AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date Thesis presented May 10, 1937

Title Hemolytic Streptococcus Bacteriophage

Redacted for privacy

Abstract Approved:
(Major Professor)

Bacteriophages for two hemolytic streptococci were isolated. Reactions of hemolytic strains of Streptococcus mastitidis and other hemolytic streptococci found in market milk with these phages were studied.

The presence of bacteriophage, specific for the hemolytic streptococci, was not demonstrated in old cultures of the organisms, in gargety milk, in fresh milk, or from the feces of cows having infectious mastitis.

Two apparently different streptococcus phages were obtained when the homologous organisms were grown in close association with organisms that were being lysed. These phages reacted differently in respect to the effect upon different organisms and also in respect to their potency. Phage (18), when diluted ten billion times, would completely lyse a standard culture of the homologous organism. Phage (16), on the other hand, could not be built up to a potency above 10^{-2}.

In studying the characteristics of streptococcus

phage, partial vacuum, anaerobic conditions, "commercial" gas and CO_2 had no depressing effect upon the lytic phenomenon, but the latter two did seem to inhibit or stop the appearance of the secondary growths.

Secondary cultures, appearing as resistant cells in the culture, following lysis proved to be a very serious handicap in the use of the test tube method of detecting the presence of the lytic agent. Regular short interval observations had to be made. Although the secondary cultures appeared to be the same culturally, morphologically, and physiologically as the unlysed culture, no lytic agent could be demonstrated for them.

When the effect of pH, in highly buffered solutions, upon bacteriophage was investigated no inactivation of the phage between pH 5 and pH 9 occurred. A very definite diminution or inactivating effect below pH 4 and above pH 10 was noted.

Further observations indicated that methylene blue inactivated phage. This dye apparently has an inactivating effect in as low a concentration as one part in ten thousand, if exposed for 8 hours at 20° C. The concentration of methylene blue to inhibit the growth of the streptococci was one part in one thousand. The phage therefore, seems to be more susceptible to the inactivating effect than the homologous organism.

The streptococcus phage passes a negatively-charged Berkefeld filter and it also passes up the full length, even in high dilutions, a negatively-charged piece of blotter paper. It thus appears that the charge was negative. However, results obtained by the electrolysis experiments indicate that the particle may be neutral. This point needs further investigation.

From the results of repeated observations it appears that surface tension may play some part in the mechanism of lysis. Lysis apparently appears near the surface of the culture. A piece of paper containing the lytic agent will produce, if floated upon the surface, complete visible lysis before one that sinks to the bottom.

Of the sixty-two hemolytic streptococci, isolated from milk, only six were lysed. Specificity was also evidenced in that phage (16) would lyse only one streptococcus. Five others were lysed by the other phage.

HEMOLYTIC STREPTOCOCCUS BACTERIOPHAGE

by

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A THESIS

submitted to the

OREGON STATE AGRICULTURAL COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF ARTS

June 1937

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A C K N O W L E D G M E N T

The writer wishes to express his thanks to Professor G. V. Copson, Head of the Department of Bacteriology of Oregon State College for his help in the arrangement and helpful criticisms in preparing this thesis, and to Professor J. E. Simmons, Associate Professor of the Department of Bacteriology, who suggested this problem and who directed the work. Thanks are also due Dr. Roger J. Williams, Professor of Chemistry, Oregon State College, for his helpful suggestions and for the use of his electrolysis apparatus. Also to Wilfrid Kennell for his aid in preparing the illustrations.

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INTRODUCTION

Historical.

The subject of "bacteriophage," or more simply "phage," the phenomenon of lysing bacteria, is of special interest to bacteriologists, and of general interest to all biologists. The study of bacteriophage promises to enlighten the philosophical consideration because it stands at the border line between catalytic reactions, on one hand, and living matter on the other. Twort in 1915 was the first to describe a curious degenerative change which he had observed in cultures of a micrococcus isolated from calf lymph. This work did not attract much attention until d'Herelle in 1917 recorded his first series of observations on the lytic properties of filtrates of broth cultures, obtained from the feces of dysentery convalescents. As a result of these observations, there have been many reports of investigators in the literature. Although it has been twenty years since the first observation of bacteriophage, its true nature, its mode of action, and its origin are still unsolved or satisfactorily explained.

Twort, d'Herelle, and other early investigators established the following observations with regard to the

behavior of bacteriophage. (1) It acts upon the homologous bacteria to bring about a partial or complete lysis of that organism. It is evidenced by the characteristic "moth-eaten"-like surfaces or edges of a colony growing upon solid media. A part or the whole colony may become clear and glassy in appearance. Microscopic examination of this film shows only broken debris. (2) By filtration through a Berkefeld, or Chamberlain candle, the lytic agent can be separated from the homologous bacteria. (3) When dilutions of more than one to a million are made, evidence of the lytic agent can be obtained. (4) It has been propagated only in the presence of young actively-growing bacterial cells. It appears that propagation of phage takes place only while the bacteria are in their logarithmic growth phase. (5) The bacteriophage is usually most active against one particular bacterial species, less active against closely related species and quite inactive against unrelated species. When lytic agents are first isolated, their range of activity varies greatly. Some appear to be very specific and some are found to be able to lyse many species of bacteria. Upon serial propagation with a single strain of bacteria, the lytic agent may become more specific toward that particular organism and may become less active against the others, while in some cases it retains power to lyse all of them. (6) As the bacteriophage seems to adapt itself to strains

of bacteria, so bacteria seem to adapt themselves to the lytic agent and resistant cultures develop, (Topley & Wilson)[62]. The resistant (secondary) colonies according to Hadley[38] undergo no further lytic changes. They are resistant to the lytic agent. d'Herelle believed these secondary colonies were the result of an adaptation undergone by the bacterium which acquired an immunity to its parasite.

Occurrence of Bacteriophage.

Hadley[39], in confirming the theory that the bacteriophage phenomenon is essentially an autolysis, found that by repeated growth and filtration through a series of 14-20 transfers in broth of an organism, a lytic filtrate could be obtained. He states, "The alleged 'sources' represent nothing more than 'liberating influences' which can force the culture into a new form of growth in which the lytic agent is generated de novo from the cells of that culture." Topley[62], in reviewing the work of Hadley, states "that this theory would clearly receive support if it could be shown that the phenomenon of transmissible autolysis occurred frequently and spontaneously in stock laboratory cultures of bacteria, which had for long been shielded from extraneous influences. Extended investigation along these lines suggest, however, that such positive results

are the exception rather than the rule, and that it is only rarely that the presence of a bacteriophage can be demonstrated in a culture which has not either been recently isolated from some natural source, or submitted to the action of some material derived from the human or animal body. Occasionally old cultures will contain the lytic principle because any strain which has come in contact with the bacteriophage will transmit the lysin through a certain number of generations without affording any obvious evidence of its activity." Topley[62] also states, "one of the readiest ways to obtain a lytic filtrate is to start from a culture derived directly from human or animal feces." Caldwell[17] has noted the ease with which lytic filtrates, active against a variety of bacteria may be obtained from sewage. Some observations of Lisbonne and Corvene (1922) recorded by Topley[62] suggest that when two organisms are grown together in the same liquid culture, one of them may produce a lytic principle active against the other. Krueger[52] in his exhaustive review of the subject of bacteriophage comments upon the theory of the generation of bacteriophage from normal bacterial cells by stating that this theory is open to criticism because lysogenic bacteria or phage-carrying strains are of much more common occurrence than previously supposed and also because phage is so widely spread in nature. He

favors the conception of the bacterial origin of bacteriophage.

Nature of Bacteriophage.

d'Herelle[41] from his earliest communications has contended that the lytic agent is a living ultramicroscopic organism, a parasite upon bacteria. He believes that the propagation of the lytic agent, through an apparently unlimited series of bacterial cultures, its increase in potency during this serial passage, and its variation in activity towards different species, suggests adaptation to varying environments. He believes that the lytic areas on solid cultural medium represent the site of development of independent lytic units which have multiplied at the expense of the surrounding culture. Many investigators are in accord with this idea.

Opposed to the ideas of d'Herelle are those who believe the lytic agent is enzymatic in nature. Bordet & Ciuca quoted by Hadley[39] in his review, were among the first to propose the chemical nature of the active substance. They state, "since the lytic agent is a chemical substance in solution, it cannot register as a single, physical unit, therefore the mechanism of phage formation must lie in the behavior of the bacterial cells." Kendall[44] believes that it is of bacterial origin because

he has been able to incite the lytic substance by the use of an autoclaved sewage. He states, "therefore it is not essentially an independent, autonomous ultra-microbe. Its resistance to $HgCl_2$ is essentially the same as certain enzymes, also produced only in the presence of specific, living cells."

In reference to the adaptation of bacteriophage to its environment, Beard[4] states, "much of the discussion in the literature concerning adaptation is based on errors in technic and interpretation." Krueger[52] writes, "Everything considered, it is the writer's opinion that clear-cut evidence of phage adaptation remains to be presented."

