A SURVEY FOR PNEUMOCOCCUS AND STREPTOCOCCUS BACTERIOPHAGES IN CITY AND HOSPITAL SEWAGE, INCLUDING THE APPLICATION OF NASCENT STREPTOCOCCUS PHAGES TO THE LYSIS OF PNEUMOCOCCI

By

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Thesis submitted to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy

1941

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I

INTRODUCTION

Sewage was selected as a source of pneumococcus and streptococcus bacteriophages in the present study, after several considerations. Since bacteriophages are entities completely parasitic on bacteria their source would be in materials with consistently luxuriant bacterial flora. The intestinal tract of man and the lower animals is a constant source of a large number of bacterial species with the result that the majority of phages reported in literature have been isolated from human and animal feces or sewage. The phages present in sewages are not necessarily limited in their specificity to normal or pathogenic intestinal species, but have been isolated for diverse other organisms such as <u>Corynebacterium diphtheriae</u> and <u>Hemophilus pertussis</u>. However, it would be expected that pneumococci might pa ss into sewage systems although survival is improbable. All sputums of pneumonia patients contain high numbers of pneumococci; Rutz (1912) isolated the organism from the stools of lobar pneumonia patients; Mathers (1916) reported their presence in the urine of infected individuals, and it is well known that there exists a high number of pneumococcus carriers (Stebbins, et al, 1940) also contributing pneumococci from the saliva and respiratory secretions.

A review of the literature revealed that Clark and Clark (1927) made the only recorded attempt to find a

principle lytic for the pneumococcus in sewage; they examined activated sludge but were unsuccessful. Brainard and Nobel (1935) attempted, without success, to induce the pneumococcus to produce a phage by passages in mixtures of heterologous phages and sewage whereby the treated pneumococcus cultures rather than the sewage were examined for phage production. In view of these results it was one of the purposes of this investigation to determine the existence of pneumophage in city and hospital sewages obtained from geographically well-separated cities during the pneumonia season.

Also the distribution in hospital sewages of previously reported streptococcus phages was determined concurrently with the search for pneumophage. There is no report in literature concerning this subject. Furthermore, positive isolations of streptococcus phage from each sewage sample were considered satisfactory controls for the technical methods employed.

In another series of isolation experiments with the same sewage samples, a new streptococcus phage was obtained, a race serologically different from any previously described in the literature. A part of this study was concerned with the isolation and identification of this lytic principle.

A few investigators have made sporadic attempts to induce lysis of pneumococci by heterologous phage filtrates, attempts which have included too few phage races or

pneumococcus types for completely conclusive findings. As a result, a more thorough investigation of this subject incorporating several phage races and all the pneumococcus types seemed expedient.

Nascent bacteriophage, or the lytic principle in the presence of sensitive bacteria, is known to possess an enhanced lytic range. The observation, mentioned by Evans (1934b), that some nascent streptococcus phages lysed the pneumococci prompted a study of the lytic activity of nascent heterologous phages for the pneumococci.

II

HISTORICAL REVIEW

Discovery of Bacteriophage: The phenomenon of bacteriophagy was first observed and described by Twort (1915). While working on the problem of cultivating filtrable viruses, he noted a translucent change occurring in micrococcus colonies isolated from glycerinated calf vaccinia. These translucent areas transferred to fresh agar either produced similar "watery" areas or no growth at all; a glassy colony cultivated with a normal colony yielded a predominance of the glassy colonies. Twort demonstrated that his "transparent material" was filtrable and thus could be separated from the micrococci from which it was obtained; it was transmissible in series, but only in the presence of the micrococci; it had no effect on dead micrococci; it was active in high dilutions (10^{-6}) after filtration; and its action was specific - "It has some action, but very much less, on Staphylococcus aureus and albus isolated from boils of man, and it appears to have no action on members of the coli group or on streptococci, tubercle bacilli, yeasts, etc." He described this agent as "an acute infectious disease of micrococci".

It was not until d'Herelle (1917) reported a similar phenomenon with B. dysenteriae Shiga that any great amount of attention was given to this subject. He found that the sterile filtrate of a culture of feces from recovering

dysentery patients clarified a heavy suspension of *B. dysenteriae*, and a trace of this dissolved suspension, in turn, cleared a second suspension of organisms. This lytic principle was transmitted in series indefinitely in the presence of living organisms, and became more active after a number of passages. D'Herelle regarded the lytic principle as an ultramicroscopic filtrable virus which he named "Bacteriophagum intestinale"; thus the lytic principles for all bacterial species came to be known as bacteriophages, and the process of lysis by bacteriophage was termed bacteriophagy. D'Herelle has devoted his scientific life to this phenomenon, and has collected much of the scientific literature in two books (1922; 1926).

