combination may be obtained by subtraction of the final titer from the control titer which was done for all combinations and those showing titer decreases of more than 3.0 have been tabulated in Table 15. The order also has been rearranged to show the close serological relationship that existed among some of the bacteriophages. Those bacteriophages which exhibited similar degrees of reactivity are considered serologically indistinguishable and form the basis of a single serological group. The seven serological groups found are set off by solid lines and identified with Roman numerals I to VII.

Table 14

Titers* Found After Action of Each of the 12 Antiphage-containing Sera Upon Each of the 12 Bacteriophage Preparations Used as Antigens

Phage no.	Control titer	Rabbit serum no. 1	2	3	4	5	6	7	8	9	10	11	12
F69	7.7	1.6**	6.8	5.9	3.8	6.2	7.5	3.6	3.2	6.5	6.8	7.2	7.4
F62	7.8	6.0	1.0**	4.2	5.2	1.0	7.5	4.0	3.8	4.0	1.0	3.7	6.4
F70	8.1	6.3	5.7	1.0**	5.5	7.4	7.9	6.3	6.7	2.7	7.0	7.1	7.8
PF2	8.5	7.6	7.4	7.3	1.0**	7.9	7.7	1.0	5.1	7.4	6.9	7.7	8.1
PF11	8.7	8.1	3.0	7.8	7.7	1.0**	8.3	8.4	7.6	7.4	2.1	7.8	8.7
F56	7.9	2.0	1.0	2.0	1.0	1.3	1.0**	1.0	1.0	2.3	1.4	2.5	7.1
PF2	8.2	6.9	6.8	7.4	1.8	6.8	7.8	1.0**	4.9	7.0	7.2	7.5	7.6
PF8	8.1	7.9	6.9	5.0	4.0	7.4	7.6	3.7	1.0**	5.8	4.8	6.7	7.3
F75	7.3	5.4	5.1	1.0	4.4	5.2	6.3	4.9	4.8	1.0**	5.3	6.7	6.8
F43	8.0	7.4	3.3	7.0	7.3	1.0	7.4	7.5	6.7	7.1	1.0**	7.2	7.4
F68	7.7	6.7	6.4	5.3	3.7	7.1	6.8	4.9	5.1	5.9	6.9	1.0**	7.4
F24	7.4	6.8	6.6	5.9	7.0	6.8	5.8	6.3	6.7	7.0	6.5	7.4	1.0**

*Decreases in titer greater than 3.0 are underscored.
**Homologous bacteriophage-antiphage reactions.

Table 15

Relationship of Various Bacteriophages
Based on Serological Cross-neutralization Reactions*

(Inactivation expressed as decrease in titer**)

Filtrate no.	Rabbit no.	Serum number 12	11	5	10	2	6	8	7	4	1	3	9
F24	12	6.4 ← III											
F68	11		6.7 ← VII			← II				4.0			
PF11	5			7.7	6.6	5.7							
F43	10			7.0	7.0	4.7							
F62	2		4.1	6.8	6.8	6.8	← IV	4.0	3.8			3.6	3.8
F56	6		5.4	6.6	6.5	6.9	6.9	6.9	6.9	6.9	5.9	5.9	5.6
PF8	8				4.3			7.1	4.4	4.1		3.1	
PF2	7							3.3	7.2	6.4	← I		
PF2	4							3.4	7.5	7.5	← VI		
F69	1							4.5	4.1	3.9	6.1	← V	
F70	3											7.1	5.4
F75	9											6.3	6.3

*Reciprocal cross-neutralization reactions only are enclosed in squares.

**Titer decreases of less than 3.0 omitted for clarity.

The figures not included with the lined areas are reactions in which the serum of one bacteriophage strain reacts with an apparently unrelated bacteriophage. This phenomenon has been observed often in the field of immunology but a satisfactory explanation has not yet been reported. F56 was neutralized by all antisera except F24 serum of group III, yet the antiserum of F56 had no effect on any bacteriophages except its own homologous group IV. This phenomena could not be related to the cross-activity tests, since in group IV all members showed complete specificity for their respective hosts.

Bacteriophage F69 also was neutralized by group I antiserum in addition to its own of group VI, although the F69 antiserum was specific for its own homologous bacteriophage and the F56 reaction described above. In the cross-activity tests, F69 also exhibited some activity toward the members of culture group 1, especially subgroup 1a. In this one case a relationship between groups I and VI was suggested. It may be reasoned that the character which contributed to the F69 susceptibility toward group I antisera also is the character which contributed to the F69 activity against the 1a culture subgroup. However, the F56 reactions do not confirm this reasoning. Possible explanation of the other reactions shown in Table 15 in which reciprocal neutralization was not demonstrated cannot be given at this time.

In Table 16 are listed numerically the results of serological testing of 54 available bacteriophage filtrates not used as antigens. Some tests were run using all twelve sera which confirmed the close relationship of the members of the various serological groups. Thereafter, when more than one test serum was available, only one was selected for use in order to conserve material, thus accounting for the obvious omissions in Table 16, notably sera 9 and 10. Filtrate F73 has not been included since it lost its activity and efforts to repropagate it have been unsuccessful.

Further uses of the serological typing method

Having developed a method by which unknown bacteriophage filtrates could be classified on a serological basis, it seemed desirable to make use of the method to further clarify the question of bacteriophage purity. The purity of biological materials usually is expressed in terms of technics used for purification. Using the same procedures outlined previously, cross-neutralization reactions were determined on nine of the original high-titer stock filtrates from which the injected antigens had been prepared. In Table 17 are listed the results obtained. In seven out of nine trials no significant differences were obtained when compared to the same bacteriophage reactions in Table 14. With F75 and F68 several additional sera had neutralized

Table 16

Titers* Found After Action of Each of the 12 Antiphage-containing Sera Upon Each of the Available Bacteriophages Not Used as Antigens

Phage no.	Control titer	Rabbit serum no. 1	2	3	4	5	6	7	8	9	10	11	12
F1	7.4	6.4	2.2	6.7	6.5	1.4	7.4		4.5			5.3	6.8
F2	8.7	7.4	7.2	7.0	4.1	6.3	7.7	2.3	2.3			7.2	7.8
F3	8.4	7.7	7.4	6.2	5.0	7.7	8.0	3.6	1.8			7.4	7.8
F4	7.6	6.6	6.8	4.8		6.4	6.9	3.0	2.0			5.9	7.4
F5	6.6	5.7	5.3	4.8	2.0	5.0	6.4	1.0	4.0			5.9	6.4
F6	5.7	4.7	4.9	4.8	2.0	5.4	5.7	1.0	1.0			5.0	5.0
F7	6.4	5.4	5.8	4.9	2.0	3.7	5.0	2.0	3.6	5.7	5.6	6.2	6.4
F8	7.2	6.2	6.4	5.8	2.0	6.7	7.1	3.2	5.1			6.8	6.6
F9	7.4	5.8	5.6	4.4		6.0	6.4	1.0	1.0			6.3	6.8
F10	7.6	6.8	1.8	5.8		1.0	5.9	5.2	4.9			7.2	7.4
F11	7.4	7.0	3.0	6.8	6.6	1.0	6.4	6.4	6.9	7.2	1.0	7.0	7.2
F12	8.2	7.6	7.4	6.8		7.4	6.9	5.0	2.0			7.8	8.0

*Decreases in titer greater than 3.0 are underscored.

Table 16 (Continued)

Phage no.	Control titer	Rabbit serum no. 1	2	3	4	5	6	7	8	9	10	11	12
F20	6.7	6.0	6.4	5.7	6.0	5.6	6.3	5.0	5.0			5.6	4.0
F21	7.6	7.2	7.0	7.8	6.3	7.0	6.0		5.8	6.4		6.6	4.8
F22	8.6	7.0	7.4	7.6	4.2	7.4	8.6	3.0	2.0			7.6	7.8
F25	6.1	6.0	5.5	4.8	5.2	3.0	4.6	6.0	6.2	5.9	4.9	4.8	5.4
F26	8.4	7.4	7.4	6.7	3.0	7.0	7.0	2.0	5.0			6.8	7.4
F27	7.0	6.2	6.0	5.8		5.8	6.4	2.0	6.0			5.8	7.2
F32	7.2	6.8	7.0	7.0	2.0	6.8	6.4	2.0	3.0			6.4	6.8
F34	7.8	6.8	6.6	6.4		7.0	7.4	3.0	4.0	5.8	5.6	6.5	8.0
F35	6.2	5.4	5.2	4.8		3.8	6.0	2.0	2.0			6.2	6.2
F42	7.6	6.7	6.8	5.9		6.4	6.7	6.8	5.8	6.8		7.2	7.4
F44	8.2	7.4	4.0	6.6	7.6	1.4	8.0	6.8	5.9		2.0	7.4	7.0
F46	7.7	7.4	3.0	6.6	7.4	1.2	7.0		6.1		2.0	5.8	7.4
F47	8.6	7.6	4.4	6.6	7.4	2.6	7.0	5.9	6.4	8.2	2.0	8.1	7.6
F48	6.2	5.8	2.0	6.4	5.7	1.0	4.6	5.2	5.6	6.0	1.0	6.0	6.0