The size of the lytic particles themselves forces one to consider their animate or inanimate nature. Although different investigators do not agree exactly upon the particle size, there is a general agreement that the difference might be explained by the accuracy of the different methods employed. Hadley[39] records the results of (1) Prousnitz, who estimated the size to be 20 $\mu\mu$. by gelatin filtration tests, (2) Van Auger, who used refraction methods, 30 $\mu\mu$., (3) Bechold, who used filtration, 4-10 $\mu\mu$. and (4) Zinsser & Tang, who used collodion membranes, 20 -100 $\mu\mu$. Krueger[52] mentions the work of Bronfenbrenner who used the diffusion coefficient method of Northrop and Anson (1929) and found the particle size

to be much smaller than had been previously thought. They found them to be only 0.4μμ. They believe the phage is carried by certain protein molecules and that this may be the difference of opinion of investigators on this subject. Elford and Andrews (Krueger)[52] have carefully studied the problem of a group of phages and a carefully controlled ultra-filtration technique. Among their phages the particle size ranged from 10μμ for the dysentery phage to a maximum size of 75μμ for several. The particle size according to data they present is uniform regardless of the bacterial substratum employed in their propagation. Nor could they detect any difference in size of purified preparations and ordinary lysates. This work has been supported by Burnet[16] using Barnard's method of micro-photography employing monochromatic ultra-violet light. Krueger[52] in summing up the investigation upon the particle size states, "It may be concluded that phage in ordinary preparations is in colloidal suspension. Various phages differ in particle size, the range in general being from 10μμ to 75μμ. It is probable that the extent of variation in particle size in any given preparation is less than had been previously assumed. There is experimental evidence both for and against the view that phage consists of an active substance carried on some inert colloidal particle; this question is still unsettled."

The conception that phage may be a rider upon some inert substance is given as an explanation of the increased susceptibility of phage in purified preparations to chemicals, heat and simple changes in pH, Krueger[52] believes they may act as protective colloids, which may well be bacterial lysis. Many references in the literature list the inactivation by chemicals such as phenol (1%), oxalic acid (2%), lactic acid (2%), formaldehyde (0.6%), glycerol (undiluted), alcohol (75%), $HgCl_2$ (0.5%), KCN (2.5%), Sodium flouride (2.5%), and chloroform. Ether however does not seem to inactivate phage. Certain dyes such as methylene blue, crystal violet and safranine have a selective action upon phage. Crystal violet according to Wells and Sherwood[63] has a selective inactivation effect on phages for the gram-positive bacteria while it has apparently no effect upon the phages for the gram-negative organisms. They state also that much greater concentrations of phenol and NaOH were required to bring about inhibition of the phages than was necessary with the dyes. The phages, according to their results, which lyse gram-negative bacteria are more resistant to deleterious substances than are those which lyse gram-positive bacteria. Inactivated phages can in some cases be re-activated. Krueger[50] has shown that phages completely inactivated by $HgCl_2$ can be reversed

by precipitation of the mercuric ions with H_2S. He believes this behavior is more compatible with the known properties of certain enzymes than with those of living protoplasm. Krueger and Baldwin[51] found that phage that had been inactivated by safranine at a pH 7.4 produces a heavy precipitate in infusion broth. The precipitate is dissolved if the pH is adjusted to 6.5 and 25% of the total phage can be reactivated.

Heat is another inactivating agent. The attenuation begins about 10 degrees below the point at which the activity is definitely lost, which is about 70° C., Hadley[39]. Burnet[15] reports that staphylococcus phages are much more readily inactivated by heat than the great majority of coli-dysentery phages. Temperatures as low as 37° C. will inactivate some staphylococcus phages. Krueger[52] reports the work of Kligler & Olitzki in 1931 where they have found that purified protein-free phage is more susceptible to heat than are ordinary impure preparations.

Experimental evidence seems to indicate that other inhibitory substances of phage are ultra-violet light, body fluids such as blood serum, bile, and bacterial extracts of the homologous organism. Enzymes such as trypsin have slight effect on the vitality of phage, (Hadley)[39]. "In fact," he states, "the principle could

be freed from the antigenic bacterial proteins by digesting the filtrates with prepared trypsin solutions." Hadley further states, "It is a known fact that proteolytic enzymes do not attack living cells."

The question of the nature of bacteriophage is therefore by no means solved.

OBJECT OF THIS INVESTIGATION

The object of this investigation was to find a bacteriophage specific for hemolytic streptococci, with special emphasis upon Streptococcus mastitis, to study the characteristics of the isolated phage and to observe its relationship to some hemolytic streptococci isolated from market milk.

It has been frequently observed that milk from cows having an active case of mastitis show few, if any bacteria present when a microscopical examination is made. The opinion was advanced that the small number of bacteria might be due to the presence of bacteriophage in the udder.

In reviewing the literature, no reference could be found dealing with this problem, and only a few could be obtained concerning streptococcus bacteriophage. Evans[27] writes, "Although it is known that bacteriophage specific for intestional bacteria may be readily isolated from sewage, the belief is common that streptococcus bacteriophage is rare and difficult to obtain." Burnet[11] concludes that it is extremely rare to obtain a phage active against streptococcus. He mentions the race isolated by Clark & Clark[18] as the only indubitable streptococcus phage so far reported. Evans[27] was able to isolate with relative ease two streptococcus bacteriophages from

sewage, and therefore came to the conclusion that streptococcus bacteriophage is widely distributed, at least during the season when streptococcus infections are prevalent.

Samples of milk were obtained from several cows suffering from mastitis, and attempts were made to obtain a lytic filtrate from them. These samples were taken from widely separated parts of the state. Attempts were made to isolate the lytic agent from fresh milk from cows that were apparently not infected. Other attempts were made to isolate the lytic agent from the feces of cows having the disease or having a history of having had the disease. All these sources failed to produce a lytic filtrate for the organisms we were using, so an attempt was made to isolate the lytic agent from city sewage.

THE ISOLATION AND PURIFICATION OF BACTERIOPHAGE

Methods of demonstrating the presence of bacteriophage.

There are two methods of demonstrating the presence of bacteriophage, (1) the plaque method, first demonstrated by Twort in 1914 and (2) the test tube method employed by d'Herelle.

The plaque method is demonstrated in a petri dish containing a suitable cultural medium. The organism to be used is taken from a young active broth culture. Three drops of this culture are transferred from a test tube by means of a sterile pipette and dispensed on to the medium in the petri dish. The medium is then evenly seeded by spreading the organism over the surface with a sterile bent glass rod. One loopful of the lytic filtrate is then streaked across the surface of the medium. The plate is incubated at 37° C. for 24 hours. After the incubation period, if phage is present there will appear, along the line of streak, clear and "moth-eaten" areas.

In the second method (test tube method) a tube of broth is inoculated with 3 drops of a young active broth culture of the desired organism. After thorough mixing, it is incubated at 37° C. until a definite turbidity appears. Usually this requires about two hours. One loopful of the lytic filtrate is then added to the culture and is further incubated until clearing of the broth takes

PLATE I

The plaque method. Cleared areas indicate lysis.

place. The lysed cultures will remain clear only for a short time, as the resistant bacteria soon make the broth turbid again. These "secondary" growths prove to be a very serious disadvantage to this method because if observations are not made while the broth is clear, no evidence of the presence of the lytic agent would be manifest. Therefore, frequent observations at regular intervals have to be made. To speed up the action of the lytic agent and therefore reduce the necessary time of observations, a slight modification of the method was employed. Instead of the one loopful of the lytic filtrate, 1 c.c. of the lytic agent was used, and observations made in 8 to 12 hourly intervals.

The medium employed in this investigation was composed of the following ingredients:

Bacto-veal infusion	1 lb.
Peptone	100 grams
Neo or Proteose peptone	50 grams
NaCl C.P.	50 grams
Yeast extract	10 grams

The volume made up to 10 liters; the pH adjusted to 7.4. If a solid media was desired, 1.2% agar was added.

The Berkefeld filters used in this investigation were grade N (medium porosity). The filters immediately after being used were steamed in flowing steam for 15 minutes, then thoroughly washed in hot tri-sodium phosphate solution. They were then assembled again and a 1%