A Proposed System of Classification of the Bacteriophages: In 1939 Holmes published a book entitled *Handbook of Phytopathogenic Viruses* in which he included a proposed system of classification and method of nomenclature of the bacteriophages.[1] This work described only those viruses which attack plants, including the bacteria. The kingdom for the phytopathogenic viruses is *Vira*; the phages fall into the division *Phytophagi* which is separated into two classes, *Schizophytophagi* - viruses pathogenic in schizophytes - and *Spermatophytophagi* - viruses pathogenic in seed plants. The former class includes only one family,

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[1]The author, less familiar with the phages, treated them in a supplement to his book.

Phagaceae - viruses pathogenic in bacteria - in which the one genus, _Phagus_, comprises 40 species and 2 varieties of bacteriophage.[1]

For each phage species, the author presents his reason for selecting the specific name and lists the following descriptions: the various synonyms for the species, the susceptible bacterial species, the geographical distribution, the induced disease,[2] the serological and immunological relationships, the thermal inactivation, "other properties" and the reference literature.

Methods of Isolating Phage from Sewage: D'Herelle (1926) planted fluids containing suspended organic matter such as feces (or sewage) in sterile broth and incubated at 37° C. overnight. The bacterial action broke down the large particles of organic matter, and the mixture was filtered through infusorial earth and a candle. He used this filtrate for specific enrichment with the bacterial strain for which a phage was being sought.

Caldwell (1927) tested city sewage filtrates for lytic activity against 67 strains of gram negative organisms and found a "potent bacteriophage for practically all

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[1]There are many other recognized "species" of phage not considered by this author, but his method of nomenclature and classification, if accepted, would apply to all species.

[2]Refers to appearance of plaques formed by the action of phage on sensitive bacteria growing on solid media.

strains of B. coli isolated from urinary infections, and for most of the other common gram negative bacilli encountered in human diseases" - (typhi, dysenteriae and pyocyaneus). There were also streptococcus and staphylococus phages in the sewage filtrates. Her technique followed d'Herelle's: 2, 10 and 30 drops of filtrate were planted in a light suspension of young organisms; the resulting filtrate was enriched with its homologous strain until definite lysis appeared.

Cowles (1931) filtered the crude sewage through paper (no candles) and enriched it with beef extract and peptone; to this highly contaminated medium he added B. anthracis, employing the usual process of alternate feeding and filtering. He obtained a phage from the third-passage filtrate. Also by planting 48-hour cultures of Cl. tetani in crude sewage contained in an equal volume of double-strength infusion broth, Cowles (1934) isolated a specific lytic principle for this anaerobe after several serial passages of the filtrates with the homologous strains.

Smith and Jordan (1931 a) employed the following technique to isolate a phage specific for C. diphtheriae: multiple inoculation of several strains was made in 9 parts of raw sewage filtrate to one part of a tenfold concentrated broth. The advantage of multiple strain inoculations was that it allowed any phage present in the filtrate to propagate itself if any one of the several strains was susceptible.

Sauer and Hambrecht (1933) secured a potent pertussis phage by a technique described by Smith and Jordan (1931b). The sewage bouillon consisted of 9 parts of sewage filtrate to 1 part of tenfold concentrated broth: it was enriched with a small amount of defibrinated blood and planted with a 24-hour culture. Lysis appeared on the third day of incubation, and plaques were numerous on solid media.

Evans (1934 a) used equal quantities of double strength broth and Berkefeld filtered sewage as a medium for isolating streptococcus phages. For the first passage in this medium the filtrate added was obtained by planting broth with unfiltered sewage, incubating overnight at 37° C. and filtering. A mixture of 10 strains was planted in one tube of sewage medium plus filtrate. (It was shown that the presence of phage resistant streptococcus strains does not inhibit lysis of phage sensitive strains in the same tube.) If only one of the 10 strains was susceptible to the phage action, the filtrate of this passage would clear the sensitive strain when tested in pure culture. The process of alternate culturing in the mixture of sewage medium and filtrate and filtering was continued until a lytic principle was isolated.

Search for Pneumococcus Phage: Clark and Clark (1927) examined activated sludge for streptococcus, pneumococcus and "colon-typhosus" phages. The various cultures were grown for 3 or 4 days in the presence of 10 per cent activated sludge in beef infusion broth, then centrifugated

and filtered through Mandler filters. Each filtrate was tested for lysis of the homologous strain (i.e., the strain employed to enrich the phage content of the activated sludge). When the strain was not lysed, the filtrate was planted with the homologous strain and 10 per cent activated sludge; this technique was repeated for at least ten serial passages or until a filtrate contained the lytic principle. Phages for the "colon-typhosus" group were most readily isolated; a second enrichment passage yielded a lytic principle active against a mucoid streptococcus, but all attempts to isolate a phage for the other streptococcus strains or the pneumococcus strains were unsuccessful. These workers attempted to adapt the mucoid streptococcus phage "to the 3 fixed types of pneumococci by all of the usual methods, and by various modifications of our own, but entirely without success".