Table 16 (Continued)

Phage no.	Control titer	Rabbit serum no. 1	2	3	4	5	6	7	8	9	10	11	12
F49	6.3	5.6	6.0	4.2	5.2	4.2	6.0	3.0	2.0	4.7	4.7	5.2	6.0
F50	5.2	3.8	2.6	2.4	2.6	1.8	4.8	4.1	2.6	3.1	2.7	3.3	3.2
F51	6.4	5.0	4.7	5.4	2.0	4.7	5.0	5.0	3.0			6.2	6.1
F52	7.6	6.2	5.8	6.2	5.4	7.1	7.0	4.0	4.0	5.8	5.8	6.2	6.4
F53	7.7	5.9	4.4	6.4	5.8	4.1		6.2	6.0	7.1	2.0	7.0	6.8
F54	6.1	6.0	5.8	5.7	4.2	5.1	5.4	5.2	5.4			6.2	6.0
F55	7.4	6.5	5.8	5.2	1.0	5.4	6.4	4.0	4.2	5.6		3.8	6.4
F57	7.0	6.2	5.0	6.5	5.0	4.2	6.6	5.4	5.8	6.3	3.5	6.2	6.0
F58	5.8	3.8	2.0	2.0		3.1	3.5	2.6	2.0			2.2	5.4
F59	7.2	6.2	6.3	7.1	7.0	6.0	5.8	5.4	6.6			6.4	6.4
F60	7.6	6.7	6.0	5.8	6.0	6.8	7.2	5.8	6.3	7.2	7.0	6.9	6.5
F61	7.0	6.9	5.7	6.6	6.4	5.2	5.4	6.7	6.7	5.9	7.2	6.6	6.4
F63	7.8	7.0	7.2	7.4	4.6	7.6	7.4	5.0	4.5			5.8	6.3
F64	7.8	7.0	7.0	5.4	4.5	7.0	7.6	5.0	5.0	5.8	7.2	6.8	6.4

Table 16 (Continued)

Phage no.	Control titer	Rabbit serum no. 1	2	3	4	5	6	7	8	9	10	11	12
F65	6.2	6.4	5.0	5.4	6.0	5.0	6.3	5.4	5.6			5.7	5.6
F66	6.2	5.7	5.0	4.9	3.0	5.6	5.8	4.7	3.2			4.8	5.7
F67	7.0	2.3	3.2	2.0	1.0	2.0	3.0	2.0	2.0			3.5	5.0
F71	6.4	5.7	6.3	5.8	1.4	5.9	6.4	2.0	4.0			4.8	5.8
F72	7.4	6.4	5.8	6.6	6.8	7.1	7.0	5.4	5.8			6.6	6.7
F74	6.8	5.8	6.2	4.9	5.7	6.4	6.0	5.7	4.0	5.2		6.1	6.0
F76	7.0	6.0	4.0	5.8		2.0	5.9	6.3	6.4			6.6	6.7
PF4	7.4	6.4	6.0	5.2	2.0	5.2	5.6	2.0	3.0			6.8	6.8
PF6	7.4	6.6	5.8	6.6	3.4	4.7	5.8	2.0	3.0		5.7	5.8	6.6
PF7	6.7	5.0	5.4	3.4	4.0	5.8	6.6	3.0	2.0	5.0	4.5	5.0	5.7
PF10	7.8	6.8	2.0	5.7		2.0	6.3	6.8	3.9			6.6	6.7
PF12	7.8	5.9	6.3	5.8	2.5	5.9	7.4	3.0	2.0			6.6	6.8
PF13	7.8	7.2	7.4	6.8	1.6	6.9	7.0	3.0	4.1	6.9	6.2	7.2	7.4
PF14	7.0	5.8	5.9	6.1	5.0	6.2	6.4	4.8	3.0	5.9	6.7	6.9	7.0

Table 17

Titers* Found After Action of Each of the 12 Antiphage-containing Sera Upon Several Original Stock Bacteriophage Filtrates**

Phage no.	Control titer	Rabbit serum no. 1	2	3	4	5	6	7	8	9	10	11	12
F69	8.0	3.0		4.6	4.0	6.0		3.4	3.4	5.4	5.6	5.7	
F70	8.7	7.7	7.6	1.0	6.9	7.3	8.2	7.6	6.0	2.9	6.0	6.5	7.5
F75	7.4	2.0	3.5	1.0	2.0	4.5	5.5	2.0	2.0	1.0	2.0	4.0	6.0
PF2	8.0	7.0	7.2	6.9	1.0	5.9	7.6	2.0	5.0	7.2	7.4	6.8	7.4
PF11	6.4	5.9	2.0	3.8	4.2	2.0	4.8	6.4	6.0	5.6	2.0	5.0	5.0
F56	8.4	3.0	1.0	1.0	1.0	1.0	1.5	2.4	1.0	2.0	2.2	1.7	6.4
PF8	7.4	5.7	6.2	4.9	4.7	7.2	7.0	3.7	1.0	4.7	4.2	5.9	6.7
F68	6.3	5.3	4.8	3.5	1.0	4.8	6.2	3.0	2.0	3.0	5.4	1.0	6.0
F24	6.4	5.0	6.0	5.0	4.8	4.7	5.2	6.4	5.7	6.0	5.4	6.0	1.0

*Decreases in titer greater than 3.0 are underscored.

**Corresponding purified filtrates may be found in Table 14.

the original filtrates. Among the other seven no further changes in neutralization of the bacteriophages had been obtained by single plaque isolations.

Another use of the serological test method was to determine whether bacteriophages from a given filtrate could be distinguished by some means other than size of plaque formed. When three of the bacteriophage types were purified by large single plaque isolations, corresponding small plaque isolations also were made from the same plate and developed into a new filtrate. These small plaque filtrates were tested against the 12 sera and the results are shown in Table 18. In all three cases the results are entirely consistent with those from large plaque isolations shown in Table 14. This showed that these filtrates prepared from both large and small plaque selections contained only one serological type of bacteriophage. The differences in plaque sizes were probably due to other factors such as technics or nutrition.

Collins (1949), investigating the nutritional aspects of bacteriophage active against S. lactis, was able to grow a selected number of S. lactis cultures on a chemically defined medium. These cultures were used as hosts on which he was able to bring about the proliferation of bacteriophage. A serological test was made of one bacteriophage strain which had been prepared in this manner. Culture W2

Table 18

Titers* Found After Action of Each of the 12 Antiphage-containing Sera Upon Three Bacteriophage Filtrates Prepared From Small Plaque Isolations**

	Small plaque filtrates												
Filtrate no.		Rabbit serum no.											
	Control	1	2	3	4	5	6	7	8	9	10	11	12
F70	8.1	7.2	6.8	2.0	5.2	6.8	7.1	7.6	7.4	2.0	5.4	6.9	7.9
PF11	7.6	7.1	2.0	6.6	6.8	2.0	7.4	7.0	7.0	6.4	2.0	6.6	6.8
F68	6.1	5.7	4.2	4.6	3.4	4.5	5.2	4.7	3.6	4.6	5.8	2.0	5.9

*Decrease in titer greater than 3.0 are underscored.

**Corresponding large plaque filtrates may be found in Table 14.

had been grown repeatedly for several months on a synthetic medium and bacteriophage F24 had been repropagated six times on these host cells after transfer from a whey filtrate. A bacteria-free filtrate of bacteriophage was made of this preparation and Dr. E. B. Collins kindly supplied a few milliliters of this filtrate for serological testing. The filtrate was diluted ten-fold in sterile skimmilk and tested against the 12 available sera. This bacteriophage preparation showed exactly the same serological pattern as that of the homologous F24 bacteriophage used to prepare the R12 sera. Rabbit sera R1 through R11 did not affect the bacteriophage, but R12 showed a strong neutralization reaction. This was the only bacteriophage available which had been propagated on a culture grown on defined medium, so only one trial was made. Although inconclusive for any general statements, it did show that in this particular instance, the bacteriophage repropagated several times on a culture grown for months on a synthetic medium had not undergone any change in serological characteristics which could be demonstrated by the usual technics. The bacteriophage had remained an antigenically constant entity during these successive repropagations.