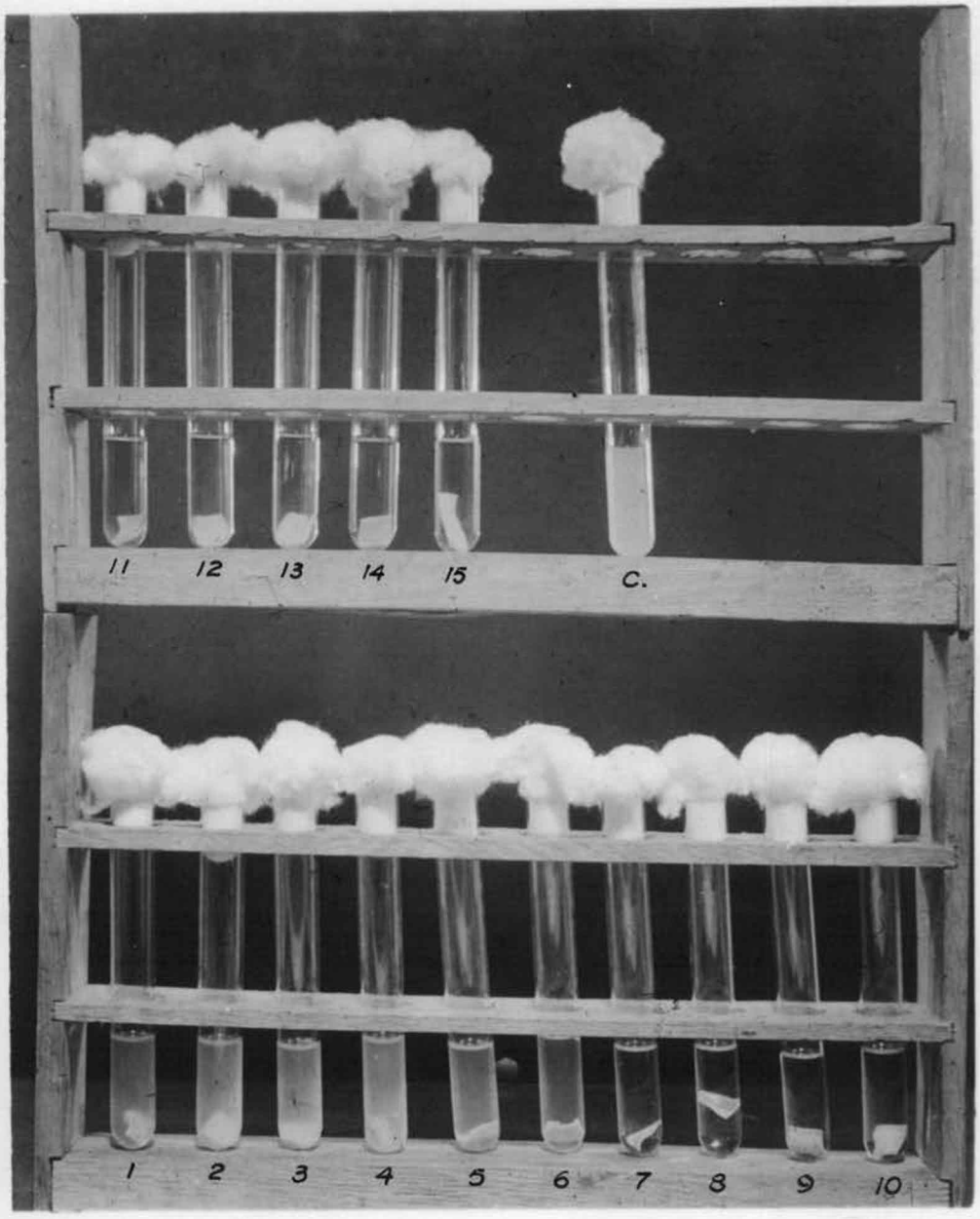

PLATE II

Bedson method of determining the electrical charge. The single tube at the top is the control. Complete clearing is observed in tubes #7-15 inclusive.

solution of $KMnO_4$ was passed through the candles, to oxidize what organic material that should have lodged there. The excess $KMnO_4$ was removed with a solution of oxalic acid (5%). The excess oxalic acid was removed by passing distilled water through the candle until the filtrate was free from acid. The filters were then dried, assembled, and sterilized in the autoclave at 15 lbs. pressure for 20 minutes.

Source of Cultures.

The cultures of streptococci originally selected for use in these studies consisted of two stock cultures of Streptococcus mastitidis obtained from the Department of Bacteriology and two streptococcus cultures isolated from milk of cows having mastitis. The two recently isolated cultures were not identified but they were selected because they appeared to have hemolytic characteristics different from the stock strains.

Later, several additional organisms were used in building the streptococcus bacteriophage. These included six gram-negative short oval rods, six gram-positive staphylococci and twenty hemolytic streptococci isolated from market milk.

All of the streptococcus cultures were carried in infusion agar that contained 0.7% agar. This medium is

termed "slush agar" and it allows for the diffusion of waste materials from the cells, thus insuring a viable culture for a much longer period of time. These cultures were kept for some time at room temperature and were transferred every two weeks, but later observations showed that by placing them in the ice box at about 4°C the cultures would remain viable for a month or more.

Table I shows the source of all the organisms used in this investigation to build up a streptococcus bacteriophage.

The isolation of phage.

Bacteriophage is reported to have been found in old broth cultures, Hadley [39]. A number of old stock cultures of Streptococcus mastitidis were filtered and the filtrates tested for the presence of a lytic agent. Many serial transfers were conducted with the hope of building up a phage that might be present in such a weakened condition that visible lysis was not produced. No active filtrates were found.

The above experiment was repeated using "fresh" cultures of the above described organisms. No active filtrates capable of lysing bacteria were found.

These results confirm the results of Topley and Wilson[62], "...it is only rarely that the presence of a

TABLE I

Source of the organisms

Culture No.	Morphology	Source
I	Streptococcus	Stock culture
II	"	Milk (garget)
III	"	" "
IV	"	Stock culture
V	Short oval rod	City sewage
VI	" " "	" "
VII	" " "	Ames: stock culture 137
VIII	" " "	" " " 244 ab
IX	" " "	Gargety milk
X	" " "	" "
XI	Staphylococcus	" "
XII	"	" "
XIII	"	" "
$XIII_1$	"	Aftergrowth of XIII
XIV	"	Class isolate
XV	"	" "
XVI	"	" "
1	Streptococcus	Market milk
2	"	" "
3	"	" "
4	"	" "
5	"	" "
6	"	" "
7	"	" "
8	"	" "
9	"	" "
10	"	" "
11	"	" "
12	"	" "
13	"	" "
14	"	" "
15	"	" "
16	"	" "
17	"	" "
18	"	" "
19	"	" "
20	"	" "

bacteriophage can be demonstrated in a culture which has neither been recently isolated from some natural source, nor submitted to the action of some material derived from the human or animal body."

A possible source of the lytic agent might be found in the gargety milk itself. Samples of gargety milk were obtained from several different dairies and attempts were made to filter these samples through Berkefeld filters. This was very unsatisfactory, as the filters would become clogged very quickly. The milk, coagulated with acid or rennet, was filtered through filter paper and later passed through the Berkefeld candle. This process however never yielded a lytic filtrate.

The above experiment was repeated using fresh milk instead of the gargety samples. Samples were taken from market milk and milk drawn directly from the teat of the cow into a sterile bottle. No bacteriophage could be demonstrated from any of the milk samples.

Bacteriophage is frequently isolated from the feces of the infected man or animal. Hadley[38] states "... mixed city sewage is the most convenient source from which to develop a wide variety of phages." Caldwell[17] was able also to obtain lytic filtrates from sewage which would lyse many species of organisms.

Samples of feces were obtained from cows having

active infectious mastitis or having a history of the infection in the past. Dilutions were made with water, and the coarse materials were removed by filtration through coarse filter paper. This filtrate was then filtered through the Berkefeld candles. In no case was there any evidence of the presence of a lytic agent for any of the four original streptococci.

A sample of the Corvallis city sewage was obtained. A small amount was filtered and 1 c.c. added to young broth cultures of the four streptococci. These were incubated at 37°C for 24 hours and then filtered. Serial transfers were made but there appeared to be no lytic agent present. However several lytic filtrates were obtained for colon and staphylococcus organisms.

Evans [27] referred to the work of Clark and Clark[18] in which they gave the technique they used in isolating their streptococcus bacteriophage.

The Technique of Clark and Clark[18].

Infusion broth (300 c.c.) was inoculated with one drop of a 12-hour old culture of each of the 37 cultures listed in Table I. After this mixed culture had incubated 2 hours, 5 c.c. of unfiltered sewage and 5 c.c. of a mixture of all the filtrates so far obtained were added. After further incubation at 37°C. for 24 hours, this

mixture was filtered through a Berkefeld N filter with a partial vacuum of 10 inches. The time of filtration was limited to 20 minutes as it was feared the "charge" of the filter might become neutralized and would let the bacteria pass through.

The theory of filtration according to Mudd[58] is, "The contact surfaces of the pores of a Berkefeld filter and the fluids bathing them is the site of an electrical potential difference, an ordinary Helmhaltz double layer, the solid walls carrying a negative charge and the fluid a positive charge. ... When solutions or suspensions are filtered, positively charged particles, ... would be absorbed on the filter wall, while negatively charged particles would be borne through by the filtration stream."

The filtrate obtained was placed in 8 oz. screw capped bottles and placed in the ice box at 4°C. ± 2°C. and labeled (B). A sample of the filtrate was incubated for 48 hours to determine the sterility.

This storage temperature was selected because Caldwell[17] reports that filtrates stored at ice box temperatures for two months were still as active as when fresh. Marshall and Paine[57] report that phages kept in sealed tubes were only slightly diminished in potency after 8 years.

The procedure was repeated using 5 c.c. of filtrate

PLATE III

Filtration apparatus

(B) instead of the sewage and mixed filtrates. After incubation of 24 hours, the mixture was filtered as before. The filtrate was lettered $(B)_1$. This procedure was repeated until $(B)_5$ was obtained. The filtrates were then tested to find the number of organisms for which a lytic agent had been produced. Tests were made by both methods, the plaque and the test tube method. The results showed a lytic agent for all the gram-negative oval rods for four of the staphylococci and six of the streptococci. Filtrate $(B)_6$ was obtained in the usual manner but at this point only the streptococci were used. This serial procedure was continued until the filtrate $(B)_{10}$ was obtained. This filtrate was then tested to find the number of streptococci which it would lyse. Both methods of testing were employed. Table II records the results of filtrate $(B)_{10}$ upon the organism used. The results show that there were no more lytic agents developed after the tests made with $(B)_5$. Therefore the procedure was discontinued.

Purification of Bacteriophage.

It is desirable when studying the characteristics of bacteriophage to have a "pure line" strain of that phage which has come from a single individual, if it is a living entity, or purified if it is inanimate. Burnet[11] writes, "The methods of obtaining 'pure' phages are analogous to

TABLE II

The lytic effect of filtrate $(B)_{10}$ and purified phage (17) upon the standard cultures

Culture	Test tube method		Plaque method	
	Filtrates		Filtrates	
	$(B)_{10}$	(17)	$(B)_{10}$	(17)
I	-	-	-	-
II	-	-	-	-
III	-	-	-	-
IV	-	-	-	-
V	+	-	+	-
VI	+	-	+	-
VII	+	-	+	-
VIII	+	-	+	-
IX	-	-	-	-
X	+	-	+	-
XI	-	-	-	-
XII	+	-	+	-
XIII	-	-	+	-
$XIII_1$	-	-	+	-
XIV	-	-	-	-
XV	-	-	+	-
XVI	-	-	+	-
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	+ ?	-	-	-
9	-	-	-	-
10	-	-	-	-
11	+	+	+	+
12	+	+	+	+
13	+	+	+	+
14	-	-	-	-
15	-	-	-	-
16	+	-	+	-
17	+	+	+	+
18	+	+	+	+
19	-	-	-	-
20	-	-	-	-

those for isolating bacteria in pure culture. The mixed phage may be spread, suitably diluted, over an agar surface inoculated with the sensitive organism. The center of an isolated plaque is touched with a sterile needle, and the trace of material so obtained is used to induce lysis in a young broth culture of the substrate bacterium. The procedure must be repeated once or twice to insure that the phage is pure."