Eddy (1928) examined specimens of pneumonia sputum for bacteriophagic action on the pneumococcus. She stated that it seemed possible that the crisis occurring in pneumonia might be caused by the rapid development and action of a phage virulent for the pneumococcus. One ml. of sputum filtrate was added to 9 ml. of a suspension of homologous pneumococci (i.e., pneumococci isolated from the sputum sample), incubated 20 hours, and filtered through a Berkefeld filter. Although fifteen serial enrichment passages were made with the filtrate and fresh suspensions of pneumococci, a bacteriophage could not be

recovered.

Grumbach (1931) described a pneumococcus colony similar in appearance to that of a phage-eaten streptococcus colony, but did not ascertain whether it was bacteriophage which produced this atypical colony form. Farago (1932) investigated the possibility of pneumococcus bacteriophage producing the dissociative processes which he observed and decided that bacteriophage action was not involved.

Brainard and Noble (1935) examined a variety of materials for pneumococcus bacteriophage without success. They used two methods to test for the presence of phage as indicated by lysis: (1) Berkefeld "W" filtrates of materials being examined were added to young surface growths of the pneumococcus on blood agar plates; and (2) the same filtrates were planted in 24-hour beef infusion broth suspensions of pneumococci. The tests were examined after overnight incubation. Stock cultures of types I, II, III and V and recently isolated strains were included in the study. Materials examined for the presence of pneumococcus phage were: One stock strain each of streptococcus and dysentery phages (by adaptation), dissociated pneumococcus colonies, and the discharges of 31 pneumonia patients and 2 persons infected with colds. Only nasopharyngeal washings were collected from the latter; 26 sputums, 20 stool and urine specimens, and 6 blood sera from the pneumonia patients were examined. The authors frequently observed clear zones on the blood agar plates

and clearing of broth cultures, but the lytic agent was never transmissible. By cultivating the pneumococcus in a broth medium containing a 10 per cent mixture of "fresh sewage" filtrate and streptococcus and dysentery phages these workers attempted, unsuccessfully, to induce lysogenicity, a process whereby certain bacterial cultures produce phages to which they themselves are resistant but to which other strains of the same species are susceptible. After a culture had been passed 10-18 times in this medium, it was planted in plain broth, incubated overnight and filtered. The filtrate was tested for pneumococcus phage with its normal (not sewage treated) homologous strain. None of the strains was found to be lysogenic.

Balsamelli (1935) reported that a bacteriophage active on the streptococcus-staphylococcus group ("gruppo streptostafilo") did not lyse the pneumococcus. Four types of pneumococci, I, II, III and IV developed normally in the presence of an approximately 1:20 dilution of this phage in broth. After 15 days at 37° C., the phage had deteriorated in the tubes containing the pneumococci and also in the phage controls without pneumococci. In about 20 to 30 days at 37° C. the pneumococcus cultures with and without phage became limpid due to autolysis, a process which dissolved the cells similarly to phage lysis. The same author examined a few sputums of lobar pneumonia patients for pneumococcus phage. The sputum was emulsified in physiological saline, filtered through a Berkefeld and then a Chamberland

filter. The homologous type pneumococcus and the other three types employed in the study were planted in broth containing sputum filtrate in an approximately 1:20 dilution. There was no lysis of the pneumococcus until autolysis appeared in 20 to 30 days in both the broth cultures containing the filtrate and the control broth cultures. Balsamelli filtered the autolysed cultures and found that the lytic agent could not be transmitted serially.

Rodigina (1938), a Russian[1], reported the first positive isolation of pneumococcus phage. His technique was the usual one: sputum samples of pneumonia patients were planted in broth, incubated for 24 hours, and then filtered. The presence of phage in the filtrates was evidenced by complete clearing of pneumococcus cultures within 12 to 18 hours and by plaque formation ("tache vierges") on solid media. The potency of the phages was determined by planting 1.0, 0.5, 0.3, 0.1, 0.05, 0.03, 0.01 and 0.005 ml. in broth containing one drop of a 24-hour broth culture of the pneumococcus. Rodigina did not describe his methods of serial passage of phage filtrates other than in the following quotation: "If at the beginning of the experiment lysis of a pneumococcic culture required 0.3 cc. of the pneumophage, later on this quantity became reduced by degrees and finally amounted to such minimum values as

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[1]An unsuccessful attempt was made to communicate with this author.

from 0.01 to 0.005 cc." He published no data showing whether or not continuous transmissibility over reasonably long periods was maintained. Three kinds of pneumococcus cultures were distinguished on the basis of their reaction to pneumophage: (1) Lysosensitive strains, (2) resistant strains made sensitive only in the presence of phage, and (3) lysoresistant strains. Each phage group contained representatives of all types studied. Cultures representing the four pneumococcus types I, II, III and IV yielded non-type specific phages. The data obtained suggested that the phage isolated from type III cultures was less potent than phages obtained from the other type cultures; likewise type III cultures were more resistant to phage action. Spontaneous lysis of cultures occurred and was apparently distinguished from true lysis, but Rodigina did not describe his method of differentiation. The pneumophage appeared in the sputum of pneumonia patients during the convalescent period, i.e., after the crisis; it also appeared sporadically in the sputum of patients "with ocular disease and pneumonia". Phages of pulmonary and ocular origin were similar in their properties. This pneumococcic lytic principle was reported to exert an "obvious curative action" on ulcus cornea serpens.

