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A study of a factor inhibiting the growth of Streptococcus lactis

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A STUDY OF A FACTOR INHIBITING THE GROWTH OF *STREPTOCOCCUS LACTIS*

by

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for the Degree of

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INTRODUCTION

The cultures commonly used in the manufacture of butter, cheese and cultured milk occasionally suffer a decrease in vitality with an attendant increase in the time required for the development of the desired flavor, aroma and acidity. Such cultures are commonly designated as "slow". If the culture fails to produce the desired change in the usual period of time, the normal sequence of plant operations is disrupted, resulting in serious loss of man- and equipment-hours. The longer periods of incubation necessary to obtain the desired acidity when a slow culture is encountered frequently permit the development of defects which would normally be restricted or prevented by the rapid development of the culture organisms. These defects may involve the body, texture, flavor and aroma of the finished product and, in some cases, the quality of the product may be so affected as to make it unsaleable.

Slow cultures are undoubtedly the result of a variety of conditions. In many cases the slow development of acid and flavor is caused by unsatisfactory methods of handling the cultures, which result in improper ripening or in contamination by extraneous organisms. Careful examination of the cultures and of the methods used in their propagation will usually reveal the cause of difficulties of this type without the expenditure of any considerable amount of time and effort. In other cases, even very careful study does not reveal the cause of the delayed acid production by the cultures, and more searching methods of investigation must be used.

Certain slow cultures are characterized by the very sudden or explosive manner in which they appear. Although a culture may be normal in all visible respects and coagulate in the normal period of time, a

transfer from it may require a ripening period of several hours greater duration than is usual. This type of delayed acid production is especially characteristic of situations in which large quantities of culture are ripened in one container in the plant, but the same phenomenon occurs occasionally in the laboratory among the mother cultures which are being carried in small bottles or flasks.

Recent studies of various investigators have shown that in some instances the causative agent of the slow coagulation appearing suddenly in cultures is apparently an ultrafilterable principle.

STATEMENT OF THE PROBLEM

The purpose of the studies reported herein was to isolate and characterize the ultrafilterable principle having an inhibitory effect upon the development of acidity by butter cultures and to study the relationships between the principle and the organisms characteristically found in butter cultures. For the sake of convenience, three subdivisions of the problem have been recognized, as follows:

Part 1. The inhibitory principle and its action upon the organisms which occur in butter cultures.

Part 2. The effect of chemical and physical agents upon the inhibitory principle.

Part 3. The characteristics of the sensitive and non-sensitive strains of Streptococcus lactis.

HISTORICAL

General observations on slow butter cultures and slow cultures of *Streptococcus lactis*

The literature contains frequent references to the slow development of acid by butter cultures. In 1915 Singleton (133) recommended the frequent replacement of old cultures with new ones, since cultures carried in the plant tended to become slow and require the use of excessive inocula.

Whitehead and Cox (144) suggested a method for the investigation of the vitality of various starters by comparing the acidities developed in whey obtained by a method simulating, to some extent, the process used in the manufacture of cheddar cheese. They pointed out that the test was merely a comparison between starters, but that it will usually detect slow butter cultures except those of the type characterized by a marked loss of vitality during the course of a single transfer. "Non-acid" milk could also be detected by this method, a normal starter being used to test a series of samples of milk.

A procedure for the detection of strains of lactic acid streptococci to be used in the making of cheese cultures has been devised by Whitehead and Cox (149). They pointed out that, since the curd is heated to 37° to 39° C. during the manufacturing process, the organisms used should be capable of producing a good clot at 37° C. as well as at 21° C. This method of selection proved satisfactory in the majority of cases, although some of the cultures chosen for their ability to grow at 37° C. would, upon occasion, suddenly become slow when put into use in the plant, the explosive type of slow culture resulting.

Kelly (73) found that there was some variation among starters with respect to their ability to grow in milk from a given source. Using one group of six butter cultures he obtained flat and yeasty cultures with

milk from one supply, while other butter cultures developed satisfactorily in milk from the same source. Using a second milk supply, the first group of cultures developed normally.

Moir (114) pointed out that there was considerable variation among the strains of Streptococcus lactis and Streptococcus cremoris in their ability to develop acid in a given supply of milk.

The milk substratum as a cause of slow butter cultures

Many of the early investigations of slow butter cultures were concerned with the influence of the milk upon the coagulation time of the cultures being studied. Baker and Hammer (7) found that milk containing a relatively high percentage of total solids tended to give cultures with comparatively high total acidities, although there were numerous exceptions to this general rule. As would be expected, less variation in acid production was found between lots of mixed herd milk than between lots of milk from individual animals. There was no parallelism between the amounts of acid developed in the butter cultures studied and the flavor scores of the individual cultures. Cultures made with normal milk were superior to those carried in normal milk to which milk ash, lactose, cream or water had been added. These workers concluded that there might be a noticeable difference between the scores of cultures made from various lots of milk with the same source of inoculum.

Knudsen and Sörensen (80) reported an instance in which the milk from a normal cow permitted only a definitely subnormal acid production by several strains of Streptococcus cremoris. Other strains of the same species produced a normal fermentation in the milk. Strains of organisms showing a normal acid production appeared as typical streptococci when

grown in the milk in question, but the abnormal strains showed a tendency toward rod and swelled forms in the atypical milk, although they appeared normal when grown in milk from other sources. The reduction in acid formation was not due to a lack of buffering action, since normal milk gave the usual amount of acid production when diluted with water to make the buffering action and nitrogen content the same as that of the abnormal milk. The feed of the cow was not a factor, since her stall mates all produced normal milk. The addition of blood serum to the abnormal milk had no effect, but the addition of 0.005 per cent of yeast autolysate, one per cent of hydrolyzed casein or 5 to 10 per cent of spinach juice made the milk suitable for the growth of these sensitive organisms which indicates a possible lack of some food accessory in the milk. One organism showed this characteristic of sensitivity with 8 out of 20 milk samples from different sources, while other lactic acid organisms showed a similar behavior but to a lesser degree.

Knudsen and Sörensen (81) noted that some milk was not as good as other milk for the growth of Streptococcus, apparently because the high bactericidal effect prevents the growth of other organisms and thus reduces the nitrogen supply for the culture organisms. Aseptically drawn milk was also considered poor because there had been no chance for the growth of proteolyzing bacteria. Streptococcus cultures were found to vary in their abilities to grow under the hypothesized condition of lack of protein. The same strains that were inhibited by raw milk were also inhibited by blood serum, and the inhibitory effect of both substances was removed by sterilization. The action of the serum was removed by heating to 63° C. for one hour. In a second article (82), the addition of autolyzed yeast, milk digested by molds, hydrolyzed milk or one of several similar sources of nitrogen, overcame the unfavorable effects of

the milk which previously had not satisfactorily supported the growth of Streptococcus cremoris.

Knudsen (79) stated that variations might occur between subcultures of the same butter culture grown in different lots of milk, these variations being especially characteristic of the milk from individual cows, although pasteurized milk from a number of different herds might show this difference also. Sometimes the culture was useless the first time it was grown in abnormal milk of this type, but ordinarily the deficiency only became evident after 2 or 3 serial transfers. Knudsen pointed out that an abnormally low solids content might cause difficulty due to slow coagulation because of the decreased acid production which might occur.

Moir (115) also pointed out that milk with a low solids content might be a cause of difficulty in starter management. He recommended the use of a curd test, comparing several samples of milk by the use of one active culture. When a herd had been incriminated as a source of unsatisfactory milk, he recommended that the test be made upon the individual animals in the herd in order to eliminate those responsible for the defect in the supply.

Harriman (64, 65) concluded that the source of the milk for the carrying of butter cultures, whether from individual cows or various herds, had no significant effect upon the coagulation time of butter cultures or of pure cultures of Streptococcus lactis used for testing purposes.

Whitehead and Cox (146) stated that "the rates of acid production in the rennet curds prepared from the milks of individual healthy cows varied to a significant extent from animal to animal and from day to day"; milk with high solids gave slower acid development but higher final acidity.

Milk from cattle suffering from mastitis has frequently been incriminated as one of the more important causes of slow butter cultures. Leitch (93, 94) pointed out that milk from cows suffering from mastitis could be inhibitory in very small amounts; a case was cited in which one pint of mastitis milk in 120 gallons of normal milk proved to be inhibitory. He stated that such milk might cause a culture to lose vigor very rapidly, one transfer upon such a substrate frequently having a lasting ill effect. Under some conditions, the use of 1.5 to 2 per cent of starter instead of the customary 0.25 to 1 per cent may help considerably in bringing about a normal development of acid in the cheese curd. Pure butter cultures were usually more sensitive than were cultures containing contaminants, but the risk of using impure starters was considered to more than offset any advantage arising because of increased resistance.

Moir stated (114, 115) that mastitis among the members of the herd supplying starter milk or cheese milk might frequently be the cause of slow cultures for cheese making, and Davis (32) also listed mastitis milk as a cause of this difficulty and pointed out that only fractional percentages of such milk might have a detrimental effect. Davis recommended testing the milk supply at frequent intervals with brom cresol purple paper for the purpose of detecting abnormal supplies.

Whitehead and Cox (146) observed that 5 million or more body cells per milliliter of raw milk inhibited the growth of lactic acid cultures, probably through phagocytosis. The inhibitory action was prevented by heating to 49° to 52° C. for 30 seconds. The cows producing milk of this type frequently were not recent sufferers from mastitis. In milk in which the number of body cells did not exceed one million, the acid development was usually greater when the number of cells present approached one million, possibly because of a stimulating substance whose secretion was

governed by the same factors which governed the presence of cells and which was stable at a temperature of 63° C. for 30 minutes.

The germicidal property of milk

The presence of a germicidal substance in most samples of freshly drawn milk is now definitely recognized by most of the workers in the field of dairy bacteriology. Heineman and Glenn (67) concluded that Streptococcus lacticus showed no lag phase caused by the germicidal property of milk, but increased from the time of inoculation if a culture in a state of active growth was used. The growth of the organism was progressively more rapid when the milk had been heated to 56°, 75° and 100° C. and then cooled prior to inoculation. Sherman and Curran (132), using fresh milk kept at 37° C. in a water bath and sterilized milk incubated at 37° C. together with 3 hour cultures of Streptococcus lactis in the logarithmic growth phase, reached the conclusion that there was no lag phase in the sterile milk but that there was usually a lag phase of about half an hour in the raw milk, rapid growth following this period.

Leitch (93) believed that slow-working curds in cheese making were usually not due to the bactericidal action of normal fresh milk.

Hammer and Baker (62) found that the heating of milk to 160° F. (71.1° C.) or above instead of the usual 145° F. (62.8° C.) for 30 minutes considerably reduced the time required for the coagulation of a culture carried in the milk; the difference amounted to 6 or 8 hours in extreme cases. The use of 150° F. (65.5° C.) instead of 145° F. (62.8° C.) resulted in a smaller but frequently considerable reduction in the time required for coagulation, the speed of coagulation at 150° F. (65.5° C.) being somewhat less than that at 160° F. (71.1° C.) or 180° F. (82.2° C.). They suggested that this effect might have been due to the destruction

within this range of the germicidal properties of milk. Possibly this phenomenon could be partially explained by the slight hydrolysis of lactose and protein which takes place at the higher temperatures and provides the organisms with a more readily available food supply.

Pasteurization at 71° C. for 30 minutes with culture milk was as satisfactory as heating at 82° C. for the same period, according to the data of Farmer and Hammer (45). They found that 6 of the 8 butter cultures studied by them were delayed in their development of acidity when grown in raw milk or milk pasteurized at either 62.5° C. or 68° C. for 30 minutes. Two of these cultures failed to coagulate raw milk even after very extended holding periods. Their results were considered to indicate that there were considerable differences between the abilities of butter cultures to withstand the germicidal property of milk.

Harriman (64, 65) found that pasteurization at 82° C. instead of 63° C. for 30 minutes reduced the coagulation time with butter cultures as much as 6 hours, although the reduction was much less in most cases.

Apparently the germicidal characteristic of milk seldom has sufficient effect upon the growth of Streptococcus lactis to cause characteristic slow acid development by that organism or by butter cultures containing Streptococcus lactis. With the present methods of culture propagation, the temperatures used for pasteurization of the milk would seem to be sufficiently high to cause the inactivation of any inhibitory principle of the usual germicidal nature.

The effect of contaminating organisms in butter cultures upon the rate of acid formation

Numerous studies have been made on the effect of contaminating organisms in the cultures or in the milk upon the rate of coagulation of butter cultures, and special attention has been given to their possible

relationship to slow cultures. Occasional butter cultures, which were of very satisfactory quality but which contained contaminating organisms, sometimes in considerable numbers, have been reported by Farmer and Hammer (45).

Baker and Hammer (7) concluded that contaminating organisms resisting pasteurization were apparently not factors in determining the quality of starters under the usual conditions encountered in practice, since there was no correlation between the score of the milk used and the score of the starter grown in the milk.

According to Leitch (93, 94), slow-working curds were usually not due to contaminating organisms. Bacillus subtilis and Bacterium coli, two of the common contaminants, were mentioned specifically as being without effect upon the speed of coagulation.

Harriman (64, 65) found that 6 unidentified cultures of bacteria from raw milk had no significant restraining effect upon the lactic fermentation. Contamination from plant equipment was also demonstrated as not being a factor under the circumstances of the experiments.

Moir (114) listed contaminating organisms which might restrain the lactic acid bacteria as one of the causes of slow starters but did not mention any special group or individual species which might have such an effect.

Using a series of gram negative organisms from milk, cow dung and human feces and two cultures from the National Type Collection (Bacterium lactis aerogenes and Bacterium acidi lactici), Whitehead (141) was unable to observe any appreciable change in the acid production of the butter cultures into which these organisms were inoculated to test their effects, especially when an active butter culture was used.

Cox and Whitehead (30) observed that Bacillus subtilis as a

contaminant in an otherwise apparently good butter culture accelerated acid production, while Bacterium coli caused an increase in the amount of acid formed in some cases and a decrease in others. A Staphylococcus had a slightly stimulative effect, and Bacterium faecalis alcaligenes had no appreciable effect.

At a later date Whitehead and Cox (145) studied the effect of the presence of Bacterium lactis aerogenes and two strains of Bacterium coli communis in a culture of a single strain of Streptococcus cremoris from a butter culture. Flasks inoculated at the same time with Streptococcus cremoris and one of the two members of the colon group showed a greater increase in acidity than did the controls. If the colon organism was allowed to grow at 30° C. for more than 16 hours before the inoculation of the milk with Streptococcus cremoris, giving a count in the hundreds of millions, the organism apparently produced some substance other than lactate and hydrogen ions which had a retarding effect upon the lactic fermentation. The inhibitory substance was more or less heat stabile, surviving exposure to 75° C. for one hour. In practice, the numbers of colon organisms sufficient to have an inhibitory effect of this type would almost never be encountered in milk.

Morgan and Curle (116) reported a case in which numerous subtilis-type organisms, probably gaining entrance to the milk as the result of plant contamination since they did not appear in any quantity in the milk from individual shippers, seemed to be the cause of the "death" of a butter culture within 2 or 3 days. Study in the laboratory showed that slight contamination of active starters with this organism had no effect, but that, if the organism once obtained a firm foothold, it would increase rapidly and the starter go "dead" in 4 or 5 days. The authors stated that Bacillus subtilis organisms gradually increased in

numbers until the starter appeared dead; then there was a rapid decrease in numbers until Bacillus subtilis seemed almost to have disappeared. After the decrease in numbers of spore forming bacilli, the Streptococcus cremoris organisms seemed to revive again and grow normally for a time, although isolated rods still appeared. These workers stated that "factory experience pointed to the probability that this was a regular cycle". Several lots of "slow" milk from various localities were examined and, with one exception, they were all contaminated to a fairly marked degree with organisms of the subtilis type. When starters contaminated with this type of organism were used in the manufacture of cheese, there was a rapid development of acidity while the curd was draining, followed by a retardation of acid production. The authors pointed out that there is another type of slow starter which, when used in cheese making, is characterized by slow acid development throughout the entire manufacturing process.

Porcher and Lambert (122) found that Bacillus subtilis inoculated into milk in considerable numbers a short time before the addition of Streptococcus lactis favored acid and curd formation, probably because of protein degradation that favored the growth of the latter organisms. Under these conditions, Streptococcus lactis gradually overgrew the bacilli.

Slow acid production, sometimes in spasmodic outbursts which usually occurred during the warm months in one out of 3 or 4 vats of milk used for cheese making, was traced by Whitehead and Riddet (151) to the milk from one producer who was furnishing the factory with about 200 gallons of milk each day. This milk was found to give almost no increase in acidity with the factory culture, the evening milk being especially pronounced in this characteristic. A gram positive coccus, indistinguishable from the lactic streptococci, was found to be present in large

numbers and further study showed that milk in which this organism had grown was inhibitory both when left raw and when pasteurized following the growth of the organism in question; the inhibition was proportional to the amount of growth of the Streptococcus. The inhibiting substance produced during growth by this organism was quite heat stable, not being destroyed by a temperature of 212° F. (100° C.) for 30 minutes. Four or five days at 30° C. were required for the organism to coagulate milk, the resulting curd being of the normal lactic acid type. The only satisfactory means of control was to keep the infected milk so cold that the organism could not grow.

Whitehead (142) reported observing a sample of milk which allowed normal lactic fermentation when in the raw state but which would not coagulate with the same culture after pasteurization at 145° F. (62.8° C.) for 30 minutes. By plating this milk on yeast whey agar, an organism was isolated which differed from Streptococcus cremoris only in its ability to prevent milk coagulation after growth and subsequent pasteurization followed by the addition of a starter. Even when the organism was present in numbers approximating 200 million per milliliter, it caused no difficulty if the milk remained unpasteurized. Bacterium coli and Bacillus subtilis were not sensitive toward the inhibitory principle. Milk which would not reduce methylene blue in 4 or 5 hours did not contain sufficient numbers of this organism to be dangerous in the cheese factory.

Further studies on two streptococci which produced substances inhibitory to Streptococcus cremoris were reported by Whitehead (143) who found that they were both lactic acid streptococci that produced d lactic acid and grew only very slowly in the absence of a fermentable carbohydrate. The ability of each organism to ferment a variety of test substances was

determined. By following the growth and acid development of lactic acid organisms in milk in which these streptococci had grown, their action was shown to be inhibitory rather than bactericidal. One inhibitory principle, which required heating to activate it, was not stable at pH 8 and was markedly inhibitory against Lactobacillus acidophilus, while the other inhibitory principle, which did not require heating for activation, did not inhibit Lactobacillus acidophilus. The organisms producing these inhibitory substances had remained stable in this characteristic for 4 and 7 months, respectively, at the time the report was written.

Cox (29) outlined a procedure for detecting "non-acid" milk, especially that caused by the growth of organisms producing inhibitory substances. He recommended pasteurization of a 20 ml. sample at 145° F. (62.8° C.) for 10 or 15 minutes, followed by cooling to 37° C. Three drops of starter and 1 ml. of methylene blue solution, as used for the reduction test, were then added and the sample incubated at 37° C. Normal milk decolorized in 2.5 hours or less, while "non-acid" milk required a considerably longer period. The method was shown to be quite sensitive in detecting milk undesirable for the manufacture of cheese.

Examples and studies of explosive type outbreaks of slow butter cultures

Whitehead and Wards (152) reported the case of a cheese factory which had difficulty in obtaining satisfactory curd formation with a series of cultures; all of these came from the Dairy Institute but had been carried in the plant for various periods of time, some of them for several months. When the inoculum for a new 20 gallon culture was obtained from a lot inoculated from the mother culture into a 20 gallon can of pasteurized milk, the culture frequently failed, usually in the second generation grown in the large containers. When a 300 ml. normal

mother culture was used each day as inoculum for the larger batch of culture, a normal clot nearly always resulted. On two occasions, mother cultures transferred in 300 ml. quantities of milk pasteurized by holding in water at 200° F. (93.3° C.) for 30 minutes failed gradually and were replaced. As a result of this observation, the use of mother cultures set in milk cooled without agitation in small cotton-plugged flasks as inocula for the large cultures was suggested as a partial remedy for slow cultures of the type experienced in the plant in question.

Another rapid-type outbreak of slow starter was exhaustively studied by Whitehead and Cox (147). They found that a transfer from an apparently normal culture grown in the plant required 3 days at 20° C. to coagulate, while a culture from the same source of inoculum grew normally in the laboratory. A culture carried in the plant in a metal can holding 3 gallons of milk was slow after the second transfer, while one inoculated from the same mother culture into a gallon of milk in a flask gave a normal subculture, both batches of milk having been pasteurized by immersion in water at 200° F. (93.3° C.) for one hour. The 2 cultures were indistinguishable by any criterion other than their activity on the following transfer. If 200 ml. quantities of milk pasteurized by the method outlined above were placed in beakers and inoculated with 6 different cultures, as many as three of the six might be slow. Further subcultures of the slow starters were also slow in bringing about coagulation. The phenomenon occurred in both raw and pasteurized milk. The temperature of pasteurization could be varied over a wide range with no difference in the results and the amount of inoculum did not appear to be significant. When flasks were used instead of beakers, no inactive cultures were obtained. Active cultures streaked on agar and examined microscopically under low power magnification showed about three-fourths smooth colonies and one-fourth

rough colonies, while the growth from inactive cultures consisted almost entirely of rough colonies. The smooth colonies could coagulate milk over night at 20° C., while the rough type required 2 or 3 days at 20° C. to accomplish the same increase in acidity. If a sensitive pure strain of the smooth-type organism was used alone in the beaker experiments, the phenomenon of slow acid production occurred in exaggerated form, some cultures failing to coagulate at all. All strains sensitive to the inhibitory factor did not show the smooth type of colony and not all smooth strains were sensitive to the inhibitory factor. Any aeration of the milk after pasteurization, no matter what type of vessel was used, caused an inhibition of growth when a sensitive strain of organism was being used, the unagitated milk in a beaker being a border line case of aeration. After the smooth strains had been observed for about 3 weeks, they suddenly became non-sensitive to the aeration of the milk and remained that way for more than 9 months. The same change occurred simultaneously in the mixed cultures from which the smooth strains had been isolated, making completion of the study impossible at that time. The change in sensitivity was not correlated with any other apparent change. At a later date the old cultures which had been insensitive regained their sensitivity and a pure culture of Streptococcus cremoris used as a cheese starter exhibited the same characteristics of sensitivity. Further studies showed that agitation of the milk in a vacuum did not render it inhibitory and that the bubbling of hydrogen through the medium did not remove the inhibitory properties conferred by agitation in the presence of oxygen. This was considered to indicate that the oxygen possibly entered into chemical combination, although the milk could be made satisfactory for growth following aeration by means of a second pasteurization, with care to avoid subsequent aeration. The authors concluded that the phenomenon was appar-

ently closely related to the oxidation-reduction reactions by which the organisms gain their energy for growth.

Other investigators (118) have indicated that there might be a correlation between rough or smooth colony type and acid production, although they have not obtained sufficient data upon which to base definite conclusions at the time of the preliminary report.

In 1933, the Iowa Experiment Station (70) reported that the addition of cultures which had coagulated very slowly to pasteurized or sterilized milk frequently caused a considerable delay in the coagulation of the milk by normal butter cultures. The cultures which had that effect apparently contained a restraining agent that could be carried through a series of transfers. The restraining agent passed a bacteria-proof filter. Microscopic and cultural examinations showed that the inhibitory filtrates were free from bacteria in the usual form. The active agent was destroyed by boiling the filtrate in which it was contained.

Harriman (64, 65) made an exhaustive study of the explosive type of slow butter culture and found that the growth of some freshly inoculated butter cultures could be quite markedly restrained by the addition of certain other cultures, especially those which were slow or had been grown in large lots. Bacteria-free filtrates were obtained by preliminary filtration of the coagulated cultures through filter paper, followed by filtration through a grade N Berkfeld ultrafilter. Using seven butter cultures and a culture of Streptococcus lactis as test cultures, 11 of the 19 filtrates from mother cultures and 22 of the 23 filtrates from large lot butter cultures caused rather marked decreases in the percentage of acid formed by at least one culture after a 16 hour period of incubation, indicating that large lot cultures were the more apt to yield inhibitory filtrates. Filtrates which were shown to be inhibitory were obtained from

37 out of 42 slow cultures and from 16 out of 27 cultures coagulating in the normal length of time. Since the different filtrates restrained different numbers of the test cultures, the suggestion was made that possibly different filtrates had different degrees of restraining ability and that different cultures had varying abilities to resist the action of filtrates, the inference being that resistance was a quantitative rather than a qualitative characteristic. Studies made upon the number of organisms developing in butter cultures retarded by the addition of filtrates revealed that the plate counts after 16 hours of incubation of the butter cultures were low, in one case only 250,000 per ml. of culture. In three cases out of twelve the only surviving organisms were S. citrovorous and S. paracitrovorous, and in several other cases the ratio of the citric acid fermenting streptococci to Streptococcus lactis was abnormally high. The surviving Streptococcus lactis strains were normal in their ability to grow and coagulate milk, the result being that after 24 hours the acidities and counts of the retarded starters usually approached those of the controls to which no filtrates had been added. Using Streptococcus lactis cultures and inhibitory filtrates, counts under 500,000 per ml. were frequently obtained at the 16 hour interval when the check cultures, without added filtrate, gave counts ranging from 219,000,000 to 1,750,000,000 per ml. Using 6 different filtrates, a butter culture was carried through seven transfers after the addition of each filtrate, and in only one case did the culture fail to return to normal by the end of the period; these results suggested that artificially induced slow cultures might recover the ability to produce a normal coagulum during an incubation period of the usual duration. Further study of the filtrates showed that in some cases a dilution of one part of filtrate in 20,000 parts of milk had a marked inhibitory effect. In 2 cases a slight restraining effect was shown in a dilution of one to 20

million. Attempts to increase the activity of the filtrates by adding them to butter cultures and recovering them after coagulation were unsuccessful. The inhibitory principle was found to be completely inactivated by heating to 60° C. for 5 minutes in all but one case, and in that 30 minutes were required. Holding at 50° C. for 30 minutes only reduced the "virulence" of the filtrate and did not result in complete inactivation. No figures for the pH level of these filtrates were given, but they were probably at a pH slightly above 4.0, since all of the filtrates were from acid-coagulated starters. Exposure of the pasteurized starter milk to the air in large evaporating dishes, either in the bacteriology laboratory or in the butter laboratory, increased the incidence of slowness, especially when certain cultures were used. Blowing a stream of air, either filtered or unfiltered, through the pasteurized milk also caused slow acid production with one starter. One starter never became slow following aeration. The conclusion was drawn that the principle responsible for slow starters came from the air.

Whitehead and Cox (148) isolated from a slow starter a bacteriophage active toward Streptococcus cremoris. They considered this bacteriophage to be the cause of the slow cultures in question. The bacteriophage was obtained in such strength after several propagations that one part in several hundred million parts of milk "sufficed to show the characteristic lysis with a susceptible streptococcus". Characteristic plaques were obtained on solid media.

Whitehead and Cox (150) recently published the results of a more complete study of their bacteriophage which was active against Streptococcus cremoris. Although the culture of Streptococcus cremoris studied by these investigators appeared normal, an aerated culture sometimes failed to produce acid in the cheese vat. Both aerated and unaerated cultures reduced

methylene blue normally, but the reduction was not permanent in the case of the aerated culture. Microscopic examination showed that the return of color was associated with lysis of the bacteria, this lysis being transmissible to a fresh culture of the sensitive organism. By smearing the aerated culture on yeast whey agar, plaque formation was demonstrated, the plaques being approximately 0.5 mm. in diameter. The bacteriophage was isolated from individual plaques and inoculated into sterile milk to which a drop of sensitive culture was added. At 30° C. lysis usually required 10 or 12 hours during this first transfer and somewhat less during succeeding transfers. Using calcium chloride and rennet to clot the lysed culture which was then filtered through linen, a bacteria-free filtrate with a usual titer of 10^{-8} or 10^{-9} was obtained by the use of a sterile Seitz filter. The titer was taken as the lowest dilution at which no plaques appeared when the filtrate and sensitive culture were smeared upon agar plates. The optimum temperature for the activity of this bacteriophage was found to be about 30° C., 20° and 37° C. being less favorable. Lysis was also demonstrated in whey broth. The original bacteriophage was almost completely destroyed by a temperature of 70° C. for 30 minutes, although a strain isolated at a later date was destroyed by 50° C. for 30 minutes. A secondary-growth organism, obtained from the action of the original bacteriophage upon the organism from which it was isolated, was quantitatively less sensitive to the action of the filtrate used in causing its appearance, 10^{-6} and 10^{-2} being the titers of the one filtrate against the 2 organisms. This organism was satisfactory in some plants but "flared up" in others, producing a bacteriophage to which this organism and the parent organism were equally sensitive. A secondary-growth organism from this partially immune organism was also partially immune to the filtrate used to produce it. Both secondary cultures gradually reverted to the original type.

Sensitive strains of Streptococcus cremoris grown in non-aerated milk generally gave no indications of the presence of bacteriophage, but growth of the same organisms in aerated milk would usually give 2 or 3 cases of high concentration of bacteriophage for each 6 cultures of the organism that were tried. Repeated purification by plating as many as 8 times would not eliminate this tendency. Some extremely sensitive cultures would produce bacteriophage when they were merely incubated at 30° C. instead of the usual 20° C. Bacteriophages flared up not only in the plant but also in the laboratory when the milk was aerated, indicating that contamination in the plant was apparently not the cause. These workers reached the conclusion that bacteriophage is usually present in very small amounts in an occluded condition and needs only the "trigger" of aeration to make it multiply. Various samples of milk were found to vary in their ability to permit this "trigger" action.

Further mentions of bacteriophages to which Streptococcus lactis is sensitive

Hadley and Dabney (57) were apparently the first to isolate a bacteriophage active against Streptococcus lactis. They obtained their bacteriophage from sewage. The maximum titer which they were able to obtain was such that one part of phage in 10 million parts of medium was inhibitory. This bacteriophage was not active against Streptococcus fecalis but did inhibit Bacterium coli. A bacteriophage which was active against Streptococcus fecalis was inhibitory to Streptococcus lactis and Bacterium coli also. McKinley (110) mentions Streptococcus lactis and Streptococcus fecalis among the organisms susceptible to bacteriophage activity.

Other attempts previous to those of Whitehead and Cox (148) to obtain a bacteriophage active against Streptococcus lactis were failures.

Major (108) found that aerogenes phage was as active in milk as in bouillon, despite the "unfavorable" pH, but none of his polyvalent aerogenes phages affected in any way the souring of the milk. He suggested the use of bacteriophage as a means of controlling the growth of organisms of the colon group in milk. Klingmüller (77) could obtain no bacteriophage against either Streptococcus lactis or Streptococcus mastiditis from cultures incubated at 37° C. for periods ranging from one day to 2 weeks or from dung filtrates. Lipska (104) reported that fresh milk, commercial milk and excrement from cattle all contained bacteriophages active against Bacterium coli, but she apparently did not attempt to recover bacteriophages active against Streptococcus lactis.

The bacteriophage and its characteristics

During the 20 years which have elapsed since the discovery of the phenomenon of bacteriophagy, the literature upon the subject has become so extensive that any attempt to discuss it with any degree of thoroughness and completeness would be beyond the scope of this literature review. Only some of those publications which have a direct relationship to the study herein reported will be discussed. For a more complete survey of the literature relating to the bacteriophage, some of the general reviews which are available, such as those written by d'Herelle (36, 37), Hadley (56), Hoder (68) and Kreuger (87), should be consulted.

Twort (137) is usually credited as having first observed the phenomenon of bacteriophage activity, his report having been published in 1915. Working with a white Micrococcus from his vaccinia experiments, he observed transparent areas at the edges of some of the colonies. Only fine granules stainable with Giemsa's stain were detectable in the cleared areas and these would not grow in any medium which he employed in his studies. A small amount of material from a cleared area smeared over the

surface of a new culture prevented the growth of the Micrococcus on the area over which the material had been smeared. The condition of induced transparency could be carried from one culture to another for an indefinite number of transfers. The number of points from which transparency started was a function of the dilution of the material used. The substance suspended in water or saline would pass the finest porcelain filters, and its activity was destroyed by heating the suspension to 60° C. for one hour. The substance had a slight action on Staphylococcus aureus and Staphylococcus albus but not on members of the colon group, streptococci, the tubercle bacillus, yeasts or a number of other organisms.

In 1917 d'Herelle (34) obtained from the intestinal contents of a patient recovering from severe bacillary dysentery a filterable lytic principle active against Bacillus dysenteriae. By adding a drop of a lysed culture of the sensitive organism to a fresh culture of the same bacterium, the principle could be carried through a number of transfers and become more actively lytic instead of losing its lytic power as a result of dilution.

Since the early experiments of Twort and d'Herelle, many studies of the principle which they observed have been made. Bacteriophage strains active against a wide variety of bacteria have been discovered and characterized more or less completely. In 1926, d'Herelle (36) listed Bacillus dysenteriae Shiga, Bacillus dysenteriae Hiss, Bacillus dysenteriae Flexner, Eberthella sanguinaria, Salmonella pullora, Pasteurella bovis, Pasteurella pestis, Eberthella typhi, Bacillus paratyphosus A, Bacillus paratyphosus B, Bacillus suipestifer, Bacillus enteriditis, Bacillus typhi murium, Escherichia coli, Encapsulatus pneumoniae, Bacillus proteus, Corynebacterium diphtheriae, Rhizobium radicicolum, Bacillus subtilis, Vibrio comma, the Staphylococcus, the Enterococcus and Streptococcus pyogenes as organisms

for which active strains of bacteriophage had been isolated. Hadley and Dabney (57) obtained bacteriophages active against Streptococcus fecalis and Streptococcus lacticus. Reports of the sensitivity of other organisms to bacteriophage action could undoubtedly be found if a more complete survey of the literature were undertaken. Further investigations of the phenomenon of transmissible lysis will undoubtedly result in the discovery of a much greater number of bacteriophages which are active against one or more organisms.

Conditions for bacteriophage activity

According to d'Herelle (36), the phenomenon of bacteriophage action will probably take place with all living unaltered sensitive bacteria, although the bacteria are apparently most vulnerable at the time of division. The minimum amount of phage which will cause the dissolution of a culture varies with the combination of bacteriophage and organism used, bacteriophages varying in their "virulence" and bacteria varying in their sensitivity to the action of bacteriophage. Dissolution of sensitive cultures has been reported over the range from 8° to 46° C., the exact range for a given combination of bacteriophage and organism being a function of the strains and species used. The optimum pH for bacteriophage activity varies somewhat but is usually slightly on the alkaline side of neutrality. An increase in the viscosity of the medium has been shown to reduce dissolution by bacteriophage. Sugars, salts and antiseptics are considered to be of influence only as they affect the bacteria which are being subjected to the action of the bacteriophage.

Hadley (56) stated that the phenomenon of bacteriophage might occur at temperatures up to 58° C., the higher temperature ranges being characteristic for those strains of bacteriophage which act upon thermophilic organisms.