This procedure was carried out with a slight modification. The six streptococci that had been lysed by the Filtrate $(B)_{10}$ were inoculated upon separate plates and one loopful of the filtrate $(B)_{10}$ was streaked over the plate. The plates were then incubated for 24 hours. After incubation, an individual isolated plaque was touched with a sterile needle and inoculated into a 2-hour old broth culture of the homologous organism. This mixture was incubated for 24 hours and filtered. A loopful of this filtrate was then streaked upon a seeded plate of the same homologous organism and the process repeated 6 times until filtrates were obtained for each organism. The filtrate for each organism then was numbered the same as its homologous organism with the addition of the parenthesis marks. For example, the purified filtrate active against organism No. 17 was numbered (17). The purified phages were then checked for their specificity toward that specific or-

ganism. For example, all the streptococcus organisms that had been lysed were checked against filtrate (17) to see if only organism No. 17 would be lysed. Table II shows the results. All of the streptococci were lysed in broth cultures, except No. 16, which did not seem to be affected. When the experiment was repeated by using the same organisms but using filtrate (18), the results were the same. Filtrate (16) would not lyse any of the organisms except No. 16 and at no time could the strength of this lytic agent be built up so that it would lyse the organism in dilutions higher than 1-100. From Table III, it can be observed that complete lysis occurred in dilutions of the lytic agent 10^{-8} with some organisms and partial lysis in dilutions 10^{-10}.

From these observations, it appears that organisms No. 11, 12, 13, 17, and 18 are the same or very similar or, that the lytic filtrate is polyvalent in nature. To make sure that the phages were "pure," filtrate (18) was checked with all the organisms used in developing the streptococcus phage. The test tube method was used for this experiment. It was found that only the streptococci No.'s 11, 12, 13, 17, and 18 were lysed. This method seems to be satisfactory in purifying the mixed phages.

Table III

Effect of dilution of filtrate (17) upon the various streptococci used in this investigation

Organism	Dilution of Filtrate (17)											Controls		
	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Broth	Filtrate	Organism
11	+	+	+	+	+	+	+	+	-	-	-	+	+	-
12	+	+	+	+	+	+	+	+	+	±+	-	+	+	-
13	+	+	+	+	+	+	+	+	±+	-	-	+	+	-
16	-	-	-	-	-	-	-	-	-	-	-	+	+	-
17	+	+	+	+	+	+	+	+	±+	-	-	+	+	-
18	+	+	+	+	+	+	+	+	+	±+	±+	+	+	-

Legend:

\+ Complete clearing of the tubes

±+ Partial " " " "

\- Turbidity

STUDIES UPON THE CHARACTERISTICS OF HEMOLYTIC STREPTOCOCCUS BACTERIOPHAGE

Tests of the potency of the filtrates.

The tests for potency, or the highest dilution at which lysis of the homologous culture can be observed, were made to determine the relative concentration of phage particles in the filtrates. It is assumed that at least one phage particle in the highest dilution will produce visible lysis. The test consists in making serial dilutions of the filtrate by taking 10 sterile test tubes and adding 9 c.c. of sterile water to each tube. Then take 1 c.c. of the filtrate to be tested and add that to the first tube. This will make a dilution of 1-10. Shake well, remove 1 c.c. and put in the second tube. This will make a dilution of 1-100. This procedure is continued until 10 dilutions are made. When the dilutions are made, 1 c.c. of each dilution is added to a separate 2-hour old broth culture of the homologous organism. During incubation, hourly observations are made to determine the highest dilution that produces visible lysis. From Table III it will be observed that a potency was obtained of 10^{-10} in respect to the lysis of organism #18. Krueger[48] criticizes this method because he advances the theory that a definite "lytic threshold" has to be reached before lysis takes

place. That is; there has to be a certain ratio of phage particles and bacteria before the cell disintegrates. He believes that in the case of some of the high dilutions the "lytic threshold" is not reached before the organisms enter the maximal growth stationary phase where lysis cannot be observed.

Secondary cultures.

Secondary cultures are those growths of organisms which appear after the culture has been lysed. d'Herelle[41] writes, "They are the result of an adaptation undergone by the bacterium which acquires an immunity to its parasite." Gratia (1921) reported by Hadley[39], believed the resistance is already present in certain cells of the culture, prior to the action of phage. Burnet (1925), also quoted by Hadley[39], concluded, "resistance depends on some variable factor in the bacteria rather than on variations in the lytic principle. A bacterium resistant to one phage might be lysed by another." Krueger[52] reports the work of Flu (1923) in which he reports the destruction of phage by resistant bacteria which have developed in the filtrate. Krueger[52] also states, "Resistance can be introduced into a normal culture by inducing dissociation with heat or chemical treatment."

The development of phage for some of the secondary

growths was not successful, although the morphological, cultural, and physiological characteristics of the organisms appeared to be the same as the original culture.

Certain observations were made during this investigation that seemed to have a bearing upon some physical relationship to the lytic phenomenon. Lysis of the broth cultures appeared always to take place at the top of the tube first and proceeded toward the bottom. The secondary growths seemed to appear first at the top and gradually the whole tube would become turbid. Schwartzmann[60] writes "If the ratio of the volume of the medium to the surface area of the culture, $\frac{\text{surface area}}{\text{volume of media}}$, was greater than 0.5, lysis and regeneration did not occur. O_2 did not effect the lytic principle." However, Larkum, whose work is quoted by Hadley[39] states, "Bubbling of O_2 through the culture increased the strength of the lytic agent. CO_2 had the reverse effect."

As there was a definite disagreement in the literature about the effect of gases upon the lytic phenomenon, a few experiments were conducted to see how filtrate (18) would react.

The first experiment consisted in inoculating several tubes of broth, that had been recently boiled and rapidly cooled, with 3 drops of culture No. 18, placed in a modified phosphorus jar, and a vacuum of 29 inches of

mercury applied. The tubes were incubated at 37°C. for 2 hours, when the negative pressure was released. 1 c.c. of filtrate (18) was added to all tubes but one, which served as a control. Two plates prepared by the plaque method were also added to the jar and a vacuum applied as before. Observations were made at short intervals to determine the effect of the reduced air pressure. From the results obtained this partial vacuum apparently had no effect upon either the rate of lysis or the rate of the appearance of the secondary growth.

In the second experiment an arrangement of a novy jar was made so that the air inside could be continuously displaced by "commercial" gas. After the gas had passed through the jar, it was burned in a Bunsen burner. The cultures were prepared as in the previous experiment. The composition of the commercial gas, as analyzed by Ervin[25] is

Carbon dioxide	1.5%
Benzene	trace to 0.1%
Ethylene	3.5%
Oxygen	0.5%
Carbon monoxide	6.5%
Hydrogen	52.0%
Methane	32.0%
Nitrogen	4.0%
	100.0%

Although there was no change in the lytic phenomenon as a result of this treatment, secondary growths did not appear even after incubation of 48 hours. After the

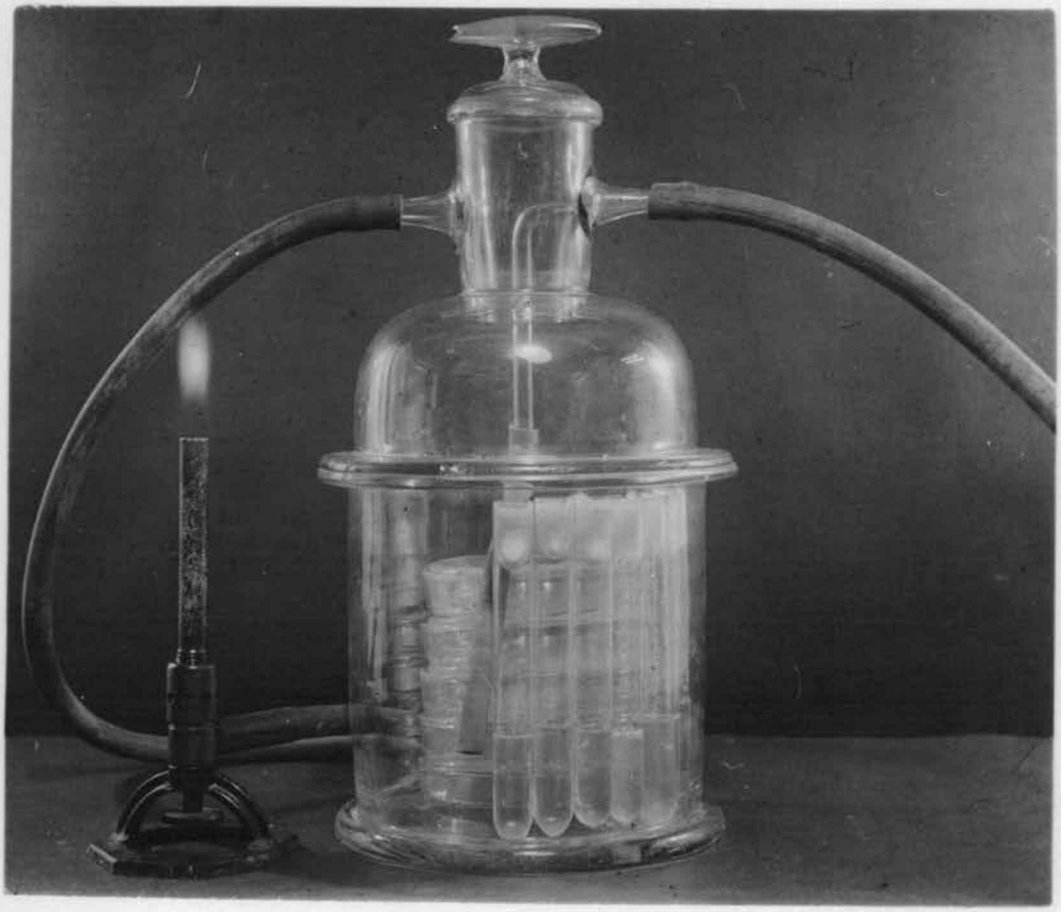

PLATE IV

Novy jar showing the arrangement of tubes and plates in the "commercial" gas atmosphere.

tubes and plates had been removed from the jar, they were placed in a locker at room temperature. The secondary growths appeared after 48 hours.