<u>Methods of Producing and Testing Antibacteriophagic Serum</u>: Bordet and Ciuca (1921) published the first paper on production of antibacteriophagic serum which they obtained by treating rabbits with several injections of a

coli phage. They demonstrated that the resulting serum was not bactericidal for E. coli, and they showed that in the presence of phage neutralized by serum E. coli grew as well as in the broth culture control. The antiserum inhibited the action of its homologous phage both in broth and on agar. In this first report, the authors found that one volume of antiserum neutralized 10 volumes of homologous phage.

Weiss and Arnold (1924) described a method for producing typhoid and staphylococcus antiphage serum. They injected rabbits intravenously 8 to 12 times at 2 to 4-day intervals. The first dose of 1.0 ml. was increased in 1.0 ml. amounts up to 6.0 ml., which quantity was inoculated for the remaining injections. The titers of the resulting antilysins were not reported. From the experiments they concluded that antilysins are the antibodies produced by bacteriophage, and that their action is highly specific.

Schultz, Quigley and Bullock (1929) prepared staphylococcus and dysenteriae-coli antilysins by injecting 16 to 21 doses of 2.0 ml. each over a period of about 4 months. Five-tenths ml. of the undiluted homologous phage filtrate was neutralized by a 1:1000 dilution of the antilysin.

Kendrick (1933) reported that she was able to produce an effective antilysin for B. suipestifer phage with a single injection of phage. Antilytic titers were influenced by individual rabbit response and immunizing

technique. This worker supported the specificity of bacteriophage units by demonstrating that "a lytic filtrate developed at the expense of a sensitive culture other than its specific one could still be neutralized by an antilysin specific for the original filtrate". The serum titer was defined as the highest dilution in which 0.01 cc. completely neutralized the test dose of approximately 100 units of bacteriophage in 24 hours at room temperature.

Evans (1934 b) differentiated four serological types of streptococcus phage with type specific antilysins prepared in rabbits injected with phage filtrates. The phage was inoculated intravenously at 3 or 4-day intervals with an initial dose of 1 or 2 ml. which was gradually increased up to 8 or 10 ml. The total quantity of antigen injected varied, but all rabbits were bled a week after the last treatment and the serum was drawn off, preserved with 0.25 per cent tricresol, and stored in the refrigerator. In determining the titer of the specific antilysins, Evans (1934 b) made serial dilutions of the serum in broth and to these dilutions added 0.1 ml. of filtered phage, diluted to contain 100 phage particles; a control tube containing broth was likewise planted with the phage dilution. The phage-serum mixtures were thoroughly shaken and incubated at 37°C. overnight. Each phage-serum mixture, the phage control and a tube of broth were then planted with a drop of homologous streptococcus culture and reincubated at 37°. overnight. The titers of the sera varied with the phage

type employed in their preparation. She differentiated four streptococcus types by cross neutralization tests with the antilysins prepared as described above; two strains of phage were regarded as belonging to the same type when antiphage serum prepared with either strain neutralized both. In support of serological type specificity of phage, Evans (1934 b) stated that "the serum prepared by treatments with a given filtrate will neutralize any lytic filtrate of the same serological type, regardless of the streptococcus which served as the substratum".

Clifton, Mueller and Rogers (1935) prepared antilysin against coli phage by injecting rabbits intravenously with repeated doses of 2.0 ml. of active phage; 24 ml. were administered in a 30-day period. Serum was drawn off from the heart's blood, inactivated at 56°C. and stored unpreserved in the refrigerator. The phage was almost completely neutralized after 72 hours by an equal volume of serum diluted 1:6400.

Sertic and Boulgakov (1935) prepared various antiphage serums by injecting rabbits intravenously at 6-day intervals with 2, 4, 8 and 20 ml.; the animals were bled 10 days after the last injection. Instead of identifying a new race of phage by neutralization test with the antisera of each known type, these workers pooled all their phages for a given species and prepared polyvalent antisera. The unknown strain of phage was mixed with equal parts of the polyvalent antiphage serum, incubated 3 hours

at 37°C. and 24 hours at room temperature, and then spread on a film of the homologous culture. The appearance of plaques was proof that the phage was a new type with no antibodies present in the polyvalent serum capable of neutralizing the phage.

<u>Nascent Bacteriophage</u>: The literature on nascent phage has been reviewed by Evans (1940) in a recent paper. The term "nascent" in relation to phage was first introduced by her (1934 b) to describe the enhanced potency or increased range of activity of bacteriophage in the presence of growing sensitive bacteria.

It has already been noted that Twort (1915) reported the inactivity of filtered lysate for dead homologous organisms; only living cells, preferably young cultures, were dissolved by filtered phage. Gratia and Rhodes (1924) were the first workers able to produce lysis of dead bacteria by phage. This was accomplished by planting a trace of living staphylococci in a mixture containing phage and a turbid suspension of dead staphylococci which cleared after a suitable incubation period. Thus the potency of the phage in the presence of living sensitive organisms was increased to the point of being lytic for the dead cells. In addition to confirming the observations of Gratia and Rhodes, Twort (1925) found phage, in the presence of its homologous organism, to be as specific in its lytic action on dead bacteria as in its lytic action on viable bacteria.