Discussion of serological typing studies

In most cases there was excellent agreement in the patterns of related bacteriophages, while there are some combinations which exhibit relatively weak reactions. It is not uncommon to find varying degrees of specificity in serological reactions, which are due in part to uncontrollable variations in the methods used, such as differences in rabbit response and concentrations of antiphage (Andrewes and Elford, 1933b). Other intermediate reactions probably are the result of differences in the antigenic material used or the bacteriophages themselves. Using an arbitrary titer difference of 3.0 as a dividing line, it can be seen that unrelated types always are below this value, related types generally show a 5.0 to 6.0 titer difference while some intermediate reactions occur in the 3.0 to 5.0 range. It is doubtful whether titer differences of less than 3.0 should be considered significant until improvements in the general technics used and more accurate methods of measurement of the bacteriophage-antiphage reaction are developed.

In Table 16 the reactions showing a titer decrease of more than 3.0 are underscored and may be considered positive reactions. Some titers which are underscored show titer drops of greater than 3.0 but the final actual titer of the mixture was still in the 2.0-4.0 range, indicating the

presence of considerable numbers of bacteriophage particles even though some neutralization had taken place. These cases usually were those in which the initial control titers were relatively high, suggesting that perhaps the concentration of the antiphage "particles" was in these cases the limiting factor in demonstrating the amount of neutralization which had taken place.

The bacteriophages exhibiting similar reactivity patterns are considered to belong to the same serological group and have been listed earlier in Table 7. When various bacteriophages were shown to exhibit similar cross-reaction patterns and similar serological reactions they were placed in the same group. Some groups contain several members, all of which showed positive reactions with very few exceptions. The members of these groups could be selected easily. Other groups were defined with greater difficulty and the members were placed in their respective groups on the basis of somewhat fewer positive reactions. Some bacteriophages and cultures did not present sufficient differentiation for group classification and have been listed as ungrouped.

It could be seen after serological testing of the 65 available bacteriophage filtrates that this method offered a workable basis for classification of related strains. In fact, judging from the similarities in results where possible duplicates were used (filtrates from the same

source, isolated on the same host at about the same time), it appeared that when used as the sole criterion the serological method of grouping is a reliable means of differentiation. This method has not as yet shown as many inconsistencies as the cross-reaction methods which have been used more widely. The serological typing of bacteria has become well recognized as a sound basis for classification and has been recognized in Bergey's Manual (1948) in the classification of some of the bacteriophages. Further study will determine whether the serological grouping or cross-reaction testing is the better. In this investigation, the serological groupings were considered the primary basis for classification, while the cross-reaction results were used for confirmation purposes. However, both tests were carried out on all bacteriophage strains considered here and the results of both were considered before any tentative groupings were established.

Heat Inactivation

Method of heating

A study was made of the different groups of bacteriophages to determine the maximum amount of exposure to heat required to inactivate the various strains. The method used for the heating was to place 10 ml. quantities of the

bacteriophage preparations into 16 x 125 mm. pyrex screw-cap test tubes followed by complete submersion in a thermostatically controlled water bath. A time lag for these tubes of 2.5 minutes was used and the temperatures were maintained within ±0.2°C. At the end of the desired heating period the tubes were cooled quickly by immersion in iced water, followed by determination of the bacteriophage activity using the tube dilution method. Several duplicate trials established the reproducibility of the individual titers to be less than 1.0 titer difference in all cases. By employing an appropriate number of tubes, which were removed at various time intervals, it was possible to study the dynamics of the thermal inactivation. Readings were made at one minute intervals for first five minutes, five minute intervals up to one hour and ten minute intervals over one hour.

The freshly-prepared bacteriophage filtrates were diluted 1:10 in skimmilk, which was the medium used for all heat determinations. Adjustment of pH was accomplished by the addition of the appropriate number of drops of sterile 10 per cent lactic acid or normal sodium hydroxide solution. Control titers of the filtrates were made just prior to heating.

Influence of pH

In order to determine the influence of pH on the length of time required to inactivate the bacteriophage, the filtrates were adjusted to pH levels of 6.0, 6.5, 7.0 and 7.5 and simultaneously exposed to heat. Figures 3, 4 and 5 show the results obtained from heating bacteriophages PF8 at 70°C. and PF11 at both 70 and 65°C. for varying lengths of time at the four different pH levels.

The experimental error was generally less than a titer of 1.0 when a smooth curve was drawn to show the death curves of the bacteriophages. With PF8, pH 7.0 was found to show the greatest heat tolerance, while increased alkalinity and especially acidity made the bacteriophages more susceptible to heat. This was confirmed by trials with PF11 which showed that pH 7.5 was about the same as pH 7.0, while a lower pH again increased the heat lability of the bacteriophage. When PF11 was heated at a five degree lower temperature, the influence of pH was the same, except that a longer time was required to inactivate the bacteriophage. At the lower temperature the death rate was diminished to the extent that complete inactivation required more than 400 minutes which was the limit of these trials. When long times were required the exact points of complete inactivation could not be determined satisfactorily. Best

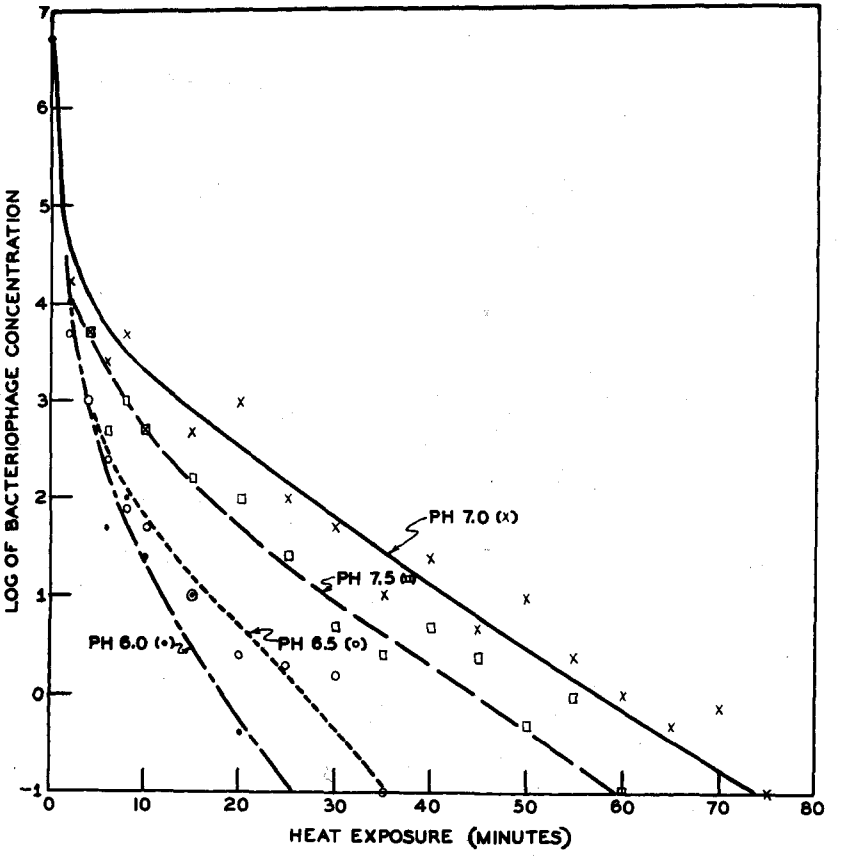


Fig. 3. Influence of pH on the Heat Inactivation of Bacteriophage PF8 at 70°C.

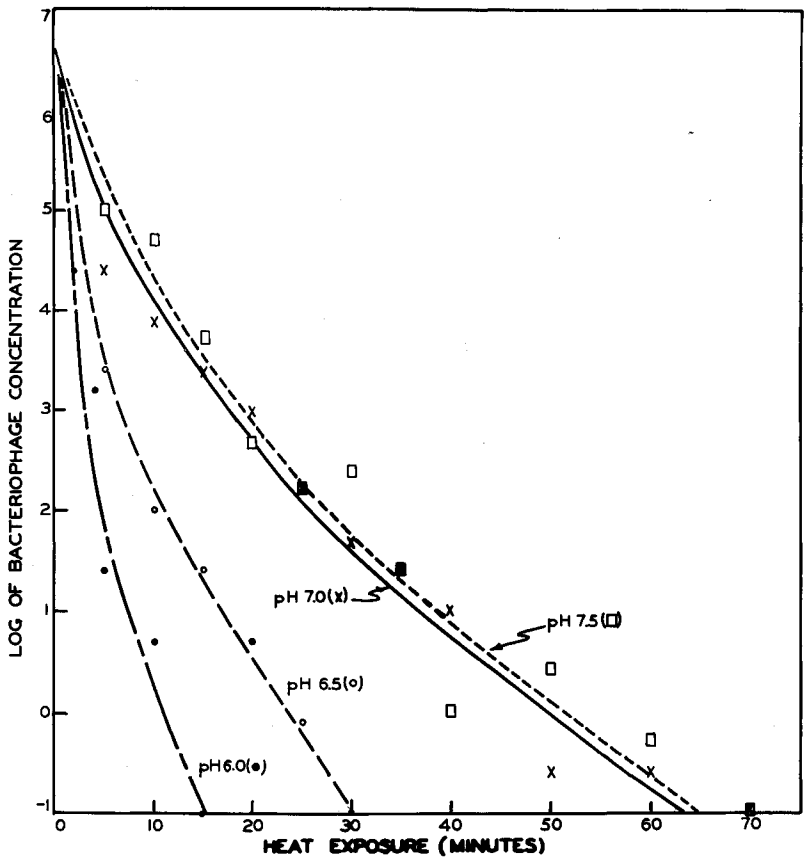


Fig. 4. Influence of pH on the Heat Inactivation of Bacteriophage PF11 at 70°C.