Arnaudi and Castellani (4) obtained complete lysis of Rhizobium radicicola by its homologous bacteriophage at pH values of 6.8, 7.0, 7.2 and 7.4 and slight lysis at pH 6.2 and pH 8.0. Beyond these points lysis was not detectable after incubation for 20 hours at 30° C.

Stages in the action of bacteriophage

d'Herelle (36) considered that the phenomenon of bacteriophagy had 2 components. "A dissolution of the bacterial cells takes place, and, in the course of this dissolution, the bacteriophage principle regenerates, reproduces itself." d'Herelle believes that the action takes place in definite stages. First the bacteria and the bacteriophage particles unite, the speed depending upon the virulence. The fixation takes place only with susceptible bacteria, although it may occur if the bacteria are dead. Following adsorption, the phage particle is considered to penetrate the bacterial cell, multiply within the cell and then burst the cell, freeing the new generation of bacteriophage particles which then repeat the process.

Burnet (19) considered that the course of bacteriophage action had been adequately demonstrated to be an adsorption of the phage particle to the surface of the bacterium followed by the entry of the bacteriophage particle into the cell, since it could not be demonstrated upon the surface of the cell after the lapse of a few minutes. After 20 or 30 minutes, a disintegration of the swollen bacterial cell and coincident liberation of from 40 to 200 new bacteriophage particles into the environment was thought to occur. The cycle would then repeat itself if unlysed sensitive organisms were still present.

Kreuger (87) summarized the action between bacteriophage and a sensitive culture as follows: "1. When phage and living bacteria are brought together the phage rapidly attaches to the cells until an equilibrium is established between the cellular fraction and that free in solution;

this linkage to the bacteria is dissociable. 2. As bacterial growth begins, the formation of phage commences; cell division seems to be the essential conditioning factor for phage formation. 3. The rate of phage production is considerably greater than the rate of bacterial production. Consequently the ratio of phage to bacteria continually increases and finally attains the critical level requisite for lysis (lytic threshold)".

Meissner (112) came to the conclusion that selective chemical combination of lysin with a component of the cell unseparable by autolysis or antiformin treatment and stable to heat of 100° C. but not 120° C. for one hour, rather than straight adsorption, was responsible for the removal of bacteriophage from an active filtrate by a sensitive organism and lack of removal by a non-sensitive organism.

Kreuger (84) found that the adsorption of a highly active Staphylococcus bacteriophage to a strain of Staphylococcus aureus at pH 7.6 was reversible when living organisms were used. When heat killed organisms were used, the adsorption reaction was irreversible and the bacteriophage could become more than 99 per cent adsorbed. Killing temperatures of 65°, 80° and 100° C. were used, and no difference in ability to irreversibly adsorb the bacteriophage could be demonstrated. Under some conditions, dead bacteria could apparently become saturated with bacteriophage.

Levine and Frisch (99) have reported that adsorption of bacteriophage by heat-killed organisms is so nearly irreversible that the residual bacteriophage may be studied both quantitatively and qualitatively without removing the adsorbing organisms. They used this method of bacteriophage adsorption for differential purposes in a study of the Salmonella group.

The polyvalency of several colon-typhoid group bacteriophages was demonstrated by Levine, Frisch and Cohen (102); some of the inhibition factors were removed by means of adsorption with various pure cultures

which had previously been killed by heating. Subsequent addition of the living test organisms to the diluted bacteriophage demonstrated the presence or absence of residual unadsorbed valences or components of the original phage. The procedure was checked by the use of an artificial "polyvalent" bacteriophage made by the mixture of 3 bacteriophages which did not lyse heterologous strains.

d'Herelle (36) concluded that the bacteriophage stimulated the growth just before dissolution of the sensitive bacterial cell.

Danbly and Bronfenbrenner (31) obtained results which they interpreted as showing that, if lysis were prevented, bacteriophage would increase the growth rate of bacteria up to a certain point. Lysis apparently set in at the point of termination of this relationship. Bacteriophages active against Bacillus coli, a Staphylococcus and the homologous organisms were used in the studies.

Eaton (40) obtained experimental results with Staphylococcus cultures which indicated that the respiration of a bacterial culture shortly after the addition of bacteriophage was out of proportion to the residual viable cells remaining in the system. By means of a constantly shaken micro-respirometer, he showed that the oxygen consumption per million cells increased sharply after bacteriophage had been added, the rate of respiration remaining for a time at a level which was normal for systems containing 50 million or more organisms per ml., although the culture became almost sterile. Carbon dioxide production was also increased, indicating true respiratory activity. Ultrafiltration destroyed the respiratory activity of the lysed bacterial cultures. No evidence of stimulation of the rate of growth by bacteriophage was obtained, the total counts decreasing rapidly in all cases soon after the addition of bacteriophage.

Using 0.75 per cent citrate agar, which inhibited the activity of a coli bacteriophage but not a Staphylococcus bacteriophage, Andrewes and

Elford (1) found that the addition of amounts of bacteriophage insufficient to cause immediate killing permitted the organisms to increase as rapidly as those in the control to which no bacteriophage had been added until the count had almost doubled, after which the count decreased rapidly. The concentration of bacteriophage necessary to kill increased as the number of organisms to be killed increased. Lysis did not begin until 20 or 30 minutes after the organisms apparently had been killed, but once started it proceeded rapidly.

Marbais (109) found that organisms lysed by bacteriophage had the same antigenic properties as organisms which were unlysed. Digestion of the organisms changed their antigenic properties, indicating that the cell-bursting by bacteriophage is not a simple enzymatic digestion.

The effect of aerobiosis and anaerobiosis upon lysis of bacteria by bacteriophage and upon the regeneration of the lytic principle has been studied by a number of investigators. Unfortunately, there has been a lack of agreement concerning the effect of this factor. Gildemeister and Herzberg (48) obtained results which indicated that the action of transmissible bacterial lysin was independent of the oxygen pressure. They used pyrogallic acid and strong alkali to obtain anaerobic conditions.

Frisbee and MacNeal (47) found that the exclusion of air had no demonstrable effect upon the activity of a bacteriophage active against Escherichia coli.

Schwartzmann (129) studied the effect of the ratio of surface area to the total volume on the regeneration of a highly diluted Bacillus coli bacteriophage in meat extract broth at pH 7.6. A ratio of 0.5 or less was necessary to furnish the degree of anaerobiosis necessary under the conditions of the experiment, an increase in this ratio interfering with regeneration. One hour of restricted air supply, if it was during the

first 3 hours of bacterial growth, was sufficient to obtain maximal regeneration. In a later paper (130), the effect of pH was brought out. At slightly acid or alkaline reactions, such as pH 6.0 or pH 8.4, aerobic or anaerobic conditions had no effect. Growth under anaerobic conditions (area/ volume ratio equals 0.5) at pH 7.0, or under aerobic conditions (area/ volume ratio equals 3.5) at pH 7.6 on meat infusion medium gave rise to numerous resistant forms and a considerable loss in the regenerating power of the bacteriophage, thus explaining the observed effect of anaerobiosis upon bacteriophage regeneration. Neither inactivation nor weakening of the bacteriophage was brought about by bubbling air or carbon dioxide through the bacteriophage at various pH levels. By varying the number of organisms added to aerobic and semi-aerobic tubes of broth containing varying amounts of bacteriophage, it was shown that the rate of bacterial growth had no relationship to the regeneration of extremely dilute bacteriophage.

Manavutty (117) found that greater exposure of the bacteria-bacteriophage system to air by means of a greater ratio of surface area to liquid volume promoted the activity of the bacteriophage. Low dilutions of both coli and Staphylococcus bacteriophage in broth at a pH of 7.8 were used.

McLeod and Govenlock (111) reached the conclusion that a free supply of oxygen was necessary for the production of antipneumococcus "bactericidin".

Hadley (56) stated Larkum found that the bubbling of oxygen through a culture increased the strength of the lytic agent while carbon dioxide had the opposite effect.

Eaton (40) obtained lysis of a Staphylococcus culture in 8 to 12 hours in an ordinary unshaken test tube, but was able to bring about complete lysis in 3 to 5 hours when the system was constantly shaken in an air atmosphere.

Hallauer (59) found that the shaking of cultures, in which the

number of phage particles was insufficient to cause lysis under normal conditions, permitted lysis to take place. In an unshaken test tube, containing 10 ml. of broth to which 1 ml. of bacteriophage with a titer of 10^{-9} had been added, only 350 million organisms per ml. could be present and still permit lysis to take place. If the tube were shaken, 3,500,000,000 organisms per ml. could be lysed. A coli bacteriophage which had been heated for 5 or 10 minutes and which was completely inactivated, according to the usual test methods, regenerated itself when the tubes were shaken in an air atmosphere. When the exposure to heat was lengthened to 15 minutes, no regeneration occurred. The indication was that a slight amount of unkilled residual bacteriophage remained after heating in the first two cases and was enabled to regenerate by the presence of sufficient oxygen in the medium. Shaking bacteriophage filtrates without added organisms, in the presence of either oxygen or air at 37° C., had no effect upon the bacteriophage titer. In shaken cultures the lag phase was shorter than in unshaken cultures. In shaken cultures the partial pressure of the oxygen was unimportant, while it was important in unshaken cultures, the oxygen used being smaller in amount when only 20 per cent of the atmosphere was oxygen. Lysis and regeneration were found to occur in a nitrogen atmosphere only if the number of organisms present was very small. Occasionally it was possible to obtain bacteriophage from previously negative cultures by the use of the shaking procedure, but the results were inconsistent as is frequently true when cultures which are apparently free of bacteriophage are used.

Ogata (119) found that oxidation conditions were apparently a factor in the development of bacteriophage from old cultures. Only cultures which had been exposed to the air and allowed to partially dry produced any lytic principle.

Kreuger (87) stated that lysis of cells could occur in bacterio-

phage suspensions under the proper conditions without bringing about any increase in the concentration of bacteriophage. He also pointed out that anything, such as reduced temperature or the maintenance of special experimental conditions, which interfered with the process of cell division in any way, also reduced the production of bacteriophage.

d'Herelle (36) found that bacteriophages, to which gram-positive cocci were sensitive, were very difficult to increase in virulence and might even be lost during one of the early transfers undertaken to build up the titer.

The biological nature of the bacteriophage

Despite the immense amount of experimental work which has been done in an effort to accurately characterize the bacteriophage, there is still little agreement among the various workers concerning the biological nature and the mode of action of the active principle.

Hadley (56) divided all theories concerning the biological nature into 3 classes: those which consider the bacteriophage as a filterable, ultramicroscopic parasite upon bacteria, a view championed by d'Herelle; those which follow the views of Kabeshima in considering the bacteriophage as a catalytic product produced by digestive glands, this product liberating or activating a diastase normally present in the bacterial cells; and those which, following the views of Bordet and Ciuca, consider the bacteriophage to be in the nature of a pathological rupture of the equilibrium existing between metabolism and assimilation which is communicable to surrounding sensitive cells, "a normal reaction carried to a pathological extreme". As would be expected, many variants of these 3 major theories have been advanced. Many of the experimental data are of such a nature that they are subject to a variety of interpretations, the result being that many theories differing only in minor details have crept into the literature.

Burnet (19) believed that the close relationship between heat inactivation of bacteriophage and the heat denaturation of proteins, the surface condition of the bacteriophage as indicated by the influence of solutions containing mono- and divalent cations on heat inactivation, the antigenic nature of bacteriophages from which bacterial antigens have been removed and the reproduction in a manner which was true to type indicated a type of organization similar to bacteria and other living organisms.

d'Herelle (36, 37) has, from the first, championed the theory of the living nature of the bacteriophage. According to his interpretations, the bacteriophage is an ultramicroscopic parasite upon sensitive bacteria, a form of life similar to a virus.

Schädler (127) was unable to obtain any evidence of independent metabolic activity for a coli phage purified by Schlesinger's method. The same negative results were obtained whether the experiments were carried out in the presence of living or dead bacteria.

Kreuger (86) considered that the existing experimental evidence did not indicate that bacteriophage possessed an independent metabolism.

Stanley's (135) work with his crystalline tobacco mosaic virus, in which he demonstrated that at least one virus was an autocatalytic protein capable of being propagated in contact with living susceptible cells, may not apply directly to bacteriophage, but it does present a possible new approach to the problem, since a similar interpretation might be applied to the phenomenon of transmissible lysis. Possibly, it might be proved that the bacteriophage is an inanimate substance of possibly protein nature, an agent destructive to certain cells, and capable of reproduction by the cells to which it is destructive in the same way as Stanley's virus.

The source of bacteriophage

The question concerning the ultimate source of the bacteriophage has been one of the most puzzling which has confronted those who have studied the bacteriophage. Twort's (137) original observations were made on a strain derived from a culture of bacteria which had shown typical growth. d'Herelle (36, 37) obtained nearly all of the bacteriophage strains which he studied from either sewage or intestinal contents. He has always contended that the presence of bacteriophage in a culture was due to contamination from some outside source, a truly "pure" culture not containing any bacteriophage. Since the work of these 2 early investigators, many attempts have been made to decide the ultimate origin of bacteriophage.

Gildemeister and Herzberg (48) reported that they were able to obtain lytic principle from a previously normal culture. They added the filtrate from the previous culture to a subculture for a number of generations and finally detected the presence of bacteriophage. This method was successful only with coli cultures.

Hadley (56) has, on various occasions, obtained a lytic principle by repeated growth and filtration through 14 to 20 generations, using several strains of coli organisms.

Ogata (119) found that 8 bacteriophage-sensitive cultures among the 10 Shiga cultures which were held for 98 days in the incubator had become sterile and contained bacteriophages. One of 5 cultures of the Flexner strain also gave rise to a sirup which contained lytic principle as a result of the partial drying undergone in an incubator. No bacteriophage could be obtained from the partially dried cultures of 4 bacteriophage-resistant strains of Schmitz-type dysentery bacilli. The results of these experiments were capable of duplication in the same laboratory at a later date. When similar tests were made using the same cultures, tempera-

tures and holding periods, no bacteriophages were obtained when the cultures were sealed in test tubes and thus kept from drying. Growth of the cultures on agar for a period of 5 months, following which the organisms were washed from the surface by the use of a physiological saline solution, did not produce any bacteriophage. The same negative results were obtained when the cells were rapidly dried in a vacuum dessicator and were held there for 2 or 3 days. The conclusion was that not only time but also the oxidation conditions played important roles in the production of bacteriophage from old cultures.

By growing dysentery bacteria of the Hiss, Flexner and Shiga strains in 5 ml. quantities of bouillon, to which varying amounts of calcium chloride solution had been added, and plating after 6 or 7 days at 37° C., Plantureux (121) obtained abnormal colonies which yielded bacteriophage on transfer to bouillon pH 7.8 and holding for 20 days at 37° C. before filtration; the phage was usually obtained at the first transfer and never later than the second.

LeMar and Myers (96) devised a technique whereby specific lytic agents, which had the characteristics of bacteriophages, were obtained from cultures of Escherichia coli, Eberthella typhosus, Salmonella enteriditis, and Staphylococcus aureus. Their procedure consisted of growth in broth for 48 hours, autoclaving for 20 minutes at 15 pounds pressure, and incubation again for 48 hours at 37° C., followed by oxidation for 48 hours at 37° C. by hydrogen peroxide. Unless the oxidation was prolonged by several days, no lytic principle was obtained when the second period of incubation was omitted. The oxidation of living cultures by the hydrogen peroxide yielded negative results.

Certain other investigators have not been able to obtain bacteriophages from sensitive cultures of bacteria. Arkwright (3) was unable to

recover a lytic principle from 6 strains of Salmonella dysenteriae Shiga, or their sensitive and non-sensitive variants, unless bacteriophage from an outside source had previously been added to the culture.

Spanedda (134) did not succeed in recovering any lytic filtrate from 32 strains of diphtheria and diphtheroid organisms which had been allowed to grow for 5 or 6 days before filtration. The absence of lytic principle was determined by the use of 59 Corynebacterium cultures from a variety of sources as test organisms.

Arnaudi and Castellani (4) reported the isolation of a bacteriophage active against Rhizobium radicicola from soils in which alfalfa had been grown for 1, 4, and 9 years. The presence of a bacteriophage of this type in the soil is pointed to as a possible source of the soil fatigue toward alfalfa that is sometimes observed.

Hadley (56) stated that sterilized sewage could cause the "liberation" of bacteriophage from bacteria, although the sewage alone, even before sterilization, did not cause plaque formation without previous passage at the expense of a sensitive organism.

Evans (43) has isolated from sewage 4 serologically different strains of bacteriophage active against hemolytic streptococci. Hadley and Dabney (57) obtained bacteriophages active against Streptococcus faecalis and Streptococcus lacticus from sewage. d'Herelle (36) points out that everything which has been exposed to excreta may contain bacteriophage and that frequent isolations of bacteriophage have been made from human, avian and animal stools.

Hadley and Klimek (58) found that pancreatin extracts in broth (pH 7.8) produced no plaques when smeared upon seeded plates of sensitive Shiga dysentery bacillus or the strain of Bacillus coli which was used. Extracts heated to 60° C. for 30 minutes and filtered through a Berkfeld N

filter gave actively lytic filtrates after 2 to 6 serial filtrations with the dysentery culture, but only one of 3 extracts brought about the production of lytic principle when Bacillus coli was used as the substrate organism. Autoclaved pancreatin produced lytic principle after 8 transfers with the dysentery bacillus, eliminating the possible presence of active enzymes as a factor in bacteriophage generation. Nine serial filtrations were required with autoclaved peptone to cause the production of lytic principle. Sterile distilled water had the same effect after 5 serial filtrations with Bacillus coli and 9 with the Shiga bacillus. Fourteen passages in broth, more broth and some culture being added after each filtration, gave a lytic principle which was eventually brought to a high titer. The conclusion was that bacteriophage was generated by the bacteria under the liberating influence of a specific environment.

Hoffstadt and Almaden (69) investigated 4 strains of Staphylococcus aureus, an undissociated strain, a dissociated smooth white form, a rough white form, and a gonidial form. K medium, plain broth and lithium chloride broth, all at a pH of 7.8, were used as substrates. Twenty-six serial filtrations, using the control culture from the previous experiment as a substratum, produced no bacteriophage in plain broth or in lithium chloride broth. The addition of the filtrates seemed to promote dissociation, however. In K medium, lysis occurred in the tube containing the dissociated smooth white strain and its homologous filtrate at the twelfth transfer and persisted except in the twenty-third and twenty-fourth transfers. The bacteriophage was specific for the homologous strain. Dissociation of the undissociated strain and 4 other strains was not accomplished by the use of polyvalent bacteriophages. These workers concluded dissociation could take place without demonstrable bacteriophage, although this did not mean that bacteriophage might not be present.

No criterion for the definite determination of the presence of bacteriophage in a culture previous to its isolation from the culture has been devised. Krueger (86) states: "At the present moment (1936) then there exists no clear-cut experimental evidence that phage can be derived from phage-free bacterial strains". Although the question concerning the ultimate source of bacteriophage remains unanswered today, the experimental evidence obtained by the majority of the investigators in this field points to the bacterial cell as the point of origin.

Classification

Because all strains of bacteriophage are not identical in their behavior toward bacteria, numerous criteria for separation into different strains have been employed. Although fairly definite groups or types have been segregated by such means, apparently no attempt has been made to classify bacteriophage strains taxonomically.

d'Herelle (36) considered the bacteriophage to be a single species which was capable of adaptation in such a manner that it would attack all species of bacteria known to be sensitive to bacteriophage action. As evidence in support of this view, he mentioned the multiple virulences possessed by many strains of lytic principle and the recorded adaptations of bacteriophage to species which were not previously susceptible. This narrow interpretation has received little support from more recent investigators.

Some of the earliest studies concerning the classification of the various strains of bacteriophages were made by Bail (6). The type of activity in broth and on agar, the ability to multiply, the speed of multiplication at the expense of the homologous organism and other organisms, and the formation of strains of organism resistant to the action of the bacteriophage in question and to other bacteriophage strains were considered

to be characteristics which might be usable for classification purposes.

Burnet and McKie (24) classified 47 bacteriophages active against a single strain of Bacillus coli communior. Upon the basis of plaque size and form, 4 groups of bacteriophages were obtained: those which gave large plaques with a wide, shelving edge; those producing moderately large plaques which were surrounded with a definite halo, narrower and more sharply differentiated than for the first group; those whose plaques were medium to small with a soft edge or a narrow halo; and those whose plaques were small to tiny and sharply delineated. Classification upon the basis of the types of organisms lysed and the nature of the resistants produced was also attempted.

While doing further work with coli-dysentery bacteriophages, Burnet (20) differentiated 12 types of bacteriophages, all differing in serological nature. Minor serological differences were found within the major groups, but intermediate types did not seem to occur. The same structural type of plaque was characteristic of all of the members of any one serological group, and the resistant organisms produced were of the same type, also. The classification upon the basis of serological character, particle and plaque size, and power to provoke resistance seemed to this investigator to be quite workable.

Burnet (21) performed experiments to determine the correlation between the serological classification and the inactivation of bacteriophages by various dyes, sodium citrate and urea. Photodynamic inactivation by means of pyronine, rivanol, methylene blue, acriflavine, and proflavine increased in the order given, the same order applying to all bacteriophages which were tested. Sodium citrate, in concentrations ranging from 0.5 to 10.0 per cent, inactivated some bacteriophages and left others completely active, and the same type of phenomenon occurred when urea was used as an inactivating agent. Among the bacteriophages in any one serological group, the reaction to any one inactivating substance was uniform, but the reactions

to any 2 tests were not parallel. Distinction of groups upon the basis of the 3 tests was considered sharp.

Among bacteriophages which cause lysis of the members of the colon group, one apparently "pure" strain which has been carried at the expense of a single organism for some time may be able to provoke lysis, not only among the various species of a single genus but also among organisms belonging to several genera. Bronfenbrenner (9) attributed such an effect to a common "element" in the compositions of the strains of organisms affected. He pointed out that some apparently pure phages could be grown and be active on any one of several rather closely related species. A coli phage carried for 6 years and repeatedly tested for purity was active against Bacillus coli, Bacillus dysenteriae, Shiga and Bacillus dysenteriae Flexner, regardless of which of these organisms was used to carry it. This strain was of special interest because, although the heat resistance under normal conditions was the same for all 3 valences, the presence of glycerine or sucrose selectively allowed the coli virulence to survive in a weakened form which would regenerate only in the presence of Bacillus coli. The regenerated bacteriophage was again active against all 3 types at normal titer after 3 passages. Mixtures of bacteriophages were not regenerated in the same manner after partial destruction.

By testing, with serologically different sensitive organisms, the residual bacteriophage remaining after adsorption of a fraction by killed sensitive organisms, Levine and Frisch (101) were able to show that certain bacteriophages from chicken stool filtrates contained 2 qualitatively different fractions, one selective for suipestifer strains and one for the typhosus-paratyphosus B group. Antiphages were obtained by rabbit injections and the results of the selective adsorptions on heat-killed organisms were confirmed by the use of the antiphages. These workers pointed out that

bacteriophages could differ markedly in composition and yet share a particular fraction in common.

In a study of 4 types of bacteriophage to which hemolytic streptococci were sensitive, Evans (43) was able to differentiate them by means of the antiphages which they produced, the 4 types being serologically different. Secondary growth occurred only when certain of the filtrates were combined with certain test organisms. The plaques on glucose meat infusion agar were usually 1 mm. or less in diameter and no differentiation upon this basis was obvious.

Italo (71) found that bacteriophages active against R, RS and S phases of typhoid and dysentery bacteria existed and could be differentiated upon the basis of heat resistance and antigenic properties.

Burnet (19) concluded that the size of the bacteriophage particle was independent of the organism used for propagation and was very nearly uniform for any one bacteriophage, making particle size a definite attribute of each strain of bacteriophage. Coli bacteriophages were capable of division into groups upon the basis of serum specificity, particle or plaque size, and resistance type, these 3 characteristics being distinct and usually unchanged by experimental procedure. The range of activity on normal bacterial species, the readiness with which the appearance of secondary cultures was allowed, and the speed with which lysis was provoked were considered as being more or less readily modified by the experimental procedures. As will be brought out later, the size of the plaques produced by a given race or strain of bacteriophage may be considerably influenced by the conditions of the experiment.

Kreuger (87) reached the conclusion that the antigenic specificity of a bacteriophage was not dependent upon the bacterial substratum upon which the bacteriophage had been propagated but was constant for each strain and was therefore a valid criterion for differentiation. He also stated

that the inactivation of bacteriophage by its specific antiserum was reversible, the bacteriophage-antibacteriophage combination being dissociable.

Sertic and Boulgakov (131) used the diameter of the plaque, the diameter of the corpuscle as determined by the porosity of the membrane limiting its passage, the temperature of inactivation, the virulence, the species of bacteria attacked, the behavior toward the secondary growths of other filtrates, and the antigenic type as bases for the classification of 75 bacteriophages which they obtained from Paris sewage. These strains, all of which were active against Eberthella typhi, were divided into 14 antigenic types.

Bronfenbrenner and Korb (11) demonstrated that the age of the culture and the conditions in the medium may greatly influence the size of the plaques formed by bacteriophages. An increase in agar concentration from 0.3 per cent to 3.0 per cent reduced the number of plaques to one-hundredth of the original number. The greater the concentration of young and susceptible bacteria, the smaller were the plaques. An increase in the proportion of old bacteria in the culture was found to decrease both the number and size of the plaques formed. When the conditions were held constant, lytic filtrates acting upon more than one species gave the same size of plaque with each sensitive species.

Yen (153) found that the number and character of the plaques could be varied by the concentration of the agar in the medium, the amount of medium and the amount of inoculum. The number of plaques was decreased when the agar content was increased above 2 per cent and a 5 per cent agar did not permit the formation of any plaques. A thin layer of agar combined with a heavy inoculum caused the formation of concentric rings of lysis around the center plaque. Under conditions which limited bacterial growth, such as growth at unfavorable temperatures or under anaerobic conditions when aerobic organisms were used, the size of the plaques was decreased.

The papers just reviewed show that when the size and the type of the plaque are used for purposes of classification, the experimental conditions must be well-standardized.

Action of chemical agents upon bacteriophage

d'Herelle (36) mentioned a number of substances which had been found by various investigators to inactivate bacteriophage. Mercuric chloride, sodium floride, potassium cyanide, copper sulfate, 90 per cent alcohol, phenol, chloroform, lactic and oxalic acids, formol, lysol, quinine salts and glycerol were included in the group of substances which inactivated bacteriophage either completely, or to an appreciable extent. Sodium chloride and sodium sulfate were listed as substances which were without effect.

McKinley (110) concluded from a survey of the literature that the resistance of bacteriophages to alcohol, acetone, ether, chloroform, 1 per cent phenol, lysol and glycerol was greater than the resistance of the homologous organisms and even greater than that of spores to the same agents.

de Poorter and Maisin (33) found that lactic, oxalic and osmic acids destroyed bacteriophage, while phosphomolybic, tungstic and citric acids did not destroy the lytic activity. They also determined the activity of a number of other compounds and concluded from their studies that bacteriophage was of the nature of an enzyme.

Lin, Kurotchkin and Bernaradsky (103) found that Shiga bacteriophage, with an original titer of 10^{-10} and held at 37° C. for 3 months, decreased to 10^{-5} in titer when no sodium chloride was added but only to a titer of 10^{-8} when the filtrate was saturated with sodium chloride. The bacteriophage which had been preserved with sodium chloride also lysed the test culture more rapidly in any active dilution than did the control held under the same conditions without added sodium chloride.

Staphylococcal bacteriophage in beef infusion broth at a pH of 7.6 and diluted with an equal quantity of $\frac{M}{325}$ potassium cyanide solution was inactivated after incubation at 37° C. for 24 hours (90). The addition of 1 ml. of 2.74 per cent silver nitrate solution and 2 ml. of 1 per cent potassium cyanide solution to 10 ml. of the inactivated bacteriophage resulted in reactivation of 78 to 100 per cent of the inactivated bacteriophage.

Krueger and Baldwin (88) succeeded in reactivating staphylococcal bacteriophage after complete inactivation, as a result of exposure for 9 days to a 2.8 per cent concentration of mercuric chloride, by precipitating the mercury with hydrogen sulfide. They considered that this indicated a structure more like an enzyme, since all known protoplasm has been shown to be much more sensitive to mercuric chloride.

Using phenyl mercuric chloride, Goldsmith (50) found that concentrations of 1:20,000, 1:40,000 and 1:80,000 had no effect upon the lytic activity of bacteriophages to which Bacillus coli and a Staphylococcus were sensitive when the period of contact was 30 minutes at room temperature. The activity of the Staphylococcus bacteriophage was completely destroyed after a 6 week period, while the activity of the Bacillus coli strain remained unaffected. The toxicity of the solutions toward the lytic principles seemed to parallel the susceptibility of the homologous organisms to destruction by the same agent. The comment was made that, since enzymes are not destroyed by this antiseptic, at least the Staphylococcus bacteriophage would not be enzymatic in nature.

Burnet and McKie (22) showed that dilute bacteriophages were more or less readily inactivated by methylene blue, toluidine blue and Janus green when divalent ions were absent, but that the presence of calcium salts or of broth prevented such inactivation. The sensitivity to inactivation by dyes and the prevention of inactivation by calcium ions varied

with the strains of bacteriophages.

The precipitation of bacteriophages, to which the Shiga and Flexner strains of Bacillus dysenteriae were sensitive, was accomplished by Bronfenbrenner and Korb (10) through the addition of 10 parts of 96 per cent alcohol to one part of bacteriophage filtrate in a tube, followed by centrifuging after different exposures. The bacteriophage was completely removed after an exposure of 6 to 24 hours at room temperature, but at 7° C., the action was much slower, usually requiring over 4 weeks. In some cases a small amount of bacteriophage would remain in the supernatant alcohol. In such cases, the residual lytic activity was always transmissible in series. The analogy to the alcoholic inactivation of certain enzymes and toxins was pointed out.

In a later report (13) the same investigators indicated that the presence of neutral salts was of significance in the inactivation of bacteriophage by alcohol. In the presence of additional neutral salts, especially those containing a cation of high valence, the alcoholic inactivation of the bacteriophage occurred more rapidly. When the salts normally present in a filtrate were removed by dialysis, the lytic principle tended to become less sensitive to alcohol, and the restoration of the original salt content seemed to restore the previous level of sensitivity. Apparently the rate of precipitation of the coaguable fractions of the medium by alcohol, and not the direct toxic action of the alcohol on the bacteriophage, was responsible for the activation of the lytic principle. The results were considered to lend support to the theory of the chemical nature of the bacteriophage.

Asheshov (5) found that bacteriophages active toward the Flexner strain of the dysentery bacillus and toward Staphylococcus aureus were very sensitive to trisodium citrate, as little as 0.5 ml. of a half normal solution per 10 ml. of bouillon preventing lysis and 0.2 ml. retarding lysis

for some hours. A third strain of bacteriophage which was active against the Flexner strain of dysentery organism was not inhibited by 0.2 ml. of the previously mentioned solution and 0.1 ml. increased the speed of lysis.

Andrewes and Elford (1) determined that even a bacteriophage with a high titer against coli organisms was 99 per cent inactivated in a few moments by 0.75 per cent citrate in the agar, even in the presence of a dilute suspension of susceptible organisms. The method was utilized to permit a greater degree of accuracy in the estimation of the number of surviving viable organisms after various periods of time.

Applebaum and Patterson (2) studied the effect of bile upon bacteriophage activity and found that the undiluted bile inhibited all 5 of the strains which they used, while a 1:10 dilution inhibited only the Streptococcus strain and had no effect upon the 2 coli, one Staphylococcus and one typhoid bacteriophages. Further tests were made using rabbit blood and serum. Bacteriophages active toward staphylocci and streptococci were more inhibited by body fluids than were those to which coli and typhoid bacilli were sensitive.

Apparently bacteriophages are sensitive to strongly oxidizing substances of various types. Lominski (105) was able to inactivate two colon, two Staphylococcus, one subtilis and one megatherium bacteriophages by small concentrations of potassium permangenate. The process seemed to be one of oxidation since, in the limiting dilutions, inactivation was accompanied by decolorization. The higher the holding temperature and the less reducing the medium, the smaller the amount of oxidizing agent which was required, one part in a thousand sufficing in saline. The inactivation was irreversible. In a later paper (106) the sensitivities of various bacteriophages and their homologous organisms to oxidation by permanganate were compared. Staphylococcus bacteriophage was killed by 0.4 part of potassium permanganate per hundred, while its homologous organism grew in 0.2 part, easily tolerated

0.4 part and partially resisted 1.0 part per hundred when held from 6 hours to 6 days at 37° C. The concentrations were reported to be essentially the same for other combinations of bacteriophage and organism which were tested, the actual quantity of reagent necessary depending upon the reducing power of the medium and the concentration of bacteria. The recommendation was made that several passages through permanganate should be made to free a culture from bacteriophage. The organisms subjected to this treatment retained their bacteriophage sensitivity during the manipulation. Lysogenic organisms remained lysogenic after treatment, this fact permitting the differentiation between lysogenic and "contaminated" organisms.

Lominski (107) reported a series of studies upon the inactivation of bacteriophages by chemical means and their subsequent reactivation, in some cases, by the use of ascorbic acid. Two Staphylococcus, two coli, one subtilis, and one megatherium bacteriophages were used. When bacteriophages were exposed to air during holding at 37° C., 2 months were required to reduce the titer by one-half. If, however, oxygen was bubbled through the filtrate, only a few hours were required to bring about the same reduction in titer. When hydrogen peroxide was used as the inactivating agent, freshly prepared bacteriophages which were rich in catalase required concentrations of one part in 100 or one part in 150 for 24 hours for complete inactivation, but the addition of the hydrogen peroxide in 2 parts at 24 hour intervals required only one-fifth as much reagent to produce the same effect. Bacteriophage preparations containing only a little catalase were almost always inactivated by 0.2 to 0.3 part of hydrogen peroxide per 100. Using Lugol's iodine solution, 30 to 50 parts per 100 were required to inactivate bacteriophage. The hydrogen peroxide and iodine solution were more active against bacteriophage than against the homologous organism and the suggestion was made that they could be used to free "contaminated" cultures from bacteriophage. The oxidations apparently were irreversible since passage on organisms,

or the dilution of the oxiding agent after its action, did not restore the activity of the bacteriophage although, in some cases, the bacteriophage inactivated by molecular oxygen or by iodine was capable of reactivation by the addition of ascorbic acid.

Schultz and Krueger (128) inactivated 2 Staphylococcus bacteriophages in from 6 to 12 hours by concentrations of methylene blue as low as 0.002 per cent. Five serial passages on susceptible staphylococci gave no evidence of residual active bacteriophage. The action was apparently selective since 8 other bacteriophages, including strains to which coli, dysentery, typhoid and proteus organisms were sensitive, were unaffected by the dye. These investigators made no attempt to explain the mechanism of the inactivation.