He suggested an increased activation of the phage by a substance originating from the lysed cells. Reynolds (1926) duplicated Twort's results with staphylococcus phage but was not able to produce lysis of dead coli or Shiga bacteria in the presence of their respective phages and a trace of living sensitive bacteria.

After a detailed confirmation of the observations of Gratia and Rhodes and those of Twort, Bronfenbrenner and Muckenfuss (1927) studied the mechanism of lysis of dead bacteria by phage in the presence of living cells. The results were elucidative. Lysis was prevented by an excess of dead bacteria due to their absorption of the phage before lysis of the live cells. Dead bacteria were lysed by phage in the presence of living organisms before, during and after the completion of lysis of the living bacteria. Dead staphylococci were lysed most easily - a species which also autolyses spontaneously. Lysis of dead _B. coli_ or _B. dysenteriae_ was indistinct. By separating the living from the dead cells with a semi-permeable membrane, it was shown that the dead cells were not dissolved even though the live cells were undergoing lysis on the opposite side of the membrane. They conclude: "The agent determining the lysis of dead bacteria is not diffusible, while the principle initiating the lysis of live bacteria diffuses fully and is demonstrably present on both sides of the membrane. The complete independence of the agent causing dissolution of dead bacteria from bacteriophage

can also be shown by separating the two agents by means of filtration, or by absorption on bacteria". Among the differences they found between phage and this "ferment-like" substance were: the latter was easily absorbed on clay filters, it was heat-labile and was inactivated on standing. The enzyme-like agent which lysed dead staphylococci was also found in spontaneously autolysing staphylococcus cultures, much later, however, than in staphylococcus cultures containing phage.

D'herelle (1926) first mentioned lysis of <u>living</u> bacteria of <u>heterologous</u> species by phage in the presence of sensitive bacteria. While previous workers had only investigated the lysis of dead homologous bacteria by phage, he reported a reduction in number of staphylococci, vibrios and streptococci in the presence of a lysing coli-phage mixture. He stated although "the results may be interpreted by the hypothesis of a lytic ferment secreted by bacteriophage, they may also be interpreted in other ways".

The Wollmans (1932) were the first to report conclusive experiments on lysis of living bacteria by nascent phage. They tested strains of living staphylococci and sarcina which were completely resistant to a filtered staphylococcus phage, and showed that in the presence of a sensitive staphylococcus strain, phage may lyse resistant[1]

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[1] i. e., resistant to the action of the filtered phage.

staphylococci and sarcina. They believed the phage-lysed sensitive cells liberated diastases or autolysins which could in turn act on the resistant strains.

Rakieten (1933) confirmed the observations of the Wollmans on the lysis of resistant strains of living bacteria by nascent phage. He also made certain other interesting observations. He believed some of his resistant staphylococcus strains actually destroyed staphylococcus lytic filtrate. In contrast to the Wollmans, who considered the secondary lytic agent an autolysin produced by the cell, he maintained that the lysis of a resistant staphylococcus by nascent phage was due to the lysin elaborated by the phage in its action on the susceptible strain. This "lysin" was not transmissible in series, but a filtrate of a resistant strain lysed by nascent phage was still active against the susceptible strain. The staphylococcus "lysin" did not dissolve Salmonella enteritidis, the meningococcus, or the pneumococcus. He tested two different races of nascent staphylococcus phage for lysis of a resistant staphylococcus and found a difference in their lytic activity. Hence, he stated that the amount of lysin produced depended primarily upon the intrinsic quality of the phage rather than upon the structure of the staphylococcus which was attacked. In 1938, Rakieten reported that pneumococcus types I, II and III had no demonstrable effect on staphylococcus or enterococcus phages when tested for their phage-absorbing

activity.

Evans (1934b) tested the sensitivity of several hundred beta hemolytic streptococci to four types of nascent streptococcus phage and reported that this technique differentiated them into well-defined groups. She also investigated the lytic action of the nascent streptococcus phages on other related species: eight pneumococcus strains, one staphylococcus, and one _Streptococcus lacticus_ strain were lysed. Five alpha streptococcus strains were unaffected.