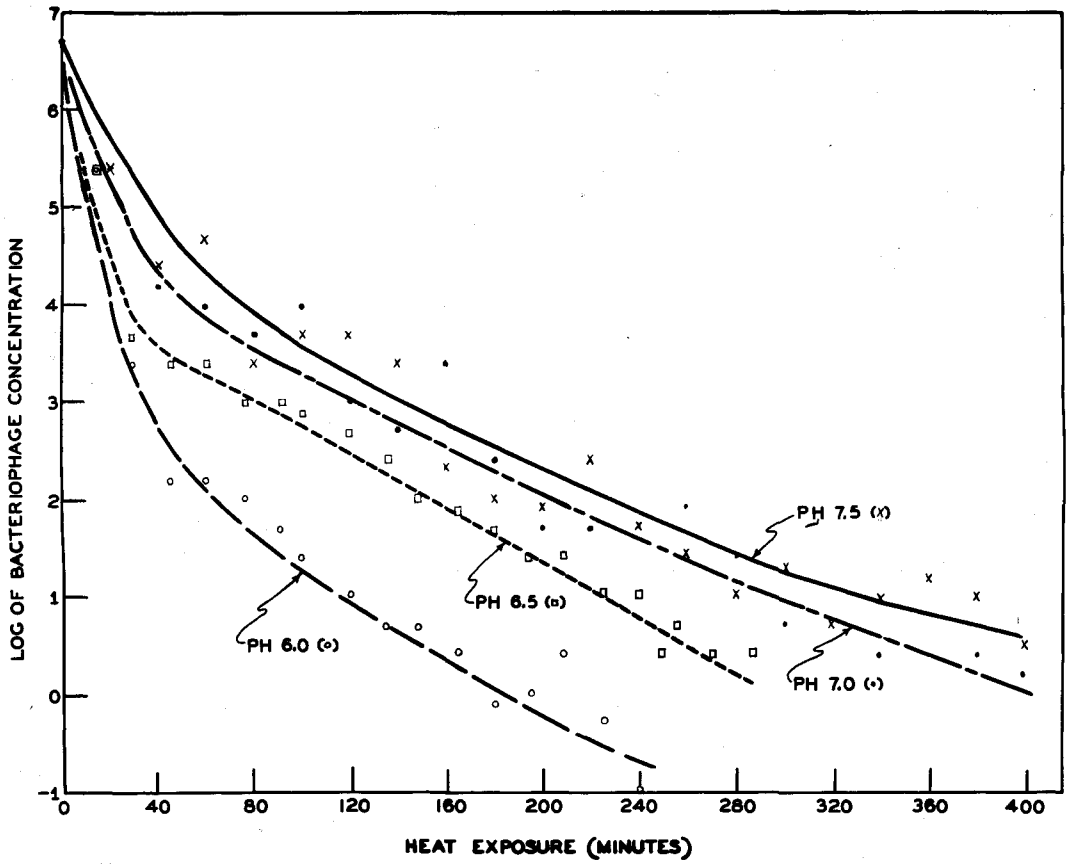


Fig. 5. Influence of pH on the Heat Inactivation of Bacteriophage PF11 at 65°C.

results were obtained by selection of temperatures which would bring about complete destruction between 10 and 100 minutes.

Two other trials were made in which the temperature differed by five degrees. In Figure 6, F68 at 65° and 70°C. is shown and in Figure 7, F62 at 70° and 75°C. is plotted. The latter curve was the only one obtained from several trials with different bacteriophages at this temperature. It was difficult to obtain curves at the higher temperatures because of the rapid rate of destruction.

In general, all curves showed a rapid drop of titer during the first portion of the heating period followed by a decreasing rate of bacteriophage destruction. In all filtrates there were many heat sensitive particles and some relatively resistant particles which tended to make the exact time of complete inactivation difficult to determine with precision. By determining the actual death curve of each strain, more reliable data were obtained for use in differentiating the various bacteriophage strains.

The representative data included in Figures 2-7 indicate that most of the bacteriophage particles were inactivated quite readily, while a few resistant particles require considerably longer heat exposures for inactivation. The plot of the logarithm of the number of survivors against

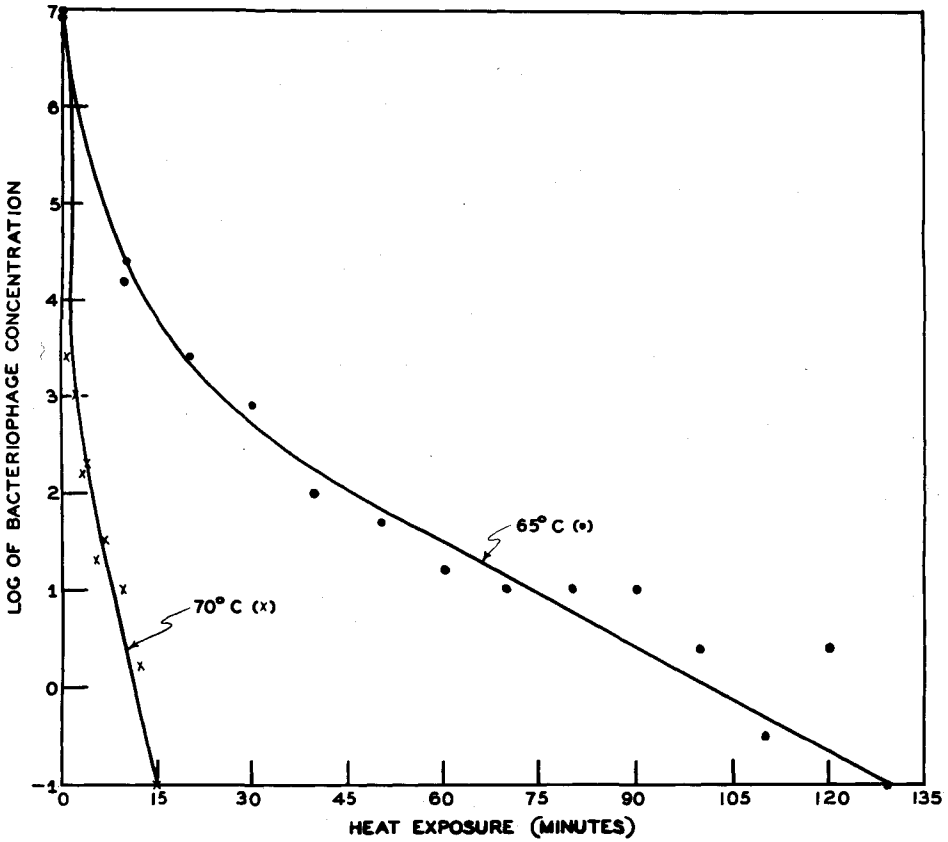


Fig. 6. Influence of Temperature on Rate of Heat Inactivation of Bacteriophage F68.