Methylene blue in a concentration of 0.05 per cent was shown by Clifton and Lawler (28) to be capable of bringing about complete inactivation of Staphylococcus bacteriophage at 37° C. In a few cases undiluted bacteriophages, or those diluted 1:10 with Martin's broth, were inactivated with concentrations of 0.005 per cent. Toluidine blue, methylene violet, methylene green, methylene azure, thionin, eosin B and phenol red were also tried, and only the first one was effective when tested at 37° C. for 24 hours or longer, the action being the same as with methylene blue. Coli bacteriophage was unaffected by either dye. The action of these dyes was considered as probably due to an adsorption phenomenon.

Clifton (27) found that, when 0.1 and 0.01 per cent concentrations of methylene blue were added to antistaphylococcus bacteriophage and the mixtures incubated for 24 hours at 37° C., exposure to indirect sunlight was necessary for about 5 minutes before incubation if inactivation was to occur; no inactivation was noted when dye and bacteriophage were mixed, incubated and tested in the dark. The evacuation of air, or its replacement with an atmosphere of hydrogen, prevented the inactivation of the bacteriophage, even

if 30 minutes had elapsed between mixing and the establishment of anaerobic conditions. The reduced or leucobase form of methylene blue was shown to have no inactivating effect. When 0.01 per cent of cysteine was added with 0.01 per cent of methylene blue, no inactivation occurred, presumably because of the protective reducing action of the cysteine. Tyrosine in considerable concentrations had no protective effect. The conclusion was that the inactivation of bacteriophage by methylene blue was due to the oxidation of the bacteriophage by photosensitized methylene blue in the presence of oxygen.

The inhibitory action of crystal violet and brilliant green upon a series of bacteriophages to which a Staphylococcus, Aerobacter cloacae, Bacillus megatherium, Bacillus coli and an Achromobacter were sensitive was investigated by Wells and Sherwood (139). Beef broth at a pH of 7.6 and 1.0 per cent agar were used as substrata with an incubation temperature of 37° C. All bacteriophage preparations were diluted 1:25,000,000 to reduce the dye concentration to a level which all of the organisms would tolerate before bacteria were added to test for the presence of residual bacteriophage. A 1:2500 dilution of crystal violet acting for 24 hours inactivated all of the bacteriophages except one of the two which were active against Bacillus coli. A 1:10,000 dilution inactivated only the Staphylococcus and Bacillus megatherium bacteriophages in 24 hours and had no effect upon the others, even after a week of incubation. After 90 hours the brilliant green in a concentration of 1:2000 partially inhibited the Staphylococcus bacteriophage and the Bacillus coli bacteriophage which had been unaffected by crystal violet and had no effect upon the others. Apparently the bacteriophages active on gram positive organisms were less resistant to the inactivating influence of certain dyes than were those acting upon gram negative organisms. The same investigators (140) later reported that in 24 hours a 1:20 concentration of phenol inactivated all eight of the bacteriophages used by them for testing

purposes. A concentration of 1:150 of sodium hydroxide inactivated all but one of the test bacteriophages within a week. Phenol was apparently more active against those bacteriophages to which gram positive organisms were susceptible, while sodium hydroxide was variable in its behavior in this respect.

Krueger and Baldwin (89) were able to obtain 98 per cent removal of Staphylococcus bacteriophage activity by adding 1.0 ml. of 0.25 per cent solution of Grübler's safranine to 9.0 ml. of bacteriophage filtrate and holding for 18 hours at room temperature before centrifuging off the precipitate containing the bacteriophage. When the precipitate from broth at a pH of 7.4 was added to broth at a pH of 6.5 and the mixture allowed to stand 2 hours before titration, 25 to 30 per cent of the original bacteriophage was capable of reactivation. The inactivation was photodynamic, the titer of the residual lytic principle in the supernatant fluid being three or four times greater if the solution were kept in the dark than when the solution was kept in the light. The bacteriophage in the supernatant fluid could be reactivated to the extent of 5 to 10 per cent of the original concentration.

Evans (42) found that, in vitro, blood, pus, ascitic fluid, bile and saliva completely inhibited bacteriophage activity. Urine also caused partial inhibition. The washed cells of blood and pus were almost entirely without effect, the sera having been shown to contain the active element. It was impossible to demonstrate any inhibiting effect of bacteriophage when it was inoculated into the blood stream, either when the Streptococcus infection was well under way or when the organisms and bacteriophage were inoculated simultaneously into the blood stream.

Levine and Frisch (97) demonstrated that a bacteriophage which was active toward Bacillus dysenteriae Shiga was inhibited by a saline extract of the homologous organism but not by a similar extract of Bacillus aertrycke. A bacteriophage against Bacillus paratyphosus B and Bacillus aertrycke was

inhibited by saline extracts of Bacillus aertrycke but not by extracts of Bacillus dysenteriae Shiga. The inhibition was also evident when Bacillus aertrycke was used as the test organism for inhibition in place of Bacillus paratyphosus B. These results were considered to demonstrate that Bacillus paratyphosus B and Bacillus aertrycke had the same heat stable components since extracts of the latter inhibited the bacteriophage which was active against the former. The experimental results were considered to be of possible significance in the study of the specificity of bacteriophages. In a later article (124) these same investigators expressed the opinion that the behavior of bacteriophages toward bacillary extracts might be used as a means of classification.

Levine and Frisch (100) found that when a crude saline extract of Bacillus aertrycke was boiled in $\frac{N}{2}$ sodium hydroxide for 5 minutes and subsequently neutralized it showed a marked increase in ability to inhibit bacteriophage activity. Treating a saline extract with sufficient $\frac{N}{5}$ hydrochloric acid to make the solution $\frac{N}{15}$, then holding at 80° C. for 30 to 90 minutes before neutralization and centrifuging to remove the precipitate increased the inhibitory effect which it had upon bacteriophage. The suggestion was made that these treatments resulted in the production of hydrolytic products in which the specific reacting groups were unmasked as disclosed by the more intense bacteriophage inhibition.

Action of physical agents

Hadley (56) concluded, after a survey of the literature, that the complete inactivation of all bacteriophages was brought about by heating the active filtrates for 30 minutes at 72° to 75° C.; partial inactivation began about 10° lower, although some bacteriophages were apparently inactivated by heating at 63° C. for 30 minutes.

d'Herelle (36) reported that all bacteriophages were inactivated

by a temperature of 75° C. maintained for 30 minutes and that many were inactivated by somewhat lower temperatures. Progressive attenuation of virulence was found to begin at about 50° C. Young bacteriophages were found to be resistant to a temperature of -180° C., while older bacteriophages were not able to withstand this temperature.

Using a heating period of one hour, Evans (43) tested the thermal stability of 4 bacteriophage strains which were active against hemolytic streptococci by placing the bacteriophages in sealed 5 mm. tubes and immersing in the heating medium. One strain was inactivated at 65° C., one strain at 63° C. and 2 strains at 60° C. In general, 50° C. caused partial inactivation of the bacteriophages tested. At refrigerator temperature and in tubes sealed with vaseline, these Streptococcus bacteriophages retained their virulence for years. Exposure to air and higher temperatures were found to be factors hastening the deterioration of the bacteriophages. One strain was very sensitive when first isolated but became more stable after several months in the laboratory.

Knorr and Ruf (78) dried a number of bacteriophages from different sources and determined that there were considerable differences in their sensitivities toward drying, some being easily inactivated in this way while others were not. This characteristic apparently was constant for a given strain of bacteriophage. Plating and dilution methods of estimation of residual bacteriophage gave comparable results. Whether the bacteriophage was dried in an atmosphere of air or carbon dioxide seemed to make no difference.

Bacteriophage active against Bacillus megatherium was dried over either phosphorous pentoxide or sulfuric acid and a suspension of sensitive organism added to the dry filtrate to determine the activity of the dried bacteriophage (138). The dried preparations were able to survive 80° C. for

30 mimutes or 100° C. for 10 mimutes but not 100° C. for 30 mimutes. All of the liquid bacteriophages were inactivated by the lowest temperature employed, 80° C. for 10 minutes. Essentially the same results were obtained when Bacillus coli, Bacillus typhi, and Bacillus paracoli bacteriophages were treated in the same way.

According to Krueger (85) the high value of the critical thermal increment indicated that protein denaturation was involved in the destruction of bacteriophage by heat. Krueger interpreted this as possibly being the result of the destruction of a carrier, the change thus produced resulting in the inactivation of the attached lytic principle. The results reported did not cover complete inactivation.

The effect of the pH of the medium upon the heat inactivation of antistaphylococcal bacteriophage containing 10^{10} activity units per cubic ml. was studied by Krueger and Scribner (91). Equal quantities of bacteriophage preparation and beef infusion broth were brought to the desired pH level with hydrochloric acid and placed at 57° C. for 30 mimutes. The residual bacteriophage activity was determined by the activity titration method of Krueger (84). In the pH range from 6.0 to 7.0, 32 to 34 per cent of the activity survived; at pH 5.75, only 3.75 per cent survived; in the range from pH 4.0 to 5.5, all but less than one per cent of the activity was destroyed; at pH 7.5, 50 per cent of the bacteriophage was destroyed; 24 per cent remained active at pH 8.0, 10 per cent at pH 8.5 and less than 1 per cent at pH 9.0 or above. These investigators concluded that: "Heat inactivation of phage has the high critical thermal increment characteristic of protein denaturation and with increases in hydrogen or hydroxyl ion concentration the inactivation reaction is favored as is the case with many enzymes".

Burnet and McKie (22) studied the effect of various ions upon the inactivation of bacteriophages active against the members of the dysentery

group by heating the bacteriophages to 60° C. for one hour. Bacteriophages which had been diluted in solutions containing sodium, potassium or ammonium salts were readily inactivated by this heat treatment. The addition of calcium, magnesium or barium salts, in the ratio of about one part of divalent ion to 20 parts of monovalent ion, partially or completely prevented inactivation by the temperature used. The same effect was observed when dyes instead of heat were used to bring about inactivation. Different bacteriophages varied in the degree to which the type of ions present influenced inactivation. These investigators called attention to the correspondence between the sensitivity of higher animals and plants and of bacteriophage to calcium:sodium ratios, the indication being that a similar protoplasmic structure was involved in each case.

The effect of the hydrogen ion concentration upon the stability of bacteriophages at lower temperatures was investigated by Bronfenbrenner and Korb (12). They used a test period of 3 hours at 7° C. in their studies. Beyond the range of hydrogen ion concentration limited by 6.3×10^{-5} and 1.6×10^{-9}, anti-staphylococcus bacteriophage showed deterioration. Anti-coli filtrate was completely resistant in the hydrogen ion concentration range from 2.7×10^{-3} to 2.5×10^{-11}. Bacteriophage active against the Shiga strain of dysentery bacillus began to be inactivated beyond the range from 1.7×10^{-4} to 1.3×10^{-11}. Filtrate of the transmissible lytic agent which attacks Bacillus pestis caviae retained full activity over ranges of hydrogen ion concentration extending from 1×10^{-3} to 3.5×10^{-12}, being the most resistant of the 4 strains examined.

Kreuger (87) stated that ultra violet rays killed bacteria and inactivated bacteriophage at about the same rate. X-rays were found to be variable in their effect upon bacteriophage.

The chemical and physical characteristics of the bacteriophage particle

Those who are engaged in the investigation of the nature of the bacteriophage and its activity nearly all agree that the active principle is particulate in nature, although some consider the particle itself to be a fragment of bacterial cell to which the actively lytic particle is attached while others consider the particle as the true bacteriophage substance.

Elford and Andrewes (41) determined the size of the bacteriophage particles by the use of a series of graded collodion membranes through which the bacteriophage preparations were forced by a nitrogen pressure equivalent to 76 cm. of mercury. The particles were found to range in size from 8 to 12 millimicrons to 50 to 75 millimicrons. The particle size was relatively constant for each bacteriophage, even if it had been propagated on various organisms. Diffusion of broth suspensions through a sintered Jena glass disc indicated the same relative sizes of the bacteriophage particles. Bacteriophages purified by lysis or by washing behaved in the same way as did unpurified preparations. The bacteriophages with the smaller size particles formed plaques which were relatively large in size.

As a result of cataphoresis experiments made with buffered broth suspensions at pH 7.4 and pH 4.2, all but one out of 20 bacteriophage strains were demonstrated to be unquestionably negative in their electrical charge (23). The one unusual strain apparently had a very slightly negative charge and it also behaved peculiarly in some other physical respects. The electrophoretic migration rates varied to a considerable extent between the various bacteriophages, and an attempt was made to use the differences as a basis for classification.

Kligler, Olitzki and Aschner (76) purified bacteriophages by adsorption on kaolin and subsequent elution with a weak ammonia solution. Approximately $\frac{N}{50}$ buffers of citrate, citrate-phosphate mixtures and sodium

hydroxide-glycocol solutions were used. In acid and decidedly alkaline suspensions, the purified bacteriophage particles were amphoteric in nature, but they were definitely negatively charged in neutral and mildly alkaline systems. When unpurified bacteriophage preparations were used, migration of the actively lytic particles was definitely to the positive pole, indicating a negative charge upon the particles within the pH range which was used.

Krueger and Tamado (92) partially purified a coli bacteriophage by cataphoretic means, the particles migrating into an agar gel from which they were extracted with physiological saline. A relatively unstable preparation having a concentration 10^{16} corpuscles per milliliter was prepared in this way from a preparation in which the concentration was only slightly above 10^{9} particles per milliliter. The purified preparation had less resistance to physical and chemical agents of inactivation than the unpurified bacteriophage.

Girard and Sertic (49) used a centrifuge which developed a sedimentation force 300,000 times greater than gravity to attain the partial removal of bacteriophage from the suspension medium after 4 hours.

High speed centrifugation was employed by Schlesinger (126) to obtain and wash a purified bacteriophage. Chemical analysis of a "purified" bacteriophage showed 42 per cent carbon, 6.4 per cent hydrogen, 13.2 per cent nitrogen and 3.7 per cent phosphorous, with sulfur undetermined, indicating a structure largely protein. No indication of carbohydrate was obtained, although there was evidence of the presence of some fat or lipin.

Kligler and Olitzki (75) employed kaolin at a pH of 5.5 as an adsorbent for bacteriophage. Elution was by means of $\frac{N}{100}$ sodium hydroxide. By means of a second adsorption and elution, followed by dialysis for 6

days under sterile conditions, they produced a suspension of bacteriophage free from protein by the usual tests.

Meyer and coworkers (113) purified bacteriophage by means of a series of precipitations of undesirable materials, precipitation and resuspension of the active principle, and final precipitation of foreign material. The purified preparation was more sensitive to chemical and physical agents than the unpurified filtrate. The xanthoproteic and Greenberg phenol reactions were positive, the Molisch test weakly positive and the biuret, Millon, glyoxallic acid, nitroprusside and alkaline lead reactions were negative. Neither glycolysis nor oxygen uptake was shown by the concentrated bacteriophage.

By means of a cataphoretic apparatus, a coli bacteriophage was purified so that it gave no chemical tests for protein substances and suffered a decrease in titer from 10^{-8} to 10^{-6} (125).

LeMar and Myers (95) used a Salmonella enteriditis bacteriophage active to the tenth tenfold dilution in their experiments upon the solubility of bacteriophage in ether. Extraction with an equal volume of ether considerably reduced the ability of the extracted filtrate to produce lysis. The solubility of the bacteriophage in the dehydrated ether extract was demonstrated by drying the extract and dissolving the residue in broth, the broth solution being capable of causing complete lysis.

The natural occurrence and artificial production of organisms which are sensitive and non-sensitive to bacteriophage

The occurrence of bacterial variants has been receiving an increased amount of attention during recent years and the relationship between bacteriophage and variation has received its share of notice. The literature dealing with the study of bacterial variation is so extensive that a comprehensive review would be impractical. Only those articles which are more definitely related to the phenomenon of transmissible lysis of bacterial cells and the production of variants by means of bacteriophage will be discussed.

By allowing an apparently normal culture of Bacillus coli to age, Gratia (51) found that vitreous areas in the colonies on the dried agar film were made up of organisms resistant to the action of the bacteriophage to which the parent strain was sensitive. Although the parent strain was non-motile, the resistant form was motile. Separation of the two strains by means of fermentation tests was not possible.

In a later article, Gratia (52) reported the isolation of seven different forms of variants from one strain of Bacillus coli by aging or by bacteriophage action. The bacteriophage-resistant organisms were divided into three groups: those which were resistant enough to survive and grow in the presence of a moderate quantity of bacteriophage but were still slightly sensitive and had a tendency to form irregular and lysogenic colonies, those which were entirely resistant and were non-lysogenic, and those which were mechanically resistant because of their mucoid nature.

Using 6 strains of Bacillus dysenteriae Shiga, 5 of which were typically smooth in growth form in broth, in stability of emulsion and in type of specific agglutination and all of which were resistant to bacteriophage activity with the possible exception of a slight amount of

sensitivity in the case of the one strain which was not typically smooth. Arkwright (3) studied the production of variants susceptible to the action of bacteriophage. Sensitive variants were obtained from 4 cultures, although aging for as much as 61 days was necessary in some cases. All of the types produced numerous variants which were resistant to bacteriophage action. The sensitive variants were all of the rough type when precipitation by salts was used as a criterion, but they varied in their form of growth in bouillon and in their ability to emulsify in water. The cultures retained their sensitivity for the duration of the experiments, as long as 3 months in some cases. The conclusion reached was that comparable resistant and nonresistant variants are produced by bacteriophage activity and by other agents such as holding for extended periods.

Breinl and Hoder (8) obtained variants by streaking bacteriophage and sensitive culture on agar or by simultaneous inoculation of broth with bacteriophage and sensitive organism, the first method being preferable. The resistant organisms obtained from the secondary growth of these cultures showed variation from the original culture of paratyphoid organism in serological relationships, in bacteriophage sensitivity and in cultural characteristics. Some of the secondary cultures obtained, especially those from the agar smears, seemed to be intermediate between the original culture and the stable mutants. Variants similar to those resulting from the action of bacteriophages had previously been obtained by means of the aging of the same original culture.

Fejgin (46) studied the organisms which appeared as secondary growth following the primary clearing of cultures of Shiga strain dysentery bacilli in meat bouillon at pH 8.2. Three cultures were obtained in this way, and all of them were resistant to the bacteriophage which

was responsible for their production. All three showed some tendency toward the "R" form, broth in which they had grown not being uniformly turbid because the cells tended to precipitate at the bottom of the tube. One culture showed the same fermentation characteristics as the parent strain, one showed slight differences in the carbohydrates which it fermented, while the third culture produced acid and gas from a considerable number of carbohydrates. The first organism had lost its power of being agglutinated by a polyvalent anti-Shiga serum, the second one was agglutinated by a serum dilution of 1:800 and the third by a dilution of 1:1600. The normal Shiga organism adsorbed from the serum all the antibodies active against any of the resistants. Feeble and slow agglutination of the normal strain by a serum dilution of 1:50 was possible after the resistant strains had adsorbed all they would from the serum.

Bacteriophages active against Bacillus pullorum, Bacillus gallinarum, Bacillus typhosus, Bacillus coli communis and Bacillus dysenteriae were obtained from sewage by Hadley (54). After being sent through numerous transfers to remove any bacteriophage which might have been carried mechanically, they were used to determine the bacteriophage sensitivities of the homologous organisms. The serological relationships of the 5 organisms were also determined. With the exception of 2 cases in which the bacteriophage action was very weak but still slightly positive, the cultures showed the same bacteriophagical and serological relationships.

Burnet (15) studied the relationship between the heat-stable agglutinogens, as demonstrated by the sensitivity to agglutination by sera from known rough and smooth strains, and the sensitivity to bacteriophages active toward Salmonella enteriditis, Bacillus typhosus and Bacillus pullorum. By the use of six strains of bacteriophage, two groups of

organisms of the smooth type and a single group of the rough type were established for Salmonella enteriditis and Bacillus typhosus and one rough and one smooth group for Bacillus pullorum. Some unstable intermediate types were obtained by bacteriophage action upon sensitive cultures. In most cases serological tests of the organisms divided them into the rough and smooth types in the same way that the bacteriophage relationships did. This parallelism was considered as evidence that the heat-stable agglutinogens were closely related to the specific adsorption of the lytic principle which constitutes the first stage in the lysis of bacteria by bacteriophage. When a change from rough to smooth or smooth to rough was induced by a bacteriophage, the serological characteristics and the bacteriophage sensitivities changed simultaneously, although an occasional organism derived in this way was not sensitive to all of the bacteriophages of the typical group.

Using 15 bacteriophages, all of which were derived from single plaque isolations from various sources but mostly from intestinal contents, and a number of Salmonella enteriditis and other strains of intestinal bacteria, Burnet (16) found that three of the bacteriophages lysed only smooth forms of bacteria, four only rough forms, two all normally occurring variants and six were irregular but, in general, lysed rough forms actively and smooth forms only weakly. Sensitivities were determined by plaque formation upon agar. The sera for agglutination tests were from pure strains of rough and smooth organisms, and the designation rough or smooth applied only to the antigenic natures rather than the appearance of the colonies. A smooth strain of Salmonella sanguinarum which would not produce rough types in old cultures yielded rough types as the result of bacteriophage activity. By the use of a strain of bacteriophage which lysed only rough forms, it was possible to cause some

rough cultures to yield subcultures which were of the smooth type. Many different types of variants were obtained from some rough cultures by the use of various bacteriophages. Apparently the "O" antigen was demonstrated to be necessary for a reversion from rough to smooth in the true antigenic sense. Using mice for test purposes, the smooth forms derived from the rough forms were found to be much more virulent than the rough forms. No effect upon the sensitivity to bacteriophage was observed as a result of the adoption of the pellicle form of growth or loss of the "H" antigen. This investigator believed that the "selection of spontaneous variants" rather than the development of immunity, as supported by d'Herelle, was the means of obtaining resistant strains by means of bacteriophage action. He believed that the principle factor which determined whether or not a certain strain of bacterium was lysed by a strain of bacteriophage was the capacity of the bacteriophage "to be specifically adsorbed by the same entity that functions in serological reactions as the heat stable ("O" or "R") agglutinogen". In support of this view, he pointed out (a) that the presence or absence of heat-labile or "H" antigen is without influence upon the behavior of a strain of bacteria toward bacteriophage, (b) that changes in cultural behavior such as the pellicle-forming "O" variants which are unassociated with antigenic change have little or no influence upon sensitivity to the action of bacteriophage, (c) that change in the nature of the heat-stable antigen from "O" to "R" is associated with a sharp change in bacteriophage reactions, (d) that the organisms which have common heat-stable antigens are very similar in the range of bacteriophages to which they are sensitive, (e) that a bacteriophage is no longer adsorbed by organisms which have become resistant to that bacteriophage, and (f) that organisms which are sensitive to bacteriophage action are still capable of adsorbing that bacteriophage even after

they have been killed by means of heating to 100° C. Spontaneous, discontimuous variation in the heat-stable antigenic struction was considered to be demonstratable by means of the change of sensitivity toward bacteriophage which the culture in question underwent. The theory was advanced that the bacteriophage probably produced resistant variant strains by selective sparing of those variants to which it was not specifically adsorbed, there being only a limited number of variants which could appear, the smooth and rough forms being two of them.

Burnet (17) investigated Salmonella gallinarum and bacteriophage-derived resistants of the same phase as the parent strains, which were both rough and smooth and were typical antigenically, morphologically and culturally. Three bacteriophages, one active against smooth forms only, one active against both rough and smooth forms equally, and one which lysed only the rough forms, were used. The original cultures were each lysed by the 2 bacteriophages which lysed the form to which they belonged, either rough or smooth. The resistant strains, which were obtained by the use of the bacteriophage which lysed both rough and smooth strains, were sensitive to the bacteriophages which lysed only the cultures of the form to which they belonged, either rough or smooth, but resistant to the bacteriophage which had caused their formation. Each strain adsorbed the bacteriophage to which it was sensitive and left the other strains undisturbed.

The types of resistant secondary growth which may appear in cultures that have undergone lysis were classified by Burnet (18). He divided them into (a) those with partial resistance, in which the organisms are resistant when the concentration of bacteriophage and growth products is high but which are sensitive in a fresh culture to which bacteriophage has just been added, (b) those which are permanently lysogenic,

growing normally but in symbiosis with a bacteriophage and producing a filtrate lytic for the sensitive parent strain, (c) those which have developed specific true resistance and do not adsorb bacteriophage or permit its multiplication at their expense, (d) those which are resistant to bacteriophage because they are mucous-secreting forms which are apparently mechanically protected, and (e) those which possess a non-specific phasic resistance because of a change in phase between rough and smooth.

Hadley (55) considered resistance to bacteriophage to be relative and not absolute, "SR" and "RR" forms which were not final or absolute but usually capable of further dissociation by the action of bacteriophage ("stronger phages") to which they are sensitive frequently being formed. High resistance to bacteriophage was apparently seldom acquired in a single step except by the action of an extremely active lytic principle.

Powers (123) reported that 6 pairs of dissociated organisms, five from the genus Escherichia and one from the genus Aerobacter, were separable by means of their bacteriophage sensitivites as well as their morphological and cultural characteristics. By this means they were separated into the smooth and rough forms. When 8 bacteriophages, of varying ranges of activity and all derived from sewage, were used to test 161 cultures of the genus Escherichia, 36 cultures were sensitive to bacteriophage, plaque formation on agar smears being used as a criterion.

In a study of bacteriophages active toward hemolytic streptococci, Evans (43) found that secondary growth only occurred with some of the combinations of sensitive organism and bacteriophages which lysed that organism. The plaques formed by the Streptococcus bacteriophages

were usually less than a millimeter in diameter.

Doorenbos (39) found that an agglutinable cholera vibrio could be transformed into a non-agglutinable one, a hemolytic organism into a non-hemolytic one, and a feeble indol reactor into a strong indol reactor by the use of bacteriophage.

Grumbach (53) believed that variations in colony form, growth in bouillon, ability to split esculin, reduction of litmus milk and hemolysis were related to the action of bacteriophage in producing Enterococcus variants. "Flatterformen" were shown in the first agar culture by 39 of 66 freshly isolated cultures and, of the 27 which showed no such forms, 16 were strongly dissociable. The conclusion was reached that the greater part of the variants which arise among the streptococci are due to the action of bacteriophage.

Chen (26) obtained smooth and non-motile, motile and rough, and non-motile and rough variants of an originally smooth and motile culture of Vibrio cholerae after growth with bacteriophage for several weeks, with transfers and additions of bacteriophage at weekly intervals. Two transfers each day in peptone water tended to cause all three of the variants to revert to the original type, but only the motile and rough form tended to revert when the transfers were made upon agar slants. Animal passage also tended to cause reversion. The rough variants were spontaneously agglutinable and gave granular growth in broth while the smooth variants were not spontaneously agglutinable and grew uniformly in broth. All of the cultures were culturally typical, although they showed slight variations serologically.

Mutants derived from a strain of apparently bacteriophage-free Salmonella enteriditis by means of an Escherichia coli bacteriophage were studied by d'Herelle and Rakieten (38). The mutants were obtained

most readily by the addition of a minute quantity of young culture and one drop of a potent bacteriophage to each of a series of tubes of bouillon and holding at room temperature for 15 to 20 days, after which the cloudiest cultures were plated and various colonies were picked. Eight mutants, all of which were comparatively stable, were used. When first isolated, all eight of these cultures were lysogenic, filtrates from them lysing the original culture. After 150 transfers, all of the cultures were still lysogenic, although the bacteriophage was so attenuated that plaque formation, rather than lysis, had to be used for the detection of the lytic principle. The bacteriophages which were associated with two of the cultures after 150 transfers were so attenuated that they would not lyse the original culture, action only being detectable upon more sensitive test organisms. After 40 months, all of the strains were still lysogenic but only when very sensitive strains of Salmonella gallinarum and Salmonella suipestifer were used, although each bacteriophage still possessed its distinctive characteristics. After 50 transfers, all mutants were still non-sensitive to the parent bacteriophage but, after 100 transfers, four were partially sensitive and four were still non-sensitive. After 150 transfers, all of the mutants had reverted sufficiently to show some degree of sensitivity and they varied from virulent to avirulent toward mice. The mutants all had the same sugar fermentation characteristics as the parent culture. The agglutinative and agglutinogenic characters of all of the mutants were different in some respect. The possibility of adding a second or even a third bacteriophage to a lysogenic organism with the resultant establishment of a multiple symbiosis was shown. These workers believed that bacterial strains were undergoing constant change as a result of the action of bacteriophages and that only isolated cultures of bacteria

maintained constant characteristics.

d'Herelle (35) found that forms of bacteria resistant toward bacteriophage developed more readily in large volumes of liquid when there were larger numbers of organisms from which variants could arise. When first isolated, the variants obtained by bacteriophage action were non-agglutinable by specific sera from the parent strain, but they regained their agglutinability after growth for some time upon gelatin.

Variation in *Streptococcus lactis*

There have been few studies of the variation in the species commonly known as *Streptococcus lactis*.

Buchanan and Truax (14) attempted unsuccessfully to obtain high and low acid strains of *Streptococcus lactis* by selection at each of 23 transfers. They found that there was no tendency for divergence from the mean acid production; in fact, continued growth under uniformly favorable conditions seemed to render the organisms less variable.

Harriman and Hammer (66), using *Streptococcus lactis*, *Streptococcus lactis* var. *maltigenes* and *Streptococcus lactis* var. *hollandicus*, obtained only 97 slow cultures from the 6,475 colonies picked from plates of 10 original cultures carried through a series of platings. Both uncoagulated cultures and old cultures of rapidly coagulating strains gave about the same ratio of slow to normal *Streptococcus lactis* cultures, as did the normally coagulated cultures. It was found that the rapidly coagulating cultures almost always produced a greater amount of amino nitrogen, indicating that the speed of acid formation probably had a physiological basis related to the nitrogenous nutrition of the cell.

Orla-Jensen (120) considered the lactic acid streptococci to be very stable in their characteristics when held for considerable periods in his laboratory. He demonstrated that there were numerous qualitative and quantitative differences among the strains of organisms commonly included under the designation *Streptococcus lactis* and he used some of these differences, especially those of carbohydrate fermentation, as bases for the creation of a number of new species.

Hammer (60) found that non-ropy cultures could frequently be obtained from ropy cultures of *Streptococcus lactis* but that ropy strains

were only rarely secured from non-ropy strains.

Hammer and Baker (61) designated four varieties of Streptococcus lactis, each of which was atypical with respect to one characteristic, and placed the organisms which resembled Streptococcus lactis except for their thermal tolerance in a separate species, Streptococcus thermophilus. Streptococcus lactis var. anoxyphilus and Streptococcus lactis var. maltigenes appeared to be relatively stable. The slowly developing strain or Streptococcus lactis var. tardus was not constant, organisms which would be placed in this group frequently being obtained from normal cultures, especially those which had been held for some time. The degree of slowness was not constant. Streptococcus lactis var. hollandicus was shown to be capable of giving rise to normal strains of Streptococcus lactis under ordinary conditions of management. Holding at 37° C. completely overcame the ropy characteristic in the cultures which were studied.

Hammer and Patil (63) demonstrated that, by means of soluble and amino nitrogen determinations made upon milk cultures, Streptococcus lactis cultures could be divided into two groups: those which were definitely proteolytic and coagulated milk rapidly at 21° C. and those which were non-proteolytic and were variable in the speed with which they coagulated milk at 21° C. The correlation between proteolysis and speed of coagulation did not hold as satisfactorily at 30° C. or 37° C. as at 21° C. The amount of proteolysis was apparently a constant characteristic of each culture studied.

Stark and Sherman (136) studied 235 cultures of Streptococcus lactis obtained from various sources and found that, while many of the biochemical characteristics were the same for all of the cultures, there was great variation among the organisms with respect to the carbohy-

drates which they fermented. Using only arabinose, xylose, sucrose and mannitol, organisms fermenting nine of the sixteen possible combinations of these 4 test substances were obtained. Streptococcus lactis cultures fermenting the other seven possible combinations of these 4 substances were found described in the literature. No statement concerning the stability of types was made.

From this brief review of the literature, it is evident that some variation occurs with the Streptococcus lactis organism. The cultures carried in pure culture under uniform laboratory conditions are apparently relatively stable. Under natural conditions of varying temperatures, growth media, symbiosis, etc., variants are seemingly more commonly found, since so many different strains or at least very closely related organisms have been isolated from a variety of habitats. Experimentally induced variation is, however, apparently unrealized at this time among the members of the species.

EXPERIMENTAL METHODS

The filtrates containing the strains of inhibitory principle which were used in the studies herein reported were obtained originally from butter cultures. The coagulated culture was first filtered through an ordinary coarse filter paper to remove the coagulum. The whey obtained in this manner was then filtered through a grade N Berkfeld filter, under negative pressure, clear filtrate resulting. When it was desired to obtain a filtrate from a culture which had not yet coagulated, sufficient dilute lactic acid was added to the culture to cause coagulation. The coagulated culture was then filtered in the same way as a naturally coagulated one. When inhibitory principle was propagated in broths, the cultures were filtered by means of a Berkfeld filter without previous treatment. All of the equipment utilized in the handling of the cultures and filtrates was sterilized at 15 pounds pressure for at least half an hour in the autoclave. The filtrates were culturally and microscopically sterile and did not develop secondary growth. Occasional contamination with mold occurred because of the prevalence of mold spores in the air of the laboratory.

Streptococcus lactis cultures were obtained by plating the material from which isolations were to be made on tomato juice agar. Incubation was at room temperature. Isolated colonies were inoculated from the plates into sterile litmus milk. If the litmus milk cultures were of the type characteristic of Streptococcus lactis, they were kept for study. In many cases, the cultures were purified by a second plating upon tomato juice agar, followed by reisolation into litmus milk. The Streptococcus lactis cultures retained for further study were transferred at 3 or 4 day intervals into fresh litmus milk and incubated and

held at room temperature.

During the summer of 1935, partially coagulated litmus milk cultures were stored in the ice cream hardening room at a temperature of approximately -15° F. (-26.1° C.). At the end of the 3 month storage period, the cultures were allowed to thaw at room temperature and were reinoculated into litmus milk. Usually 2 transfers sufficed to bring about the restoration of the normal growth rate. The stock cultures stored in this manner apparently retained their cultural and sensitivity characteristics.

The activity of the filtrates containing inhibitory principle usually was determined by the addition of 0.1 ml. of the filtrate to approximately 8 ml. of litmus milk, followed by the inoculation of the milk with a drop of a 24 hour culture of the test organism grown in litmus milk or occasionally in tomato juice broth. A control culture which was inoculated with bacteria but to which filtrate was not added was made each time. Observations of the cultures were usually made at 2 hour intervals during the period from 16 to 30 hours after inoculation and at less frequent intervals thereafter. The sequence of the changes occurring in the milk and the time required for coagulation were used as the criteria for the determination of inhibition of the culture by the principle present in the filtrate. If the culture to which the filtrate had been added required an appreciably longer time, 10 hours or more, to coagulate than the control culture required or if the test culture containing filtrate remained unchanged for some time after the control culture became either coagulated or acid and reduced, the filtrate was considered to be inhibitory to the strain of organism used for testing purposes. The second condition was necessary because, in some cases, the growth of secondary organisms, which occurred as a result of

the action of inhibitory principle, was so rapid that coagulation occurred in almost normal time. The same method was also used to determine the sensitivities of S. lactis and of other organisms to the action of the inhibitory principle, filtrates of known activity being used.