III

AN EXTENSIVE SEARCH FOR PNEUMOCOCCUS AND STREPTOCOCCUS BACTERIOPHAGES IN CITY AND HOSPITAL SEWAGE DURING THE PNEUMONIA SEASON

Sewages and polluted waters have afforded a prolific source of bacteriophage for a wide variety of bacterial species including _B. dysenteriae Shiga_ (Dumas, 1920), _Eberthella typhi_ (Becherich and Hauduroy, 1922), a thermophilic strain (Koser, 1927), a psychrophilic strain (Elder and Tanner, 1927), streptococci (Clark and Clark, 1927; Evans, 1934 a), staphylococci (Bulgakov and Sertic, 1930), _C. diphtheriae_ (Smith and Jordan, 1931 a), _H. pertussis_ (Sauer and Hambrecht, 1933), and the strict anaerobe - _Cl. tetani_ (Cowles, 1934). The present study was undertaken to determine the presence or absence of pneumococcus phage and the distribution of streptococcus phages in sewage. Ten samples, 7 of which were hospital sewages obtained from different cities in eastern, northern, and southern parts of the United States, were examined for pneumococcus and streptococcus phages.

Experimental Procedures

Materials: In searching for a pneumococcus phage a special low-heat beef infusion broth, used by Dr. Lloyd Felton for culturing the pneumococcus, was employed. It was hoped that a highly favorable medium for pneumococcus growth might simplify phage isolations. The double

strength, low-heat broth was prepared in the same way as the single-strength medium but with half the volume of water.

Low-heat beef infusion broth for pneumococci
(Dr. Lloyd Felton)

Constituents:

Beef steak (freed of fat and bone)	500	grams
Peptone (bacteriologic) Parke Davis and Co.	20	"
Sodium chloride, C.P.	5	"
Sodium phosphate, dibasic $Na_2HPO_4 12H_2O$	5	"
Distilled water	1000	cc.

The reaction was adjusted to pH 8.0 before final sterilization in an autoclave at 15 pounds pressure for 15 minutes.

Another broth employed was a 2% neopeptone beef infusion with a pH of 7.6.

Blood agar was prepared by adding 5% fresh rabbit blood to 2% beef infusion agar.

The samples of sewage were obtained during the pneumonia season from December through March from the following geographically well-distributed cities. Samples were taken from the Pumping Station located at Second and N Streets, S. E., Washington, D. C. They consisted of domestic waste and street wash. The liter of raw sewage received from Cincinnati, Ohio, was collected near the mouth of a large city sewer; it was of the combined type and contained some industrial waste as well as domestic waste and street wash. The other sewage samples, supplied

by 6 U. S. Marine Hospitals and a local hospital, were collected at the final clean-out traps before the sewage entered the city system. The samples were sent in liter pyrex bottles by railroad express; no sample was in transport more than 3 days. All samples were adjusted to pH 7.0, stored in the cold room (10° to 12°C.) and examined for phage content before the lapse of a week.

Doctor H. E. Trimble, Medical Officer in Charge, U. S. Marine Hospital in Galveston, kindly supplied sewage samples from three hospitals. These were pooled and treated as one representative Galveston sample. Because a strong disinfectant odor was noted in the Boston hospital sample, a filtrate of the sewage was tested for inhibition of the pneumococcus. The pneumococcus grew satisfactorily in the presence of the filtrate, hence the sample was treated like the others.

After a sewage sample was centrifugated to throw down the suspended matter, the cleared sewage was filtered through large "N" Berkefeld candles. Eight ml. of each sewage filtrate, the amount employed per tube for phage isolation, was tested for sterility. Sterile sewage filtrates were stored in rubber-stoppered flasks in the cold room; contaminated sewage filtrates were refiltered until sterile and then stored.

Clean, clear test tubes of uniform diameter were used in all tests. Serial enrichment passages in the sewage medium were made in 6 x 3/4" tubes; 6 x 1/2" tubes were

used for all other purposes.

All filtrations were made through "N" Berkefeld candles. Small quantities of material were filtered through 2-1/2" candles stoppered to 250 ml. flasks; large amounts of sewage were filtered through 5" candles equipped with liter flasks.

Maintenance of cultures: Thirty pneumococcus types, I through XXXII, exclusive of XXVI and XXX, were maintained in stock in 2% rabbit blood broth (low heat beef infusion). The pneumococcus grew very rapidly in this medium and lost much of its viability if incubated more than 18 hours. The young blood broth cultures were sealed with sterile vaseline and stored at 4 to 6°C. for one month; monthly transfers to fresh blood broth were made to insure viability. Fresh mouse strains of the commoner pneumococcus types were frequently added to the stock. Thus both smooth and rough stock strains and virulent mouse strains were included in the attempt to isolate either a type specific or species specific phage.

Beta hemolytic streptococcus strains (N.I.H. designation) 751, 563, 594, and 693, sensitive to the action of phages A, B, C and D, respectively, were stocked in 10 per cent rabbit blood broth sealed with vaseline.

Isolation Methods: Enrichment passages:

The "sewage medium" used in the search for phage contained equal parts of fresh sewage filtrate and double

strength low-heat beef infusion broth.

Thirty types of pneumococci were employed in the search for pneumophage in the sewage samples, i.e., Types I through XXXII, exclusive of Types XXVI and XXX. All types were cultured in 1% rabbit blood broth for 10 to 16 hours before planting in the sewage medium.