In order to eliminate any possibility of the gas acting as an inhibiting agent, a culture of this secondary growth as well as a culture of the original unlysed organism were exposed to the gas for a corresponding period. The original culture grew but the secondary culture was apparently inhibited in the presence of this gaseous mixture. This point needs further investigation.

Similar results were obtained when CO_2 was used instead of the "commercial" gas.

The effect of anaerobic condition was studied. A "phosphorus jar", shown in the illustration, was prepared. The principle of this jar is that all of the O_2 is combined with phosphorus forming P_2O_5. In preparing the jar, a small amount of NaCl solution is put in the bottom. The materials to be tested are then placed in a metal rack and lowered into the jar. A small porcelain cup, in which $CaCO_3$ is placed in the bottom, is placed in a rack at the top. A small piece of phosphorus is prepared by drying slightly and placed upon the $CaCO_3$. A porcelain lid is placed over the cup and then a heavy metal covering over that to prevent cracking of the jar by the heat generated. The cover of the jar is then clamped down. Excess phosphorus is used to maintain anaerobic conditions so that when

the jar is opened, there will be some phosphorus that has not been oxidized.

Broth cultures as well as plates were made in the usual manner and placed along the sides of the jar so that observations could be made at regular intervals without having to open the jar. Lysis of the organism appeared in the usual manner. The secondary growths apparently were not inhibited. The results of the above experiments can be observed from the following Table.

TABLE IV

The effect of partial vacuum, "commercial" gas, CO_2 and anaerobic conditions upon the phage phenomenon.

Test Agent	Organism	Filtrate	Lysis	Secondary Growth	Control
Partial Vacuum	18	(18)	-	+	turbidity
Commercial Gas	18	(18)	-	-	turbidity
CO_2	18	(18)	-	-	turbidity
Anaerobic	18	(18)	-	+	turbidity

In another experiment, the phage was adsorbed on blotting paper. Some of the pieces of paper floated upon the surface of the liquid. Complete lysis in these tubes appeared earlier than in the tubes in which the paper sank to the bottom. In some cases lysis occurred many hours

PLATE V

Phosphorus jar showing arrangement of test tubes.

before other cultures in the series which contained a greater concentration of phage particles. From these observations, it appears that there is some physical relationship of surface tension to the rate of lysis.

The influence of pH upon the activity of bacteriophage.

Krueger[52] cites the work of Bronfenbrenner and Korb (1925) in which they found the effect of pH varied with different strains of bacteriophage. They found that staphylococcus phage was completely inactivated at pH below 4.3 and above 8.8 in 3 hours at 70° C. The coli phages were inactivated at pH below 1.55 and above pH 10.6 under the same environment. The question might be raised as to whether the inactivation was due to the pH or to the temperature employed. Krueger[52] also cites the work of Muramatsu (1931) who records observations in which phage resisted exposure to H^+ ion concentrations between pH 4.0 to pH 11.0 with little loss in activity.

Buffered solutions were prepared and checked by the quinhydrone electrode. The pH of the buffered solutions were 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. To a 50 c.c. portion of each buffered solution, 1 c.c. of the lytic filtrate was added. The mixture was shaken thoroughly and 1 c.c. removed and added to active broth cultures of No. 18, as controls. At hourly intervals, 1 c.c.

was removed and a check made to see if the phage had been inactivated. The temperature used for the buffered solutions was 20° C.

Table V records the results of this experiment. It appears from these results that a pH of 5 - 9 has very little effect upon the activity of the phage exposed for 4 hours but a marked inactivating effect at pH 4 and 10.

Apparently there were other deleterious effects upon the phage than that of the pH. From the 26-hour exposure it was observed that the time for complete lysis was greatly increased even in the pH range where the phage was not apparently affected.

Inactivation of streptococcus bacteriophage by the use of methylene blue.

The observation was made that when a 1% solution of methylene blue was passed through the Berkefeld filter that the dye was adsorbed upon the filter and the filtrate was crystal clear. If the methylene blue was of the opposite charge to that of the filter candle, which let the phage pass, that possibly the methylene blue would inactivate the phage.

Serial dilutions of 1% solution of methylene blue to a dilution 10^{-9} were made. One c.c. of the lytic filtrate (18) was added to each dilution. At 1, 4, 8,

TABLE V

The effect of pH upon the inactivation of hemolytic streptococcus bacteriophage (18)

Exposure	pH	Lysis	Hours Partial Lysis	Hours Complete Lysis	Control
Control	4	+		Complete lysis in less than 1 hour	Clear
	5	+			"
	6	+			"
	7	+			"
	8	+			"
	9	+			"
	10	+			"
1 hr.	4	+	2	3	"
	5	+	Less than 2 hours	2	"
	6	+		2	"
	7	+		2	"
	8	+		2	"
	9	+		2	"
	10	+	2	3	"
2 hrs.	4	+	4	24	"
	5	+	1	< 7	"
	6	+	1	< 7	"
	7	+	1	2	"
	8	+	1	< 7	"
	9	+	-	2	"
	10	+	3	< 7	"
3 hrs.	4	+	8	> 24	"
	5	+	1	6	"
	6	+	1	6	"
	7	+	-	1	"
	8	+	1	6	"
	9	+	1	6	"
	10	+	3	8	"
4 hrs.	4	+	5	24	"
	5	+	1	2	"
	6	+	1	5	"
	7	+	1	5	"
	8	+	1	5	"
	9	+	1	5	"
	10	+	5	7	"
26 hrs.	4	-	-	-	"
	5	+	4	< 24	"
	6	+	5	< 24	"
	7	+	2	< 24	"
	8	+	2	24	"
	9	+	2	24	"
	10	+	2	< 24	"

and 24 hour intervals, 1 c.c. of the mixture was added to an active broth culture of the homologous organisms.

No growth of the streptococcus occurred in the first two dilutions. There was apparently enough of the dye carried over in the 1 c.c. amount transferred to inhibit the growth of the organism. The results showed complete lysis in tube 3 which was a dilution of 10^{-2} with an exposure of 1 hour, but only a partial lysis with an exposure of 4 hours. In the 8-hour exposure apparently complete inactivation of the phage occurred. A check upon the effect of the concentration of methylene blue upon the bacteria revealed that a 1% solution would completely inhibit the growth of the organism.

The reactions of ether extracts of bacteriophage.

Hadley[39] reports that Feggin, Stassano, Beufort and Nakashima found that bacteriophage extracted with ether showed no loss of activity. LeMar and Myers[54] state, "Bacteriophage can, apparently, be extracted wholly or in part unharmed, from aqueous solutions by ether." They state however, "Unless there is a prolonged period of contact, some bacteriophage remains in the water phase."

Fifty c.c. of filtrate (18) were mixed with an equal amount of ether in a separatory funnel. The mixture

was shaken well and placed in the ice box at 4° C. The mixture was shaken several times during the 21 days that the ether was in contact with the filtrate. This time was selected because of the reported slow extraction of the phage. The separation was made by drawing off the non-ether soluble fraction through the funnel. The ether extract was evaporated at 20° C. When the residue became an oily viscous mass, it was taken up with 75 c.c. of sterilized distilled water. The mixture was then filtered through a Berkefeld filter. No apparent evidence of the presence of the lytic filtrate could be obtained in the ether fraction. In the non-ether soluble fraction, there was a very small amount of active agent present, but it had apparently been greatly inactivated. This needs further investigation.

Determination of electrical charge.

In the literature, opinion is divided upon the matter of the electrical charge carried by phage particles. Todd[61] reports, "Migration of a bacteriophage for B. shiga at a pH 3.6 to 7.6 the migration was toward the anode therefore having a negative charge." Hadley[39] quotes Eliava and Suarez as writing, ". . . using Bechold-Konig assembly, we found that phage filtered readily unless the membranes were made positive, then

adsorption was great and filtration rendered difficult. This suggests a negative charge carried by the corpuscles." Frankl and Schultz noted that the bacteriophage was never adsorbed by substances possessing a positive charge. Koch, recorded by Hadley[39], confirms the work of Frankl and Schultz by cataphoresis experiments. Krueger[52] found the phage to carry a negative charge between pH 3.4 to 9.0 but below 3.4 they assumed a positive charge. Krueger[52] mentions also the work of Kligler, Olitzki and Aschner (1931) in which they observed that between pH 4.0-12.0 the phage carried a negative charge but as the increase of H^+ ions the charge was changed. They also believe that proteins alter the charge. Natorajau and Hyde (1930) mentioned by Krueger[52] state that the migration is toward the anode but two phages were amphoteric in slightly alkaline solutions. They found between pH 3.4 to pH 12.0 the charge is negative, but below pH 3.4 the phage tends to become positive. The H^+ ions tend to inactivate the phage.