A second method which was used to a limited extent in the study of the sensitivity of S. lactis cultures to inhibitory principle and also to determine the presence of the principle in filtrates was the determination of plaque formation upon solid media. From 12 to 14 ml. of tomato juice agar (pH 6.8) were poured into each petri plate and allowed to harden and dry before several drops of young culture of bacteria were spread as evenly as possible over the surface. The plate was again allowed to dry before loopfuls of the filtrates were placed upon the surface of the agar in areas which were marked off by wax pencil upon the backs of the plates. The loops delivered a little less than 0.01 ml. of filtrate. Except in the case of the lactobacilli, incubation of the plates was at room temperature. Observations were made at the end of 24 and 48 hour intervals. The presence of plaques, which are areas free from the growth of bacteria, at the spot where the filtrate was placed was considered as evidence of the presence of inhibitory principle to which the test organism was sensitive.

The dilution method was used to determine the titers of the filtrates containing inhibitory principle. Although the method has its disadvantages, the principal one being that only the minimum amount of filtrate which will cause inhibition and not the absolute amount of inhibitory principle present in the filtrate is determined, the advantages of simplicity and general adaptability to filtrates of varying titers and virulences make it preferable to the other methods which have been suggested. Ordinary 99 ml. sterile water blanks were used, 1 ml. of the

filtrate or of a previous dilution of the filtrate being added to the blank. 0.1 and 1 ml. quantities of diluted filtrate were added to 8 ml. quantities of litmus milk which were then inoculated with 24 hour cultures of the test organisms. Thorough shaking of the culture before incubation, which was always at room temperature, was found to be absolutely necessary. The smallest quantity of filtrate, expressed in milliliters, which would cause a significant retardation of typical acidification and reduction or coagulation was considered to be the titer of that filtrate. If 0.000000001 ml. of filtrate was found to be the limiting active dilution, the titer of the filtrate was expressed as 10^{-9}. The tests were always made in duplicate. If one duplicate indicated a titer of 10^{-9} and the other indicated 10^{-10}, the titer of the filtrate in question was designated as $10^{-9.78}$. The results obtained by this method of estimation were almost invariably duplicable, indicating that the method was reasonably accurate for its purpose.

The litmus milk which was used in these studies was prepared from pasteurized skim milk to which a sufficient amount of aqueous infusion of litmus cubes had been added to impart a slight but definite blue color. Sterilization was at 15 pounds of steam pressure for 25 minutes.

The tomato juice agar was made by using 20 per cent clear tomato juice, 1 per cent Bacto proteose peptone, 1 per cent Bacto peptonized milk, 1.5 per cent agar and water to make 100 per cent. The pH was adjusted colorimetrically and checked electrometrically after sterilization, if the effect of pH entered into the experiment. If a buffer was desired, $\frac{M}{20}$ phosphate was added prior to the adjustment of the pH. Tomato juice broth was made in the same way, except that the agar was omitted.

Beef infusion broth was made from 0.5 per cent Bacto peptone,

50 per cent beef infusion and water to make 100 per cent. The beef infusion was prepared by infusing one pound of lean ground beef in 500 ml. of distilled water at 0° to 10° C. overnight and expressing the infusion by pressure after first heating to 45° C. The pH was adjusted colorimetrically and checked electrometrically after sterilization.

Buffered milk digest broth was made by adding 1.5 per cent Bacto peptonized milk to distilled water. Potassium acid phthalate was used for buffering at pH levels below 6 and disodium phosphate was used above pH 6, the concentrations being $\frac{M}{20}$ in each case. The pH was adjusted colorimetrically in each case and checked electrometrically following sterilization.

Unbuffered milk digest broth was prepared by adding 2.5 per cent of peptonized milk to distilled water.

THE SOURCES OF MATERIALS

In order to make them readily available in one place, the sources of the filtrates which are specifically mentioned by a numerical or alphabetical designation in the section upon experimental results are shown in table I. The sources of the cultures of Streptococcus lactis are shown in table II.

Table 1.

The sources of the filtrates

Filtrate number	Date secured	Source	Remarks
1	10-19-34	BC232	Slow 10 gal. culture in creamery.
2	10-19-34	BC232	Slow 10 gal. culture in creamery.
3	10-19-34	BC232	Slow first transfer of source of filtrate 1.
5	10-25-34	BC232	Normal laboratory culture.
6	10-25-34	BC232	Slightly slow in milk exposed in laboratory.
7	10-25-34	BC232	Slow third transfer of source of filtrate 1.
9	10-25-34	BC15/3	Slow laboratory culture.
10	10-25-34	BC232	Slow in milk exposed in creamery.
13	10-27-34	BC15/3+F191	
14	10-27-34	BC232+F1	
16	10-27-34	BC15/1	Slow laboratory culture.
17	10-27-34	BC15/1+F191	
21	11-13-34	BC103A	Slow laboratory culture.
29	11-23-34	S42+F10	
30	11-23-34	S42+F14	
32	11-23-34	S42+F191	
35	12-16-34	S42+F29	Incubated 12 hrs. before addition of filtrate.
36	12-16-34	S42+F29	Incubated 18 hrs. before addition of filtrate.
41	1-16-35	BC15/3	Slow 10 gal. culture in creamery.
44	1-18-35	S97+F21	
48	1-25-35	BC15/3	Normal in milk exposed in laboratory.
49	1-25-35	BC26	Normal in milk exposed in laboratory.
50	1-25-35	BC232	Normal in milk exposed in laboratory.
51	1-25-35	BC15/1	From source of inoculum from which F52 was obtained.
52	1-25-35	BC/151	Slow laboratory culture.
53	1-25-35	BC15/1	Slow in milk exposed in creamery.
54	1-26-35	BC232	Slow in milk exposed in creamery.
55	1-26-35	BC15/3	Slow in milk exposed in creamery.
61	2-13-35	BC15/3	Slow and ropy in 1 gal. can.
62	2-14-35	S113+F3	Plaque isolation.

Table 1 (continued)

69	2-19-35	BC146	Slow laboratory culture.
72	2-28-35	S161+F61	
73	2-28-35	S163+F191	
78	3-9-35	S83+F72	
79	3-9-35	S163+F73	
80	3-9-35	S42+F73	
82	3-9-35	S42+F44	
92	3-14-35	S161+F72	
96	3-14-35	BC15/3	Grown in raw milk.
97	3-14-35	BC232	Grown in raw milk.
98	3-14-35	BC15/3	Grown in raw milk.
104	3-15-35	BC232	Grown in raw milk.
108	3-16-35	BC15/3	Normal laboratory culture.
110	3-26-35	BCCR	
118	4-11-35	S99+F191	
123	4-11-35	S99+F191	30° C. incubation.
124	4-11-35	S99+F191	30° C. incubation for 4 hr. before filtrate addition.
127	4-22-35	S99+F118	
128	4-22-35	S99+F123	30° C. incubation.
129	4-22-35	S147+F191	30° C. incubation.
130	4-22-35	S147+F124	30° C. incubation.
132	5-2-35	S147+F129	
134	5-2-35	S111+F54	
135	5-2-35	S97+F110	
138	5-2-35	S111+F54	Tomato juice broth at pH 7.8 used.
142	5-6-35	S99+F127	
152	5-27-35	S147+F132	
153	5-30-35	S97+F135	
154	5-30-35	S99+F142	
157	5-31-35	S99+F142	
158	5-31-35	S147+F132	
159	9-26-35	BC232	Slow in 400 gal. vat.
160	10-9-35	S97+F135	
162	10-9-35	S99+F157	
164	10-9-35	S147+F158	
166	10-11-35	S97+F160	
167	10-11-35	S97+F161	
168	10-11-35	S99+F162	
185	10-21-35	S97+F62	
191	Unknown	BC15/3	Obtained from Harriman, Iowa State College.
192	10-24-35	S163+F134	3 propagations.
198	10-26-35	S163+F192	
204	10-30-35	S213+F191	
205	11-5-35	S97+F167	5 propagations.
206	11-5-35	S97+F61	5 propagations.
207	11-5-35	S97+F185	3 propagations.
208	11-5-35	S147+F164	6 propagations.
209	11-5-35	SR+FR	FR obtained from Whitehead, N. Z. Institute for Dairy Research.

Table 1 (continued)

210	11-5-35	SRW+FRW	FRW obtained from Whitehead, N. Z. Institute for Dairy Research.
211	11-7-35	S97+F206	
212	11-7-35	S97+F207	
213	11-7-35	S147+F208	
215	11-7-35	SRW+F210	
218	11-11-35	S163+F198	2 propagations.
235	11-16-35	S99+F168	4 propagations.
236	11-16-35	S147+F61	2 propagations.
246	11-23-35	BC122	Slow culture from sample from a commercial creamery.
246a	11-26-35	S122FE+F191	2 propagations.
247a	11-23-35	BCM_1	Slow laboratory culture.
251	11-26-35	SR+F206	2 propagations.
256	12-12-35	S153A+F191	
257	12-12-35	S1530+F138	
265	12-14-35	S1530+F257	
266	12-17-35	BCFL	Slow and ropy laboratory culture.
273	1-6-36	BCA	Slow laboratory culture.
274	1-6-36	BCB	Normal laboratory culture.
275	1-6-36	BCC	Slow laboratory culture.
276	1-9-36	S97+F212	2 propagations.
278	1-9-36	S147+F213	2 propagations.
296	1-20-36	S97+F61	Plaque isolation propagated twice.
300	1-24-36	S97+F3	Plaque isolation propagated 3 times.
310	1-29-36	S97+F276	2 propagations.
311	1-29-36	S147+F278	2 propagations.
313	2-3-36	SRW+F210	4 propagations.
315	2-6-36	BC122F	Slow and ropy 10 gal. culture in creamery.
345	2-14-36	BC103	Slow and ropy laboratory culture.
359	2-19-36	BC232	Laboratory culture saturated with oxygen.
363	2-19-36	BC122F	Laboratory culture saturated with oxygen.
367	2-21-36	BC16	Normal laboratory culture.
368	2-21-36	BC15/3	Normal laboratory culture.
369	2-21-36	BC146	Normal laboratory culture.
370	2-21-36	BC19/1	Normal laboratory culture.
371	2-21-36	BCC_4	Normal laboratory culture.
372	2-21-36	BCG	Normal laboratory culture.
373	2-21-36	BC103	Normal laboratory culture.
374	2-21-36	BCF_5	Normal laboratory culture.
375	2-21-36	BCH_5	Normal laboratory culture.
384	2-26-36	BCLOL	Normal laboratory culture.
385	2-26-36	BC233	Normal laboratory culture.
386	2-26-36	BC92	Normal laboratory culture.
387	2-26-36	BC66	Normal laboratory culture.
388	2-26-36	BCM_1	Normal laboratory culture.

Table 1 (continued)

389	2-26-36	BCH_4	Normal laboratory culture.
A	1-13-36	S97+F206	
B	1-13-36	S147+F213	2 propagations.

BC = butter culture
F = filtrate
S = S. lactis culture

Table 2

The sources of the Streptococcus lactis cultures

Culture number	Source	Date isolated	Remarks
22C	BC22		
25	BC15/1	11-8-34	
27	BC15/1	11-8-34	
37	BC232	11-8-34	
42	BC232	11-8-34	
47	BC232	11-8-34	
57	BC19/1	11-15-34	
68	Raw milk whey	11-15-34	
72	Sour raw milk	11-15-34	
75	Sour raw milk	11-15-34	
83	BC15/3	11-15-34	
88	BC15/3	11-15-34	
97	BC232	11-15-34	
99	BC232	11-15-34	
101	BCH	11-15-34	
103A	BC103	11-14-35	
111	BC146	12-13-34	
113	BC232	1-14-35	
122FE	BC122F	11-4-35	
146B	BC146	10-17-35	
146C	BC146	10-17-35	
147	BCS	1-14-35	
148	BCS	1-14-35	
151C	BC15/1	12-9-35	
151K	BC15/1	12-9-35	
153A	BC15/3	11-24-35	
153C	BC15/3	11-24-35	
161	S113+F9	2-13-35	Isolated from secondary growth in a plaque.
163	S99+F54	2-13-35	Isolated from secondary growth in a plaque.
167	S42+F54	2-13-35	Isolated from secondary growth in a plaque.
176	BC15/3	2-20-35	Slightly ropy.
178	S42+F61	2-25-35	
180	S111+F61	2-25-35	
183	S113+F3	2-25-35	Isolated from secondary growth in a plaque.
185	S113+F7	2-25-35	Isolated from secondary growth in a plaque.
197	S161+F21	3-8-35	
201	S176+F55	3-8-35	

Table 2 (continued)

206	S185+F61	3-11-35	
213	S185+F41	3-11-35	
214	S185+F54	3-11-35	
217	S42+F72	3-16-35	
222	S178+F17	3-16-35	
224	S185+F72	3-16-35	
227	S185+F73	3-16-35	
229	S97+F1	3-19-35	
231	S197+F61	3-19-35	
232	S206+F73	3-19-35	
232T	BC232	11-24-35	
233F	BC233	11-24-35	
236	S214+F73	3-19-35	
239	S214+F80	3-19-35	
251	S99+F80	4-7-35	
261	S217+F191	4-7-35	
263	S224+F17	4-7-35	
269	S97+F72	4-13-35	
273	S97+F110	4-13-35	
277	S163+F191	4-13-35	
281	S185+F191	4-13-35	
286	S99+F123	4-23-35	
288	S263+F119	4-23-35	
289	S281+F118	4-23-35	
295	S148+F41	5-1-35	
299	S273+F72	5-1-35	
301	S281+F128	5-1-35	
305	S288+F128	5-1-35	
331	S288+F142	6-3-35	
335	Sour raw milk	10-6-35	
363	S146B+F191	12-11-35	
364	S122FE+F134	12-11-35	
365	S97+F206	12-11-35	
380	S151C+F41	12-14-35	
382	S151C+F138	12-14-35	
384	S151C+F168	12-14-35	
386	S233F+F191	12-14-35	
388	S151C+F191	12-16-35	
391	S363+F14	12-16-35	
392	S363+F41	12-16-35	
394	S363+F51	12-16-35	
396	S363+F138	12-16-35	
a	S233F+F315	3-4-36	
b	S233F+F54	3-4-36	
c	S97+F315	3-4-36	
d	S97+F211	3-4-36	
FLJ	BCFL	11-20-35	
R	--	--	Obtained from Whitehead, N. Z. Institute for Dairy Research.
RW	--	--	Obtained from Whitehead, N. Z. Institute for Dairy Research.

BC = butter culture F = filtrate
S = S. lactis culture

EXPERIMENTAL RESULTS

Part I. The inhibitory principle and its action upon the organisms which occur in butter cultures.

The characteristics of the filtrates obtained from various normal and slow butter cultures.

The various bacteria-free filtrates which were obtained from a variety of normally and slowly coagulating butter cultures were tested for the presence of inhibitory principle by addition to litmus milk simultaneously inoculated with various cultures of S. lactis which were used for test organisms. The cultures which were used were not the same in all cases, because the S. lactis strains with which the tests were made were chosen to include the greatest possible range of sensitivity to the action of various strains of inhibitory principle. Since constant additions were made to the culture collection, new strains of S. lactis gradually replaced many of the cultures which had been used for earlier determinations.

In order to compare the inhibition characteristics of the filtrates from slow butter cultures with those of filtrates from normal cultures and to compare the strains of S. lactis inhibited by one filtrate with the strains inhibited by other filtrates derived from the same butter culture, the data concerning a number of filtrates are assembled in table 3. The filtrates are grouped according to the butter culture from which they were obtained. The filtrates from a single strain of butter culture are also separated into those which were obtained when the culture was in a slow condition and those which were obtained when the culture was in a normal condition.

The filtrates obtained from slow butter culture 232, while

showing a certain degree of group relationship, were quite diverse in their behavior toward the same test organisms. The one common characteristic with respect to inhibition shown by these 6 filtrates is the ability to retard the growth of S. lactis culture 97. Two groups can be distinguished, those which inhibited only organism 97 and possibly a few very closely related cultures and those which inhibited a considerable group of organisms. In the second group, the 3 filtrates are all slightly different, there being slight variations in the strains of S. lactis inhibited by the individual filtrates.

Filtrates which were divisible into 2 groups were obtained from butter culture 232 which coagulated in normal time. Three filtrates which were unable to inhibit any of the organisms with which they were tested were included in the first group. Filtrate 50 of this group was obtained at the same time that filtrate 54 was obtained from a slow transfer of the same butter culture. Although a number of organisms was sensitive to the action of filtrate 54, none was affected by filtrate 50. The second type obtained from a normally coagulating transfer of butter culture 232 was represented by filtrate 359 which inhibited organisms 97 and 327, thus showing its relationship to other inhibitory filtrates from the same strain of butter culture.

The filtrates from slow cultures of butter culture 15/3 were all characterized by their ability to inhibit a large number of strains of S. lactis. No two of these filtrates were active against all of the same test organisms, although the 4 filtrates did inhibit a considerable number of organisms in common. This is especially noteworthy because 3 of the filtrates were obtained within a period of a month while the fourth filtrate had been obtained a year earlier.

Filtrate 48, which was obtained from a normally coagulating

Table 3

A comparison of the inhibition characteristics of the filtrates obtained from normal and slow butter cultures

Filtrate number	Date of isolation	Butter culture Number	Type	S. lactis cultures inhibited	S. lactis cultures not inhibited
1	10-19-34	232	S	97,113,147	42,47,72,75,83,88,99, 111,161,185
2	10-19-34	232	S	97	47,68,72,75,83,88, 110,111
3	10-23-34	232	S	42,47,97,99,111,113, 147,180,185	72,75,88,161,163,176, 183
10	10-25-34	232	S	37,42,47,83,88,99,97, 111	25,27,68,72,75,110, 113
54	1-26-35	232	S	42,47,83,97,99,111, 176,180,185	27,72,75,88,113,147, 161,163,183
159	9-26-35	232	S	97	42,47,75,83,88,99, 111,113,147,180,185
50	1-25-35	232	N	---	42,47,72,88,97,99, 111,113
97	3-14-35	232	N	---	42,75,97,111,113,161, 163,176,185
104	3-15-35	232	N	---	42,75,97,111,113,161, 163,176,185
359	2-19-36	232	N	97,327	22C,83,99,122FE,146C, 147,151C,1530,185, 232T,233F,386,FLJ, R,RW
41	1-16-35	15/3	S	22C,42,47,83,97,99, 111,113,122FE,147, 1530,180,185,386	72,88,151C,161,163, 183,R,RW
55	1-26-35	15/3	S	22C,97,99,111,151C, 1530,176,232T,233F, FLJ	42,47,72,88,113,161, 163
61	2-13-35	15/3	S	22C,42,47,83,88,97, 99,111,113,147, 1530,180,185,232T, 233F,FLJ	72,75,163,183,R,RW
191	Unknown	15/3	S	22C,83,99,122FE,146C, 147,151C,185,233F,	97,1530,183,232T,FLJ, R,RW

55	1-26-35	15/3	S	22C,97,99,111,151C, 1530,176,232T,233F, FLJ	42,47,72,88,113,161, 163
61	2-13-35	15/3	S	22C,42,47,83,88,97, 99,111,113,147, 1530,180,185,232T, 233F,FLJ	72,75,163,183,R,RW
191	Unknown	15/3	S	22C,83,99,122FE,146C, 147,151C,185,233F, 386	97,1530,183,232T,FLJ, R,RW
48	1-25-35	15/3	N	22C,42,83,99,147, 185,233F,386	75,97,122FE,146C,151C, 1530,183,232T,327, FLJ,R,RW
96	3-14-35	15/3	N	---	42,75,97,111,113,161, 163,176,185
98	3-14-35	15/3	N	97,113,146C,327, FLJ	22C,83,99,122FE,147, 151C,1530,183,185, 232T,233F,386,R,RW
108	3-16-35	15/3	N	97,113	42,75,111,163,176,185
368	2-21-36	15/3	N	146C,233F,386	22C,83,97,99,122FE,147, 151C,1530,183,185, 232T,327,FLJ,R,RW
21	11-13-34	103	S	97,113,180,233F	42,75,83,111,147,1530, 161,163,176,185
345	2-14-36	103	S	146C,327,386	22C,97,99,147,183,FLJ
373	2-21-36	103	N	---	22C,83,97,99,122FE,146C, 147,151C,1530,183,185, 232T,233F,386,FLJ,R,RW
52	1-25-35	15/1	S	42,88,99,122FE, 1530,232T,233F, 386	22C,47,72,75,83,97,99, 146C,151C,185,FLJ
51	1-24-35	15/1	N	42,47,99	72,75,83,88,97,111,113, 147,161,185
315	2-6-36	122F	S	22C,97,122FE,146C, 151C,1530,232T, 233F,327,386	83,99,147,183,185,FLJ, R,RW
363	2-19-36	122F	N	386	22C,97,99,146C,147,183, 327,FLJ
69	2-19-35	146	S	---	22C,83,97,99,122FE,146C, 147,151C,1530,185,232T, 233F,R,RW
369	2-21-36	146	N	97	22C,83,99,122FE,146C,147, 151C,1530,185,232T, 233F,327,R,RW

				233F,327,386	
363	2-19-36	122F	N	386	22C,97,99,146C,147,183,327,FLJ
69	2-19-35	146	S	---	22C,83,97,99,122FE,146C,147,151C,1530,185,232T,233F,R,RW
369	2-21-36	146	N	97	22C,83,99,122FE,146C,147,151C,1530,185,232T,233F,327,R,RW
247a	11-26-35	M_1	S	97,327	22C,83,99,122FE,146C,147,151C,1530,185,232T,233F,R,RW
388	2-26-36	M_1	N	---	22C,83,97,99,122FE,146C,147,151C,153C,185,232T,233F,327,R,RW
53	1-25-35	26	S	42,47,146C,233F,386	22C,83,97,99,122FE,147,151C,1530,183,185,232T,327,FLJ,R,RW
49	1-25-35	26	N	122FE,233F,386	22C,47,75,83,97,99,147,151C,1530,183,185,232T,327,FLJ,R,RW
246	11-23-35	122	S	---	83,122FE,151C,1530,185,232T,233F,327,FLJ,R,RW
266	12-17-35	FL	S	146C	22C,83,97,99,122FE,147,151C,1530,183,185,232T,233F,327,R,RW
273	1-6-36	A	S	97,99,146C,183,233F,327,386	22C,83,122FE,147,151C,1530,185,232T,FLJ,R,RW
275	1-6-36	C	S	---	22C,97,99,146C,147,161,183,327,386,FLJ
110	3-26-35	CR	N	97	42,75,111,161,163,180,185
274	1-6-36	B	N	97,146C,183,327	22C,99,122FE,147,151C,1530,185,232T,233F,386,FLJ,R,RW
367	2-21-36	16	N	---	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
370	2-21-36	19/1	N	---	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
371	2-21-36	C_4	N	327	22C83,97,99,122FE,146C,147,151C,1530,183,185,

275	1-6-36	C	S	---	22C,97,99,146C,147,161,183,327,386,FLJ
110	3-26-35	CR	N	97	42,75,111,161,163,180,185
274	1-6-36	B	N	97,146C,183,327	22C,99,122FE,147,151C,1530,185,232T,233F,386,FLJ,R,RW
367	2-21-36	16	N	---	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
370	2-21-36	19/1	N	---	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
371	2-21-36	C_4	N	327	22C83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,386,FLJ,R,RW
372	2-21-36	G	N	386	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,FLJ,R,RW
374	2-21-36	F_s	N	97	22C,83,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
375	2-21-36	H_s	N	---	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
384	2-26-36	LOL	N	---	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
385	2-26-36	233	N	146C	22C,83,97,99,122FE,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
386	2-26-36	92	N	---	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
387	2-26-36	66	N	---	22C,83,97,99,122FE,146C,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW
389	2-26-36	H_4	N	146C	22C,83,97,99,122FE,147,151C,1530,183,185,232T,233F,327,386,FLJ,R,RW

S=slow
N=normal

transfer of butter culture 15/3 during the same period as were several of the filtrates from the same butter culture in a slow condition inhibited many of the test organisms against which the filtrates from slow cultures were active. Filtrates 96 and 98, which were obtained from different transfers made at the same time from a single culture demonstrate the variations which may occur under such conditions. The differences in the handling of the cultures which yielded these filtrates will be discussed in a later section. Filtrate 368, which was obtained almost a year later than the last prior filtrates from the same culture had entirely different inhibition characteristics.

The 2 filtrates which were obtained at a 15 month interval from transfers of butter culture 103 which failed to coagulate in a normal period of time were quite different in their abilities to inhibit the various strains of S. lactis which were employed for test purposes. The normal transfer of butter culture 103 yielded a filtrate which, although obtained only 7 days after active filtrate 345 had been secured, was inactive, apparently containing no inhibitory principle.

Of the 21 filtrates obtained from slow cultures, only three contained no demonstratable inhibitory principle when pure cultures of sensitive strains of S. lactis were used as test organisms. These 3 filtrates were 246 which was obtained from butter culture 122, 69 which was obtained from culture 146, which only once in 18 months showed any of the visible characteristics of a typically slow butter culture, and 275 from culture C whose history was unknown, it being a commercial culture. Of 28 filtrates from butter cultures which coagulated normally, eleven apparently contained no inhibitory principle. Most of the remaining 17 filtrates inhibited only one organism or a few closely related organisms. In most instances, each of the filtrates was slightly

different from any of the other filtrates obtained from butter cultures, indicating that the inhibitory principle in butter cultures is apparently non-static in character from both qualitative and quantitative standpoints.

The effect of exposure of the milk substratum to air upon the production of inhibitory principle by butter cultures

To determine the effect of the exposure of the milk to be used for starter making to the air, quantities of skim milk, usually about 3 l., were sterilized in the autoclave at 15 pound pressure for 30 minutes, cooled and then divided into either 2 or 3 parts. One part was placed in a 2 l. evaporating dish and the uncovered dish set on a table in the butter culture room in the college creamery. A second portion was also exposed to the air in a similar container in the bacteriology laboratory. The uncovered dishes were left exposed for various periods of time before the milk was placed in 140 ml. quantities in glass-stoppered bottles and inoculated with approximately 0.6 per cent of the various butter cultures. Usually a third portion of the milk was immediately placed in bottles and held at 21° C. to be inoculated as controls for the cultures grown in the lots of milk exposed to the air. When a culture grown in one of the exposed lots of milk was slow to coagulate, filtrates were obtained from the culture and from the other transfers of the same butter culture and the characteristics of the different filtrates determined and compared.

The development of acidity by a number of cultures seemed to be unretarded when they were grown in the milk exposed to air. In one series of experiments, the 2 lots of milk were exposed to the air for 2½ hours prior to inoculation with butter cultures 15/1, 15/3, 22, 26, 103 and 146. All of the cultures, except 15/3 in the milk exposed in

the culture room in the creamery, coagulated as rapidly in the exposed milk as in the unexposed milk. At the time that filtrates 5, 6 and 10 were obtained from butter culture 232, cultures 103, H, 15/1, 15/3 and 19/1 were also used but did not suffer any appreciable retardation of acid production when grown upon the milk exposed to air before inoculation. Cultures G, G_3, 17, H, 19/1 and 22 grown at another time in milk exposed for 16 hours in the culture room and in the laboratory coagulated as rapidly as cultures inoculated into unexposed milk from the same lot of sterilized milk. Two months later, butter cultures X, 3, 22, 103, 233, G_4, 17, H_2 and 19/1 were inoculated into the lots of milk exposed to air that were used to obtain slow cultures of 15/3, 26 and 232. All of the members of the group coagulated in the normal period of time.

The data concerning the filtrates obtained from the cultures which were slow in milk exposed to air or from the control cultures which coagulated in a normal period of time are given in table 4. In 3 of the 4 comparisons, the culture exposed to the air in the butter culture room in the creamery yielded a filtrate which was inhibitory to a greater variety of strains of S. lactis than the filtrate from the culture which was exposed to the air of the bacteriology laboratories. The difference was especially noticeable in the case of filtrates 6 and 10. The former, from a culture in milk exposed to the air of the laboratory, did not inhibit a single test organism while the latter, from a culture which had been exposed in the culture laboratory in the creamery, was active against the majority of the organisms with which it was tested. The situation was reversed in the case of butter culture 15/3, the transfer in the milk exposed in the laboratory yielding a filtrate which inhibited the growth of a greater variety of organisms than was inhibited by the filtrate from the culture exposed in the

Table 4

The effect of exposure of sterilized milk to air upon the production of inhibitory principle in butter cultures

Filtrate number	Butter culture	Place	Exposure Time (hr.)	Coagulation time (hr.)	*S. lactis* cultures inhibited by filtrate	*S. lactis* cultures not inhibited by filtrate
5	232	---	---	14	---	42,47,57,72,88,97,99, 101,111,113
6	232	Laboratory	2½	16	---	42,47,57,72,88,97,99, 101,111,113
10	232	Creamery	2½	30	42,47,88,97,99, 101,111	57,72
48	15/3	Laboratory	16	15	42,47,88,99,101, 111	57,72,97,113
55	15/3	Creamery	16	27	97,111	42,47,57,72,88,99, 101,113
49	26	Laboratory	16	15	42,101	47,57,72,88,97,99, 111,113
53	26	Creamery	16	20	97,111,113	42,47,57,72,88,99, 101
50	232	Laboratory	16	15	---	42,47,57,72,88,99, 101,111,113
54	232	Creamery	16	32	111	42,47,57,72,88,97, 99,101,113

The results of these experiments are inconclusive. Although the exposure of the sterilized milk to the air of the butter culture room in the creamery did increase the incidence of slow cultures, exposure to the air of the bacteriology laboratory had no such effect. No reason for this difference was observed, the temperatures, chances for aerial contamination and other factors with the possible exception of relative humidity being apparently the same in both cases. The differences in inhibition characteristics between the filtrates from each pair or group of cultures were apparently qualitative rather than quantitative, although the same mother culture was used as inoculum for each group. This fact may be of significance in the final solution of the problem, although the available data do not seem to afford a basis for interpretation of the phenomenon.

The effect of bubbling pure gases through freshly inoculated butter cultures upon the rate of coagulation and the presence of inhibitory principle

An experiment was made to determine the effect of the bubbling of oxygen, nitrogen and hydrogen through the milk upon the incidence of slowness among butter cultures and also upon the production of inhibitory principle by the cultures. Sterile litmus milk in 140 ml. quantities was placed in 250 ml. Erlenmeyer flasks. The rubber stoppers of these flasks were pierced by two holes, one containing a cotton-plugged vent tube and the other containing a tube the lower end of which reached almost to the bottom of the flask and the upper end of which was plugged with cotton. After 4 lots of milk had been inoculated with one drop of butter culture 232 and 4 other lots with butter culture 122F, oxygen, hydrogen and nitrogen gas were each bubbled through one flask from each series for 10 minutes, the rate being as rapid as was possible without

excessive foaming of the milk. At the end of the period, clamps were placed upon the outlets of the tubes to prevent the entry of air after disconnection. The cultures were incubated at 21° C. After coagulation, filtrates were obtained by the usual method.

The data are presented in table 5. Only the culture of 122F through which oxygen had been bubbled was slow in coagulating. The inhibition characteristics were the same for all 4 filtrates obtained from the cultures in each series. The variations in titer between the filtrates in each series were not sufficiently large to be significant, especially since the relationships between the titers of the filtrates from the cultures treated with the various gases were not the same in both series. The filtrate from culture 232 treated with hydrogen had the highest titer in its group, and the titer of the filtrate from culture 122F treated with hydrogen was the lowest in its series. The titer of the filtrate from the culture which developed acidity somewhat slowly was normal for the group indicating that the retarded acid formation, apparently as a result of treatment with oxygen, was not associated with an increase in the titer of the inhibitory principle contained in the culture.

The same procedure of bubbling gases through freshly inoculated cultures in litmus milk was repeated except that 10 ml. of the filtrate from the previous culture treated with the same gas was in each case added before inoculation. Again all of the cultures except one, 122F through which oxygen had been bubbled, coagulated normally. The one culture required an extra 6 hours to coagulate, indicating that the delayed acid formation in the first series was probably not a circumstantial occurrence.

Saturation with various gases of the butter cultures freshly

Table 5

The effect of bubbling gases for 10 minutes through freshly inoculated litmus milk cultures of butter cultures upon the production of inhibitory principle

Butter culture	Gas	Coagulation time (hr.)	Filtrate titer	*S. lactis* cultures inhibited by filtrate	*S. lactis* cultures not inhibited by filtrate
232	Oxygen	20	10^{-3}	97	22C,99,146C,147,161, 183,327,386,FLJ.
	Nitrogen	20	10^{-2}	97	22C,99,146C,147,161, 183,327,386,FLJ.
	Hydrogen	20	$10^{-3.78}$	97	22C,99,146C,147,161, 183,327,386,FLJ.
	---	20	$10^{-2.78}$	97	22C,99,147,161,183, 327,386,FLJ.
122F	Oxygen	26	10^{-2}	386	22C,97,99,147,161,183, 327,FLJ.
	Nitrogen	20	$10^{-2.78}$	386	22C,97,99,147,161,183, 327,FLJ.
	Hydrogen	20	$10^{-1.78}$	386	22C,97,99,147,161,183, 327,FLJ.
	---	20	$10^{-1.78}$	386	22C,97,99,147,161,183, 327,FLJ.

inoculated into litmus milk followed by sealing against the entry of air from outside the system seemed to have no qualitative or quantitative effects upon the development of inhibitory principle in the cultures. The retardation of one culture by oxygen may have been caused by the effect upon the inhibitory principle contained in the culture or it may have been the result of some factor unrelated to the ultrafilterable principle. Probably the effect of exposure of the milk used for the propagation of butter cultures to the atmosphere is not a simple question of the effect of the normal air constituents but is the result of some factor or factors which are not yet determined.

The effect of raw milk upon the production of inhibitory principle by butter cultures

Samples of raw milk from the shipments of various patrons were obtained at the receiving platform in sterile containers. These lots of milk were placed in sterile bottles in 150 ml. quantities. One bottle of milk from each lot was allowed to stand at room temperature until natural coagulation occurred. Other bottles were inoculated with butter cultures 15/3 and 232, although both cultures were not used in every case. After the cultures had coagulated, filtrates were obtained from each. The inhibition characteristics of the individual filtrates were determined by the litmus milk culture method.