In following a multiple strain inoculation method of enriching any pneumococcus phage present in a sewage filtrate, the young pneumococcus type cultures were grouped as follows: Strains of Types I through VIII were designated group A; those of Types IX through XVI, group B; those of Types XVII through XXIII, group C; and those of Types XXIV through XXXII, group D. The cultures of each group were mixed by adding 2 or 3 drops of each type to 9.0 ml. of broth. By this technique, the 30 strains were included in four series of sewage filtrates to alternately enrich and filter. Time, media, and filters were economized in this way.

In addition to examining the hospital sewages for pneumococcus phage, a new technique (not used in the city sewage samples) was included which allowed for simultaneous isolation of streptococcus phages. The finding of streptococcus phages would check the efficacy of the phage isolation methods if there were no positive pneumophage isolations, and data would be obtained on the distribution of streptococcus phages in sewage. Therefore, strains of young streptococcus culture sensitive to the action of four

known races of streptococcus phage originally isolated from sewage were planted with the 4 groups of pneumococci, one drop each, as follows: Strain 751 sensitive to streptococcus phage A was planted with group A of mixed pneumococcus types; Strain 563, sensitive to phage B, with group B; Strain 594, sensitive to phage C, with group C; and 693, sensitive to phage D, with group D.

Each serial passage[1] contained 2 possible sources of phage: (1) the sewage filtrate making up the sewage medium, and (2) the filtrate of the previous serial passage. Each sample of sewage was examined for pneumococcus and streptococcus phages by the same technique which was as follows: The sewage medium was prepared fresh daily by placing 8.0 ml. of sewage filtrate and 8.0 ml. of double-strength low-heat broth in each of four large test tubes. In many experiments, the sewage filtrates were enriched in duplicate, i.e., one series was incubated in large test tubes and the other series in 300 ml. Erhlenmeyer flasks which allowed greater aeration. In the first serial passage in a given sewage filtrate medium, 4.0 ml. (20%) of sterile sewage culture filtrate was added as the second possible source of bacteriophage. It was obtained by planting raw, unfiltered sewage in broth, incubating overnight at 37°C., centrifuging and filtering. Theoretically, on incubation

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[1]i.e., passage of sewage filtrate with mixed cultures of pneumococci and streptococci in sewage medium.

of the bacteria present in a sewage sample, the various phages present in the sewage, specific for these bacteria, would be enriched and as a result the filtrate of such a culture would contain more potent phages.

Therefore, in the first serial passage of a given sewage filtrate, there were present in each of four tubes (1) 16 ml. of sewage medium and (2) 4 ml. of sewage culture filtrate. To one of these tubes was added a drop of group A mixed culture of pneumococci and streptococcus 751; to the 3 remaining tubes a drop containing mixtures of group B plus streptococcus 563, C plus streptococcus 594, and D plus streptococcus 693, respectively. The tubes were labeled I_{A+751}, I_{B+563}, I_{C+594} and I_{D+693}. "I" designated the first passage of sewage filtrate with the mixed culture of pneumococci and streptococci, "A +751" designated the particular group of pneumococci and streptococcus, etc. Broth controls were prepared by planting the same inocula in tubes containing 10 ml. of double strength broth diluted with 10 ml. of distilled water. The cultures were incubated at 37°C. for 16 hours (overnight) or less. After incubation, the tubes were examined for evidence of lysis by comparing the turbidity which had developed in the sewage medium with the turbidity of the same inoculum in plain broth. After recording lysis, the four sewage medium mixed cultures were filtered, labeled "Filtrate I_{A-751}" etc., and 4 ml. of each filtrate was tested for sterility.

The second and succeeding serial filtrate passages with the 4 groups of mixed cultures were made in fresh sewage medium (8 ml. of sewage filtrate and 8 ml. of double-strength broth), to which was added the filtrates of the preceding passage. For example, for the second passage cultures 4 ml. of filtrate I $_{A+751}$ were added to 16 ml. of fresh sewage medium, and the combination was planted with a drop of young group A pneumococci plus 751; 4 ml. of filtrate I $_{B+563}$ was added to sewage medium and inoculated with a drop of young group B pneumococci plus 563, etc. The latter cultures were labeled II $_{A+751}$, and II $_{B+563}$, etc. They were incubated at 37°C., examined for lysis with suitable controls, and filtered. The filtrates obtained were employed in preparing the third passages with the pneumococci and streptococci. This was the process employed of alternately specifically enriching (by feeding) and filtering. At least 5 passages were made for each series; in several series a greater number of passages were made.

<u>Testing of Mixed Sewage Culture for Phage Content</u>: Filtrates of the third and fifth serial passage were routinely tested for lytic activity with the individual strains as described below. When lysis of a mixed culture of pneumococci and streptococci appeared, the lysed culture was filtered and the filtrate tested for lytic activity on the individual cultures which made up the enrichment

group; the third and fifth passage filtrates were tested regardless of lysis. For example, Galveston sewage culture I $_{D+693}$ showed a definite clearing after incubation and was immediately filtered; 0.5 ml. of filtrate was added to each of 9 tubes containing 4.5 ml. of broth. Seven tubes were planted as follows: two drops of young pneumococcus culture of Type XXIV, XXV, XXVII, XXVIII, XXIX, XXI, or XXXII; the eighth tube was planted with one drop of young streptococcus culture 693; the ninth tube was left uninoculated to serve as a sterility control for the filtrate. Broth culture controls were made by planting the same amount of each inoculum into 4.5 ml. amounts of broth. The cultures were incubated at 37°C. and read at frequent intervals during the day to prevent any possibility of secondary growth developing before lysis had been observed. Readings were also made the following day after the tubes had stood overnight in the cold room.