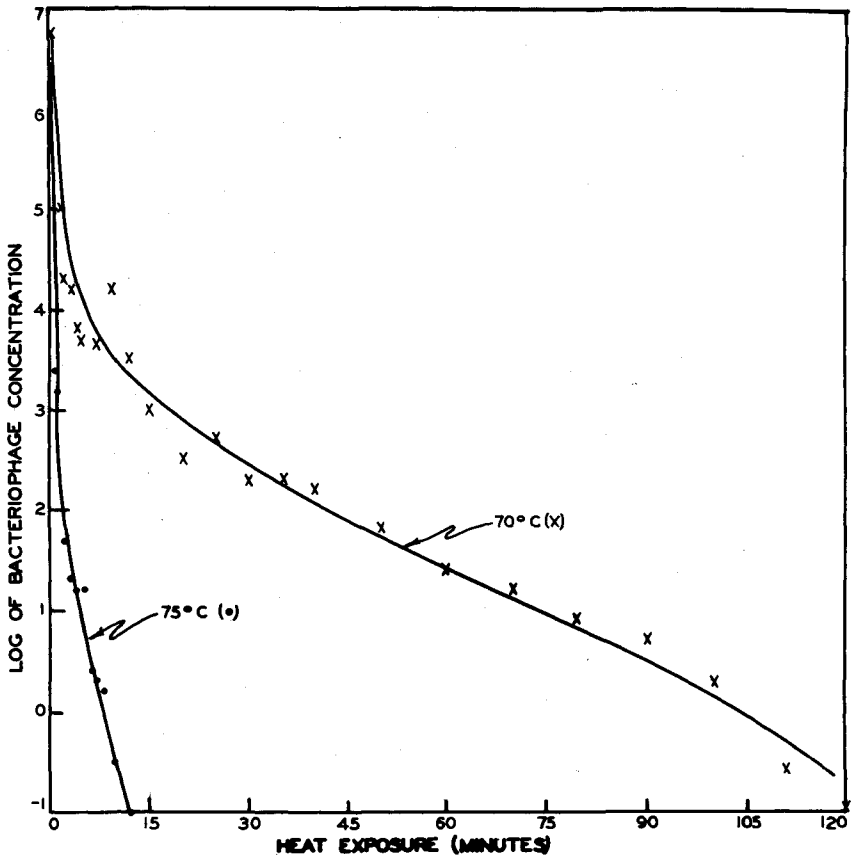


Fig. 7. Influence of Temperature on Rate of Heat Inactivation of Bacteriophage F62.

unit time definitely does not give a straight line over the entire time necessary for complete inactivation.

Discussion of heat inactivation studies

In Table 19 the different bacteriophages are listed in respect to their thermolability. Using five degree temperature increments, only one reliable death curve could be obtained in some cases. Higher and lower temperatures resulted in too short or too long time periods for accurate determinations. In some cases two time-temperature figures were obtained and in one case, PF11, three figures are presented covering a ten degree range. However, the lower temperature value was extrapolated from the data shown in Figure 5. The most heat resistant strain was F62, which withstood 75°C. for 12 minutes before complete inactivation. The least heat resistant bacteriophage was F24 which was inactivated at 60°C. for 45 minutes.

All bacteriophages used in these heat studies were freshly propagated filtrates. Earlier preliminary trials on some filtrates on which the exact age was not recorded but which were known to be several months old are listed in Table 19 in parentheses. These comparisons suggest that the older bacteriophage are somewhat more susceptible to heat than freshly prepared preparations, but further studies

Table 19

Heat Inactivation Times at Various Temperatures
of Representative Bacteriophage Strains

Group	Filtrate	Time (minutes) for complete inactivation at temperatures (°C.) of: 57.5	60	62.5	65	70	75	Source
I	PF8					75		I.S.C.
	PF2					50		I.S.C.
	PF13					45		I.S.C.
II	F62					120 (70)*	12	New Zealand
	F43					50		I.S.C.
	F10					50		I.S.C.
	F44					45		I.S.C.
	PF11				600	65 (40)	3	I.S.C.
III	F24	140	45	10				I.S.C.
	F20		30					I.S.C.
	F21		>200	50				I.S.C.
IV	F56				30	3		London, England
	F58				35			New Zealand
	F67				55			Reading, England
V	F70				90	10		Canada
	F75				120	15		Canada
VI	F69					55		New Zealand
VII	F68				130 (70)	15 (6)		Reading, England

*Preliminary trials on old filtrates in parentheses.

along this line would be desirable. Also, studies on whether there exist in nature any highly heat resistant bacteriophages would be of value.

In Figure 8 some of the data on heat resistance of bacteriophages from Table 19 were plotted in a form used in studies with bacteria. In cases where points at more than one temperature were determined on one bacteriophage strain, such points have been joined by straight lines. In general, the slopes of the lines connecting the points are very much the same. In the range between ten and a few hundred minutes it was possible to show the time-temperature relationship of the heat destruction of some of the bacteriophages. The differences in heat tolerance noted in the various different types affords to a limited degree a means of differentiating the various bacteriophage types. The five members of group II all were among the most heat resistant. The members of group I and group VI also exhibited similar high heat resistance. The two Canadian bacteriophages F70 and F75 of group V were of the medium heat resistance range and group VII (F68) also was of this range. Three bacteriophages, F67, F58 and F56, of the same serological group IV, from Reading, New Zealand, and London, respectively, all exhibited similar sensitivities to heat and belonged in the medium heat resistance range. The most heat sensitive filtrates, group III (F21, F24 and F20),

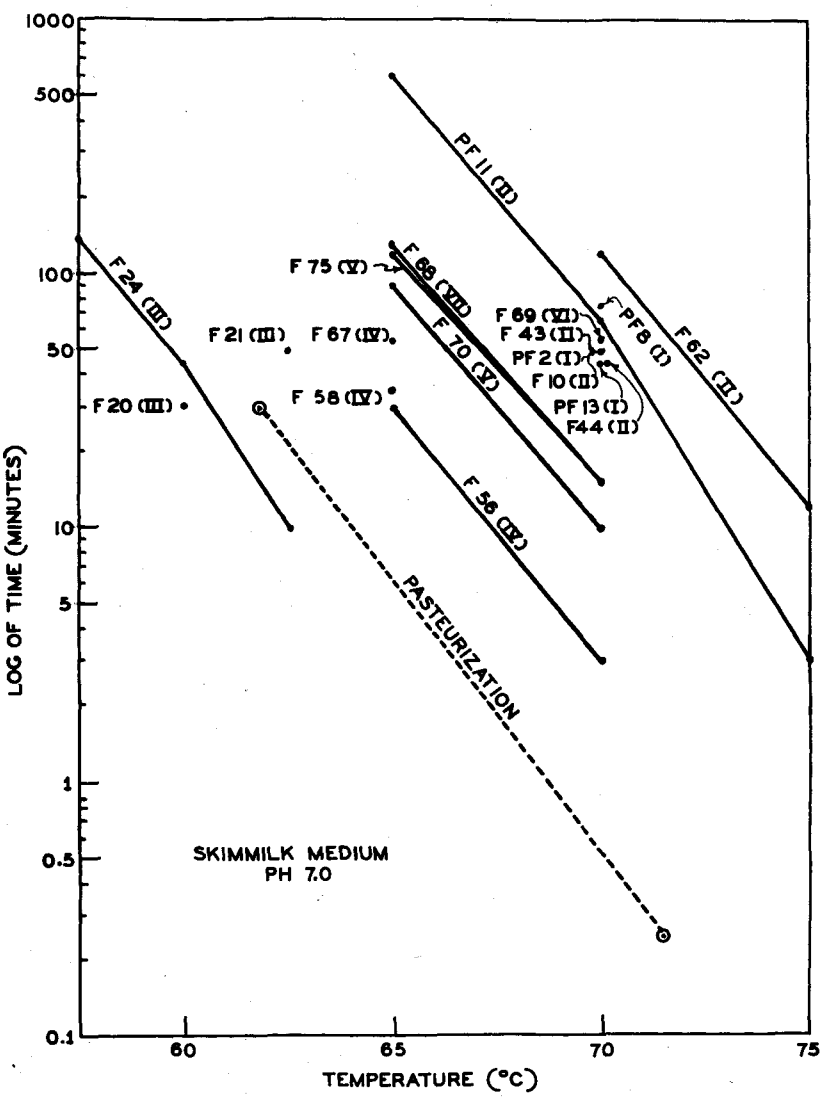


Fig. 8. Time-temperature Relationships for Complete Inactivation of Several Bacteriophage Strains.

also showed similar heat sensitivities. It is significant that the respective members of the different serological and cross-reaction groups also exhibited similar heat sensitivites. Of the 18 different filtrates tested, none was placed in a different heat range than the other members of the same group. However, for a given time the inactivation of all bacteriophages covered only about 12 degrees of temperature. Within that range a high, medium and low resistance can be used as a guide to further classification. Temperature data by itself would be of limited value, since three groups were found in the upper range, three in the middle range and one in the lower range. When used with the serological typing and cross-reaction testing, heat resistance of bacteriophages appears to be a characteristic by which members of various bacteriophage groups may be identified.

DISCUSSION

Opposed to the concept of d'Herelle that bacteriophages are one indistinguishable, homogenous group, and in accord with the more recent views, bacteriophage strains apparently can be distinguished by differential tests as belonging to various groups defined by correlated characteristics. The experimental evidence supports the existence of differentiable bacteriophage groups and thus provides a workable basis for rudimentary classification of bacteriophages.