The data from the experiment are assembled in table 6. No filtrate obtained from a naturally soured raw milk showed any indications that it contained inhibitory principle active against any of the test strains of S. lactis. The same was true of all of the filtrates obtained from cultures of 232, all of which coagulated within a normal period of time. The filtrate obtained from culture 15/3 grown in lot 1 of raw milk was also non-inhibitory but the filtrates from culture 15/3

Table 6

The effect of raw milk upon production of inhibitory principle by butter cultures

Raw milk	Butter culture	Coagulation time at 21° C. (hrs.)	*S. lactis* cultures inhibited by filtrate	*S. lactis* cultures not inhibited by filtrate
1	----	37	-------	42,75,97,111,113,146,161,163, 176,185
1	15/3	16	-------	42,75,97,111,113,146,161,163, 176,185
1	232	16	-------	42,75,97,111,113,146,161,163, 176,185
3	----	31	-------	42,75,97,111,113,146,161,163, 176,185
3	232	18	-------	42,75,97,111,113,146,161,163, 176,185
4	----	47	-------	42,75,97,111,113,146,161,163, 176,185
4	15/3	16	97,113	42,75,111,146,161,163,176,185
4	232	16	-------	42,75,97,111,113,146,161,163, 176,185
7	----	47	-------	42,75,97,111,113,146,161,163, 176,185
7	232	18	-------	42,75,97,111,113,146,161,163, 176,185
33	----	54	-------	42,75,97,111,113,146,161,163, 176,185
33	232	18	-------	42,75,97,111,113,146,161,163, 176,185
--	15/3	16	97,113	42,75,111,146,161,163,176,185
--	107	16	-------	42,75,97,111,113,146,161,163, 176,185

grown in raw milk from lot 4 and from the culture in milk pasteurized in the usual way for the propagation of butter cultures inhibited the growth of organisms 97 and 113.

Since inhibitory principle is sometimes obtained from normal cultures of 15/3 and is not obtained at other times, the variation between the 3 cultures grown on the 3 lots of milk is probably not of significance. The results of the experiment do show that the cultures did not produce additional inhibitory principle as a result of growth upon various lots of raw milk.

Attempts to obtain inhibitory principle from pure cultures of Streptococcus lactis

At various times filtrates were obtained from different sensitive strains of S. lactis grown in 100 ml. quantities of various media under a variety of conditions. The results of the tests made by the litmus milk culture method to determine the presence of inhibitory principle in these filtrates are assembled in table 7.

Although 10 filtrates were obtained from 8 different strains of S. lactis, not a single filtrate showed any tendency to retard the growth of the 10 or more test organisms of widely different sensitivities.

At another time 5 strains of S. lactis were grown in milk digest broth at 30° C. and filtered after 24 hours of incubation. 10 ml. of the resulting filtrate were added to 100 ml. of fresh broth which was then inoculated with the organism from which the filtrate had been obtained. This procedure was repeated 7 times. The filtrates which were obtained from the seventh culture were tested for the presence of inhibitory principle by the litmus milk culture method. The results were negative.

Table 7

The results of attempts to obtain inhibitory principle from pure cultures of Streptococcus lactis

Date	Culture	Treatment	Test cultures, all of which were not inhibited by the filtrate
12-16-34	42	Grown 60 hr. at 21° C. on litmus milk	42,47,57,72,88,97,99,101,111, 113
2-19-35	97	Grown 48 hr. at 37° C. on litmus milk	42,75,83,97,111,113,146,147, 161,185
4-11-35	99	Grown 48 hr. at 21° C. on litmus milk	42,75,88,97,99,111,147,180, 185,263
4-11-35	99	Grown 48 hr. at 30° C. on litmus milk	42,75,88,97,99,111,147,180, 185,263
12-12-35	103A	Grown 48 hr. at 21° C. on litmus milk	88,97,99,147,161,163,213,269, 295,327,151C.
2-6-36	97	Grown 24 hr. at 21° C. on tomato juice broth (pH 6.8)	22C,97,99,146C,147,161,183, 327,386,FLJ.
2-8-36	FLJ	Grown 20 hr. at 30° C. on tomato juice broth (pH 6.8)	22C,97,99,146C,147,161,183, 327,386
2-8-36	232T	Grown 20 hr. at 30° C. on tomato juice broth (pH 6.8)	22C,97,99,146C,147,161,183, 327,386,FLJ.
2-8-36	151K	Grown 20 hr. at 30° C. on tomato juice broth (pH 6.8)	22C,97,99,146C,147,161,183, 327,386,FLJ.
2-8-36	386	Grown 20 hr. at 30° C. on tomato juice broth (pH 6.8)	22C,97,99,146C,147,161,183, 327,386,FLJ.

The filtrates mentioned in the preceding paragraph were added in 10 ml. quantities to 100 ml. of milk digest broth which was then inoculated with one of the 5 strains of S. lactis from which the filtrates were obtained and held at 30° C. for 9 days, at the end of which time they were filtered. The filtrates were unable to retard the acid development of any of the 11 cultures of S. lactis with which they were tested.

The results obtained in the experiments indicate that inhibitory principle is not present in a detectable form in pure cultures of sensitive strains of S. lactis and is not produced in the cultures when held for 9 days at 30° C. in unbuffered milk digest broth, conditions which apparently are very favorable for regeneration of the principle as will be shown later.

Attempts to obtain inhibitory principle from other sources

The absence of factors active against S. lactis in 8 representative bacteriophages active against members of the colon group was determined. The bacteriophages were obtained from M. J. Powers of the Bacteriology Department of Iowa State College. Cultures FLJ, R, 83, 97, 99, 122FE, 146C, 147, 151C, 153C, 233F, 327 and 386 were used for test purposes and the litmus milk culture method of determination of active principle was chosen. None of the cultures showed any tendency to be inhibited by any of the 8 strains of bacteriophage.

Because the inhibitory principle is associated with butter cultures and apparently is not found in pure cultures of S. lactis, attempts were made to obtain the principle from pure cultures of citric acid fermenting streptococci. Litmus milk cultures of 3 typical organisms obtained from M. B. Michaelian of the Dairy Industry Department of

Iowa State College were incubated for 3 days at room temperature, coagulated with lactic acid and filtered. The absence of inhibitory principle in the resulting filtrates was demonstrated by the litmus milk culture method, using S. lactis cultures 220, 97, 99, 146C, 147, 151C, 161, 163, 269, 295 and 327.

The lack of inhibitory principle in the 3 cultures of citric acid fermenting streptococci indicates the probability that such organisms are not more than an environmental factor in the incidence of slow starters. The number of cultures examined was not sufficiently large to permit the drawing of definite conclusions.

The method of increasing the titer of the inhibitory principle found in the filtrates obtained from butter cultures

Since the titers of the filtrates obtained from butter cultures were usually rather low, many of them being in the range of 10^{-2} and 10^{-3}, attempts were made to increase them by propagation at the expense of sensitive strains of S. lactis. Litmus milk in 100 ml. quantities in cotton-stoppered 160 ml. medicinal ovals was usually used as the medium, although tomato juice broth and milk digest broth, both at a pH of 6.8 in most cases, were also used. The litmus milk cultures frequently did not coagulate during the 48 hour incubation period at 21° C. which was used in almost all cases. In these cases, sufficient dilute lactic acid was added to cause coagulation before the culture was submitted to the preliminary filtration.

The effects of the propagation of several strains of inhibitory principle for several generations at the expense of sensitive cultures of S. lactis grown in litmus milk upon the titers of the resulting filtrates is shown in table 8. In the case of filtrate 191 propagated at

Table 8

The change in titer resulting from propagation of strains of inhibitory principle upon sensitive cultures of *Streptococcus lactis*

Filtrate number	Propagation conditions *S. lactis*	Filtrate	Titer	Remarks
191	---	---	10^{-5}	Filtrate from butter culture 15/3
118	99	191	$10^{-8.78}$	
127	99	118	$10^{-7.78}$	No incubation prior to filtrate addition.
142	99	127	10^{-9+}	
157	99	142	$10^{-8.78}$	
162	99	157	10^{-9}	
168	99	162	$10^{-9.78}$	
10	---	---	10^{-1}	Filtrate from butter culture 232.
29	42	10	$10^{-6.78}$	
36	42	29	10^{-7+}	
62	113	3	10^{-1}	Plaque isolation from filtrate from butter culture 232.
185	97	62	10^{-2}	Filtrates 189, 196 and 201 were obtained from successive propagations of filtrate 185.
207	97	201	$10^{-5.78}$	
276	97	216	$10^{-8.78}$	Filtrates 212 and 216 were obtained from successive propagations of filtrate 207.

the expense of culture 99, the titer of the filtrate was relatively high at the beginning and increased to almost the maximum in the course of one generation. After that, the conditions of propagation seemed to influence the titer somewhat, although nearly all of the values obtained were very close to 10^{-9}. The culture from which filtrate 127 was obtained was not incubated for an appreciable period between the inoculation with S. lactis and the addition of the filtrate, while nearly all of the other cultures in the series were incubated for 4 to 10 hours before filtrate was added. The influence of this factor will be shown later.

Although filtrate 10 had a very low original titer, it rapidly increased in titer as a result of propagation at the expense of S. lactis 42 against which it was very active.

The strain of inhibitory principle represented by filtrate 62 was isolated from a single plaque upon the surface of the agar of a plate used for the determination of filtrate activity by the agar plate plaque method. The original filtrate had a very low titer and 5 propagations only increased the titer to a value of $10^{-5.78}$; 3 further propagations brought the titer to $10^{-8.78}$, which was found to be about the normal value for most strains of inhibitory principle.

Occasionally filtrates with titers as high as 10^{-11} were obtained, but it was impossible to maintain them at that high level through a series of propagations. The majority of the filtrates which were at what might be called "maximal titer" were active in dilutions of 10^{-9} or 10^{-10}.

As indicated previously, the amount of growth made by a culture or the number of cells per unit volume in the culture at the time the filtrate to be propagated was added was a factor in the regeneration of the inhibitory principle. To demonstrate this effect, 100 ml. quantities

of litmus milk in cotton-plugged medicinal ovals were inoculated with drops of sensitive strains of S. lactis. The desired filtrate was then added in 1 ml. quantities after various periods of incubation, the condition of the culture being noted at the time of filtrate addition. The cultures were then allowed to stand at either 21° or 30° C. for a period which was usually 48 hours in duration, after which filtrates were obtained from them. The titers of the filtrates were determined by the dilution method.

The results of several series of experiments are summarized in table 9. Except in the case of filtrate 191 propagated upon S. lactis 99, the filtrates obtained from cultures which had been allowed to grow for a short time before inhibitory principle was added had the highest titers. If growth was permitted to progress too far before the principle was added, the multiplication of the organisms was not stopped and the culture coagulated within 24 hours. The titers of the filtrates obtained from the coagulated cultures were usually considerably less than those of the filtrates from cultures which had not coagulated. The filtrates obtained from cultures which had definitely changed reaction at the time of the addition of inhibitory principle and which coagulated soon afterward were usually either of very low titer or entirely lacking in inhibitory principle.

The numbers of viable organisms in cultures which had been allowed to grow for a time before the addition of filtrate were followed after the addition of filtrate by plating the cultures at intervals. Tomato juice agar was used as the growth medium. Counts were made of the culture immediately before the addition of filtrate, 10 minutes, 1 hour and 4 hours after the addition of filtrate and at various periods thereafter. The 100 ml. quantities of litmus milk were inoculated with

Table 9

The effect of the period of incubation of a sensitive culture of Streptococcus lactis before the addition of filtrate upon the titer of the resulting filtrate

Time from inoculation to addition of filtrate (hrs.)	S. lactis culture	Number	Filtrate Titer	Temp.	Appearance of the Culture: At time of filtrate addition	Appearance of the Culture: 24 hours after inoculation	Titer of resulting filtrate
0	99	191	10^{-5}	21° C.	Unchanged	Unchanged	$10^{-8.78}$
4					Unchanged	Unchanged	$10^{-5.78}$
12					Partially reduced	Coagulated	10^{-4}
24					Coagulated	Coagulated	0
0	99	191	10^{-5}	30° C.	Unchanged	Acid, reduced	10^{-7}
4					Very slightly acid	Coagulated	10^{-8}
12					Sl. acid, reduced	Coagulated	0
0	99	127	$10^{-7.78}$	21° C.	Unchanged	Unchanged	$10^{-7.78}$
5					Unchanged	Unchanged	$10^{-8.78}$
8½					Partially reduced, v. s. acid	Sl. acid	10^{-9}
12					Reduced, sl. acid	Moderately acid	10^{-7}
0	99	128	$10^{-6.78}$	30° C.	Unchanged	Very slightly acid	$10^{-7.78}$
5					Partially reduced, sl. acid	Partially reduced, sl. acid	10^{-9}
8½					Reduced, moderately acid	Coagulated	$10^{-3.78}$
12					Reduced, quite acid	Coagulated	10^{-2}
12	42	29	$10^{-6.78}$	21° C.	Unchanged	*Unchanged	10^{-7}
18					Sl. reduced	Sl. reduced	10^{-7}
36					Reduced, sl. coag.	Reduced, sl. coag.	0
60					Coagulated	Coagulated	0

* 36 hours after inoculation

a drop of S. lactis culture and 1 ml. of filtrate was added. At the termination of the experiment, a filtrate was obtained from each culture and the titers of the filtrates were determined.

The data from the experiment are presented in table 10. The culture of S. lactis 97 to which filtrate 216 was added remained unchanged in reaction during the entire period of the experiment. The same was true of S. lactis 147 to which filtrate 217 was added after 11 hours of incubation. S. lactis culture 147 was almost completely reduced and quite acid when the filtrate was added after 15.5 hours and remained reduced for more than 10 hours. In one culture, no definite reduction in count occurred until more than an hour after the filtrate had been added. The viable count of the other 2 cultures decreased slightly within 10 minutes after the addition of filtrate and decreased considerably by the end of an hour. Except for the culture whose count was almost half a billion at the time the filtrate was added, 4 hours of incubation after the addition of filtrate sufficed to reduce the number of viable organisms per milliliter to less than a thousand. Culture 97 began to increase in count by the time of the 13½ hour interval and contained appreciable numbers of organisms at the 24 hour interval, apparently as the result of the growth of resistant types. The same sequence of events took place in culture 147 to which filtrate was added at the end of 11 hours. At the end of 20 hours, culture 147 to which filtrate 217 was added after 15½ hours contained less than 100 viable organisms per ml., indicating almost complete killing of the S. lactis cells in the culture and only a very slight tendency for resistant organisms to begin growth.

The results obtained in the experiments discussed in this section indicate that regeneration of inhibitory principle and progressive

Table 10

The effect of the addition of inhibitory principle upon the plate counts of sensitive cultures of Streptococcus lactis

Period	Plate counts of cultures (per ml.) Culture 97 plus filtrate 216 after 11 hours incubation	Culture 147 plus filtrate 217 after 11 hours incubation	Culture 147 plus filtrate 217 after 15½ hours incubation
Before filtrate addition	44,500,000	48,700,000	425,000,000
10 min. after filtrate addition	40,300,000	16,750,000	307,000,000
1 hour after filtrate addition	53,000,000	13,250,000	165,000,000
4 hours after filtrate addition	750	150	9,850,000
13½ hours after filtrate addition	3150	40	100 (9 hours)
24½ hours after filtrate addition	114,000	3550	100 (20 hours)
Titer of filtrate obtained	10^{-9}	$10^{-8.78}$	10^{-10}

increase in the titers of filtrates are brought about by the propagation of the principle in cultures of living sensitive bacteria. While the particles of principle are multiplying, the number of viable organisms in the culture is decreasing. Apparently the multiplication of the principle is at the expense of the living cell of the sensitive bacterium. The greater the number of bacteria present per unit volume of culture, up to the point where the inhibitory principle is unable to stop the growth of the culture, the higher is the titer of the filtrate obtained from the culture. If the number of cells present is too great, multiplication of the principle is much retarded or does not occur at all. Sometimes this may be the result of unfavorable reaction of the medium, but the ratio of cells of the sensitive bacterium to particles of the inhibitory principle is undoubtedly a factor of importance.

The purification of strains of inhibitory principle

Propagation of inhibitory principle upon strains of sensitive bacteria and the picking of plaques were the 2 methods used for purification. The procedure for the first method has already been described. Purification by means of plaque isolation was accomplished by touching a flamed and cooled inoculating needle to an isolated plaque in a smear of sensitive organism on which filtrate had been spotted and rinsing the needle in a 100 ml. quantity of sterile litmus milk which was then inoculated with a small drop of 24 hour culture of the organism used for the making of the smear in which the plaque had appeared. To obtain a filtrate of any titer, it was necessary to propagate the principle for several generations on a sensitive strain of S. lactis. The filtrates obtained by these 2 methods were tested by the litmus milk culture method to determine their abilities to retard the growth of a number of strains

of S. lactis and the results are shown in table 11.

In general, the purification procedures resulted in the elimination of certain inhibition characteristics found in the original filtrates and in the strengthening of the remaining abilities to inhibit the growth of certain strains of S. lactis. The apparent discrepancy in the case of filtrates 110 and 166 is due to the larger number of cultures used to test the latter filtrate. In this instance, the original filtrate was able to inhibit only a single culture of those with which it was tested; propagation on that organism did not seem to change the nature of the inhibitory principle derived from the original filtrate.

When different strains of sensitive S. lactis were used in the purification procedure, different inhibition abilities were retained by the purified filtrates. This is illustrated by filtrates 92, 211 and 236 obtained from filtrate 61 by the use of cultures 161, 97 and 147, respectively. The combinations of cultures inhibited by these 3 filtrates were all different, although culture 327 was sensitive to all of them. Filtrates 235, 246a and 311 obtained from filtrate 191 by propagation of that filtrate on cultures 99, 122FE and 147, respectively, were even more diverse with respect to the strains of S. lactis which they would inhibit. These 3 organisms were much more diverse in their sensitivities to the action of inhibitory principles than were cultures 97, 147 and 161. This fact undoubtedly explains the differences in the behavior of the 2 groups with respect to the inhibition factors which they are able to propagate.

Although derived from different butter cultures, organisms 220 and 147 were apparently able to propagate the same inhibitory factors. This was demonstrated by filtrates 249, 260 and 311, which were obtained by the use of these organisms. The 3 filtrates inhibited only

Table 11

The results of the purification of strains of inhibitory principle by propagation at the expense of sensitive strains of Streptococcus lactis and by the picking of plaques

Filtrate number	Propagation conditions A	B	C	Plaque isolation	Cultures inhibited	Cultures not inhibited
3	-	-	0	no	42,47,97,99,111,113,147,180, 185	88,161,163,176,183
300	97	3	3	yes	97,327,FLJ	22C,83,99,146C,147,151C, 1530,233F,327,386,R,RW
61	-	-	0	no	22C,83,97,99,146C,147,151C, 1530,122FE,185,233F,327, 386,FLJ	183,R,RW
92	161	61	2	no	97,327,FLJ	22C,83,99,146C,147,122FE, 151C,1530,183,185, 233F,386,R,RW
211	97	61	6	no	97,147,327	22C,83,99,122FE,146C, 151C,1530,183,185,233F, 386,FLJ,R,RW
236	147	61	2	no	183,327	22C,83,97,99,122FE,146C, 147,151C,1530,185,233F, 386,FLJ,R,RW
251	(97 (R	61	5 2	no) no)	97,327,FLJ,R	22C,83,99,122FE,146C,147, 151C,1530,183,185,233F, 386,RW
296	97	61	2	yes	97,327,FLJ	22C,83,99,122FE,146C,147, 151C,1530,183,185,233F,386, R,RW
54	-	-	0	no	83,97,99,122FE,146C,151C, 1530,185,233F,327,386	22C,147,183,FLJ,R,RW
218	(111 (163	54	1 5	no) no)	83,99,151C,185	22C,97,122FE,146C,147,183, 233F,327,386,FLJ,R,RW
265	(111 (1530	54	1 2	no) no)	97,122FE,1530,185,233F, 327,386,FLJ	22C,83,99,146C,147,151C, 183,R,RW
110	-	-	0	no	97	42,111,161,163,180,185
166	97	110	3	no	22C,97,147,327,FLJ	99,122FE,146C,151C,1530, 183,185,233F,386,R,RW
159	-	-	0	no	97,146C,327	22C,83,99,122FE,147,151C,

54	-	-	0	no	83,97,99,122FE,146C,151C, 1530,185,233F,327,386	22C,147,183,FLJ,R,RW
218	(111	54	1	no)	83.99.151C,185	22C,97,122FE,146C,147,183, 233F,327,386,FLJ,R,RW
	(163		5	no)		
265	(111	54	1	no)	97,122FE,1530,185,233F, 327,386,FLJ	22C,83,99,146C,147,151C, 183,R,RW
	(1530		2	no)		
110	-	-	0	no	97	42,111,161,163,180,185
166	97	110	3	no	22C,97,147,327,FLJ	99,122FE,146C,151C,1530, 183,185,233F,386,R,RW
159	-	-	0	no	97,146C,327	22C,83,99,122FE,147,151C, 1530,183,185,233F,386, FLJ,R,RW
205	97	159	7	no	97,327	22C,83,99,122FE,146C,147, 151C,1530,183,185,233F, 386,FLJ,R,RW
191	-	-	0	no	22C,83,99,122FE,146C,147, 151C,185,233F,327,386	97,1530,183,FLJ,R,RW
235	99	191	10	no	83,99,151C,185	22C,97,122FE,146C,147, 1530,183,233F,327,386, FLJ,R,RW
246a	122FE	191	2	no	122FE,146C,233F,386	22C,83,97,99,147,151C, 1530,183,185,327,R,RW
249	(147	191	4	no)	22C,147	83,97,99,122FE,146C,151C, 1530,183,185,233F,327, 386,FLJ,R,RW
	(295		2	no)		
260	(BC 15/3	191	1	no)	22C,147	83,97,99,122FE,146C,151C, 1530,183,185,233F,327,386, FLJ,R,RW
	(22C		2	no)		
293	99	191	2	yes	83,99	22C,97,122FE,146C,147, 151C,1530,183,185,233F, 327,386,FLJ,R,RW
311	147	191	15	no	22C,147	83,97,99,122FE,146C, 151C,1530,183,185,233F,327, 386,FLJ,R,RW

A = Streptococcus lactis
B = filtrate
C = generations
BC = butter culture

the 2 cultures in the test group and were apparently alike in every respect.

The use of the same organism does not guarantee that the filtrates obtained by its use will be exactly the same in characteristics. Filtrates 211 and 296 were both obtained from filtrate 61 by the use of S. lactis 97, the former by propagation alone and the latter by means of plaque isolation followed by 2 generations of propagation. Filtrate 211 inhibited cultures 97, 147 and 327 and filtrate 296 inhibited cultures 97, 327 and FLJ. The difference was small, but definitely demonstrable. A similar situation was apparent with filtrates 235 and 293 which were obtained from filtrate 191 by the use of S. lactis 99. Filtrate 235 was obtained by propagation for 10 generations and was active against cultures 83, 99, 1510 and 185. Filtrate 293 was a plaque isolation strain propagated twice on culture 99 and inhibited cultures 83 and 99 but not cultures 1510 and 185. The filtrates obtained from a number of original filtrates by the use of one organism are frequently even more diverse in their characteristics, as comparison of filtrates 300, 211, 166 and 205 shows.

In only one instance was an ability to inhibit a culture not inhibited by the parent filtrate acquired by a filtrate as a result of propagation. Filtrate 206, which is not mentioned in the table, was the result of the propagation of filtrate 61 on S. lactis 97 for 5 generations. This filtrate was able to inhibit organism R which was uninhibited by filtrate 61. Filtrate 61, which is mentioned in the table, was the result of the propagation of filtrate 206 for 2 generations upon organism R. Since all equipment used for handling cultures and filtrates was sterilized, contamination does not seem to offer a satisfactory explanation for the appearance of the added inhibition factor.

Many of the filtrates obtained from butter cultures apparently contained more than one type or strain of inhibitory principle, the criterion for differentiation of strains being the inhibition of different strains of S. lactis. Each organism of a different sensitivity type seems to selectively propagate one strain or a few closely related strains of principle to which it is sensitive and permit other strains to be removed by dilution in the propagation procedure and segregation in the plaque isolation method. Individual strains of S. lactis varied markedly in the degree of purification which they would effect. Culture 1530 was definitely unselective and cultures 220 and 147 were very selective. Such differences in behavior are undoubtedly reflections of the relative complexities of the groups of factors in the cells which determine the sensitivities of the cells to inhibitory principle. If 2 cultures of S. lactis possess the same sensitivities to inhibitory principles, they apparently propagate the same strains of principle. Organisms 220 and 147 demonstrate this fact very clearly.

No attempt was made to obtain the maximum possible number of separate strains of inhibitory principle from a single filtrate, although the indications are that a considerable number of individual strains could be obtained if different organisms definitely selective in nature were used. The segregation would be more complete if plaque isolations rather than propagation were used, since more than one type of principle active against the organism being used may be propagated on that organism. If widely separated plaques were picked and the isolations repeated once or twice, the chances for inclusion of more than one strain of principle would probably be small.

Comparisons of the litmus milk culture method and the agar plate plaque method for the demonstration of inhibitory principle in filtrates

Upon several occasions, a group of S. lactis cultures and a group of filtrates were tested against each other by the use of both the litmus milk culture and agar plate plaque methods to obtain comparisons of the effectiveness of the 2 methods for the determination of the presence of inhibitory principle in filtrates and for the demonstration of the sensitivities of the S. lactis cultures to the filtrates. Representative results of the experiments are shown in tables 12 and 13.

The 2 methods agreed only moderately well in the case of the organisms and filtrates used for the experiments reported in table 12. In most cases where agreement was lacking, the litmus milk method indicated the presence in the filtrate of inhibitory principle active against the organism in question when the result of the agar plate plaque method was negative, although the situation was sometimes reversed. All of the filtrates used had been isolated directly from slow butter cultures and some of them were of low titer and slight activity by either method of determination. The use of the 0.1 ml. quantity of filtrate for the litmus milk method and of the 0.01 ml. quantity or less for the agar plate plaque method may explain some of the discrepancy in results, because inhibitory principle may have been present in the larger and not in the smaller quantity of filtrate. The discontinuous form of growth of S. lactis made the detection of plaque formation very difficult in many cases in which the area to which the filtrate was applied was not completely cleared. Occasional plaques were undoubtedly overlooked or unrecognizable, although they actually were present. The plaques ranged in size from 0.3 to 2.0 mm. in diameter, although the majority of them were approximately 1 mm. in diameter. The size of the plaque appeared relatively constant for each

Table 12

Comparisons of the litmus milk culture method and the agar plate plaque method for the demonstration of inhibitory principle in filtrates

Organism	Test method	Filtrate 3	7	9	41	53	54
42	M	+	-	+	+	+	+
	A	-	-	-	+	+	+
47	M	+	-	+	+	+	?
	A	-	-	-	+	+	+
57	M	-	-	-	-	-	-
	A	-	-	-	-	-	-
72	M	-	-	-	-	-	-
	A	-	-	-	-	-	-
88	M	-	-	-	-	-	-
	A	-	-	-	+	-	-
97	M	+	+	+	+	+	+
	A	+	?	+	+	-	+
99	M	+	-	+	+	+	+
	A	-	-	-	+	+	+
101	M	+	-	+	+	+	+
	A	-	-	-	+	+	+
111	M	+	-	+	+	+	+
	A	-	-	-	?	?	?
113	M	-	+	-	+	+	-
	A	+	+	+	+	-	+

+ = inhibition
- = no inhibition
? = questionable inhibition
M = litmus milk culture method
A = agar plate plaque method

purified strain of inhibitory principle.

The reasons for the differences of the type which occurred in the cases of organism 88 and filtrate 41 and of organism 113 and filtrates 3, 9 and 54 are less obvious. Possibly the concentration of inhibitory principle in the filtrates was so small and the virulence of the principle so low for the organisms in question that the principle was unable to overcome the organisms in the litmus milk culture but was able to inhibit growth sufficiently to bring about the development of plaques under the conditions of the agar plate method.

With the combinations of organisms and filtrates used for obtaining the data found in table 13, fewer discrepancies between the 2 methods occurred. This situation was probably the result of 2 factors, the use of filtrates of higher titer and greater virulence and the employment of cultures of greater sensitivity. The determinations were made at a later date than those in the preceding group, and greater latitude in the selection of cultures and filtrates was possible.

The litmus milk culture method was more satisfactory than the agar plate plaque method because it demonstrated the presence of inhibitory principle in a greater number of border-line cases and because it more closely simulated the conditions in a butter culture. Considerable time was saved by the use of the litmus milk method, permitting the completion of a greater number of tests. The agar plate plaque method was of value for determining the possible sensitivities of organisms which would not produce characteristic changes in litmus milk.

The lysis of Streptococcus lactis by inhibitory principle and the factors which affect lysis

After preliminary experiments had shown that inhibitory principle would lyse cultures of sensitive strains of S. lactis, a series of

Table 13

Further comparisons of the litmus milk culture method and the agar plate plaque method for the demonstration of inhibitory principle in filtrates

Organism	Test Method	Filtrate 41	52	53	54	55	61	98	159	191	235	247a	251	311	312	313	315	369
R	M	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-
	A	-	-	-	?	?	-	-	-	-	-	-	+	-	+	-	-	-
RW	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	A	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
22C	M	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	+	-
	A	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-	-
83	M	+	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-
	A	+	-	-	+	+	+	-	-	?	+	-	-	-	-	-	-	-
97	M	+	-	-	+	+	+	+	+	-	-	+	+	-	-	-	+	+
	A	+	-	-	+	+	+	+	?	-	-	?	-	-	-	-	+	-
99	M	+	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-
	A	+	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-
122FE	M	+	+	-	+	+	+	-	-	+	-	-	-	-	-	-	+	-
	A	?	+	-	+	+	+	-	-	+	-	-	-	-	-	-	+	-
146C	M	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	+	?
	A	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-
147	M	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-	-
	A	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-	-
151C	M	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	+	-
	A	-	-	-	+	+	?				+	-	-	-	-	-	+	-
153O	M	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-
	A	+	?	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-
327	M	+	-	-	+	+	+	+	+	?	-	?	+	-	-	-	+	-
	A	+	-	-	+	+	+	+	?	?	-	?	?	+	-	+	+	-
386	M	+	+	+	+	+	+	-	-	+	-		-	-	-	-	+	-
	A	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	-

+ = inhibition
- = no inhibition
? = questionable inhibition
M = litmus milk culture method
A = agar plate plaque method

experiments was undertaken to determine the optimum conditions for the occurrence of the phenomenon. Tomato juice broths at pH levels of 5.41, 6.77 and 7.45, unbuffered milk digest broth at pH 6.81, 1.5 per cent milk digest broth buffered with $\frac{M}{20}$ phthalate pH 5.83 and with $\frac{M}{20}$ phosphate at pH 6.68 and pH 7.31 and beef infusion broths adjusted at pH levels of 5.93, 7.16 and 7.70 were used. Broths at pH 4.8 were not used because preliminary experiments showed that neither growth nor lysis seemed to occur at that pH level. 0.1 ml. of 24 hour tomato juice broth culture was added to each 8 ml. quantity of broth, that amount of culture having been found to cause a definitely perceptible cloudiness in the otherwise clear media. The filtrates used were all of high titer and had recently been propagated for several generations upon the strains of S. lactis which were used in the experiments. Filtrate in 0.2 ml. quantity was added to 3 tubes of each inoculated medium and not added to 3 other tubes. One tube containing filtrate and organism and one containing organism alone were placed at 37° C., another pair was placed at 30° C. and the third pair at 21° C.

The results obtained with S. lactis 147 and filtrate 278 are summarized in table 14, the data obtained when S. lactis 147 and filtrate 235 were used are summarized in table 15 and table 16 shows the results which were secured with S. lactis 97 and filtrate 276. Except in the case of the phosphate-buffered milk digest broth, a pH in the neighborhood of 6.8 was the most favorable for lysis. Possibly because of the presence of the phosphate ion, buffered milk digest broth adjusted to pH 6.68 permitted the occurrence of lysis in only one of the 3 combinations studied. The beef infusion broth at pH 7.16 permitted lysis to occur much more frequently than in the broths at the pH levels of 5.93 or 7.70. In the case of buffered milk digest broth, pH 5.83

Table 14

The effect of medium, temperature and pH upon the lysis of *Streptococcus lactis* 147 by filtrate 278

Medium	pH	Inoculum	Appearance of culture held at 37° C. for 5½ hr.	37° C. for 18 hr.	30° C. for 5½ hr.	30° C. for 18 hr.	21° C. for 5½ hr.	21° C. for 18 hr.
Tomato juice broth	5.41	C+F	-	-	-	-	-	-
		C	?	-	++	++++	+	++++
	6.77	C+F	?	-	-	-	-	-
		C	+	?	+++	++++	++	++++
	7.45	C+F	-	-	-	-	+	-
		C	?	-	?	++++	+	++++
2.5% milk digest broth, unbuffered	6.81	C+F	-	-	-	-	-	-
		C	+++	+++	+++	++++	+++	++++
1.5% milk digest broth, phthalate buffered	5.83	C+F	-	-	-	-	-	-
		C	++	+++	++	++++	+	++++
1.5% milk digest broth, phosphate buffered	6.68	C+F	?	-	-	-	-	-
		C	++	+	+++	++++	++	++++
	7.31	C+F	+	?	+	+	+	+
		C	+	+	++	++++	++	++++
Beef infusion broth	5.93	C+F	+	+	?	+	?	-
		C	+	++	?	++	?	++
	7.16	C+F	-	-	-	-	?	-
		C	?	?	?	++	-	+
	7.70	C+F	+	++	+	?	?	?
		C	?	+	?	+	-	?