A method suggested by Bedson[6] for the determination of the electrical charge upon virus particles was modified and used in this experiment. Blotter paper was cut into strips 1 cm. wide and 16 cm. in length. Each strip was marked at 1 cm. intervals with a pencil. The strips were then sterilized in the autoclave for 20 minutes at

15 lbs. pressure. The electrical charge of the paper was checked with a 1% solution of methylene blue. Methylene blue according to Bedson[6] is positively charged. Therefore if the paper has the opposite charge, the dye will be adsorbed in the first few centimeters above the line of the liquid when it is immersed 1 cm. in the solution of methylene blue. If the charge is the same, the color will follow the moisture line up the paper. The paper chosen for this experiment was negatively charged because the dye was visible only 1 cm. above the liquid. A strip was immersed 2 cm. into filtrate (18). It required 1.5 hours for the liquid to reach the top of the strip. The strip was removed from the filtrate and cut into centimeter strips with a pair of sterilized scissors starting at the top. The top strip was cut off and placed in a standard broth culture of the homologous organism. This tube was numbered 1. The second centimeter was added to tube 2, etc. The last 2 centimeters that had been immersed in the filtrate were added to tube 15. They were incubated in the usual manner. Lysis appeared in all tubes at the end of 3 hours.

The concentration of the lytic filtrate might have been so great that the charge on the filter paper could not stop them. Serial dilutions of the lytic filtrate were made both in water and in broth up to 10^{-6}. A test

was made from each dilution. The results are recorded in Table VI. Apparently from these results, in dilutions of 10^{-5}, the lytic agent was in such concentration as to cause complete lysis 12 cm. above the surface of the liquid. The electrical charge according to this method is negative. Bedson[6] however, does not consider in his publication the question of the particle that does not carry an electrical charge.

If the lytic agent was neutral then when methylene blue was added to the solution and filtered, the phage would not be retained on the filters, but if it was negatively charged, it might be removed from the filtrate. One c.c. of filtrate (18) was mixed with 9 c.c. of a 0.1% broth solution of methylene blue. The mixture was shaken and then filtered. The filtrate did not show any evidence of the presence of phage.

Williams[65] suggests fractional electrical transport as a tool in biochemical research. It appeared that this method might indicate the electrical charge carried by phage particles. As described by Williams and Truesdail[64] it consists essentially of four or six glass cells connected by large glass siphons. Distilled water is placed in each cell and the siphons are filled by vacuum. The platinum electrodes are placed in the end cells and a direct electric current is applied. The current used in

TABLE VI

A Comparison of Dilution of Filtrate (18) in Broth and in Water upon its Ability to Carry Phage up a Strip of Filter Paper

medium	dilution	Strip Number															Control
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Broth	10^{-0}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	10^{-1}	+	⊕	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	10^{-2}	+⁻	+	+	+	⊕	+	⊕	+	⊕	+	+	+	+	+	+	-
	10^{-3}	-	+	+	+	+	+	⊕	+	+	+	+	+	+	+	+	-
	10^{-4}	-	-	-	+⁻	+	+	+	⊕	+	+	+	+	+	+	+	-
	10^{-5}	-	-	+	+	+⁻	+	+	+	+	+	+	+	+	+	+	-
	10^{-6}	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-
Water	10^{-0}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	10^{-1}	+	+	+	+	+	⊕	+	+	+	+	+	+	+	+	+	-
	10^{-2}	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
	10^{-3}	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	10^{-4}	-	-	-	+	+	+⁻	⊕	+	+	+	+	⊕	+	+	+	-
	10^{-5}	-	-	-	-	-	-	-	-	-	+⁻	+⁻	+⁻	+⁻	+	+	-
	10^{-6}	-	-	-	-	-	-	-	-	-	-	-	-	+⁻	+	+	-

Legend:

\+ Complete lysis

+⁻ Partial lysis

\- No lysis

⊕ Tube where paper floated on surface of the liquid and lysis appeared first in the series.

this experiment was 9.75 volts and 5 milliamperes. The usual practice is to continue electrolyzing until the ammeter registers zero. Preliminary tests, however, indicated that the phage was inactivated in two to three hours, although at the end of this time there was no diminution of the current. Electrolysis was therefore discontinued after four hours.

During the electrolysis, there is a separation of the acidic and the basic substances. The acidic substances migrate to the anode, which is the positive electrode and the basic substances pass to the cathode or the negative electrode. The cells were numbered from the acidic end of the system; hence cell #1 was the cell which contained the anode and cell #4 or #6, depending upon the number of cells used, contained the cathode. The pH in cell #1 became as low as pH 3.4 in a short time during the electrolysis. On the other hand, the pH in the cell containing the anode was near the other end of the range of the quinhydrone electrode which was used to determine the pH. Readings were obtained from pH 8.4 to pH 9.6. In the intermediate cells, the range of pH was between the two extremes. Table VII shows the average of the pH of the different cells.

If the substance under investigation has an isoelectric point within this range of pH, the substance will

TABLE VII

The Effect of Electrolysis upon Migration of Phage.

No. of Cells Used	No. of Cell Inoculated	No. of Tests	No. of Cells phage Migrated	pH of cell Inoculated	Time Phage was Inactivated
4	1	6	0-0-0 0-0-1*	3.5	2
	2	1	1	6.5	3
	3	1	1	7.3	2.5
	4	3	1-0-0	8.7	3
6	1	-	-	3.5	-
	2	3	3-1-0	5.0	3
	3	3	0-1-0	5.6	3
	4	1	0	5.9	3
	5	1	1	6.7	3
	6	-	-	8.7	-

* Just a slight evidence of presence of phage in cell No.2

migrate to that pH and can thus be obtained from that cell. By this method, many impurities can be removed from the material. If the substance does not have an isoelectric point in this pH range, it will migrate to the electrode carrying the opposite charge.

Because of the inactivating effect of electrolysis upon the phage, 1 c.c. samples were removed from each cell at regular 30-minute and hourly intervals. This portion was then added to a "standard" culture of the homologous organism. A young active broth culture of the organism is spoken of as a "standard" culture. When a standard culture of the organism and a 1 c.c. portion from the cell were incubated in the usual manner, the usual clearing of the broth culture occurred when the lytic agent was present. As shown in Table VII, there is some evidence of a slight migration toward the cathode while in the majority of instances there is no apparent migration. It is possible that this apparent migration might be due to diffusion as a result of the removal of so many 1 c.c. portions from the cells. When the samples were taken at 15-minute intervals, the lytic agent could be detected in the four immediate cells from the one inoculated. If diffusion is concerned with the migration, then glucose, which is electrically neutral, could be added to the same cell as the phage. Glucose should not be detected in any

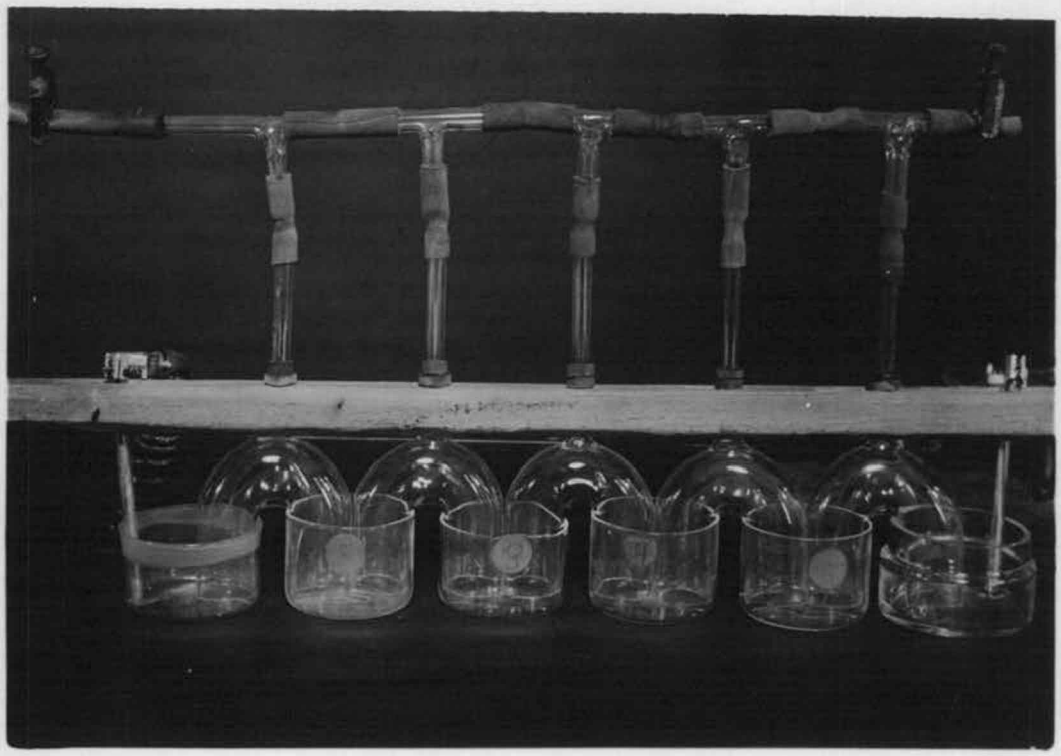

PLATE VI

Electrolysis apparatus

cell other than that in which it was placed. There was no evidence of glucose in any other cell than the one to which it was added. In view of the fact that the phage was apparently completely inactivated, further investigation of this matter was made.

Krueger[52] concludes that the active lytic agent might be associated with some sort of vehicle or carrier which might act as a protective colloid. If the phage were a rider upon some such substance, then electrical force might cause them to separate thus appearing in different cells. If this hypothesis were true, it would seem that combining these different portions, the phenomenon of lysis would appear again. The reaction of each cell (4 cell system) was adjusted to pH 7.1±0.4. The cells were combined in all possible arrangements and checked for the presence of phage. From no combination of the cells could the presence of the phage be detected. The evidence so far obtained by this method in regard to the electrical charge of phage is not conclusive enough to make any positive statements. There are several problems brought forth by this study that would merit further investigation.