Up to the present time, most attempts to classify many of the various bacteriophages (Bergey's Manual, 1948) have been based primarily on lytic activity upon organisms of different susceptibility types, plaque sizes, antigenic activity, heat resistance and morphology. In the present study these characteristics have been employed in an attempt to differentiate in an orderly manner a number of bacteriophage strains active against S. lactis and S. cremoris.

From the present investigations seven bacteriophage groups are suggested. Fifty-one of the 66 strains studied were placed in the groups which are summarized on the following pages. These seven groups are not to be considered as representing all possible groups, as 15 strains were

not grouped by the technics used and the collection used undoubtedly does not include representatives of all possible groups. Until the ungrouped strains are used as antigens and the reactions of the resulting antisera determined, conclusions cannot be reached as to whether other groups should be recognized among the available strains of bacteriophage active against lactic streptococci. Additional bacteriophage strains from different sources also will need to be studied by these same technics.

Group I

Serological basis: Antiphage sera R4, R7 and R8 prepared with bacteriophage strains PF2(H1-2)* and PF8(H1-5) exhibited cross-neutralization of the two strains and also neutralized other members of this group but not most members of groups II-VII. The three sera also neutralized F69 of group VI and F56 of group IV. Sera R7 and R8 neutralized F62 of group II and serum R4 neutralized F68 of group VII.

Bacteriophages of group I (29 strains):

Subgroup IA (11 strains): F2, F3, F4, F8, F9, F12, F32, F52, PF6, PF7, PF8.

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*Numbers in parentheses are cultures used for preparation of filtrates.

Subgroup IB (10 strains): F7, F22, F26, F34, F35, PF2, PF4, PF12, PF13, PF14.

Subgroup IC (3 strains): F5, F6, F27.

Subgroup ID (5 strains): F49, F51, F63, F64, F66.

Bacteriophage group IA active against culture group 1a, only; IB against both 1a and 1c; IC against 1c; and ID against 1d.

Sensitive cultures of group 1 (17 strains):

Subgroup 1a (10 strains): H1-1, H1-5, H1-6, H1-7, H1-9, H1-11, H1-12, H1-13, H1-14, 459.

Subgroup 1c (3 strains): H1-2, H1-3, H1-4.

Subgroup 1d (4 strains): H1-8, H1-15, E8-1, FH8.

Most members of culture groups 2-7 and some of of the ungrouped cultures were not sensitive to group I bacteriophages.

Heat inactivation at pH 7.0 in skimmilk at 70°C.: PF8 in 75 min., PF2 in 50 min. and PF13 in 45 min. All belong to the most heat resistant group.

Median plaque diameter: PF2, 1.32 mm.; PF8, 1.21 mm.

Sources of bacteriophage strains: England (London), F52; New Zealand, F63, F64, F66; and United States (Iowa) all others.

Group II

Serological basis: Antiphage sera R2, R5 and R10 prepared with bacteriophage strains PF11(H1-10), F43(122-1) and F62(K2) exhibited cross-neutralization of the three strains and also neutralized other members of this group but not most members of groups I and III-VII. The three sera also neutralized F56 of group IV and serum R10 neutralized PF8 of group I.

Bacteriophages of group II (12 strains): F1, F10, F11, F43, F44, F46, F47, F48, F62, F76, PF10, PF11.

Sensitive cultures of group 2 (12 strains): H1-10, 122-1 to 5, 146-1 to 5, K2.

Most members of culture groups 1 and 3-7 and some of the ungrouped cultures are not sensitive to group II bacteriophages.

Heat inactivation at pH 7.0 in skimmilk: PF11, 3 min. at 75°C., 65 min. at 70°C. and about 600 min. at 65°C.; F62, 12 min. at 75°C. and 120 min. at 70°C.; F43, 50 min. at 70°C.; F70, 50 min. at 70°C.; and F44, 45 min. at 70°C. All belong to the most heat resistant group.

Median plaque diameter: F43, 1.32 mm.; PF11, 1.10 mm.; and F62, 1.10 mm.

Sources of bacteriophage strains: New Zealand, F62; and United States (Iowa) all others.

Group III

Serological basis: Antiphage serum R12 prepared with bacteriophage strain F24(W2) exhibited specific neutralization and also neutralized other members of this group but not members of groups I, II and IV-VII. No neutralization of bacteriophages in other groups or by antisera prepared from bacteriophages of other groups.

Bacteriophages of group III (3 strains): F20, F21, F24.

Sensitive cultures of group 3 (3 strains): W2, W4, W8. Most members of culture groups 1, 2 and 4-7 and some of the ungrouped cultures are not sensitive to group III bacteriophages.

Heat inactivation at pH 7.0 in skimmilk: F24, 10 min. at 62.5°C., 45 min. at 60°C. and 140 min. at 57.5°C.; F20, 30 min. at 60°C.; F21, 50 min. at 62.5°C. and over 200 min. at 60°C. All belong to the lowest heat resistant group.

Median plaque diameter: F24, 0.33 mm. or smaller.

Sources of bacteriophage strains: All from the United States.

Group IV

Serological basis: Antiphage serum R6 prepared with bacteriophage strain F56(712) exhibited specific neutralization and also neutralized other members of this group but not most members of groups I-III and V-VII. Bacteriophage strain F56 also was neutralized by sera of groups I, II and V-VII, but the bacteriophages of these groups were not neutralized by the R6 antiserum.

Bacteriophages of group IV (3 strains): F56, F58, F67.

Sensitive cultures of group 4 (3 strains): 712, HP, 144F. Most members of culture groups 1-3 and 5-7 and some of the ungrouped cultures are not sensitive to group IV bacteriophages.

Heat inactivation at pH 7.0 in skimmilk: F56, 3 min. at 70°C. and 30 min. at 65°C., F58 in 35 min. and F67 in 65 min. All belong to the medium heat resistant group.

Median plaque diameter: F56, 1.21 mm.

Sources of bacteriophage strains: England (London), F56; England (Reading), F67, and New Zealand, F58.

Group V

Serological basis: Antiphage sera R3 and R9 prepared with bacteriophage strains F70(5) and F75(6) exhibited cross-neutralization of the two strains but not most members of groups I-IV, VI and VII. The two sera also neutralized F56 of group IV and F62 of group II and the R3 antiphage serum neutralized PF8 of group I.

Bacteriophages of group V (2 strains): F70, F75.

Sensitive cultures of group 5 (2 strains): 5, 6.

Most members of culture groups 1-4, 6 and 7 and some of the ungrouped cultures are not sensitive to group V bacteriophages.

Heat inactivation at pH 7.0 in skimmilk: F70 in 10 min. at 70°C. and 90 min. at 65°C.; F75 in 15 min. at 70 and 120 min. at 65°C. All belong to the medium heat resistant group.

Median plaque diameter: F70, 1.43 mm. and F75, 1.43 mm.

Sources of bacteriophage strains: Canada (Quebec).

Group VI

Serological basis: Antiphage serum R1 prepared with bacteriophage strain F69(ML1-1) exhibited specific neutralization but not most members of groups I-V and VII. The serum also neutralized F56 of group IV.

Bacteriophages of group VI (1 strain): F69.

Sensitive cultures of group 6 (1 strain): ML1-1.

Most members of culture groups 1-5 (except the H1-1 series and 459) and 7 and some of the ungrouped cultures are not sensitive to group VI bacteriophages.

Heat inactivation at pH 7.0 in skimmilk: F69, 55 min. at 70°C. This strain belongs to the most heat resistant group.

Median plaque diameter: F69, 1.54 mm.

Sources of bacteriophage strains: New Zealand

Group VII

Serological basis: Antiphage serum R11 prepared with bacteriophage strain F68(IP5) exhibited specific neutralization but not most members of groups I-VI. The serum also neutralized F56 of group IV and F62 of group II.

Bacteriophages of group VII (1 strain): F68.

Sensitive cultures of group 7 (1 strain): IP5.

Most members of culture groups 1-6 (except the 122-146 series) and some of the ungrouped cultures are not sensitive to group VII bacteriophages.

Heat inactivation at pH 7.0 in skimmilk: F68 in 15 min. at 70°C. and 130 min. at 65°C. This strain belongs to the medium heat resistant group.

Median plaque diameter: F68, 1.54 mm.

Source of bacteriophage strains: England (Reading).