C = culture of *S. lactis*
C+F = culture of *S. lactis* + filtrate

- = no cloudiness
? = questionable cloudiness
+, ++, +++, ++++ = increasing cloudiness

Table 15

The effect of medium, temperature and pH upon the lysis of *Streptococcus lactis* 147 by filtrate 235

Medium	pH	Inoculum	Appearance of culture held at 37° C. for 5 hr.	37° C. for 20 hr.	30° C. for 5 hr.	30° C. for 20 hr.	21° C. for 5 hr.	21° C. for 20 hr.
Tomato juice broth	5.41	C+F	++	+++	++	++++	+	++++
		C	++	+++	++	++++	+	++++
	6.77	C+F	+++	++++	+	+++	?	?
		C	+++	++++	+++	+++	++	++++
	7.45	C+F	+++	++++	+	++++	?	?
		C	+++	++++	+++	++++	++	++++
2.5% milk digest broth, unbuffered	6.81	C+F	+++	++++	++	++++	+	++++
		C	+++	++++	++	++++	++	++++
1.5% milk digest broth, phthalate buffered	5.83	C+F	++	++++	+	+	?	-
		C	++	++++	++	++	+	++++
1.5% milk digest broth, phosphate buffered	6.68	C+F	+++	++++	+	++++	+	++++
		C	+++	++++	+++	++++	++	++++
	7.31	C+F	+	++++	+	++++	+	++++
		C	+++	++++	+++	++++	++	++++
Beef infusion broth	5.93	C+F	++	+++	+	++	+	+++
		C	++	++	+	++	+	+++
	7.16	C+F	-	-	-	-	-	-
		C	++	++	+	++	+	++
	7.70	C+F	+	+++	-	+	?	-
		C	+	+	+	++	+	++

C = culture of *S. lactis*
C+F = culture of *S. lactis* + filtrate

- = no cloudiness
? = questionable cloudiness
+, ++, +++, ++++ = increasing cloudiness

Table 16

The effect of medium, temperature and pH upon the lysis of Streptococcus lactis 97 by filtrate 276

Medium	pH	Inoculum	Appearance of culture held at 37° C. 6 hr.	37° C. 26 hr.	30° C. 6 hr.	30° C. 26 hr.	21° C. 6 hr.	21° C. 26 hr.
Tomato juice broth	5.41	C+F	+	+	-	-	?	-
		C	+	++	+++	+++	++	++++
	6.77	C+F	?	-	-	-	-	-
		C	++	+	++++	++++	++++	++++
	7.45	C+F	?	-	-	-	?	-
		C	?	?	+++	++++	+++	++++
2.5% milk digest broth, unbuffered	6.81	C+F	-	-	-	-	-	-
		C	+++	+++	+++	++++	+++	++++
1.5% milk digest broth, phthalate buffered	5.83	C+F	+++	+++	++	?	++	-
		C	+++	+++	++	++++	++	++++
1.5% milk digest broth, phosphate buffered	6.68	C+F	++	++	++++	++++	+++	++++
		C	++	++	++++	++++	+++	++++
	7.31	C+F	++	++	++++	++++	+++	++++
		C	++	++	++++	++++	+++	++++
Beef infusion broth	5.93	C+F	+	+	+	+	+	-
		C	+	+	++	++	++	++
	7.16	C+F	-	-	-	-	-	-
		C	+	+	+	++	+	++
	7.70	C+F	?	-	?	?	+	+
		C	+	?	+	++	+	+

C = culture of S. lactis
C+F = culture of S. lactis + filtrate

- = no cloudiness
? = questionable cloudiness
+,++,+++,++++ = increasing cloudiness

was much more suited to lysis than pH 7.31. When tomato juice broth was used, a pH of 7.45 was slightly more favorable than a pH of 5.41, although the difference was not great. In beef infusion broth, pH 5.93 was more suited to the occurrence of lysis of the culture than pH 5.93, and the difference was considerable.

A temperature of 21° C. was the most favorable for the occurrence of lysis under all the conditions of pH and medium. 30° C. was somewhat less favorable and 37° C. was definitely unfavorable in many cases. Reclouding of the medium by secondary growth was noted in numerous instances where lysis took place at 21° C. The clouding was usually apparent within 3 days after the addition of filtrate. The cultures lysed at 30° and 37° C. did not show this tendency.

The unbuffered 2.5 per cent milk digest broth at a pH of 6.81 was the most consistent of the media tested in permitting lysis at all temperatures, although the beef infusion broth at a pH of 7.16 was almost as favorable. Tomato juice broth at pH 6.77 ranked third among the media under all conditions. None of the other broths permitted lysis in a sufficient number of trials to make them utilizable in the routine determination of the lytic ability of inhibitory principles active against S. lactis.

The titers of a number of the lysed cultures obtained in the experiments just reported were determined by the dilution method, using the strain of S. lactis which had been lysed as the test organism.

The data assembled in table 17 show that, with the exception of the 2 lysed cultures obtained at 37° C., there were very small differences in the amounts of inhibitory principle present in the various lysed cultures. As long as the cultures were lysed and remained clear for some time, the type and pH of the medium seemed to be of little

Table 17

The titers of inhibitory principle obtained from cultures of *Streptococcus lactis* lysed under different conditions

S. lactis	Filtrate	Medium	pH	Temperature (° C.)	Titer
97	276	Beef infusion broth	7.16	37	10^{-7} or less
				30	10^{-8}
				21	$10^{-7.78}$
			5.93	21	10^{-8}
		Tomato juice broth	6.77	37	10^{-7} or less
				30	$10^{-8.78}$
				21	$10^{-8.78}$
			7.45	30	10^{-8}
				21	10^{-8}
			5.41	30	$10^{-8.78}$
				21	10^{-8}
		Buffered milk digest broth	5.83	21	10^{-9}
		Unbuffered 2.5% milk digest broth	6.81	30	10^{-9}
147	278	Buffered milk digest broth	5.83	21	10^{-9}
			6.68	21	$10^{-8.78}$
			7.31	21	10^{-8}
		Tomato juice broth	5.41	30	$10^{-8.78}$
			6.77	30	10^{-9}
			7.45	30	$10^{-8.78}$
		Unbuffered 2.5% milk digest broth	6.81	30	10^{-9}
		Beef infusion broth	7.16	30	10^{-8}

influence upon the amount of principle produced in the culture. Had the lysed cultures been carried through several propagations on sensitive organisms added to the various media, differences in the abilities of the various strains of principle to propagate in the various media might have been demonstrated.

The action of the inhibitory principle on other organisms closely related to Streptococcus lactis

Six strains of lactobacilli, which had been isolated from cheese, were obtained from C. B. Lane of the Dairy Industry Department at Iowa State College and were tested by the litmus milk culture method for sensitivity to active filtrates 14, 16, 17, 29, 30, 35, 45 and 191. In no case was any retardation of growth evident when the cultures to which filtrate had been added and control cultures to which no filtrate was added were incubated at 37° C. until coagulation took place. The agar plate plaque method was also used to test 2 of the cultures. Litmus milk cultures smeared upon tomato juice agar plates were unaffected by drops of filtrates 3, 7, 9, 41, 53 and 54 placed upon the surfaces of the plates.

The lack of sensitivity of 8 strains of citric acid fermenting streptococci was demonstrated by the agar plate plaque method. Litmus milk cultures of the organisms were smeared upon the surfaces of previously poured tomato juice agar plates and drops of filtrates 3, 7, 9, 41, 53 and 54 were placed upon the smeared surfaces. The plates were examined after 18 and 42 hour intervals, but no plaques were visible.

Filtrates 17, 21, 49, 51, 61, 66, 80, 82, 94 and 191 were used to test the sensitivities of 8 representative cultures of Streptococcus liquefaciens obtained from H. F. Long of the Dairy Industry Department at Iowa State College. The litmus milk culture method was used. No

inhibition of growth as a result of the addition of filtrate was evident in any case.

Apparently the inhibitory principle found in filtrates obtained from some butter cultures is unable to influence the growth of organisms closely related to S. lactis. This is to be expected, since the filtrates are only able to act upon a part of the strains of organisms included in the species S. lactis.

The variations in the sensitivities of the organisms and the distribution of strains of Streptococcus lactis which are sensitive to the inhibitory principle

The results of tests made, by means of the litmus milk culture technique previously outlined, to determine the susceptibilities toward inhibitory principle of a number of strains of S. lactis isolated from a variety of sources are shown in table 18.

The cultures of S. lactis obtained from either milk or whey which had been allowed to sour without the addition of butter culture were never found to be susceptible to the inhibitory action of any of the filtrates utilized in the study. The filtrates indicated in the table are only representative of a large number that were tested in an effort to find one or more filtrates to which various organisms of this group were sensitive. From these results, it would appear that naturally-occurring S. lactis organisms are not susceptible to inhibition by the ultrafilterable inhibitory principle active toward the organisms that appear to be non-sensitive could be found if a sufficiently large number of strains of the principle from a greater range of sources were used for testing purposes.

Examination of the results obtained with the cultures of S. lactis isolated from butter cultures reveals that some butter cultures yielded only strains which were subject to inhibition by one or more

Table 18

Summary of tests to determine the susceptibility of Streptococuc lactis strains to inhibition by bacteria-free filtrates

Source of S. lactis	Date of isolation	No. of cultures isolated Total		Filtrates which inhibited	Filtrates which did not inhibit
Raw milk whey	11-5-34	8		---	10,14,30,32,35,53,61,124, 134,135,157,159,191
Soured raw milk	11-5-34	10		---	10,14,30,32,35,53,61,124, 134,135,157,159,191
Soured raw milk P1	10-6-35	5		---	13,21,52,53,61,134,135,157, 158,159,191
Soured raw milk P5	10-6-35	5		---	13,21,52,53,61,134,135,157, 158,159,191
Soured raw milk P6	10-6-35	6		---	13,21,52,53,61,134,135,157, 158,159,191
Soured raw milk P7	10-6-35	6		---	13,21,52,53,61,134,135,157, 158,159,191
Soured raw milk P8	10-6-35	6		---	13,21,52,53,61,134,135,157, 158,159,191
B.C. 146	11-6-35	6	2	---	13,21,52,53,191,205,206, 207,208,209,210
			1	191	13,21,52,53,205,206,207, 208,209,210
			2	13,191	21,52,53,205,206,207,208, 209,210
			1	13,52,53,191	21,205,206,207,208,209,210
	11-13-35	18		---	35,134,157,191,192,205,206, 207,208,209,210
	3-4-36	13	7	---	35,55,134,157,191,206,208, 315
			4	55,134,315	35,157,198,206,208
			1	55,315	35,134,157,191,206,208
			1	55,134,206	35,157,191,208,315
B.C.122F	11-13-35	15		134,191	35,157,192,205,206,207,208, 209,210
	3-4-36	9	1	315	35,134,157,191,206,208,315
			3	55,191,208	35,134,157,206,315
			3	55,191,315	35,134,157,206,208
			2	55,134,191,315	35,157,206,208
B.C. 232	11-8-34	9		10,14,30,32,35,41,53, 61,134,157,191	2,21,54,132,135,159

B.C.122F	11-13-35	15	1	134,191	35,157,192,205,206,207,208,209,210
	3-4-36	9	1	315	35,134,157,191,206,208,315
			3	55,191,208	35,134,157,206,315
			3	55,191,315	35,134,157,206,208
			2	55,134,191,315	35,157,206,208
B.C. 232	11-8-34	9		10,14,30,32,35,41,53,61,134,157,191	2,21,54,132,135,159
	11-15-34	5	4	10,14,30,35,54,61,134,135,157,191	32,159
			1	10,14,30,54,61,134,135,159	32,35,157,191
	1-14-35	3	2	29,35,53,61,134,135,157,191	30,159,92
			1	41,53,61,92,134	29,30,35,54,135,157,159,191
B.C. 232	12-4-35	22	21	---	13,14,35,41,51,134,157,191,206,209,210
		1	1	41,134,191,257	13,14,35,51,157,206,209,210
B.C. 15/3	11-15-34	5	2	14,32,191	2,10,30
			1	10,14,30,35,41,53,61,157,191	2,32,159
			2	10,32,35,41,53,61	2,14,30,157,159
	3-18-35	1		13,53,61,134,157,191	21,52,135,158,159
	12-3-35	25	21	---	13,14,35,41,51,134,157,191,206,209,210
			2	191,256	13,14,35,41,51,134,157,206 209,210,257
			2	41,134,257	13,14,35,51,157,191,206 209,210,256
	3-4-36	5	3	---	35,55,134,157,191,206,208,315
			2	315	35,55,134,157,191,206,208
B. C. A	4-8-35	4		---	13,21,52,53,61,134,135,157,158,159,191
B.C. LOL	3-4-36	14		---	35,55,134,157,191,206,208,315
B.C. M_1	11-19-35	25		---	13,14,35,41,51,134,157,191,206,209,210
B.C. 103	11-13-35	25		---	35,134,157,191,192,205,206,207,208,209,210
B.C. 19/1	11-15-34	5		---	10,14,35,41,53,61,132,134,135,157,158,159,191
B.C. H	11-15-34	7	4	---	2,10,14,30,32,52,53,61,134,135,157,158,159,191
			1	134	2,10,14,30,32,52,53,61,135,157,158,159,191
			2	10,14,30,32,53,61,	2,132,135,158,159

					206,207,208,209,210
B.C. 19/1	11-15-34	5		---	10,14,35,41,53,61,132,134, 135,157,158,159,191
B.C. H	11-15-34	7	4	---	2,10,14,30,32,52,53,61, 134,135,157,158,159,191
			1	134	2,10,14,30,32,52,53,61, 135,157,158,159,191
			2	10,14,30,32,53,61, 134,157,191	2,132,135,158,159
B.C. S	1-14-35	4	1	13,61,135,158,191	21,35,51,52,53,80,82, 134,157,159
			1	80,82,191	21,35,52,53,61,134, 157,158,159
			2	13,41,61,80,135,158, 191	21,35,52,53,134,157, 159
B.C. 233	12-7-35	12	11	---	13,14,35,41,51,134, 157,191,206,209,210
			1	13,41,191,256,257	14,35,51,157,206,209, 210
B.C. 15/1	12-10-35	23	4	---	13,14,35,41,51,134, 157,191,206,209,210
			13	13,14,41,134,157,191,256	35,51,206,209,210, 257
			2	13,41,134,191,256,257 (13 neg. for one cult.)	14,35,51,157,206,209, 210
			4	13,14,134,157,191 (14 neg. for one cult.)	35,41,51,206,209,210
	2-4-36	14		---	35,55,134,157,191,206, 208,315
B.C. FL	11-25-35	26	22	---	13,14,35,41,134,157,191, 206,209,210
			2	41,134,206	13,14,35,51,157,191, 209,210
			2	14,41,134,206,257	13,35,51,157,191,209, 210,256
B.C. 22	12-6-35	23	7	---	13,14,35,41,51,134, 157,191,206,209,210
			2	13,41,191,206,256	14,35,51,134,157,209,210, 257
			14	41,134,257	13,14,35,51,157,191, 206,256

P = patron

B.C. = butter culture

strains of inhibitory principle used in this study, some yielded only non-susceptible strains, while both sensitive and non-sensitive strains were obtained from the majority of the butter cultures studied in any detail.

The results of the studies upon the organisms isolated from butter culture 146 will be discussed in some detail, because they are more or less characteristic of the results obtained with the organisms isolated from a number of other butter cultures during the studies. Among the 6 cultures obtained at the time of the first isolations, 4 different sensitivity types were recognizable. Two of the cultures were not sensitive to any strain of inhibitory principle then available; one culture was sensitive to filtrate 191 only; 2 cultures were sensitive to 2 filtrates, 13 and 191; and the sixth culture was inhibited by filtrates 13, 52, 53 and 191 and uninhibited by filtrates 21, 205, 206, 207, 208, 209 and 210, which constituted the remainder of the test group. One week after the first isolations were made, 18 other strains of S. lactis were obtained from butter culture 146 which had been transferred daily on whole milk pasteurized by holding above 90° C. for half an hour or longer. This second group of organisms was apparently identical with the non-sensitive group obtained at the time of the previous isolations, since 11 representative strains of inhibitory principle were without effect upon the cultures. After a period of approximately 4 months, 13 additional cultures were isolated from the same butter culture. Of this last group of organisms, seven were uninhibited by any strain of inhibitory principle used, four were inhibited by filtrates 55, 134 and 315 and uninhibited by filtrates 35, 157, 191, 206 and 208, one was inhibited by filtrates 55 and 315 and one was sensitive to filtrates 55, 134 and 206.

Butter culture 146 thus was shown to change from a condition in which the S. lactis organisms present were mostly sensitive types, with only a small percentage of non-sensitive or resistant types, to a condition in which all of the cultures picked were of the non-sensitive type. Following this, there was another change after which sensitive and non-sensitive types were present in almost equal numbers. Other changes undoubtedly took place during the intervals between the observations. The sensitive organisms from a single series of isolations made at one time varied in their ranges of sensitivity toward the filtrates containing inhibitory principle. The organisms obtained at any one time usually showed a group similarity, all sensitive cultures in the group being sensitive toward one strain of inhibitory principle, filtrate 191 in the case of the first group isolated and filtrate 55 in the case of the third group. The organisms from the first group were not sensitive to the same strains of inhibitory principle as were those from the third group. This would seem to indicate that a definite change in the sensitivity characteristics of the organisms in the butter cultures occurred during the period the culture contained only non-sensitive organisms.

The S. lactis strains in butter culture 232 also underwent considerable change in sensitivities. During a period of over a year, this culture changed from a condition in which all the organisms were of a single type sensitive to a large number of strains of inhibitory principle to a condition in which all but one of 22 isolations made at the last observation were non-sensitive to any strain of inhibitory principle used. In the case of this butter culture, the changes in sensitivity were somewhat less than in culture 146 and the variation among the cultures in the range of sensitivity was not as great, more than 2 sensitivity types

never having been encountered at one plating. Similar statements can be made concerning the component S. lactis organisms of butter cultures 15/1 and 15/3 except that in the latter 2 cases there were apparently slightly greater differences in sensitivity among the organisms isolated at any one time.

Butter culture 122F was of special interest because, when it was first observed, it contained only organisms of a single sensitivity type. For 2 years preceding the first plating of this culture it had been held in an ice cream hardening room. After the culture was thawed, only sufficient time to permit the reestablishment of normal growth elapsed before the first group of organisms was obtained from it. Four different sensitivity types were included in a second series of organisms isolated 3 months after the first group had been obtained. None of the second group of cultures was of the same sensitivity type as were the earlier isolations.

Only organisms of the non-sensitive type were obtained from butter cultures A, M, 103, LOL and 19/1. These cultures were examined but once. If they had been studied at other intervals, it is quite possible that types sensitive to at least one strain of inhibitory principle might have been isolated.

Butter cultures H, S, 233, EL and 22 each was found to contain one or more types of sensitive S. lactis. Since they were examined but once, no information concerning the changes in sensitivity characteristics which these cultures might undergo was obtained. The organisms obtained from the butter cultures in this group represented very diverse sensitivity types, ranging all the way from strains which were uninhibited by any strain of inhibitory principle available for testing purposes to

strains which were sensitive to a considerable number of strains of inhibitory principle.

No close relationship was evident between the record of a butter culture with respect to its tendency to become slow at times and the types of sensitive or non-sensitive organisms obtained from it. Although they had been typically slow at least once under laboratory conditions during the 18 months they were observed, butter cultures M, A and 103 apparently did not contain S. lactis organisms sensitive to any strain of inhibitory principle available for testing purposes. Examination of these cultures at other times might have resulted in the isolation of sensitive cultures. The use of strains of inhibitory principle other than those which were available might have shown that some of the cultures isolated were sensitive to certain types of principle. Butter cultures 233, H, S and 22 had shown no tendency toward retarded acid production during the 18 month period of observation, but sensitive strains of S. lactis were isolated from them. Possibly they could have become noticeably slow under the proper circumstances, since they apparently possessed at least one of the necessary potentialities in a latent form and needed only the proper supplementary condition or conditions to bring about the characteristic loss in vitality. This condition might be that necessary to bring about the spontaneous origin, from the bacterial cell, of an inhibitory principle active against the strain or strains of S. lactis in the culture, or it might be the addition to the culture of the proper strain of inhibitory principle from an outside source.

Of the 363 S. lactis cultures obtained from various samples of milk, whey and starter, the 46 which were obtained from spontaneously soured raw milk and from raw milk whey were resistant to the

action of various strains of inhibitory principle derived from slow butter cultures. Of the 317 strains obtained from 14 butter cultures at various times, 170 were sensitive to one or more strains of inhibitory principle. There was frequently considerable variation in range of sensitivity among the strains of S. lactis isolated from a butter culture at one time and also among the strains of S. lactis obtained from one butter culture over a period of time. Butter cultures were encountered from which only sensitive organisms were obtained and others from which only non-sensitive types were isolated, but the majority of the cultures examined were non-stable mixtures of sensitive and resistant strains of S. lactis.

The sensitivity characteristics of the secondary growth organisms obtained from cultures of Streptococcus lactis inhibited by various filtrates

When cultures of S. lactis which had been inhibited by the addition of filtrate were held for some time, reduction and coagulation usually occurred. The period of time required for the appearance of growth varied from 2 days to more than 30 days. When the coagulated cultures obtained in this manner were plated upon tomato juice agar and typical colonies were isolated into sterile litmus milk, the resulting cultures had the cultural and morphological characteristics of the strain from which they originated. In a few instances, colonies appeared in the previously cleared areas caused by the placing of a drop of filtrate on a film of organisms smeared upon the surface of an agar plate. Isolations were made from the colonies into sterile litmus milk in which the organisms grew as typical cultures of S. lactis.

The results of the determinations of the sensitivities of the secondary growth organisms are summarized in table 19. The changes in sensitivity which were caused by the various filtrates are well demon-

Table 19.

The sensitivity characteristics of the secondary growth organisms obtained from cultures of Streptococcus lactis inhibited by inhibitory principle .

S. lactis culture	Parent culture	Filtrate used	Filtrates which inhibit	Filtrates which do not inhibit
42	--	--	10,13,17,35,36,41,51,52,61, 72,73,80,82,191	1,21,53,54,98,110,125
167	42	54	191	7,10,17,35,41,49,53,54,61
178	42	61	191	17,21,35,41,54,61
217	42	72	61,80,82,191	9,17,35,41,54,72,73,78,79
222	178	17	---	9,17,35,41,54,61,72,73,78,79, 80,82,191
261	217	191	---	17,21,49,51,61,80,82,94,191
97	--	--	1,10,21,53,55,61,72,75,92, 98,110,138	16,17,41,49,54,73,80,82,118, 131,191
229	97	1	---	1,14,54,61,72,73,78,79,80,82, 86,92,94
273	97	110	61,72	54,79,110,118,123,125,191
299	273	72	---	21,35,41,61,72,110,126,127, 130,191
365	97	206	---	13,14,36,41,51,138,168,191, 206,209
c	97	215	55,211	191,215,218,235,246a,273,315, 368
d	97	211	273	55,191,211,215,218,235,246a, 315,368
99	--	--	10,16,35,36,51,52,61,72,79, 80,82,118,129,138,191	1,53,54
163	99	54	191	10,17,35,41,49,53,54,61,79, 92,98,110
251	163	191	35,53,61,124,134,157,191	31,132,135,158,159
277	99	80	---	13,21,52,53,54,61,72,79,110, 118,123,125,138,160,162,164, 167,191
286	99	123	---	13,21,41,52,53,61,72,73,123, 138,160,162,164,167,191

251	163	191	35,53,61,124,134,157,191	31,132,135,158,159
277	99	80	---	13,21,52,53,54,61,72,79,110, 118,123,125,138,160,162,164, 167,191
286	99	123	---	13,21,41,52,53,61,72,73,123, 138,160,162,164,167,191
113	--	--	3,7,9,14,21,41,53,61,72	1,10,16,17,35,36,49,51,52,54, 79,80,82
161	113	9*	61,72	1,7,9,16,21,35,41,49,51,52,53, 54,79,80,82,98,110
183	113	3*	---	3,7,9,35,41,53,54,61,124,132, 134,135,157,158,159,191
185	113	7*	3,9,35,41,54,61,72,79,80, 82,127,191	1,7,21,49,51,52,98,110
197	161	21	61,72,73	17,21,35,41,62,191
206	185	61	73,79,80,82	41,54,61,72,78
214	185	54	73,80,82	41,54,61,72,78,79
224	185	72	17,80,82,191	9,35,41,54,61,72,73,78,79
227	185	73	---	9,17,35,41,54,61,72,73,78,79, 80,82,191
231	197	61	---	1,41,54,61,72,73,78,79,80,82, 92,94
232	206	73	---	1,41,54,61,72,73,78,79,80,82, 92,94
236	214	73	---	1,41,54,61,72,73,78,79,80,82, 92,94
239	214	80	---	1,41,54,61,72,73,78,79,80,82, 92,94
263	224	17	14,17,61,80,82,94,124, 134,157,191	21,49,51,132,135,158,159
281	185	191	118,123	21,52,54,61,72,79,110,125,134, 160,162,164,167,191
288	263	118	82,119,123,127,128,129, 142,191	21,41,54,61,72,73,94,110,130
289	281	118	---	13,21,52,53,54,61,82,94,126,127, 130,134,160,162,164,167,191
301	281	128	---	13,21,35,41,52,53,61,72,110,126, 127,130,134,160,162,164,167,191
305	288	128	29,191	14,35,53,54,61,124,131,132,134, 135,152,153,154,157,158,159,191
331	288	142	---	13,14,21,29,52,53,54,61,131,134, 152,153,154,160,162,164,167,191
122FE	--	--	134,191	35,168,192,205,209,210,211,212, 213
364	122FE	134	---	13,14,36,41,51,134,168,191,206, 209

305	288	128	29,191	14,35,53,54,61,124,131,132,134, 135,152,153,154,157,158,159,191
331	288	142	---	13,14,21,29,52,53,54,61,131,134, 152,153,154,160,162,164,167,191
122FE	--	--	134,191	35,168,192,205,209,210,211,212, 213
364	122FE	134	---	13,14,36,41,51,134,168,191,206, 209
146B	--	--	13,191	21,52,53
363	147	213	13,14,41,51,138,191	36,168,206,209
391	363	14	13,191	3,14,36,41,49,51,138,168,206
392	363	41	13,191	3,14,36,41,49,51,138,168,206
394	363	51	13,191	3,14,36,41,49,61,138,168,206
396	363	138	13,191	3,14,36,41,49,51,138,168,206
151C	--	--	13,14,41,138,168,191	3,49,36,51,206,209,210
380	151C	41	191	3,13,14,36,41,49,51,138,168, 206
382	151C	138	191	3,13,14,36,41,49,51,138,168, 206
384	151C	168	191	3,13,14,36,41,49,51,138,168, 206
388	151C	191	---	3,13,14,36,41,49,51,138,168, 191,206
176	--	--	13,21,52,53,54,55,91,138, 140,191	3,7,16,21,49,62,90,92,131
201	176	55	---	17,21,35,41,61,62,72,73,191
323	176	140	---	13,14,21,29,52,53,54,61,131,138, 152,153,154,191
325	176	145	---	13,14,21,29,52,53,54,61,131,138, 152,153,154,191
233F	--	--	13,41,138,191	14,35,51,168,206,209,210
386	233F	191	41,191	3,13,14,36,49,51,138,168,206,
a	233F	315	---	55,191,211,215,218,235,246a,273, 315,368
b	233F	54	---	55,191,211,215,218,235,246a,273, 315,368

* Secondary growth in the cleared areas obtained by the agar plate plaque method.

strated by the organisms derived from S. lactis 42, and this group will be discussed in some detail as it is characteristic. Although S. lactis 42 was inhibited by a considerable number of filtrates, the secondary growth culture obtained by the use of filtrate 54 was only sensitive to filtrate 191. The culture obtained from S. lactis 42 acted upon by filtrate 61 was also only sensitive to filtrate 191. The culture obtained by the use of filtrate 72 was sensitive to filtrates 61, 80, 82 and 191. Filtrate 72 had been obtained from filtrate 61 by propagation for one generation upon organism 161 and did not possess some of the inhibition factors present in filtrate 61. Apparently the same group of factors was present and active in filtrates 54 and 61, because both filtrates produced the same type of secondary growth organism with the same sensitivities to inhibition by filtrates. At least one group of factors seemed to have been eliminated in filtrate 72. The secondary growth organism arising from the action of the filtrate therefore retained a group of sensitivities which probably would have been eliminated by the presence of the group of factors which had been removed by propagation. Organism 178 was an unstable organism from the standpoint of sensitivity to inhibitory principle, a phenomenon which will be discussed in a later section. It became sensitive to filtrate 17 and organism 222 was isolated from the secondary growth from the combination of filtrate and organism. Organism 222 was resistant to all of the filtrates which were used to test it. Organism 261, which was obtained from the secondary growth arising from a culture of organism 217 to which filtrate 191 had been added, had lost the sensitivity to the action of filtrate 191 which was the only sensitivity which filtrate 61 had permitted it to retain from its parent strain, culture 42.

The same type of progressive elimination of factors permitting

the action of inhibitory principle was undergone by the other strains of S. lactis whose secondary growth cultures were studied. In each case, the sensitivities possessed by the original culture were removed group by group until a non-sensitive organism was obtained.

If the inhibitory principle does not entirely kill the culture, it apparently causes some change to occur in the weakened survivors which permits those survivors to grow in the presence of the principle active against the parent strain of S. lactis. The length of time required, in many instances, for the appearance of secondary growth indicates that the process of elimination of sensitivity factors may require deep-seated changes in the organism. The resulting secondary growth organism is nearly always resistant to the strain of inhibitory principle which caused its metamorphosis. Exceptions occur, however. Apparently the organism may become destabilized as a result of the changes which it undergoes and revert to the sensitivity type of its parent culture, as seemed to happen with culture 197 in the group derived from S. lactis 113, or it may even acquire sensitivities which preceding generations of secondary growth organisms or even the original culture did not have, as was the case with cultures 206, 214 and 224 of the group of organisms derived from S. lactis 113 by the use of filtrates. Possibly at the time of isolation these cultures were resistant to the filtrates which caused their appearance, but they changed to the more sensitive forms before they could be tested, since they were usually grown through 2 transfers on litmus milk to increase their vigor before they were tested for sensitivities.

Quite obviously, the character of the filtrate used is the factor that determines which sensitivity factors are eliminated. This being the case, filtrates that cause the same type of secondary growth

to appear from a single strain of sensitive S. lactis must have some of the same inhibitory factors in common. By this criterion, filtrates 14, 41, 51 and 138 all possess a common type of factor active against culture 363 which was derived from culture 146B. The same thing is true of filtrates 41, 138 and 168 which all produced the same type of secondary growth organisms from culture 151C. This does not mean, however, that the other inhibitory factors present in the filtrates are the same because there are numerous cases in which the members of the groups of filtrates just mentioned do not inhibit the same organisms. If applied to a sufficiently large number of trials with a large variety of organisms, the types of secondary growth organisms produced by a series of filtrates might be a means of classification of the filtrates upon the basis of the strains of inhibitory principle which they contained.

The spontaneous changes in sensitivity which occur in cultures of Streptococcus lactis

Inconsistencies with respect to the sensitivities of certain cultures of S. lactis to inhibition by some filtrates were occasionally noted during the course of the work herein reported. Although no specific experiments were undertaken to study the phenomenon of variation in sensitivity, tabulation of the results obtained from other experiments showed that the situation was more regular than it at first appeared to be. Representative data are presented in table 20. The filtrate sensitivities which were found to change have been underlined in each case.

Organism 97 was relatively stable in its sensitivity characteristics; the only change was with respect to filtrate 53 which at first inhibited the culture but was later unable to exert any effect. Organism 99 showed no change in sensitivity during the 16 month period of observation. The few available comparisons which could be made in the case of organism 178 revealed no changes in susceptibility to

Table 20.

The sensitivities of Streptococcus lactis cultures grown in litmus milk for a considerable number of transfers

Organism number	Date of tests	Filtrates which inhibit	Filtrates which do not inhibit
97	1-9-35	10,14,30	16,17,32,191
	1-25-35	---	48,49,51,52
	1-30-35	---	29,35,36,41
	2-13-35	3,7,9,41,53,54,61	---
	3-7-35	72	48,51,52,70,73
	5-3-35	134,135,138	131,132,133,136,137
	10-1-35	14,61,134,135,159	35,53,124,132,157, 191
	12-18-35	3	48,49
	3-3-36	92	191
	3-5-36	3,41,54,55,61,98,159	21,48,49,52,53
99	11-24-34	10,14,16,17,30,32,191	2
	1-25-35	48,51,52	49
	1-30-35	29,35,36,41	---
	2-13-35	3,9,41,53,54,61	7
	3-28-35	35,41,54,61,72,73,78, 79,80,82	17
	5-3-35	134,138	131,132,133,135, 136,137
	10-1-35	14,35,53,61,124,134, 157,191	132,135,159
	3-5-36	41,48,54,55,61	3,21,49,52,53,98, 159
178	3-3-35	191	3,17,21,35,41,54, 61,62
	10-28-35	191	13,21,52,53,61,134, 160,162,164,167
180	3-3-35	3,21,35,41,54,61,191	17,62
	4-24-35	72,191	41,61,73
	10-7-35	53,61,124,134,191	31,35,132,135,157, 158,159
	10-28-35	---	13,21,52,53,61,134, 160,162,164,167, 191
185	3-3-35	3,9,35,41,54,61,191	7,62
	3-11-35	80,82	21,49
	10-1-35	14,35,53,61,124,134, 157,191	132,135,158,159
	3-5-36	41,48,54,55,61,191	3,21,49,52,53,159

Table 20 (continued)

197	3-11-35	61,72,73	17,21,35,41,62,191
	10-28-35	---	13,21,52,53,61,134, 191
224	3-28-35	17,80,82,191	35,41,54,61,72,73, 78,79
	10-1-35	14,35,53,61,124,157,191	132,135,158,159
229	3-27-35	---	41,54,61,72,73
	4-24-35	41,61,72,110	73,191
	10-29-35	61,134	13,21,52,53,191
251	4-10-35	---	17,21,49,51,61,80, 82,191
	10-7-35	35,53,61,124,134,191	31,132,135,158,159
281	4-17-35	118,123	54,61,72,110,191
	5-3-35	---	131,132,133,134,135, 136,137,138
	10-28-35	13,53,61,134,162,191	21,52,160,164
386	12-16-35	41,191	3,13,14,36,49,51, 138,168,206
	1-7-36	3,13,41,49,191,138	14,36,51,168,206
	3-5-36	3,41,48,49,52,53,54, 55,61	21,98,159

inhibition by filtrates. The first 2 organisms in this group were obtained directly from butter culture 232 and organism 178 was an isolation from secondary growth, as were the remainder of the organisms for which comparisons were made.

Sensitivities to 5 different filtrates were lost by culture 180 during an 8 month period. In the 52 days which elapsed between the first and second times the culture was tested, the sensitivities to filtrates 41 and 61 were lost. At some time during the first 7 months, the culture became resistant to filtrate 35. The sensitivity to filtrate 61 was regained during the period between the second and third observations. Within the 21 days between the last 2 periods at which tests were made, the culture underwent changes which eliminated the sensitivities to filtrates 53, 61 and 191. Organisms 185 and 197 also lost the abilities to be inhibited by filtrates which had been able to inhibit them before, although the changes were comparatively small in each case.

Organism 224 gained sensitivities to 2 filtrates by which it had previously been uninhibited. Within a period of less than a month, organism 229 became sensitive to 3 filtrates to which it had previously been resistant. Organisms 251 and 281 also acquired new sensitivity characteristics, but the time required for them to appear was not determined. Four new sensitivity characteristics appeared in organism 386 within a month after the culture was isolated.

Sensitivities to filtrates 3, 21, 35, 41, 53, 61, 72, 138 and 191 were involved. The more general use of this group of filtrates for testing purposes is probably the reason for their position in this group, although other factors may have been involved.

Cultures of S. lactis are apparently able to lose or acquire sensitivities to the action of inhibitory principle without the inter-

vention of any outside agency at the time of change. Although the data are not sufficiently extensive to permit the drawing of definite conclusions, the phenomenon of sensitivity variation seems to be associated primarily with the cultures obtained as secondary growth organisms from cultures of S. lactis upon which inhibitory principle has acted. As was suggested in a previous section, the inhibitory principle may have a destabilizing effect upon the cell at the time the changes involved in the loss of sensitivity characteristics are effected. The destabilized cell can then undergo either a gain or a loss of the ability to be inhibited by various filtrates, the direction of the change being conditioned by factors unknown at the present time. This hypothesis seems much more plausible than an explanation which would involve changes in the inhibitory factors present in the filtrates. The abilities of the filtrates to inhibit the majority of the cultures against which they are active remain unchanged, indicating relative stability of the filtrates. Changes involving the loss of some characteristics and the gain of others in a relatively stable system seem improbable, although such changes would have to occur in several of the filtrates if the observed changes in sensitivity were to be explained upon the basis of filtrate instability.