THE PHAGIC RELATIONSHIP OF HEMOLYTIC STREPTOCOCCI FROM MARKET MILK

In a study of the presence of hemolytic streptococci in market milk, forty isolates were made. These isolates, in addition to the twenty-four hemolytic streptococci used in building the bacteriophage, were tested against the phages isolated. The purpose of this was to check the lytic relationship of these organisms.

To a standard culture of each of the organisms, was added 1 c.c. of filtrate (18) and incubated in the usual manner. Observations were made at hourly intervals. The potency of filtrate (18) toward its homologous organism at the time of the experiment was 10^{-9}. In no case was there any evidence of lysis in any of the cultures other than the ones already found sensitive to this phage.

After 24 hours incubation, all the cultures, except 11, 12, 13, 17 and 18 which were previously lysed by filtrate (18), were filtered, and 1 c.c. of the filtrate was added to another standard culture and checked for lysis. No evidence of a lytic filtrate in any case was produced.

The above experiment was repeated using filtrate (16). In no case could a culture be lysed except #16. Filtrate (17), was then checked with all the streptococci

with the same results as those from the use of filtrate (18). It appears from these results that filtrates (17) and (18) are the same. From the results of combining this large group of streptococci with these lytic filtrates it appears that streptococcus bacteriophage is very specific in its reactions upon the hemolytic streptococci found in market milk. The lytic filtrate (16) was exceptionally specific. No effort has been made, other than a few single tests, to differentiate them. It is evident from the results obtained that the work of Burnet is confirmed. Hemolytic streptococcus bacteriophage is rare and hard to obtain.

DISCUSSION AND CONCLUSION

Although it has been more than thirty years since the phenomenon called bacteriophage was first observed, many phases about its characteristics are still quite unsatisfactorily explained. One of these is its occurrence. The results reported in this investigation seem to confirm the results of Caldwell[17] in which he was able to obtain a lytic filtrate from sewage which was active against several species of bacteria. This applies very satisfactorily for the colon and staphylococci groups of organisms. The observations of Lisboune and Carvene reported by Topley[62] seem also to be confirmed. They record "When two organisms are grown together in the same liquid culture, one of them may produce a lytic principle active against the other". The method of Clark and Clark[18] is based upon this principle and it was by their method that the streptococcus bacteriophages were produced.

Hadley[39] reports that one of the best sources of bacteriophage is an old culture of the organism. He bases this conclusion upon his theory that bacteriophage is an antolytic enzyme developed within the cell and liberated upon the death of the cell. This did not prove a satisfactory source of phage for streptococci. Many attempts to obtain phage by filtrations of young and old broth cultures were made but not in a single case was any

lytic agent obtained.

Since it was not possible to secure phage from old cultures, gargety milk was used. Gargety milk, received directly from cows infected with mastitis, might possibly be a most likely source of the lytic agent, especially if it was concerned with the decrease in the numbers of organisms in the milk. Many filtrates were made but no evidence of the phage could be obtained. Fresh milk was next tried but no evidence of phage was observed. This phage might be adsorbed upon the protein particles of the milk and thus become inactivated. However sterilized milk, to which phage had been added, did not inactivate the phage even though they had been mixed together for several days.

Feces of the infected animal has often been quoted as a good source of phage. Many experiments, in which feces was obtained from non-infected as well as infected cows, revealed no lytic filtrates.

The first evidence of streptococcus bacteriophage came as a result of the stimulation from other phages which had been isolated from sewage. These observations are not in themselves conclusive because sewage was used in building these phages. The lytic agent may have been present in the sewage at the beginning but in such a weakened condition or in such a small concentration that lysis could not be produced.

After the isolation of the streptococcus phage it was necessary to purify it. At first the filtrate contained lytic agents against many organisms other than streptococci. After several successive transfers, the bacteriophage was purified and all of the phages other than the one for the homologous organism used were eliminated.

Of the two methods used to indicate the presence of phage, the plaque method appears to be the most sensitive in detecting the lytic agent. Several phages were shown to be present by this method that were not observed by the test-tube method. When the plaque method is used, no difficulty is experienced with the appearance of the secondary growths. They appear as small compact colonies inside of the lysed area, while in the test-tube method frequent observations are necessary or the observer might not detect the clearing of the broth before the appearance of secondary growths. An advantage of the test-tube method over the plaque method is in determining the potency of the filtrate as standard amounts of filtrate as well as culture can be used. The potency of some of the filtrates tested by this method was 10^{-10}. That is, the original filtrate could be diluted 10 billion times and still 1 c.c. would produce visible evidence of lysis. In the purification of the phages both methods were employed. Cultures

of the secondary growths were isolated and studied. The cultural, morphological and physiological characteristics appeared to be the same as the culture of organism from which it was obtained. However it was different in that no lytic agent could be developed for it. The effect of CO_2 and "commercial" gas upon the appearance were also studied. These gases had a retarding influence upon the secondary growths, while partial vacuum and anaerobic conditions apparently had little or no effect.

The effect of pH in highly buffered solutions upon bacteriophage was investigated. The works of Bronfenbrenner and Korb[22] (1925) and Muramatsu[52] (1931) were confirmed. There was apparently no inactivation of the phage between pH 5 and pH 9, but a very definite diminution or inactivating effect below pH 4 and above pH 10. However, after being exposed to the buffered solutions for 26 hours, lysis was delayed indicating that the activity of the phage had been decreased. This decrease apparently was due to influences other than pH, because the inactivation was just as noticeable at pH 7 as at pH 5 or pH 9.

Further observations indicated that methylene blue inactivated phage. This dye apparently has an inactivating effect in as low a concentration as 1 part in 10,000, if exposed for 8 hours at 20°C. In a concentration 1 in 1,000 growth of the streptococci was inhibited. When this

same concentration of the dye was used, there was no appearance of the secondary growths.

Attempts were made to obtain a lytic filtrate free from protein by the extraction by ether as suggested by LeMar and Myers[54]. A very potent filtrate (18) was used. In this procedure equal amounts of ether and filtrate were mixed in a separatory funnel. The extraction was continued for three weeks at ice box temperature. No evidence could be obtained from the ether extract that any phage was present in that fraction. When the non-ether soluble fraction was tested, it was observed that the phage had been almost completely inactivated. It might be concluded then that the streptococcus bacteriophage reacted differently than the coli phage used in the experiments of LeMar and Myers.

The matter of the electrical charge carried by phage was investigated. The streptococcus phage passes a negatively-charged Berkefeld filter and it also passes up the full length, even in high dilutions, of a negatively charged piece of blotter paper. This would lead to the conclusion that the charge is negative. This does not take into consideration, however, neutral particles. The results obtained by electrolysis on the other hand indicates that the latter might be the case. Of the 19 tests made by the use of this method, evidence of migration

was observed in 7 cases. This apparent migration might be explained on the grounds of currents set up in the liquid as a result of the removal of so many 1 c.c. portions from the system. Table VII shows one instance in which apparent migration was detected in 4 cells from the one inoculated. In this case, samples were removed every 15 minutes. A further study of the electrical charge and the reasons for the inactivation of the phage in such a short period and at such a low current, would be desirable.

An observation subsidiary to the main problem was the improvement in the method of keeping stock cultures. These streptococcus cultures would not live longer than two weeks at room temperature and thus necessitated frequent transfers. By preparing a "slush" infusion agar (agar 0.7%) and storing the cultures at 4°C., their viability could be maintained for at least three months. This "slush" agar apparently allows for the diffusion of the waste products away from the cell.

It was also observed that the visible appearance of phage lysis commenced at the surface of the broth cultures. Whenever a piece of paper containing the phage floated on the surface or near the surface, complete lysis appeared considerably in advance of any other tubes in the series where the paper sank to the bottom of the liquid. There is apparently some surface-tension phenomenon connected

with the lysis. In many of the tubes the concentration of phage was much less than that in the tubes in which the paper sank to the bottom. However, lysis would appear in some cases many hours before it did in the other tubes.

The matter of the specificity of the streptococcus phage was evident in this experiment. Of the 62 hemolytic streptococci, isolated from milk, only 6 were lysed. The fact that 56 of the cultures were not lysed by either of the phages, would indicate that these were different. There also appears to be differences in those that were lysed. One of these, number 16, was lysed only by one phage. This phage was active only against its homologous streptococcus. The other 5 cultures were sensitive to the other phage. Phage (16) was also different from the other in that the potency of this phage could not be built up greater than 10^{-2}.

SUMMARY

Two distinct races of hemolytic streptococci bacteriophage were isolated. One was very specific and lysed only one culture of the entire group. The other was able to lyse five cultures of the group.

No lytic filtrates were obtained from old cultures, gargety milk, fresh milk, nor from the feces of animals having infectious mastitis.

Lytic filtrates were obtained that produced visible lysis even when diluted ten billion times.

No phage was found which would lyse the secondary growths.

Partial vacuum and anaerobic conditions (absence of O_2) did not have any apparent effect upon the lytic phenomenon nor upon the appearance of the secondary growths.

"Commercial" gas and CO_2 had no depressing effect upon the lytic phenomenon but did inhibit or stop the appearance of the secondary growth.

The H^+ ion has a deleterious effect upon phage (18) at pH 4 and pH 10, but has no apparent effect between pH 5 and pH 9.

Methylene blue inactivates streptococcus bacteriophage in dilutions as high as 1 part in 10,000 parts of water.

Attempt to obtain a purified phage by ether extraction was not successful.

The electrical charge of phage (18) seems to be either negative or neutral.