In establishing these seven groups the serological characteristics of the bacteriophage were considered the primary basis of classification, while the activities measured by use of cross-reaction testing methods were used for confirmation purposes. In the serological testing quantitative measurements of the degree of neutralization were made. Neutralization of bacteriophage by homologous sera showed titer decreases considerably greater than 3.0, which was the minimum considered significant in this work. Unknown filtrates which subsequently were classified in the same group also generally showed titer decreases greater than 3.0, but there were borderline cases in which some difficulty was encountered in establishing whether the degree of reaction for a particular combination in question was significant for group classification. Also, there were several instances in which apparently unrelated bacteriophages and sera reacted, as has been pointed out in Table 14. In these cases one bacteriophage strain was neutralized by one or more apparently

unrelated sera, while the bacteriophages used to produce these sera were not, in turn, neutralized by the antiserum of the first strain. These reactions suggest that certain strains have partial common antigenic characteristics. Such characteristics prevent complete separation of various bacteriophages into distinct serological groups. Techniques for segregation of possible antigenic components were not employed. To lessen the possible influence of these partial reactions, the bacteriophages generally were tested against all 12 available sera. First the 12 strains (which included one duplicate) used to prepare the sera were tested to establish the serological patterns to be used as the basis for classification. A group was established on the basis of complete cross-neutralization of all members of the group. Each member was expected to produce a serum which in turn reacted with every member of the group before it was included in that group. When an unknown filtrate was neutralized, for example by sera R2, R5 and R10, and not affected by the other nine sera, all 12 reactions contributed to the final decision to place the bacteriophage strain in group II. Groups I and II had three members each and group V had two members, in which complete cross-neutralization was found. However, the other four groups (III, IV, VI, VII) each contained but one serum prepared from one series of bacteriophage injections which was used

to establish a possible group.

In each of these four cases the individual serum did not cross-neutralize most of the bacteriophages of any other groups. The different characteristic serological patterns exhibited by these four sera formed the basis of four additional serological groups.

Group III was entirely specific, serum for this group not reacting with any other bacteriophage group and the bacteriophage strains of this group not being affected by any of the other sera tested. Both group VI and VII sera neutralized group IV (F56) bacteriophage, but since serum prepared from strain F56 was strain specific, all three groups could be differentiated on that basis, since no common cross-neutralization reactions were demonstrated.

When the various strains were tested serologically with the 12 available sera, those exhibiting similar patterns were considered belonging to the same group. In this way 51 bacteriophage strains could be placed into the seven established groups. In general, the serological method of grouping did not show as many inconsistencies as the cross-reaction testing method and the data obtained in the serological studies was more quantitative. Serological differentiation only appeared to be reliable as one criterion on which to establish various groups.

In addition to classification, the serological method of testing bacteriophage filtrates has been used in three additional experiments with satisfactory results. In one case, the question of purity required further clarification. Some filtrates of questionable purity were purified by single plaque isolations. Both the original and purified filtrates then were subjected to the action of the 12 available antisera. In two cases (F68 and F75) single plaque isolations produced filtrates which exhibited serological patterns different from those shown by the original filtrates. In the seven other filtrates (Table 17) no further change in the serological patterns was effected by single plaque isolations.

In a second experiment a similar use of the serological method of bacteriophage differentiation was used to determine whether small and large plaque isolations resulted in different serological types. The three strains considered each produced exactly the same serological reactions which defined the group to which they belonged.

In a third experiment, two bacteriophage filtrates of the same origin but grown in different types of media were tested for differences. They were shown to be of the same serological group. In general, the serological test method of strain identification is a definite and accurate method of measurement. The various bacteriophages could be

identified clearly and shown to be either related or not related. Apparently, the serological method eventually will find a wide variety of uses in bacteriophage identification and classification.

In cross-reaction studies, possible grouping of some strains of bacteriophage as well as their differentiation from other groups was suggested. Several inconsistencies have been noted, but generally the strains placed within a given group exhibited similar cross-reaction patterns. The qualitative method used for cross-reaction determinations of simply placing one drop each of bacteriophage and culture in litmus milk and observing culture inhibition, even when using the best established technics, did not always show consistently reproducible results. Since the single reactions were somewhat inconsistent when a number of test organisms were used, the resultant patterns also could not be reproduced consistently. The pattern determinations were repeated several times and only those individual combinations of the patterns which showed consistent results, or definite tendencies toward reproducibility, were used for establishing group relationships.

When an unknown filtrate was active against all cultures of a given group and was not active against representative cultures of other groups on several occasions of testing, the resulting patterns, in a general way, indicated the

activity of the bacteriophage was specific for a given culture group. There were exceptions to this generally specific action. F68 (group VII) was active against the 122-146 cultures of group 2 and F69 (group VI) was active against the H1-1 series and 459 of group 1a. Filtrate F52 was found to belong to serological group I and was active against the H1-1 series of cultures in addition to 459, its homologous culture, yet the other filtrates of group IA did not lyse culture 459. Similarly, F62 lysed the 122-146 series of cultures of group 2, yet the group II bacteriophages did not lyse K2, the homologous culture of F62.

In general, the bacteriophage strains of the seven serological groups were specific for the cultures of their respective groups. This general strain specificity further tends to limit the value of cross-reaction determinations for group classification of bacteriophages, such as the case of group IV in which the three bacteriophage strains belonged to the same serological group and yet were specific in their activity upon cultures. Also, all filtrates in group I were not active against all group 1 cultures; the specificity in this case was used to further subdivide the bacteriophages and cultures beyond divisions established by serological means. The cultures of group 1 appeared to fall into four distinct subgroups in respect to their

sensitivities toward the group I bacteriophages. This subdivision was based entirely upon cross-reactions with lactic streptococci of various sensitivities. In this case the cross-reaction procedure was more sensitive for maximum separation of bacteriophage strains than was the serological method of differentiation.

There was good correlation of the groups established by serological methods with the general cross-reaction patterns exhibited by the members of the groups. It is recognized that the cross-reaction method of bacteriophage identification has been the most widely used and undoubtedly will continue to play an important part in identification of lactic streptococcus bacteriophages. However, because of the inability to obtain consistently identical patterns and a general tendency toward strain specificity, when used alone the cross-reaction method is not considered a satisfactory method of classification. When used in conjunction with serological grouping studies, the combined methods form a means of differentiating various culture and bacteriophage strains.

Several of the ungrouped bacteriophage strains had wide ranges of activity which suggested the possibility of their being mixed filtrates because they were active against cultures of several groups. Since no logical grouping could be made and no satisfactory relationship to the other

groups could be established, they necessarily were placed in the "ungrouped" category. The fact that they could not be classified by the cross-reaction procedure used here to group all of the available strains further shows that this procedure is of definitely limited scope.

The ungrouped bacteriophage strains were so designated because they did not fit well into either the serological or cross-reaction patterns. Several were strain-specific in their activity toward host cells, while others exhibited rather wide ranges of activity. Their varied cross-reaction patterns alone were insufficient basis for establishing groups. Serological testing showed that the ungrouped strains were not sensitive to any of the available sera that had been prepared by use of other bacteriophage strains. No antisera were prepared for these ungrouped strains. By using the ungrouped strains as antigens in preparing additional sera, further grouping might be possible.

The use of heat inactivation studies for establishing bacteriophage groups is of limited value. The seven groups established by serological and cross-reaction testing could not be completely distinguished from one another by their differences in resistance to heat. The inactivation curves have shown the presence of some heat resistant particles in a given filtrate, but the point of complete inactivation was found to be nearly the same for the members of a given

group. However, all strains from the most heat resistant to the most sensitive were inactivated within a range of about 12°C. for a given time of heating. The various strains could be identified as of high, medium or low resistance within that range, but more exact strain differences could not be shown. Of the seven groups established by serological and cross-reaction tests, three groups were in the upper heat range, three groups in the middle range, and one group in the lower range. In those cases where the members of several groups occur in the same general heat range it is doubtful whether they could be further separated by more accurate heat inactivation studies.

The plaque sizes were of less value in bacteriophage differentiation than the serological, cross-reaction and heat inactivation characteristics discussed above. In general, most of the strains exhibited almost the same size plaques. However, F24 (group III) consistently produced characteristic small size plaques which clearly distinguished it from the others. This characteristic correlated with the low heat resistance of this group. In some cases, such as this one, the plaque size information may be of value in differentiation but, on the whole, it does not appear to be of much value for group classification.

With respect to morphology, the nine bacteriophages reported by Parmelee et al. (1949) included representative

members of five of the seven groups proposed here. The strains and groups (the latter given in parentheses) included F22(IB), F66(ID), F43(II), F67(IV), F70(V), F68(VII) and also F54, F57 and F72 belonging to the ungrouped series. Since that time, Parmelee (1949) has examined with the electron microscope representative members of the other two groups, including F69(VI) and F24(III), as well as additional members of other previously examined groups, including PF2(IB), F56(IV), F68(VII), F70(V) and F75(V). Examination of these additional bacteriophage filtrates has confirmed the earlier observations that the various strains apparently are so nearly alike in size and shape that they could not be differentiated satisfactorily on the basis of morphology.