Part II. The effect of physical and chemical factors upon the activity of the inhibitory principle

The action of physical factors upon the activity of the inhibitory principle

Temperature inactivation of the inhibitory principle

In order to determine the effect of heat upon strains of the inhibitory principle, 1 ml. quantities of the filtrates were added to 100 ml. quantities of sterile litmus milk. After thorough shaking of the litmus milk dilutions, 2 ml. quantities were pipetted into sterile 10 x 60 mm. serological test tubes and the tubes sealed in a blast lamp. These sealed tubes were then suspended in a water bath maintained at the desired temperature and stirred during the heating period. At the end of the period, the tubes were removed from the bath and immediately placed in ice water. The presence or absence of active inhibitory principle was then determined by the procedure previously outlined for the determination of the activity of filtrates.

The results of the heat inactivation experiments are shown in table 21. The susceptibilities of the strains of inhibitory principle varied considerably; filtrates 204 and 236 were inactivated by an exposure of 5 minutes at 65° C., the lowest temperature and the shortest time interval used in the experiments, while filtrates 310, 311 and 313 required an exposure of 15 minutes at 70° C. to bring about complete inactivation. The other strains of inhibitory principle which were subjected to the heat treatments were intermediate in their sensitivities to heat inactivation. The titers of the various filtrates after they had been subjected to the heat treatments were not determined; thus, the results are merely qualitative.

Although only 9 filtrates were tested to determine the exposure

Table 21

The inactivation of various strains of inhibitory principle by heat
(Filtrates diluted 1:100 with sterile litmus milk)

Temp. (°C.)	Time of Exposure (Min.)	Effect of heat on filtrate								
		A	B	204	235	236	251	310	311	313
65	5	-	-	+	-	+	-	-	-	-
	10	-	-	+	+	+	-	-	-	-
	20	-	-	+	+	+	+	-	-	-
	30	±	-	+	+	+	±	-	-	-
70	1	-	-	+	-	+	-	-	-	-
	3	±	-	+	+	+	+	-	-	-
	5	+	±	+	+	+	+	-	-	-
	10	+	+	+	+	+	+	-	-	±
	15	+	+	+	+	+	+	+	+	+
Test organism		97	147	99	99	147	R	97		

\+ = destruction

\- = no destruction

± = one of the duplicates showed reduction and one showed survival.

necessary to completely inactivate them, an almost continuous gradation in degree of sensitivity from the low resistance to heat shown by filtrates 204 and 236 to the high resistance shown by filtrates 310, 311 and 313 indicated that, for the particular type of inhibitory principle which was studied, there was no sharp and characteristic point which marked the limit of survival of the principle when subjected to heat treatment. The differences in heat stability were apparently not due to characteristics of the organisms upon which the principles had been propagated, but rather were due to the characteristics of the particular strain of principle. This was best demonstrated by strains B and 236 which were both active against S. lactis 147, although strains A and 310 to which S. lactis 97 was sensitive also demonstrated the same point.

It is significant that the inhibitory principles were all inactivated by exposures to heat which have been found necessary for the destruction of the activity of bacteriophages. This furnishes further evidence that the principle is of the nature of a bacteriophage.

Filtrates A, B, 311 and 313, diluted 1:100 in litmus milk and placed in 2 ml. quantities in sealed serological test tubes, were held for 7 days in an ice cream hardening room at a temperature ranging from -10° C. (-23.3° F.) to -20° C. (-28.9° F.). The thawed preparations were titrated at once and the titers were found to be the same as they had been before freezing, 10^{-6}, 10^{-7}, $10^{-9.78}$ and $10^{-9.78}$, respectively, indicating that freezing and holding at low temperatures has no appreciable effect upon the activity of the 4 representative strains of inhibitory principle.

The effect of pH upon heat inactivation

For the purpose of determining the effect of the pH of the medium upon the heat inactivation of inhibitory principle, filtrates A

and B were diluted 1:100 with 1.5 per cent milk digest broths adjusted to various pH values. Potassium acid phthalate, in M/20 concentration, was used to buffer the broths at pH 4.8 and pH 5.8 and M/20 phosphate to buffer those at pH 6.7 and pH 7.3. The broth at pH 7.3 had been adjusted to pH 7.8 before sterilization, but it underwent a change in reaction during the sterilization process. The diluted filtrates, in 2 ml. quantities, were sealed in sterile serological test tubes and treated in the manner outlined in the previous section.

The results of the experiments to determine the effect of pH upon the heat inactivation of inhibitory principle are shown in table 22. At pH 4.8, exposure to 60° C. for only 5 minutes brought about complete inactivation, although exposure to the same temperature for 30 minutes had no effect upon either of the filtrates when the reaction was in the range from pH 5.8 to pH 7.3. The inhibitory principle in filtrate A was more heat-labile than that in filtrate B. Filtrate A was inactivated by all exposures and at all pH levels by a temperature of 70° C. Although the experimental results were not conclusive, filtrate A was apparently somewhat more stable at pH 6.7 than at either pH 5.8 or pH 7.3 when a temperature of 65° C. was used, inactivation not occurring after 30 minutes in the first case but taking place in 15 minutes in both of the latter cases. In the cases of filtrate B, the effect of the reaction of the medium was not as great at 65° C. At pH 5.8, filtrate B was apparently only partially inactivated by an exposure to 65° C. for 30 minutes but the filtrate was completely inactivated by 70° C. after a period of 5 minutes. At pH 6.7 or pH 7.3, neither 65° C. for 30 minutes nor 70° C. for 5 minutes would inactivate filtrate B, 70° C. for 15 minutes being the least drastic exposure which rendered the filtrate inactive.

The difference between filtrates in the effects of changes in

Table 22

The effect of pH upon inactivation of inhibitory principle by heat (Filtrates diluted 1:100 in 1.5% milk digest broth of the designated pH)

Heating conditions		Effect of heat on Filtrate A at pH				Effect of heat on Filtrate B at pH			
Temp. (°C.)	Time of exposure (Min.)	4.8	5.8	6.7	7.3	4.8	5.8	6.7	7.3
60	5	+	-	-	-	+	-	-	-
	15	+	-	-	-	+	-	-	-
	30	+	-	-	-	+	-	-	-
65	5	+	-	-	-	+	-	-	-
	15	+	+	±	+	+	-	-	-
	30	+	+	-	+	+	±	-	-
70	5	+	+	+	+	+	+	-	-
	15	+	+	+	+	+	+	+	+
	30	+	+	+	+	+	+	+	+
Test organism		97				147			

+ = destruction

- = no destruction

± = one of the duplicates showed destruction, one showed survival.

pH level upon the thermal tolerance of the inhibitory principle was clearly demonstrated, even though only 2 strains were used in the experiments. The difference in the breadth of the zone of maximum resistance was of interest. The results obtained in the studies showed the importance of the pH of the system in the determination of the thermal lability of various strains of inhibitory principle and demonstrated the necessity for the use of a single hydrogen ion concentration if comparable results are to be obtained in the study of a series of filtrates containing the principle.

Comparison of the heat inactivation of the inhibitory principle and of the homologous organism

As shown in the 2 preceding sections, when the pH level is comparable to that of fresh milk, the inhibitory principle, in at least some cases, is able to survive the heat exposures which approximate those commonly employed in market milk plants for pasteurization purposes, exposures which destroy S. lactis very readily. The results obtained when the inhibitory principle was exposed to heat at a pH level of approximately 4.8 indicated there was a possibility that the principle would be less stable than the homologous organism under certain conditions at that pH level.

Filtrates A and B and 24 hour tomato juice broth cultures of the organisms upon which they had been propagated, S. lactis strains 97 and 147, respectively, were added in 1 ml. amounts to 100 ml. quantities of 1.5 per cent milk digest broth at about pH 4.8. The electrometrically determined pH values for these suspensions were all within the range from 4.82 to 4.89. Sealed tubes containing 2 ml. of these dilutions were heated as before, 60° C. being the temperature used.

Table 23 gives the data obtained. Both of the cultures of S. lactis were destroyed by an exposure of 1 minute, while the 2 strains

Table 23

The inactivation of inhibitory principles and homologous organisms by a temperature of 60° C. at pH 4.8 (Filtrates and 24-hour cultures diluted 1:100 in milk digest broth)

Time of exposure (Min.)	Effect of heat on Inhibitory principle A	Inhibitory principle B	*S. lactis* cultures 97	*S. lactis* cultures 147
½	-	-	-	-
1	-	-	+	+
1½	±	-	+	+
2	+	-	+	+
2½	+	-	+	+
3	+	±	+	+
3½	+	±	+	+
4	+	+	+	+

\+ = destruction

\- = no destruction

± = One of the duplicates showed destruction; one duplicate showed survival.

of inhibitory principle required 2 minutes and 4 minutes for complete inactivation. Additional trials indicated that with these combinations of principle and sensitive organism, the freeing of a culture from the principle could not be achieved by the use of heat within the pH range of 4.8 to 7.3.

The effect of pH and temperature upon the stability of filtrates containing inhibitory principle

To determine the effect of pH and of the temperature of holding upon filtrates containing active inhibitory principle, the filtrates were diluted 1:100 with 1.5 per cent buffered milk digest broths, sealed in serological test tubes and placed in regulated incubators for various periods before they were examined to determine the titer of the surviving principle. The titer determination was by the method outlined in the discussion of methods.

The results of the studies are shown in table 24. At pH 4.8, both strains of inhibitory principle were relatively unstable, decreasing considerably in titer even at 21° C. which was the least detrimental temperature of the three which were utilized. At 37° C., 8 days were sufficient to completely inactivate the system when the pH was 4.8. At 30° C., under the same conditions of pH, only partial inactivation took place in 8 days, but inactivation was complete in 45 days with the one filtrate whose titer was determined under this set of conditions. At 21° C., there was a little inactivation at pH 4.8 in the case of one of the filtrates after a holding period lasting 8 days, and considerable inactivation of both filtrates during a 45 day period. At pH 5.8, filtrate A was relatively stable, although a decrease in titer from $10^{-6.78}$ to 10^{-4} was noted after 45 days at 30° C.; no comparable decrease in titer occurred at 21° C. and at 37° C. At pH 6.7, filtrate A was possibly slightly more

Table 24

Titers of filtrates containing inhibitory principle after they had been held at different pH levels for various periods (Filtrates diluted 1:100 in 1.5% milk digest broth)

Filtrate	pH	Original titer	Titer after holding at 21° C. for 8 days	21° C. for 45 days	30° C. for 3 days	30° C. for 8 days	30° C. for 45 days	37° C. for 1 day	37° C. for 3 days	37° C. for 8 days
A	4.8	10^{-6}	10^{-5}	10^{-2}	$10^{-5.5}$	10^{-3}	---*	$10^{-3.5}$	$10^{-1.5}$	0
	5.8	$10^{-6.5}$	10^{-7}	10^{-6}	$10^{-6.5}$	$10^{-5.5}$	10^{-4}	10^{-7}	10^{-5}	10^{-5}
	6.7	10^{-6}	$10^{-6.5}$	10^{-6}	$10^{-5.5}$	10^{-6}	$10^{-5.5}$	$10^{-6.5}$	10^{-5}	10^{-5}
	7.3	$10^{-6.5}$	10^{-6}	$10^{-5.5}$	$10^{-6.5}$	$10^{-6.5}$	$10^{-4.5}$	$10^{-6.5}$	10^{-6}	10^{-5}
B	4.8	10^{-7}	$10^{-7.0}$	$10^{-3.5}$	10^{-6}	10^{-4}	0	10^{-4}	10^{-2}	0
	5.8	10^{-7}	$10^{-7.0}$	$10^{-6.5}$	$10^{-6.5}$	$10^{-6.5}$	10^{-5}	10^{-7}	10^{-6}	10^{-5}
	6.7	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	$10^{-5.5}$	10^{-7}	$10^{-6.5}$	$10^{-5.5}$
	7.3	10^{-7}	$10^{-6.5}$	10^{-7}	10^{-7}	10^{-7}	10^{-6}	10^{-7}	10^{-5}	$10^{-4.5}$

* not determined.

stable than at either pH 5.8 or pH 7.3, although the difference in titer was small in all cases. Filtrate B apparently was just as stable at pH 5.8 or pH 7.3 as at pH 6.7. As the temperature increased, the rate of inactivation of both filtrates also increased to some extent; inactivation at 21° C. was much slower than at 37° C., and 30° C. was intermediate in this respect.

Filtrates which were held at approximately 6° C. for as long as 2 years apparently did not decrease significantly in titer during that time, although actual data upon that point are lacking. The filtrates held at that temperature retained their "virulence" very satisfactorily and the stock filtrates were stored at that temperature with no apparent ill effects.

Apparently the lower the temperature, at least down to freezing, at which filtrates are stored and the closer the pH level of the filtrate is to 6.8, the longer filtrates containing the principle inhibitory to S. lactis can be held without marked decrease in titer occurring. Holding at 6° C. proved very satisfactory in practice.

The effect of drying upon filtrates containing the inhibitory principle

In the first series of experiments undertaken to determine the effect of the drying of filtrates upon the activity of the inhibitory principle contained, 15 ml. quantities of filtrates 31 and 35 were dried for 5 days over concentrated sulfuric acid. Filtrate 31 had been propagated in tomato juice broth which was originally at pH 7.45 but which unquestionably was more acid at the time of drying, although the pH was not determined, and filtrate 35 had been propagated in litmus milk and was probably at a pH of approximately 4.3 at the time of drying, since the culture had been coagulated with lactic acid in order to obtain the

filtrate. The dried filtrates were reconstituted to approximately the original volume with sterile distilled water. The dried matter of filtrate 31 went into solution easily, but small flocks remained in suspension even after thorough agitation in the case of filtrate 35. The titers of the reconstituted filtrates were determined by the usual dilution method, S. lactis 42 being used as the test organism.

The titer of filtrate 31 was $10^{-4.78}$ before drying and zero after drying and reconstitution. The titer of filtrate 35, which originally was 10^{-7}, was so reduced by drying that the use of a 1 ml. quantity of reconstituted filtrate was necessary to demonstrate the presence of residual inhibitory principle. Under the conditions of the experiments, the active principle was almost completely destroyed by the drying of the filtrates.

In a second series of experiments, the pH of the filtrates which were dried was more carefully controlled and the drying agent was phosphorous pentoxide. Two undiluted filtrates, 311 and 313, which had been propagated in tomato juice broth and which were at a pH of approximately 6.7 and 2 filtrates, A and B, which had been propagated in litmus milk and diluted 1:100 in 1.5 per cent milk digest broth at pH 6.7 were employed. Using sterile petri plates as containers, 2 ml. quantities of the 4 filtrates were dried for 7 days, all visible moisture having disappeared in 3 days, and were then reconstituted with sterile distilled water. No difficulty was experienced in getting the solid matter to redissolve. The titers of the reconstituted filtrates were determined by the usual dilution method.

The results of the experiments are tabulated in table 25. In all 4 cases, a slight but definitely measurable and apparently significant reduction in titer resulted from the drying of the filtrates. There

Table 25

The effect of drying over phosphorous pentoxide upon the titer of filtrates adjusted to pH 6.7

Filtrate preparation	Titer of filtrate			
	A	B	311	313
Not dried	10^{-6}	10^{-7}	$10^{-9.78}$	$10^{-9.78}$
Dried and reconstituted	10^{-5}	$10^{-5.78}$	$10^{-8.78}$	$10^{-8.78}$

was no appreciable difference between the behaviors of the various strains of principle, the results being surprisingly uniform. This was probably due to the uniform pH level, since that was the only common factor in the four cases, different media, different strains of propagating and testing organisms and different concentrations of principle all having been used.

Although no definite series of studies was made to demonstrate the effect of pH upon the ability of a filtrate containing active inhibitory principle to survive drying, the results of the experiments which were made indicate that a pH of approximately 4.3 is less favorable to undiminished activity after drying than is a pH of 6.7. Apparently the strains of principle which are active against S. lactis are less stable to adverse physical factors when the pH level is 5 or below than when the system containing the principle is nearly neutral in reaction.

The action of chemical substances upon the activity of filtrates containing inhibitory principle

In order to determine the effects of certain substances upon the inhibitory principle, 1 per cent aqueous solutions of crystal violet, methylene blue and safranine were made up and sterilized. Measured amounts of these solutions and of 30 per cent hydrogen peroxide were added to 8 ml. quantities of filtrates which had been diluted 1:100, either with milk digest broth adjusted to pH 6.7 and buffered with M/20 phosphate or with litmus milk. After thorough shaking to insure complete mixture, the tubes containing the solutions were placed at either 21° or 30° C. for 3 days. At the end of the holding period, 0.1 ml. quantities of the treated filtrates were used in testing the inhibition activity by the litmus milk culture method previously described. Unless

the culture to which the treated filtrate had been added coagulated as rapidly as a culture to which no filtrate had been added, complete destruction of the filtrate was not considered to have taken place.

The results of the first series of experiments made to determine the destructive effects of dyes and hydrogen peroxide are tabulated in table 26. Crystal violet in all concentrations tried inactivated both strains of the inhibitory principle in broth but not in litmus milk. The inactivation was the same at 21° and 30° C. Since the lower limit for the inactivating concentration of this substance was not determined, there might be some effect of temperature in establishing the limiting inactivation concentration. This hypothesis is strengthened by the results obtained with the other substances which were tested, as will be brought out later.

In the case of methylene blue, none of the concentrations used were able to inactivate either strain of the principle when litmus milk was used as the medium in which the tests were made. Although the results obtained with concentrations of 0.024 and 0.059 per cent methylene blue in broth at 21° C. were somewhat anomalous, in that one of the duplicate tests showed the presence of active inhibitory principle in each case while the second test did not, a concentration of 0.2 per cent methylene blue in broth was apparently necessary to definitely inactivate each of these strains of principle when they were held at 21° C. With a holding temperature of 30° C., strain A was inactivated by all 4 concentrations of methylene blue, while strain B was only completely inactivated by a concentration of 0.11 per cent, the concentration of 0.059 per cent being just on the border line of inactivation under the conditions of the experiment. Thus filtrate A was shown to be more resistant than filtrate B to inactivation by methylene blue at 30° C. under

Table 26

The effect of exposure for 3 days to various dyes and to hydrogen peroxide upon the activity of inhibitory principle

Substance tested	Conc. per cent	Effect of exposure on Filtrate A in Milk digest. broth 21°C.	Filtrate A in Milk digest. broth 30°C.	Filtrate A in Litmus milk 21° C.	Filtrate A in Litmus milk 30°C.	Filtrate B in Milk digest. broth 21°C.	Filtrate B in Milk digest. broth 30°C.	Filtrate B in Litmus milk 21°C.	Filtrate B in Litmus milk 30°C.
Crystal violet	0.024	+	+	-	-	+	+	-	-
	0.059	+	+	-	-	+	+	-	-
	0.11	+	+	-	-	+	+	-	-
	0.20	+	+	-	-	+	+	-	-
Methylene blue	0.024	±	+	-	-	-	-	-	-
	0.059	±	+	-	-	-	±	-	-
	0.11	-	+	-	-	-	+	-	-
	0.20	+	+	-	-	+	+	-	-
Safranine	0.024	-	-	-	-	-	-	-	-
	0.059	-	-	-	-	-	-	-	-
	0.11	-	-	-	-	-	-	-	-
	0.20	-	-	-	-	-	-	-	-
Hydrogen peroxide	0.15	±	+	+	+	+	+	-	-
	0.47	-	-	+	-	-	-	-	-
	1.25	-	-	-	-	-	-	-	-

+ = destruction

- = no destruction

± = one of the duplicates showing destruction, one showing survival.

the experimental conditions used, although the 2 strains were practically identical in their resistance at 21° C.

Safranine was without effect upon either strain of inhibitory principle. Had the precipitate which formed in each case been removed by centrifugal means or by filtration, inactivation might have been demonstrated in some cases, however.

Hydrogen peroxide was somewhat irregular in its behavior. In many cases the lower concentrations seemed to cause inactivation while the higher concentrations did not. This phenomenon was undoubtedly due to the inhibitory effects of the higher concentrations on the bacteria when the treated filtrates were added to cultures of S. lactis to determine the residual activity. Later studies demonstrated that even when 0.1 ml. of a broth, containing the lowest concentration of hydrogen peroxide used in this series of experiments (0.15 per cent) and no inhibitory principle, was placed in 8 ml. of litmus milk into which the S. lactis strains used for testing purposes were subsequently inoculated, the rate of acid formation and coagulation was significantly decreased. More than 30 hours were required while the check cultures needed only 24 hours. In the series of experiments at present under discussion, inactivation by a hydrogen peroxide concentration of 0.15 per cent was apparently complete in all cases except that of filtrate B diluted with litmus milk.

Further studies on the action of crystal violet, methylene blue and hydrogen peroxide in various concentrations were made at a later date, using 4 strains of inhibitory principle rather than the 2 strains which had been employed previously. The same stock solutions of test substances were used again and a 5 per cent aqueous solution of potassium permanganate was also used. In these trials, 1 ml. of

Table 27

Further studies of the effect of exposure for 3 days to various substances upon the activity of inhibitory principle

Substance tested	Conc. per cent	Effect of exposure on filtrate			
		A	B	311	313
Potassium permanganate	0.56	+	-	-	-
Crystal violet	0.024	+	+	-	+
	0.012	-	-	-	-
	0.006	-	-	-	-
Methylene blue	0.2	+	+	-	-
Hydrogen peroxide	0.17	-	-	-	-
	0.073	-	-	-	-
	0.037	-	-	-	-

\+ = destruction

\- = no destruction

each filtrate was diluted with 100 ml. of 2.5 per cent unbuffered milk digest broth. The final pH in each case was 6.8. The temperature used was 21° C.

The results of the second series of tests are shown in table 27. The 0.56 per cent potassium permanganate inactivated only one principle, strain A, out of the four which were tested. Crystal violet in a concentration of 0.024 per cent totally inactivated strains A, B and 313 but did not inactivate strain 311. Lower concentrations of crystal violet had no effect upon any of the 4 strains. The 2 per cent methylene blue inactivated filtrates A and B but not 311 and 313. The hydrogen peroxide concentrations studied did not bring about the inactivation of any one of the 4 strains of inhibitory principle.

In this second series of experiments, quantities of the treated broths equal to the quantities of treated diluted filtrates were added to the control litmus milk cultures of the organisms used for testing purposes to determine the effect of the test substances alone upon bacterial growth. Only in the case of hydrogen peroxide was any growth inhibition demonstrated, and then only in the cases when the concentration of hydrogen peroxide in the broth, which was later diluted, was 0.17 per cent. No effect was noticeable when the concentration was only 0.073 per cent.

All 4 strains of inhibitory principle which were used in the experiments showed different resistances and combinations of resistances to the 4 substances employed to test them. The data are insufficient to permit the drawing of conclusions as to whether the differences in resistance were qualitative or quantitative.

A study of the effect of pH and temperature on the action of potassium permanganate inhibitory principle during a 3 day incubation

period was made. Filtrates A and B were used, 1 ml. of each being added to 100 ml. quantities of 1.5 per cent milk digest broth buffered with M/20 phthalate or phosphate. The resulting dilutions were distributed in 8 ml. quantities into sterile test tubes to which measured amounts of sterile 5 per cent aqueous solution of potassium permanganate were added.

The results of the studies are shown in table 28. In the case of filtrate A, the inactivation observed at pH 4.8 was probably partially due to the pH effect, as demonstrated in an earlier section, since inactivation did not occur at pH 5.8 and did occur at pH 6.8 under the same conditions. For this reason, the trials at pH 4.8 will not be included in the discussion. At 21° C., strain A was only inactivated by 0.56 per cent of permanganate at pH 6.7. At 30° C., the same concentration brought about inactivation at pH 6.7 and pH 7.3. At 37° C., inactivation was obtained by 0.24 per cent permanganate at pH 6.7 in addition to partial inactivation by 0.56 per cent concentration at pH 5.8 and complete inactivation by the same permanganate concentration at pH 6.7 and pH 7.3. None of the temperature and concentration combinations which were investigated was able to bring about the inactivation of either strain of principle when the medium employed was litmus milk. Filtrate B in broth was inactivated by 0.56 per cent potassium permanganate at pH 6.7 or 7.3 at all 3 temperatures, and at 37° C. was partially destroyed by the same concentration at pH 5.8. The destruction at pH 4.8 with a concentration of 0.56 per cent permanganate was probably largely a result of the pH effect rather than because of the added oxidizing agent.

The inactivation of inhibitory principle by means of crystal violet, methylene blue, hydrogen peroxide and potassium permanganate was

Table 28

The effect of pH and temperature on the action of potassium permanganate upon the inhibitory principle after a 3 day period

Filtrate	Medium	pH	Effect of exposure at 21° C.			30° C.			37° C.		
			Concentration of $KMnO_4$ (per cent)								
			0.13	0.24	0.56	0.13	0.24	0.56	0.13	0.24	0.56
A	MDB*	4.8	-	-	+	-	-	+	-	-	+
		5.8	-	-	-	-	-	-	-	-	±
		6.7	-	-	+	-	-	+	-	+	+
		7.3	-	-	-	-	-	+	-	-	+
	LM**	---	-	-	-	-	-	-	-	-	-
B	MDB	4.8	-	-	-	-	-	-	-	-	+
		5.8	-	-	-	-	-	-	-	-	±
		6.7	-	-	+	-	-	+	-	-	+
		7.3	-	-	+	-	-	+	-	-	+
	LM	---	-	-	-	-	-	-	-	-	-

\+ = destruction
\- = no destruction
± = one of the duplicates showed destruction, one showed survival.

*1.5 per cent milk digest buffered with $\frac{M}{20}$ phthalate or phosphate.
**Litmus milk.

accomplished. Safranine was without demonstrable effect. The influence of the reaction of the medium and of the temperature upon the inactivation of inhibitory principle was again demonstrated. Under the experimental conditions employed, no one substance was able to inactivate all of the filtrates which were used for testing purposes. The filtrates varied in their qualitative sensitivities to chemical destruction, no 2 strains of inhibitory principle having the same resistance characteristics. The quantitative effects of the test substances were not determined.

Part III. The characteristics of sensitive and non-sensitive strains of Streptococcus lactis

The cultural and morphological characteristics

All of the strains of S. lactis used in the studies were typical representatives of the species as far as could be determined. Both sensitive and non-sensitive cultures caused the characteristic reduction and acid coagulation of litmus milk. The time required for coagulation varied from culture to culture, but both sensitive and non-sensitive organisms were included among the more rapid and the slower producers of acid. Occasionally some of the sensitive cultures, among them organisms 22C, 99, 146C, 147, 151C, 153A, 161, 163, 269, 295 and 327, remained unchanged for approximately 24 hours after transfer from a normally coagulated culture into fresh litmus milk. These cultures coagulated within 48 hours from the time of inoculation and produced a normal coagulum. The situation sometimes persisted for several transfers, but usually the culture returned to normal after one or 2 transfers. Demonstrable inhibitory principle was not obtainable from the cultures, and no changes in sensitivity were detectable.

The form of growth in tomato juice broth at a pH of 6.8 varied considerably. A number of the sensitive cultures produced no visible growth in the medium in 24 hours but caused the broth to become very cloudy and produced a moderate precipitate during 48 hours of incubation at 21° C. With the exception of the slow development of some of the sensitive culture, no correlation between the type of growth in tomato juice broth at a pH of 6.8 and the sensitivity type of the strains of S. lactis was observed. Both resistant and non-resistant types of organisms grew as uniformly cloudy cultures and as almost clear cultures containing a considerable amount of precipitate, indicating no difference between the

types on the basis of "smooth" and "rough" types as indicated by growth in broth.

Microscopic examination of smears made from litmus milk cultures and stained by the Gram procedure showed slight differences in the morphology of the cultures. Variations in the size of cell, the shape of cell and the arrangement of the cells with respect to each other were encountered, but the differences were not correlated with the sensitivity types.

Macroscopically the colonies of all of the cultures were very similar, only slight variations in size being evident. Under the microscope with a magnification of approximately 100 diameters, slight differences appeared. Colonies which were smooth, entire and uniformly slightly granular and which transmitted light moderately well were designated type I, and other colonies which were entire, very slightly pitted upon the surface and uniformly moderately granular and which appeared quite dark by transmitted light were called type II. The differences between the colonies were only in the degree of the characteristics mentioned, and many cultures produced colonies which were somewhat intermediate in character. During the classification of the colony types of 48 representative cultures of S. lactis, they were arbitrarily placed in one or the other of the 2 groups, although some of the characteristics of part of the cultures were somewhat intermediate. Of the 26 sensitive organisms examined, the colonies of 16 cultures were of type I and the colonies of 10 cultures were of type II. Four non-sensitive cultures formed type II colonies and 18 formed type I colonies. Although very few cultures of the resistant type produced type II colonies, no division of sensitive and non-sensitive cultures could be accomplished upon the basis of colony type.

The fermentation of carbohydrates by the sensitive and non-sensitive strains of Streptococcus lactis

The abilities of 50 representative cultures of sensitive and non-sensitive strains of S. lactis to ferment arabinose, dulcitol, fructose, galactose, glucose, glycerol, inisitol, imulin, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbital, starch and sucrose were determined by the use of one per cent solutions of the carbohydrates in one per cent Bacto tryptophane broth to which brom cresol purple indicator had been added. Observations were made after one day, 3 days, 9 days and one month of incubation at room temperature.

None of the organisms were able to produce acid from dulcitol, glycerol, inisitol, imulin, raffinose, rhamnose and sucrose. All cultures fermented fructose, galactose, glucose and lactose, although 3 days were frequently required for the medium containing fructose to become acid. The abilities to ferment arabinose, maltose, mannitol, salicin and starch varied among the organisms and the fermentation of these substances usually required more than 3 days. The results obtained with these 5 substances are shown in table 29. Among the 50 cultures tested, 3 non-sensitive organisms were the only ones that could ferment mannitol; the 20 other non-sensitive organisms were unable to ferment the substance. Although there were 4 groups, separated on the basis of carbohydrate fermentation, which contained only non-sensitive organisms and one group which contained only sensitive organisms, the members of these groups constituted such a small fraction of the total number of organisms that their significance is very doubtful. The major portion of the organisms examined were included in the 5 fermentation groups made up of both sensitive and non-sensitive organisms.

The fermentation of carbohydrates offered no basis for the

Table 29

The fermentation of carbohydrates by sensitive and non-sensitive strains of Streptococcus lactis

Type of organism	Number of cultures	Acid production in Arabinose	Maltose	Mannitol	Salicin	Starch
Sensitive	2	-	-	-	-	-
Non-sensitive	4	-	-	-	-	-
Sensitive	6	+	-	-	-	-
Non-sensitive	1	+	-	-	-	-
Sensitive	7	+	-	-	+	-
Non-sensitive	2	+	-	-	+	-
Sensitive	3	+	+	-	+	-
Non-sensitive	2	+	+	-	+	-
Sensitive	4	+	+	-	+	+
Non-sensitive	5	+	+	-	+	+
Sensitive	5	-	+	-	+	-
Non-sensitive	2	+	+	+	+	+
Non-sensitive	1	-	+	+	+	+
Non-sensitive	3	-	+	-	+	+
Non-sensitive	3	-	-	-	+	-

+ = acid formation

- = no acid formation

separation of S. lactis organisms into types on the basis of sensitivity to the action of inhibitory principle.

The suitability of non-sensitive strains of Streptococcus lactis for the making of butter cultures

Cultures of S. lactis which had proven resistant to all of the filtrates against which they had been tested were examined to be sure that they were free from characteristics which would be detrimental to butter cultures made from them. From the group selected in this manner, 13 organisms were chosen for combination with 6 cultures of citric acid fermenting streptococci. Drops of the citric acid fermenting organisms were inoculated into 8 ml. quantities of litmus milk and incubated for approximately 12 hours at room temperature. A drop of the desired litmus milk culture of S. lactis was then introduced into each tube of milk and the cultures kept at 21° C. until coagulation occurred. The cultures were transferred daily in 8 ml. quantities of litmus milk in test tubes. After several weeks, they were transferred into milk pasteurized by holding above 90° C. for 30 minutes. Following the second transfer in whole milk, they were graded by an experienced judge of butter cultures.

The results of the butter culture gradings are shown in table 30. The dashes indicate cultures which coagulated so slowly that they were of no value and were not examined. The deficiencies of butter cultures made with organisms 27, 57, 183, 216, 233H and 323 were due to the slowness of growth which characterized the strains of S. lactis used in them. A sufficiently large number of satisfactory butter cultures was obtained from the other strains of S. lactis to show that the non-sensitive strains of the organism can be used for the making of satisfactory cultures. Several of the more satisfactory combinations

Table 30

The criticisms of the butter cultures made with non-sensitive strains of *Streptococcus lactis*

S. lactis culture	Citric acid fermenting streptococcus A	B	C	D	E	F
22A	good	poor	poor	poor	good	good
27	--	poor	--	--	--	--
57	--	poor	--	poor	--	--
103A	fair	fair	fair	fair	good	good
151K	good	poor	poor	poor	fair	good
183	--	poor	--	poor	poor	fair
216	--	poor	--	poor	--	--
233A	poor	poor	poor	poor	fair	good
233H	--	poor	--	poor	--	--
323	--	poor	--	poor	--	--
335	fair	fair	poor	poor	fair	good
MV	fair	fair	fair	poor	fair	good

of organisms were transferred regularly for 3 months and continued to be satisfactory butter cultures.

DISCUSSION OF RESULTS

The characteristics of the inhibitory principle active against S. lactis are those commonly reported for bacteriophages. The principle is capable of passing through an ultrafilter of the type used for obtaining bacteriophage preparations. Propagation of the principle on cultures of sensitive organisms is possible under a variety of conditions. Plaque formation in smears of sensitive organisms on the surfaces of agar plates is demonstrable for almost all of the filtrates tested, and reasons for the failure of plaques to develop have been advanced to cover the anamolous cases. Lysis of sensitive organisms is accomplishable under a variety of conditions by the addition of filtrates containing the active inhibitory principle to suspensions of the organisms in various broths. Secondary growth organisms obtained from cultures inhibited but not completely killed are almost always resistant to the strain of principle which caused their appearance. The principle is unaffected by freezing and only slightly affected by drying, provided the preparation is at the proper pH level. At reactions which are approximately neutral, some strains of the principle are resistant to 70° C. for 10 minutes but not for 15 minutes and some of the strains are inactivated by somewhat less rigorous heat treatments. The principle is more resistant to heat than is its homologous organism. The principle is usually inactivated by crystal violet, methylene blue and hydrogen peroxide and, at times, by potassium permanganate. These are all characteristics which have been reported for at least one strain of bacteriophage. The only differences between the inhibitory factor investigated in the experiments reported and known

bacteriophages are quantitative and not qualitative. The inhibitory principle has its optimum activity at neutral or very slightly acid reactions, and most bacteriophages are favored by slightly alkaline reactions. The virulence of the principle is apparently somewhat less than that characteristic for bacteriophages active against the members of the colon group but is equal to that of bacteriophages active against pathogenic streptococci. The titer developed by filtrates containing the principle is equal to that reported for all but the most virulent bacteriophages.