BIBLIOGRAPHY

1. Arnold, Lloyd and Weiss, Emil. Isolation of bacteriophage free from bacterial proteins. Jour. Inf. Dis., vol. 37, 1925.

2. Bail, O. Der Stand und Ergebnisse of Bakteriophagen forschung. Centralbl. f. Bakterial, 1, 1925.

3. Beard, Paul J. Decreased resistance of purified bacteriophages to disinfectants. Proc. Soc. Exp. Biol. Med., vol. 26, 1929.

4. Beard, Paul J. Bacteriophage adaptation. Jour. Inf. Dis., vol. 49, 1931.

5. Beard, Paul J. Role of bacteriophage in sewage purification. Jour. Inf. Dis., vol. 52, 1933.

6. Bedson, S. P. and Bland, J. O. W. A simple method for determining the electrical charge carried by virus particles. Brit. Jour. of Exp. Pathology, vol. 10, 1929.

7. Bronfenbrenner, J. J. Virus Diseases of Bacteria. Filterable Viruses, Rivers, T. M. Pub. by Williams and Wilkins, Baltimore, 1928.

8. Bronfenbrenner, J. J. and Daubly, J. Stimulation of bacterial metabolism by bacteriophage. Proc. Soc. Exp. Biol. Med., vol. 30, 1933.

9. Bronfenbrenner, J. J. The mechanism of bacteriophage lysis. Amer. Jour. of Public Health, vol. 24, April, 1934.

10. Burnet, F. M. A method for the study of bacteriophage multiplication in broth. British Jour. of Exp. Path., vol. 10, 1929.

11. Burnet, F. M. Bacteriophage and cognate phenomena. A System of Bacteriology, vol. 7, 1930, London.

12. Burnet, F. M. Bacteriophage activity and the antigenic structure of bacteria. Jour. of Path. and Bact., vol. 33, 1930.

13. Burnet, F. M. The phage-inactivating agent of bacterial extracts. Jour. of Path. and Bact., vol. 38, 1934.

14. Burnet, F. M., and Gough, G. A. C. The chemical nature of the phage-inactivating agent in bacterial extracts. Jour. of Path. and Bact., vol. 38, 1934.

15. Burnet, F. M. Inactivation and absorption of phages by heat killed staphylococcus. Jour. of Path. and Bact., vol. 40, 1935.

16. Burnet, F. M. The bacteriophages. Biol. Rev., vol. 9, 1936.

17. Caldwell, Janet Angerson. Sewage filtrate as a source of bacteriophage. Jour. Inf. Dis., vol. 40, 1927.

18. Clark, Paul F., and Clark, Alice Schiedt. A bacteriophage active against a virulent hemolytic streptococcus. Proc. Soc. Exp. Biol., vol. 24, 1927.

19. Clifton, Chas. E. The kenetics of bacteriophage. Jour. of Bact., vol. 31, 1936.

20. Colvin, M. G. Behavior of bacteriophage in body fluids and in exudates. Jour. Inf. Dis., vol. 51, 1932.

21. Craigie, James, and Brandon, K. F. Bacteriophage specific for the "O"-resistant "V" form of B. typhosus. Jour. of Path. and Bact., vol. 43, 1936.

22. Craigie, James. A bacteriophage for the "V" form of Salmonella typhi. Proc. Soc. Am. Bact., Jan., 1936.

23. Dreyer, Geo., and Campbell-Renton, Margaret L. The quantitative determination of bacteriophage activity and its application to the study of the Twort-d'Herelle phenomenon. Jour. of Path. and Bact., vol. 36, 1933.

24. Dutton, L. O. Role of bacteriophage in streptococcus infections. Jour. Inf. Dis., vol. 39, 1926.

25. Ervin, Harold. Personal communication.

26. Evans, Alice C. Inactivation of antistreptococcus bacteriophage by animal fluids. Public Health Reports, vol. 48, April 21, 1933.

27. Evans, Alice C. The prevalence of streptococcus bacteriophage. Science, vol. 80, July 13, 1934.

28. Evans, Alice C. Streptococcus bacteriophage. Public Health Reports, vol. 49, Nov. 23, 1934.

29. Evans, Alice C. Streptococcus bacteriophage and its usefulness for the identification of strains of hemolytic streptococcus. Jour. Bact., vol. 27, 1934.

30. Evans, Alice C. A comparative study of streptococcal immunity produced in rabbits by heat-killed cultures, by active bacteriophage and by inactivated bacteriophage. Public Health Reports, vol. 50, Feb. 8, 1935.

31. Evans, Alice C. Studies on hemolytic streptococci. Jour. of Bact., vol. 31, April 1936.

32. Evans, Alice C. Studies on hemolytic streptococci. Jour. of Bact., vol 31, June 1936.

33. Evans, Alice C. Studies on hemolytic streptococci. Jour. of Bact., vol. 32, Nov. 1936.

34. Frobisher, Martin J. The action of bacteriophage in producing filtrable forms and mutations of bacteria. Jour. Inf. Dis., vol. 42, 1928.

35. Gildemeister, E. Weitere Untersuchungen uber das d' Herelleschen Phanamen. Centralbl f. Bakteriol., 93, 1924.

36. Hadley, Philip. Proliferative reaction to stimuli by the lytic principle (bacteriophage) and its significance. Jour. Inf. Dis., vol. 37, 1925.

37. Hadley, P. and Klimek, John. The so-called "origin" or "source" of the bacteriophage. Proc. Soc. Exp. Biol. and Med., vol. 25, 1927.

38. Hadley, Philip. Microbic dissociation. Jour. Inf. Dis., vol. 40, 1927.

39. Hadley, Philip. The Twort-d'Herelle phenomenon. Jour. Inf. Dis., vol. 42, 1928.

40. Hadley, Philip and Jemenez, Beunaventura. Production of bacteriophage by enforced dissociation. Jour. Inf. Dis., vol. 48, 1931.

41. d'Herelle, F. The bacteriophage and its behavior. Pub. by The Williams and Wilkins Co., Baltimore, 1926.

42. Jern, H. L., and Meleney, F. L. Methods of maintaining and testing the potency of bacteriophage. Jour. Lab. and Clin. Med., vol. 20, 1935.

43. Kendall, A. I., and Walker, A. W. Observations upon the "Prezone" of certain bacteriophages. Jour. Inf. Dis., vol. 58, 1936.

44. Kendall, Arthur I. The elicitation of specific phage from autoclaved (lifeless) material. Jour. Inf. Dis., vol. 59, 1936.

45. Kendrick, Pearl, and Hollon, Harriet C. Seralogic and bacteriophagic relationship in a group of fecal streptococci. Jour. Bact., vol. 21, 1931.

46. Koch, K. Untersuchungen uber Bakteriophagen katophonec. Centrobl. f. Bakteriol, 99, 1926.

47. Krueger, A. P. Quantitative determination of phage. Science, vol. 72, Nov. 14, 1930.

48. Krueger, A. P. Critique of the serial dilution method for quantitative determination of phage. Science, vol. 75, May 6, 1932.

49. Krueger, A. P., and Elberg, S. Reversible inactivation by KCN. Proc. Soc. Exp. Biol. Med., vol. 31, 1934.

50. Krueger, A. P., and Baldwin, D. M. The reversible inactivation of bacteriophage by bichloride of mercury. Jour. Gen. Physiol., vol. 17, 1934.

51. Kreuger, A. P., and Baldwin, D. M. The reversible inactivation of bacteriophage with safranine. Jour. Inf. Dis., vol. 57, 1936.

52. Krueger, A. P. The nature of bacteriophage and its mode of action. Physiological Reviews, vol. 16, No. 1, 1936.

53. Larkum, N. W., and Semmes, Margaret F. Filtration of bacteriophage. Jour. Bact., vol. 19, 1930.

54. LeMar, John D., and Myers, John T. The artificial production of a specific lytic agent which behaves like bacteriophage. Jour. Bact., vol. 27, p. 49, 1934.

55. LeMar, John D., and Myers, John T. Studies on the nature of bacteriophage. Jour. Inf. Dis., vol. 57, p. 1, 1935.

56. Marshall, Max S. Observations on d'Herelles bacteriophage. Jour. Inf. Dis., vol. 37, p. 126, 1927.

57. Marshall, M. S., and Paine, F. S. Survival of bacteriophage. Proc. Soc. Exp. Biol. Med., vol. 28, pp. 606-607, 1931.

58. Mudd, S. The role of electrical adsorption. Am. Jour. Physiol., vol. 63, p. 429, 1923.

59. Schuurman, C. Der Bakteriophage ein lebender organisms. Centralbl. f. Bakteriol, 95, 1925.

60. Schwartzman, Gregory. Studies on streptococcus bacteriophage. Jour. Exp. Med., vol. 46, pp. 497-509, 1927.

61. Todd, Charles. The electrical behavior of the bacteriophage. British Jour. Exp. Path., vol. 8, p. 369, 1927.

62. Topley, W. W. C., and Wilson, G. S. The principles of bacteriology and immunity. Pub. by William Wood and Company, N. Y., vol. 1, 1929.

63. Wells, Alvin, and Sherwood, Noble P. Selective action of dyes and of other disinfectants on bacteriophage. Jour. Inf. Dis., vol. 55, 1934.

64. Williams, R. J., and Truesdail, J. H. The use of fractional electrolysis in the fractionation of the "Bios" of Wildiers. Jour. Am. Chem. Soc., vol. 53, 1931.

65. Williams, Roger J. Fractional electrical transport as a tool in biochemical research. Jour. Biol. Chem., vol. 110, no. 3, 1935.

66. Zinsser, Hans, and Bayne-Jones, Stanhope. A textbook of bacteriology. Pub. by Appleton-Century Co., New York City, 1934.