The source of the bacteriophage strains was of some value in identification of the members of the various groups. Twenty-five of the group I filtrates have been isolated at Iowa State College since 1945 and are active against H1-series cultures. Group I also contained four filtrates from foreign countries. Group II contained 11 filtrates isolated at Iowa State College on 122 and 146 cultures while only one (F62) was from New Zealand active against culture K2. All three members of group III were isolated at Iowa State College. The two in group V both came from Quebec, Canada. In group IV each of the three

strains came from a different source. Most of the bacteriophages known to be of the same source and isolated on related cultures at about the same time generally showed identical serological patterns when tested with the twelve antisera. The cross-reaction patterns also were similar. In general, there was good correlation of the groups established by serological and cross-reaction methods with the origin of the bacteriophage strains. However, exchange of filtrates and cultures between investigators, as well as widespread distribution of closely related culture strains by commercial culture laboratories, has tended to obscure the original sources of the strains against which the bacteriophages are active.

Some information concerning whether the cultures used in this study were S. lactis or S. cremoris was available. Parmelee (1949) found that cultures W2, W4 and W8 produced ammonia from peptone but did not grow at 40°C., in four per cent sodium chloride or at pH 9.2. Cultures 565 and 712 grew at 40°C. but were negative for the other three tests. Thus these five cultures failed to show positive reactions for all of the four tests which, according to Sherman's (1937) classification, are characteristic of S. lactis.

Collins (1949) reported cultures 712, 565 and W2 as S. lactis and considered 22 other cultures, all of which also have been used in this study, to be S. cremoris.

He had included W4 in the S. cremoris group but later data (Parmelee, 1949) have shown that of 15 single colony isolates from W4, 11 produced ammonia from peptone, one grew at pH 9.2, and none of the 15 grew in four per cent sodium chloride or at 40°C.

Difficulties have been encountered when positive identification of these strains have been attempted because "intermediates" occur which do not agree consistently in all reactions. However, it appears that cultures W2, W8, 565, 712, and possibly 5 and W4, tested more nearly like the wild S. lactis, while all others used in this study may be considered S. cremoris by their differential reactions.

The bacteriophage strains active against W2, W4 and W8 all belonged in bacteriophage group III. Filtrate F54, active against culture 565, did not fall in any of the seven groups. Filtrate F56, active against culture 712, belonged to group IV, to which also belonged F58 and F67, both active against S. cremoris cultures. This was the only instance in which the filtrates active against both S. lactis and S. cremoris species were placed in the same group. Possibly over a period of time some of the cultures may have changed in their reactions to the differential tests while the bacteriophages may have retained permanently the same antigenic characteristic which has resulted in their being placed in the same serological group.

It has been suggested by Hunter (1946), Nichols and Hoyle (1948), Collins (1949) and Parmelee (1949) that the lactis types are gradually transformed to the more fastidious cremoris types during several years of laboratory cultivation. Hunter (1946) also had shown a general lack of specific bacteriophage action against the S. lactis organisms in marked contrast to definite tendencies toward strain specificity in bacteriophage strains which attack S. cremoris organisms

The apparent differentiation based solely on cross-reactions of the various culture group 1 subgroups to the serologically homogeneous bacteriophage group I may possibly be due to an adaptation in the culture susceptibility. Whether the change in reaction is due to a change in character of the organism or the bacteriophage has not been determined. Further studies concerning variations or possible mutations of both bacteriophages and cultures in respect to each other would be of considerable value. However, to show whether any variations or mutations occur in the bacteriophage has needed a stable method of measurement to which all subsequent variations could be compared. Perhaps serological testing may now offer this needed method of measurement.

Nichols and Hoyle (1948) isolated some culture strains which did not react with diluted filtrates active against

other cultures, but they were able to obtain bacteriophages for these "resistant" cultures by addition of undiluted phage suspensions. If any lysis appeared when "neat" bacteriophage was applied to previously unattacked cultures growing on agar, the new bacteriophage was propagated on that strain. By this method they were able to isolate bacteriophages active against a majority of 116 cultures for which no bacteriophage previously had been isolated. They did not establish whether an adaptation of the bacteriophage had taken place, since purity of their undiluted filtrates was not established, but considered this only a method for obtaining active bacteriophage strains for apparently resistant cultures.

It seems desirable to compare the number of suggested groupings reported in this investigation with the number of groupings reported for other bacteriophages. Burnet (1933) established 12 groups for the dysentery-coli bacteriophages from 48 strains, five of which were not neutralized by the available sera. The bacteriophages active against hemolytic streptococci have been classified into five serological groups by Evans (1934) and Evans and Sockrider (1942). The seven "T"-system E. coli bacteriophages belong to four serological groups (Delbrück, 1946). Rountree (1948) established six serological groups from 31 staphylococcal bacteriophages.

The classification of the bacteriophages active against the lactic streptococci in general compares favorably with studies on other bacteriophages. In this investigation 51 out of 66 bacteriophages have been classified into seven groups, while the taxonomic positions of 15 remaining strains were not established completely, since none of the available sera had any neutralizing effect on these 15 filtrates. When further purified and tested possibly some also may belong to these seven groups while others may form the basis for additional groups.

CONCLUSIONS

1. When the bacteriophages are cross-reacted for activity against various lactic streptococcus cultures, the resulting patterns may be used in a general way to identify similar strains of bacteriophages.

2. Cross-reactions of bacteriophages and cultures are not entirely reliable for classification when used as the sole basis of differentiation, but become more significant when considered in conjunction with other differential tests, especially serological reactions with which a relatively good relationship has been shown.

3. The plaques of the various bacteriophage types under standardized conditions are approximately the same general size, which limits their value as a means of classification. For comparative purposes plaque size determinations are of value for recognizing strains producing characteristic larger or smaller plaques.

4. There was no general correlation of plaque sizes with any other general characteristics of bacteriophages, except one bacteriophage (F24, group III) exhibited unusually small plaques and also was quite readily inactivated by heat.

5. Inactivation of bacteriophage progresses rapidly during the initial stages of heat treatment, but presence of a number of resistant particles in the population prevents a linear relationship between the logarithm of survivors and time of heat treatment.

6. Various strains of bacteriophages exhibit differences in heat inactivation ranging from 75°C. for 12 minutes down to inactivation by exposures no more rigorous than normal pasteurization.

7. Heat resistance studies may be used to distinguish bacteriophage groups of high, medium and low resistance; such groups showed a general correlation with the groups established by serological and cross-reaction studies.

8. The bacteriophage strains active against S. lactis and S. cremoris are capable of inducing the formation of antiphage antibodies in rabbits.

9. A measurable bacteriophage-antiphage neutralization reaction can be demonstrated by reacting high titer bacteriophage preparations and immune sera, using the resultant decrease in the bacteriophage activity as a measure of the antiphage activity.

10. Serological cross-neutralization testing of various bacteriophage groups is a satisfactory method for differentiation and classification of lactic streptococcus bacteriophage strains.

11. Although the work of others has indicated that bacteriophages active against the lactic streptococci all have similar morphological characteristics, the present study shows that the antigenic characteristics, patterns of activity against selected test organisms, differences in heat inactivation and variations in plaque diameters permit differentiation and grouping of various bacteriophage strains. Some degree of correlation exists between these characteristics and thus these characteristics provide bases for classification of bacteriophages active against lactic streptococci.

SUMMARY

A number of bacteriophage filtrates active against Streptococcus lactis and Streptococcus cremoris cultures have been isolated at the Iowa Agricultural Experiment Station or acquired from New Zealand, Quebec and England (Reading and London). An investigation of some of the characteristics of these bacteriophage strains has been undertaken with a view toward possible classification. They have been characterized on the basis of (1) cross-reaction activity, (2) sizes of plaques, (3) serological activity and (4) heat resistance. Morphological data, based upon electron micrographs, has been available.

Cross-reaction studies with the various bacteriophages and host cultures have indicated the existence of several differentiable types. A method of serological grouping was developed, based on the specificity of the antigenic reactions exhibited by the different bacteriophage groups. When 12 prepared antiphage sera were cross-tested against various unknown bacteriophage strains, specific cross-neutralization of the bacteriophage activity was exhibited by serologically related strains of bacteriophage. Most of the different types of bacteriophages could not be differentiated on the basis of plaque sizes which was of value primarily for

comparative purposes. The thermal inactivation of the various types was sufficiently varied for differentiation.

A direct relationship of the cross-reaction and serological groups was found. Plaque sizes and thermal inactivation served as additional criteria by which the identity of the various groups could be established. The combined use of these four characteristics proved satisfactory for differentiation of the various bacteriophage groups and appeared to offer a satisfactory basis for systematic classification. Of the 66 strains of bacteriophages studied, 51 of them have been classified into seven groups, with the 15 remaining strains tentatively being considered as unclassified.

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