Many of the filtrates obtained from the butter cultures are able to inhibit a considerable number of the test organisms. When the inhibition factors active against single cultures of sensitive organisms are segregated, either by propagation for several generations or by plaque isolation, a number of filtrates containing individual strains of principle can frequently be obtained from the original filtrate. The various types of inhibitory principle can be distinguished upon the basis of their differences in ability to inhibit the growth of various cultures of S. lactis and to cause the appearance of secondary growth organisms of different sensitivity types and upon the basis of their differences in resistance to chemical and physical factors. Differentiation by serological methods was not attempted. The multiplicity of types of inhibitory principle is probably related in some way to the diversity of the sensitivity types of the organisms which is frequently observed in butter cultures, since different types of filtrates require different organisms for their propagation. Undoubtedly, further examination of slow butter cultures would yield additional strains of inhibitory

principle, and further isolations of S. lactis cultures would probably result in the discovery of additional types sensitive to the strains of inhibitory principle which have already been obtained.

The qualitative and quantitative differences between filtrates from slow butter cultures and those from the same strain of butter culture in a normal condition are obvious, but the results of the experiments made in an effort to determine some of the factors responsible for the rise and fall of the inhibitory principle in butter cultures were inconclusive. The demonstration of principle in cultures which appeared normal and had been normal for some time indicates the possibility that the principle may be present in the cultures at all times and only require the action of the proper factors to make it become evident in the form of a slow culture. At the times when the principle was not demonstrable in the butter cultures, it may have been present in quantities too small to be detectable by the methods used. The effect of the exposure of culture milk to air upon the rates of coagulation was not constant, and the bubbling of oxygen, which was the only gas found to have an effect, through freshly inoculated butter cultures influenced only one culture, although the results with that one culture were duplicable.

The intimate relationship between the sensitive organism and the inhibitory principle is shown by the ability of the organism to propagate the same strains in a group of strains of inhibitory principle, such as is found in many of the filtrates from slow butter cultures, that are active against the organism. At the same time, the organism has no effect upon the strains of principle which do

not influence it and such strains are diluted out or segregated, depending upon the purification procedure used. The inability of secondary growth organisms to propagate the strain of principle which caused their origin is further evidence of the interdependence of organism and principle.

The isolation of various types of sensitive organisms from a butter culture at one time and of other types at different times indicates that the organisms in the culture are undergoing changes in sensitivity. Part of this change may be due to the elimination of sensitivities from the organisms by the small amounts of principle which are present in the cultures at least part of the time. This will not explain the acquisition of new sensitivities which frequently occurs, however. This instability of some S. lactis cultures, particularly those which have originated as a result of the action of inhibitory principle upon a sensitive culture, may be the explanation for the type of organism with more sensitivities to inhibitory principle than were possessed by the organisms previously found in the culture, since the unstable organisms are apparently able to either gain or lose sensitivities.

The favorable effect of oxygen upon the regeneration of bacteriophage was discussed in the literature review, as were reports of the effect of oxygen upon the supposed isolations of bacteriophage from organisms which had previously appeared to be free from bacteriophage. In the studies here reported, air and oxygen had a slight effect in some cases in producing slow butter cultures from previously normal cultures, and other investigators have reported similar

results. There is a possibility that the oxidative action of the oxygen of the air could have such an effect upon an occasional unstabilized cell that the inhibitory principle could be generated from the cell. The principle originating in this way could then be propagated upon sensitive organisms and cause a slow culture if a sufficiently high percentage of sensitive organisms was present in the culture. This is admittedly an hypothesis, but it fits the reported and observed experimental facts in all respects and offers an explanation for almost all of the observed phenomena which occur in slow butter cultures. Much more experimental work would be needed to prove or disprove the hypothesis.

Apparently the differences in sensitivity which occur between cultures of S. lactis are not correlated with other characteristics of the organisms, the resistance to the action of the inhibitory principle being an independent variable.

CONCLUSIONS

The characteristics of the inhibitory principle contained in most of the bacteria-free filtrates from slow butter cultures and in some of the filtrates from apparently normal butter cultures indicate that the principle is a bacteriophage active against Streptococcus lactis.

Upon the bases of ability to inhibit the growth of various strains of organisms which are sensitive to the principle, of ability to cause the appearance of secondary growth organisms of different sensitivity types and of variation in resistance to the action of physical and chemical agents, differences between strains of the principle can be recognized.

Different cultures of Streptococcus lactis have various sensitivities to the principle. Cultures occurring in natural products are resistant to the activity of the principle. The organisms in some butter cultures are apparently destabilized by an unknown factor that makes part of them sensitive to the action of the inhibitory principle. Some of the destabilized organisms are able to undergo spontaneous changes in sensitivity and all of the sensitive organisms are apparently subject to changes in sensitivity when acted upon by inhibitory principle.

The instability of some of the sensitive organisms forms the basis for a possible explanation of some of the phenomena characteristic of slow butter cultures. Stabilization in the direction of greater sensitivity would account for the organisms with sensitivities previously not encountered in the culture which are frequently isolated.

The generation of inhibitory principle from the unstabilized organisms by oxidative action agrees with the facts reported in the literature concerning the possible origin of bacteriophage from cultures of sensitive organisms and also explains the influence of exposure to air and of large containers upon the incidence of slow butter cultures.

SUMMARY

1. Filtrates containing a principle inhibitory to the growth of Streptococcus lactis were obtained by the ultrafiltration of some butter cultures.

2. The inhibitory principle was demonstrated in 8 of the 21 filtrates from slow butter cultures and in 16 of the 28 filtrates from normal butter cultures.

3. The active filtrates from slow butter cultures were usually able to inhibit a greater variety of Streptococcus lactis cultures than were the active filtrates from normal butter cultures.

4. The inhibition characteristics of the filtrates obtained from a single strain of butter culture were not the same in all respects, indicating a changing condition within the culture.

5. Although exposure of the milk in which the butter cultures were later grown to the air of the creamery increased the incidence of slow cultures, exposure to the air of the bacteriology laboratory had no such effect.

6. The data concerning the qualitative and quantitative effects of exposure of the milk for butter culture propagation to air for some time before inoculation are inconclusive.

7. The bubbling of pure oxygen gas through freshly inoculated cultures retarded the acid production of one butter culture but not of a second culture, but oxygen, hydrogen and nitrogen gases apparently had no effect upon the presence of inhibitory principle in the cultures.

8. Raw milk had no effect on the incidence of slowness or on the presence of inhibitory principle in butter cultures.

9. Inhibitory principle was not recovered from any pure cultures of Streptococcus lactis under any conditions.

10. Representative bacteriophages active against members of the colon group did not contain a factor inhibitory to the growth of Streptococcus lactis.

11. Bacteria-free filtrates from citric acid fermenting streptococci did not inhibit the growth of Streptococcus lactis.

12. The titers of the filtrates were increased by propagation of the inhibitory principle on sensitive strains of Streptococcus lactis.

13. The maximum titer for all strains of principle studied was approximately 10^{-9} or slightly higher.

14. Propagation of the principle was associated with the killing of the sensitive organisms used.

15. The various strains of inhibitory principle present in many of the filtrates obtained directly from butter cultures could be segregated by series of propagations on organisms sensitive to the different strains of principle or by the picking of plaques from plates prepared with organisms of various sensitivities.

16. Agreement between the litmus milk culture method and the agar plate plaque method for the demonstration of inhibitory principle was not always obtained and the litmus milk culture method was chosen for the routine demonstration of the inhibitory principle.

17. Lysis of cultures of Streptococcus lactis by inhibitory principle was accomplished in several media.

18. A temperature of 21° C. and pH of approximately 6.8 were most favorable for lysis.

19. The inhibitory principle active against Streptococcus

lactis had no effect upon cultures of lactobacilli, Streptococcus liquefaciens and citric acid fermenting streptococci.

20. Only non-sensitive strains of Streptococcus lactis were obtained from naturally soured milk and whey.

21. Both sensitive and non-sensitive strains of Streptococcus lactis were obtained from a series of butter cultures.

22. More than one type of sensitive Streptococcus lactis culture was frequently obtained from a butter culture at one time.

23. The sensitivity types of the cultures of Streptococcus lactis isolated from a single butter culture at various times were quite different, indicating that the butter cultures were not stable with respect to their sensitivities to inhibition.

24. The organisms obtained from the secondary growth following the inhibition of a culture of Streptococcus lactis by inhibitory principle were typical Streptococcus lactis organisms which were, with a few exceptions, resistant to the filtrate which caused their appearance.

25. Changes in sensitivity occurred in some pure cultures of Streptococcus lactis, especially among those isolated from the secondary growth of inhibited organisms.

26. The inhibitory principle diluted with litmus milk was destroyed by heating to 70° C. for 15 minutes, and some strains were destroyed at lower temperatures.

27. The inhibitory principle was readily destroyed by heat when at a pH of 4.8 and was most stable at an approximately neutral reaction.

28. The inhibitory principle was more stable to heat than the homologous organism.

29. The inhibitory principle was most stable if stored at low

temperatures in a medium at approximately neutral reaction.

30. The principle withstood freezing without decrease in titer.

31. If a preparation at a pH level of approximately neutrality was used, the inhibitory principle could be dried with very little loss in activity.

32. The inhibitory principle in milk digest broth was inactivated by 0.02^{4} per cent of crystal violet, 0.2 per cent of methylene blue, 0.15 per cent of hydrogen peroxide and, in one case, by 0.56 per cent potassium permanganate when the substances were allowed to act for 3 days at room temperature or at 30° C.

33. Potassium permanganate had the greatest inactivating effect at a pH of 6.7.

34. Sensitive and non-sensitive types of Streptococcus lactis could not be differentiated upon the bases of cultural and morphological characteristics or by differences in carbohydrate fermentations.

35. Resistant strains of Streptococcus lactis which would coagulate milk sufficiently rapidly were suitable for use in butter cultures.

36. The experimental results indicate that the inhibitory principle is a bacteriophage active against Streptococcus lactis.

37. An explanation of the phenomenon of slow butter cultures, based upon the instability of the sensitivities of certain Streptococcus lactis types is advanced.

BIBLIOGRAPHY

1. Andrewes, C. H. and W. J. Elford.
The "killing" of bacteria by bacteriophage. Brit. J. Exp. Path. 13:13-21. 1932.

2. Applebaum, M. and M. B. Patterson.
The effect of bile on the bacteriophage phenomenon. J. Infectious Diseases 58:195-203. 1936.

3. Arkwright, J. A.
The source and characteristics of certain cultures sensitive to bacteriophage. Brit. J. Exp. Path. 5:23-33. 1924.

4. Arnaudi, C. and E. Castellani.
Sur le bactériophage du Rhizobium radicicola. Soc. intern. mikrobiol., Boll. sez. ital. 6:317-21. 1934.

5. Asheshov, I.
Action du citrate de soude sur le bacteriophage. Compt. rend. soc. biol. 94:687-9. 1926.

6. Bail, O.
Versuche über die Vielheit von Bakteriophagen. Z. ImmunitÄts., Orig. 38:57-164. 1923.

7. Baker, M. P. and B. W. Hammer.
Influence of the milk on the starter made from it. Iowa Agr. Exp. Sta. Research Bull. 92. 1926.

8. Breinl, F. and F. Hoder.
Bakteriophagenwirkungen in der Paratyphusgruppe. Centr. Bakt. Parasitenk., I. 96:1-8. 1925.

9. Bronfenbrenner, J.
True polyvalence of pure bacteriophages. Proc. Soc. Exp. Biol. Med. 30:729-32. 1933.

10. Bronfenbrenner, J. and C. Korb.
Studies on the bacteriophage of d'Herelle. II. Effect of alcohol on bacteriophage of d'Herelle. J. Exp. Med. 42: 419-29. 1925.

11. Bronfenbrenner, J. and C. Korb.
Studies on the bacteriophage of d'Herelle. III. Some of the factors determining the number and size of plaques of bacterial lysis on agar. J. Exp. Med. 42:483-97. 1925.

12. Bronfenbrenner, J. and C. Korb.
Studies on the bacteriophage of d'Herelle. IV. Concerning the oneness of the bacteriophage. J. Exp. Med. 42:821-8. 1925.

13. Bronfenbrenner, J. and C. Korb.
Studies on the bacteriophage of d'Herelle. V. Effect of electrolytes on the rate of inactivation of bacteriophage by alcohol. J. Exp. Med. 43:71-86. 1926.

14. Buchanan, R. E. and R. Truax.
Non-inheritance of impressed variations in Streptococcus lacticus. J. Infectious Diseases 7:680-97. 1910.

15. Burnet, F. M.
The relationships between the heat-stable agglutinogens and sensitivity to bacteriophage in the Salmonella group. Brit. J. Exp. Path. 8:121-9. 1927.

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

17. Burnet, F. M.
Further observations on the nature of bacterial resistance to bacteriophage. J. Path. Bact. 32:349-54. 1929.

18. Burnet, F. M.
Bacteriophage and cognate phenomena. Chapter XXXIX of A system of bacteriology, edited by Medical Research Council. H. M. Stationery Off., London.

19. Burnet, F. M.
Recent work on the biological nature of bacteriophages. Trans. Roy. Soc. Trop. Med. Hyg. (London) 26:409-16. 1933.

20. Burnet, F. M.
The classification of dysentery-coli bacteriophages. II. The serological classification of coli-dysentery phages. J. Path. Bact. 36:307-18. 1933.

21. Burnet, F. M.
The classification of dysentery-coli bacteriophages. III. A correlation of the serological classification with certain biochemical tests. J. Path. Bact. 37:179-84. 1933.

22. Burnet, F. M. and M. McKie.
Balanced salt action as manifested in bacteriophage phenomena. Australian J. Exp. Biol. Med. Sci. 7:183-98. 1930.

23. Burnet, F. M. and M. McKie.
The electrical behavior of bacteriophages. Australian J. Exp. Biol. Med. Sci. 7:199-209. 1930.

24. Burnet, F. M. and M. McKie.
The classification of dysentery-coli bacteriophages. I. The differentiation by Bail's methods of phages lysing a typical B. coli strain. J. Path. Bact. 36:299-306. 1933.

25. Chambers, W. H.
Bacterial inhibition. I. Germicidal action in milk. J. Bact. 5:527-41. 1920.

26. Chen, W. K.
Dissociation of V. cholerae by bacteriophage. Proc. Soc. Exp. Biol. Med. 29:1160-2. 1932.

27. Clifton, C. E.
Photodynamic action of certain dyes on the inactivation of staphylococcus bacteriophage. Proc. Soc. Exp. Biol. Med. 28: 745-6. 1931.

28. Clifton, C. E. and T. G. Lawler.
Inactivation of staphylococcus bacteriophage by toluidine blue. Proc. Soc. Exp. Biol. Med. 27:1041-2. 1930.

29. Cox, G. A.
A simple method for detecting non-acid milk. New Zealand J. Agr. 49:231-4. 1934.

30. Cox, G. A. and H. R. Whitehead.
The influence of other bacteria on the production of acid by lactic streptococci in milk. J. Dairy Research 2:164-75. 1931.

31. Danbly, J. and J. Bronfenbrenner.
Stimulation of bacterial metabolism by bacteriophage. Proc. Soc. Exp. Biol. Med. 30:732-5. 1933.

32. Davis, J. G.
Slow starters in cheesemaking. Agr. Progress 12:138-44. 1935.

33. de Poorter, P. and J. Maisin.
Contribution à l'étude de la nature du principe bactériophage. Arch. intern. pharmacodynamie 25:473-84. 1921.

34. d'Herelle, F.
Sur un microbe invisible antagoniste des bacillus dysenteriques. Compt. rend. acad. sci. 165:373-5. 1917.

35. d'Herelle, F.
Sur la résistance des bactéries a l'action du microbe bactériophage. Compt. rend. soc. biol. 83:97-9. 1920.

36. d'Herelle, F.
The bacteriophage and its behavior. Williams and Wilkins, Baltimore. 1926.

37. d'Herelle, F.
The bacteriophage and its clinical applications. C. C. Thomas, Springfield, Illinois. 1930.

38. d'Herelle, F. and T. L. Rakieten.
Mutations as governing bacterial characters and serologic reactions. J. Infectious Diseases 54:313-38. 1934.

39. Doorenbos, W.
Étude sur la symbiose du vibrion cholérique avec le bactériophage. Ann. inst. Past. 48:457-69. 1932.

40. Eaton, M. D., Jr.
A quantitative study of the respiration of staphylococcus cultures lysed by bacteriophage. J. Bact. 21:143-56. 1931.

41. Elford, W. J. and C. H. Andrewes.
The sizes of different bacteriophages. Brit. J. Exp. Path. 13:446-56. 1932.

42. Evans, A. C.
Inactivation of antistreptococcus bacteriophage by animal fluids. U. S. Pub. Health Rep. 48:411-26. 1933.

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

44. Evans, A. C.
The prevalence of streptococcus bacteriophage. Science 80: 40-1. 1934.

45. Farmer, R. S. and B. W. Hammer.
Studies on the development of butter cultures from mixtures of organisms. Iowa Agr. Exp. Sta. Research Bull. 146. 1931.

46. Fejgin, B.
Contribution à l'étude des races résistantes du Bacille de Shiga-Kruse. Comp. rend. soc. biol. 89:1381-3. 1923.

47. Frisbee, F. C. and W. J. MacNeal.
The behavior of Escherichia coli and its specific bacteriophage in urine. J. Infectious Diseases 46:405-12. 1930.

48. Gildemeister, E. and K. Herzberg.
Ueber das d'Herellesche Phänomen. III. Mitteilung. Centr. Bakt. Parasitenk., I. 91:12-9. 1923.

49. Girard, P. and V. Sertic.
Action de hauts champs centrifuges sur diverses cellules bactériennes sur différents bactériophages et la lysine diffusible d'un bactériophage. Compt. rend. soc. biol. 118:1286-8. 1935.

50. Goldsmith, N. R.
Differences in effect of phenyl mercuric chloride upon different races of bacteriophage and similarity in effect upon a phage and its homologous organism. J. Bact. 30:237-42. 1935.

51. Gratia, A.
Studies on the d'Herelle phenomenon. J. Exp. Med. 34:115-26. 1921.

52. Gratia, A.
The Twort-d'Herelle phenomenon. II. Lysis and microbic variation. J. Exp. Med. 35:287-302. 1922.

53. Grumbach, A.
Ueber Streptokokkenbakteriophagen und Dissoziationserscheinungen an Streptokokken. Centr. Bakt. Parasitenk., I. 118: 206-16. 1930.

54. Hadley, P.
Parallelism between serologic and bacteriophagic response in B. typhosus and certain avian paratyphoids. Proc. Soc. Exp. Biol. Med. 23:443-6. 1926.

55. Hadley, P.
Microbic dissociation: the instability of bacterial species with special reference to active dissociation and transmissible autolysis. J. Infectious Diseases 40:1-312. 1927.

56. Hadley, P.
The Twort-d'Herelle phenomenon. J. Infectious Diseases 42: 263-434. 1928.

57. Hadley, P. and E. Dabney.
The bacteriophagic relationships between B. coli, S. fecalis and S. lacticus. Proc. Soc. Exp. Biol. Med. 24:13-8. 1926.

58. Hadley, P. and J. Klimek.
The "source" of the lytic principle. Proc. Soc. Exp. Biol. Med. 25:34-7. 1927.

59. Hallauer, C.
Die übertragbare Lyse als Funktion des bakteriellen Gaastoffwechsels. I. Mitteilung: Ueber Aktivierung der übertragbaren Lyse durch Sauerstoff. Zentr. Bakt. Parasitenk., I. Orig. 129:265-74. 1933.

60. Hammer, B. W.
Studies on ropiness in cultures of Streptococcus lactis. Iowa Agr. Exp. Sta. Research Bull. 74. 1923.

61. Hammer, B. W. and M. P. Baker.
Classification of the Streptococcus lactis group. Iowa Agr. Exp. Sta. Research Bull. 99. 1926.

62. Hammer, B. W. and M. P. Baker.
Studies on starters. Iowa Agr. Exp. Sta. Research Bull. 106. 1928.

63. Hammer, B. W. and V. H. Patil.
Proteolysis by Streptococcus lactis with special reference to butter cultures and butter. Iowa Agr. Exp. Sta. Research Bull. 123. 1930.

64. Harriman, L. A.
Causes of slow acid production in butter cultures. Ph. D. thesis, Iowa State College Library. 1934.

65. Harriman, L. A.
Causes of slow acid production in butter cultures. Iowa State Coll. J. Sci. 9:155-7. 1934.

66. Harriman, L. A. and B. W. Hammer.
Variations in the coagulation and proteolysis of milk by Streptococcus lactis. J. Dairy Sci. 14:40-9. 1931.

67. Heineman, G. P. and T. H. Glenn.
The agglutination power of milk serum. J. Infectious Diseases 5:534-41. 1908.

68. Hoder, F.
Der gegenwärtige Stand der Bakteriophagenforschung. Archiv. Mikrobiol. 4:589-635. 1933.

69. Hoffstadt, R. E. and P. Almaden.
Dissociation of Staphylococcus aureus. III. Relation of bacteriophage to the dissociation of Staphylococcus aureus. J. Infectious Diseases 54:253-8. 1934.

70. Iowa Agricultural Experiment Station.
Report on Agricultural Research for the Year Ending June 30, 1933, p. 63. 1933.

71. Italo, P.
Recherche des réactions d'immunité sur le bactériophages du bacille typhique et dysentérique de Shiga dans leurs phases "R" et "S". Soc. intern. mikrobiol., Boll. sez. ital. 7:275-6. 1935.

72. Jones, F. S.
The properties of the bactericidal substance in milk. J. Exp. Med. 47:877-88. 1928.

73. Kelly, C. D.
Is all milk equally suitable as a medium for the preparation of starters? Sci. Agr. 10:328-32. 1930.

74. Kendall, A. I. and A. W. Walker.
The effect of ozone upon certain bacteria and their respective phages. Studies on bacterial metabolism, CV. J. Infectious Diseases 58:204-14. 1936.

75. Kligler, I. J. and L. Olitzki.
Purification of phage by adsorption and elution. Proc. Soc. Exp. Biol. Med. 30:1365-7. 1933.

76. Kligler, I. J., L. Olitzki and M. Aschner.
Studies on protein-free suspensions of viruses. II. Cataphoresis experiments with protein-free suspensions of a bacteriophage and fowl-pox virus. Brit. J. Exp. Path. 12: 178-82. 1931.

77. Klingmüller, O.
Untersuchungen über Unterschiede des Streptococcus cremoris und Streptococcus mastiditis. Milchwirtschaft. Forsch. 10: 431-54. 1930.

78. Knorr, W. and H. Ruf.
Phagtrocknung und Phagauswertung. Zentr. Bakt. Parasitenkund., I. Orig. 133:289-98. 1935.

79. Knudsen, S.
Starters. J. Dairy Research 2:137-63. 1931.

80. Knudsen, S. and A. Sörensen.
Über Milch als Nahroboden für gewisse Milchsäurbakterien. Centr. Bakt. Parasitenk., II. 71:500-7. 1927.

81. Knudsen, S. and A. Sörensen.
Beiträge zur Bakteriologie der Säurewecker. Zentr. Bakt. Parasitenk., II. 79:75-85. 1929.

82. Knudsen, S. and A. Sörensen.
Bidrag til syrevaekkerries Bakteriologi. K. Vet.-og Landbohöjskole, Copenhagen, Aaraskrift 1929:64-138. English summary, pp. 131-7. 1929.

83. Krueger, A. P.
Quantitative determination of bacteriophage. J. Gen. Physiol. 13:557-64. 1930.

84. Krueger, A. P.
The sorption of bacteriophage by living and dead susceptible bacteria. I. Equilibrium conditions. J. Gen. Physiol. 14: 493-516. 1931.

85. Krueger, A. P.
The heat inactivation of antistaphylococcus phage. J. Gen. Physiol. 15:363-8. 1932.

86. Krueger, A. P.
Critique of the serial dilution method for the quantitative determination of bacteriophage. Science 75:494-5. 1932.

87. Krueger, A. P.
The nature of bacteriophage and its mode of action. Physiol. Rev. 16:129-72. 1936.

88. Krueger, A. P. and D. M. Baldwin.
The reversible reaction of bacteriophage by bichloride of mercury. J. Gen. Physiol. 17:499-506. 1934.

89. Krueger, A. P. and D. M. Baldwin.
The reversible inactivation of bacteriophage with safranine. J. Infectious Diseases 57:207-11. 1935.

90. Krueger, A. P. and S. Elberg.
Reversible inactivation of bacteriophage by KCN. Proc. Soc. Exp. Biol. Med. 31:483. 1934.

91. Krueger, A. P. and E. J. Scribner.
The effect of pH on heat inactivation of bacteriophage. Proc. Soc. Exp. Biol. Med. 33:21-3. 1935.

92. Krueger, A. P. and H. T. Tamado.
The preparation of relatively pure bacteriophage. J. Gen. Physiol. 13:145-51. 1929.

93. Leitch, R. H.
Cheddar cheese-making. Scottish Agricultural Publishing Co., Glasgow. 1932.

94. Leitch, R. H.
L'arrêt de la fermentation lactique dans la preparation du fromage. Lait 13:529-41. 1933.

95. LeMar, J. D. and J. T. Myers.
Studies on the nature of bacteriophage. I. Extraction of bacteriophage by ether. J. Infectious Diseases 57:1-5. 1935.

96. LeMar, J. D. and J. T. Myers.
Studies on the nature of bacteriophage. II. The artificial production of a specific lytic agent which seems to behave like a bacteriophage. J. Infectious Diseases 57:6-11. 1935.

97. Levine, P. and A. W. Frisch.
Specific inhibition of bacteriophage by bacterial extracts. Proc. Soc. Exp. Biol. Med. 30:993-6. 1933.

98. Levine, P. and A. W. Frisch.
Further observations on specific inhibition of bacteriophage action. Proc. Soc. Exp. Biol. Med. 31:46-8. 1933.

99. Levine, P. and A. W. Frisch.
Note on adsorption of phage by heat-killed bacilli. Proc. Soc. Exp. Biol. Med. 32:339-41. 1934.

100. Levine, P. and A. W. Frisch.
Observations on phage inhibition by bacillary extracts. Proc. Soc. Exp. Biol. Med. 32:341-3. 1934.

101. Levine, P. and A. W. Frisch.
Polyvalency demonstrated by antiphages. Proc. Soc. Exp. Biol. Med. 32:886-8. 1935.

102. Levine, P., A. W. Frisch and E. V. Cohen.
On adsorption of phage by bacilli. J. Immunol. 26:321-5. 1934.

103. Lin, F. C., T. J. Kurotchkin and C. V. Bernaradsky.
Use of saturated sodium chloride solution for preservation of viruses. Proc. Soc. Exp. Biol. Med. 33:332-4. 1935.

104. Lipska, I.
Le phénomène d'Herelle dans le lait. Lait 12:88-95. 1932.

105. Lominski, I.
Inactivation du bactériophage par oxydation. Compt. rend. soc. biol. 119:952-4. 1935.

106. Lominski, I.
Sensibilitiés comparée des bactériophages et des bactéries homologues a l'oxydation. Compt. rend. soc. biol. 119:1090-2. 1935.

107. Lominski, I.
Inactivation du bactériophage par oxydation; réactivation par l'acide ascorbique. Compt. rend. soc. biol. 119:1345-8. 1935.

108. Majer, G.
Untersuchungen über Bakteriophagen und Käseblähung. Milchwirtschaft. Forsch. 9:179-85. 1929.

109. Marbais, S.
Le bacteriophage et la chronaxie. Soc. intern. mikrobiol., Boll. sez. ital. 6:343-57. 1934.

110. McKinley, E. B.
The Twort-d'Herelle phenomenon: the bacteriophage. In Agents of disease and host resistance, ed. by F. P. Gay. C. C. Thomas, Springfield, Illinois. pp. 1274-82. 1935.

111. McLeod, J. W. and P. Govenlock.
The production of bacteriociduis by microorganisms. Lancet 200:900-3. 1921.

112. Meissner, G.
Die Bindungsverhältnisse zwischen Bakteriophagen und Bakterien. Centr. Bakt. Parasitenk., I. 93:489-95. 1925.

113. Meyer, M., R. Thompson, D. Khorazo and J. W. Palmer.
Experiments on purification of a bacteriophage, and a respiratory pigment in Escherichia coli communis. Proc. Soc. Exp. Biol. Med. 33:129-33. 1935.

114. Moir, G. M.
Starters for cheese making: methods of preparation and maintenance. New Zealand J. Agr. 47:81-8. 1933.

115. Moir, G. M.
Starters for cheese making. Methods of preparation and maintenance. New Zealand J. Agr. 47:143-9. 1933.

116. Morgan, G. F. V. and J. Curle.
Slow development of acidity in cheese making. New Zealand J. Agr. 37:305-8. 1928.

117. Nanavutty, S. H.
The role of oxygen in bacteriophagy. Brit. J. Exp. Path. 11:5-9. 1930.

118. National Institute for Research in Dairying.
Starters—slow working. Nat. Inst. for Research in Dairying, Reading. Ann. Rep., 1931:61. 1931.

119. Ogata, N.
Zur Entstehung des Bakteriophagen in alten Kulturen. Centr. Bakt. Parasitenk., I. 93:329-34. 1924.

120. Orla-Jensen, S.
The lactic acid bacteria. Mém. de l'acad. roy. d. sci. et d. lettres de Danemark, Copenhagne. Ser. d. sci. Ser. 8, 5:81-196. 1919.

121. Plantureux, E.
Sur la nature des "bacteriolytes" agents de la lyse transmissible. Compt. rend. soc. biol. 103:387-9. 1930.

122. Porcher, C. and L. Lambert.
Action favorisante du Bacillus subtilis dans la fermentation lactique. Lait 10:640-54. 1930.

123. Powers, M. J.
Differentiation of species of the genus Escherichia on the basis of metabolism. M. S. thesis, Iowa State Coll. 1934.

124. Rogers, L. A. and E. O. Whittier.
Limiting factors in the lactic fermentation. J. Bact. 16: 211-29. 1928.

125. Scalfi, A.
A propos de l'épuration du bacteriophage par la cataphorèse. Soc. intern. mikrobiol., Boll. sez. ital. 7:159-63. 1935.

126. Schlesinger, M.
Zur Frage der chemische Zusammensetzung des Bakteriophagen. Biochem. Z. 273-306-11. 1934.

127. Schäler, H.
Stoffwechsel und Fermentuntersuchungen an Bakteriophagen. Biochem. Z. 276:254-61. 1935.

128. Schultz, E. W. and A. P. Krueger.
Inactivation of staphylococcus bacteriophage by methylene blue. Proc. Soc. Exp. Biol. Med. 26:100-1. 1928.

129. Schwartzmann, G.
Studies on regeneration of bacteriophage. I. The influence of partial anaerobiosis upon regeneration of a highly diluted principle. J. Exp. Med. 42:507-16. 1925.

130. Schwartzmann, G.
Studies on regeneration of bacteriophage. II. The influence of oxygen on the behavior of Bacillus coli toward lytic principle. J. Exp. Med. 43:743-51. 1926.

131. Sertic, V. and N. Boulgakov.
Classification et identification des typhi-phages. Compt. rend. soc. biol. 119:1270-2. 1935.

132. Sherman, J. M. and H. R. Curran.
The germicidal action of milk. Proc. Soc. Exp. Biol. Med. 22:15-7. 1924.

133. Singleton, W. M.
The preparation of starters from commercial cultures. New Zealand Dept. Agr. Bull. 10. 1915.

134. Spanedda, A.
Recherches sur le bactériophage anti-diphtérique. Soc. intern. mikrobiol., Boll. sez. ital. 7:394-6. 1935.

135. Stanley, W. M.
Isolation of a crystalline protein possessing the properties of tobacco-mosaic virus. Science 81:644-5. 1935.

136. Stark, P. and J. M. Sherman.
Concerning the habitat of Streptococcus lactis. J. Bact. 30:639-46. 1935.

137. Twort, F. W.
An investigation on the nature of ultramicroscopic viruses. Lancet 2:1241-3. 1915.

138. Vedder, A.
Die Hitzresistenz von getrockneten Bakteriophagen. Centr. Bakt. Parasitenk., I. 125:111-4. 1932.

139. Wells, A. Y. and N. P. Sherwood.
Selective action of crystal violet and of brilliant green on bacteriophages. J. Infectious Diseases 52:209-13. 1933.

140. Wells, A. Y. and N. P. Sherwood.
Selective action of dyes and of other disinfectants on bacteriophage. J. Infectious Diseases 55:195-8. 1934.

141. Whitehead, H. R.
The influence of bacilli of the colon group on cheddar cheese. J. Dairy Research 2:76-80. 1930.

142. Whitehead, H. R.
Further observations on slow development of acidity in cheese manufacture. New Zealand J. Agr. 47:376-8. 1933.

143. Whitehead, H. R.
A substance inhibiting bacterial growth, produced by certain strains of lactic streptococci. Biochem. J. 27: 1793-1800. 1933.

144. Whitehead, H. R. and G. A. Cox.
A method for the determination of vitality in starters. New Zealand J. Sci. Tech. 13:304-9. 1932.

145. Whitehead, H. R. and G. A. Cox.
The influence of bacilli of the colon group on the production of acid by lactic streptococci in milk. J. Dairy Research 4:74-80. 1932.

146. Whitehead, H. R. and G. A. Cox.
Observations on some factors in milk of individual cows which modify the growth of lactic streptococci. Biochem. J. 27:951-9. 1933.

147. Whitehead, H. R. and G. A. Cox.
Observations on sudden changes in the rate of acid formation in milk by cultures of lactic streptococci. J. Dairy Research 5:197-207. 1934.

148. Whitehead, H. R. and G. A. Cox.
The occurrence of bacteriophage in cultures of lactic streptococci. A preliminary note. New Zealand J. Sci. Tech. 16:319-20. 1935.

149. Whitehead, H. R. and G. A. Cox.
Selection of starter cultures for cheese manufacture--a preliminary note on a new method. New Zealand J. Agr. 50:107-9. 1935.

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

151. Whitehead, H. R. and W. Riddet.
Slow development of acidity in cheese manufacture. New Zealand J. Agr. 46:225-9. 1933.

152. Whitehead, H. R. and L. Wards.
Loss of vitality in dairy starters. A cheese factory investigation. New Zealand J. Agr. 47:218-21. 1933.

153. Yen, A. C. H.
Pour plate study of bacteriophage. Proc. Soc. Exp. Biol. Med. 32:1006-10. 1935.

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