When a pneumococcus or streptococcus passage culture appeared less turbid than its broth culture control, the lysate and its broth culture control were streaked on blood agar in order to observe any plaque formation, and, next, the lysate was diluted with an equal quantity of sterile broth, filtered and retested for lysis in dilutions of 10^{-1}, 10^{-3} and 10^{-5} with fresh homologous broth culture. This range of dilutions was selected to avoid any pseudo-negative readings which might result from the zone phenomenon in which there is failure of lysis in the

low dilutions of the phage with complete lysis in higher dilutions.

Filtrates of pneumococcus broth-control cultures were tested for lysis of the homologous strain in dilutions of 10^{-1}, 10^{-3} and 10^{-5} in parallel tests.

Results

Table I gives a diagrammatic picture of the technique employed in searching for phages present in Evansville sewage. It may be observed that none of the multiple cultures in sewage filtrate media showed evidence of lysis; yet the filtrates of the third and fifth passages "inhibited" pneumococcus growth and contained streptococcus phages. For this reason, the filtrates of the sewage medium cultures were routinely tested for lytic activity toward the individual strains after the third and fifth serial passages. However, in the case of other hospital sewage filtrates, lysis of mixed serial cultures was often evident.

The four types of streptococcus phage as differentiated and identified by Evans (1934b) were easily isolated by the technique employed in this work. Table II indicates that phage D occurred in every sewage sample examined; phages A and C were found in four out of seven samples (57 per cent of sewages); and phage B occurred in three out of seven (43 per cent incidence). Since samples of sewage filtrates vary in their streptococcus phage content

a series of samples should be examined before the search for a phage is abandoned.

Data on the isolation of pneumococcus inhibiting agents and streptococcus phages are collected in Tables III and IV. Approximately 230 serial filtrates were either examined for evidence of lysis or tested for lytic activity on the individual strains before passing. Table III shows that many of the streptococcus phages were isolated from the filtrate after the same number of passages. For example, three phages were found in third filtrate passages of the Evansville sewage; probably phage D had developed in the filtrate before the third passage, but since there was no evidence of lysis in the mixed culture the filtrate was not tested for lysis of individual strains until after the third passage.

Enriched filtrates obtained from each hospital sample and from one city sample of sewage were capable of inhibiting pneumococcus growth; Table IV describes (1) the source and passage number of the filtrates which prevented growth of the pneumococcus inoculum; (2) the number of times the agents were isolated for each sensitive type, and (3) the number of times the inhibiting agent could be transmitted.

It has already been stated that enriched filtrates were tested for lytic activity on the individual strains making up the enrichment group by adding two or three drops of pneumococcus culture to a 10^{-1} dilution of filtrate.

Employing this technique all the pneumococcus types, with the exception of types X, XIII, XVI, XVII, XVIII, XX, XXVII and XXXII were apparently "lysed" by one or more enriched sewage filtrates. Types III and XII were particularly sensitive. The cultures, in the presence of filtrate, usually developed only a slight turbidity, whereas the broth culture controls become definitely cloudy in five or six hours. However, the fact that none of these pneumococcus "lysates" ever demonstrated plaques in pneumococcus blood agar cultures and the failure to obtain indefinite transmissibility conclusively proved that this phenomenon was not induced by a pneumophage.

At the same time that a pneumococcus culture, appearing clearer than the culture control, was diluted and filtered for re-testing, a filtrate of the broth culture control was likewise tested for lytic action by the same technique. Control culture filtrates never inhibited or lysed the pneumococci--to the contrary, growth was often stimulated in the 10^{-1} dilution. The appearance of inhibiting agents was referable, then, to some property derived from the sewage rather than from the pneumococcus culture.

Discussion

The finding of phage D in all samples of sewage agreed with the unpublished findings of Dr. Evans, who secured a D type phage in practically all city sewage samples

studied. Furthermore, she observed that, as in this work, phages A and C were secured with considerable more difficulty in fewer sewage filtrates. Phage B was present in a greater per cent of city sewages than phages A and C, but the reverse was true in hospital sewage. The difference was an interesting one, in view of the fact that phage B is known to lyse many animal strains of streptococcus but few, if any, human strains, whereas phages A and C lyse many human types. Possibly these facts might account for the relative incidence of the phages in sewage from the two sources.

The ease with which streptococcus phages were isolated indicates that a lytic principle specific for the pneumococcus was not present in the sewage samples examined, or else that the technique employed was not applicable to pneumophage isolations. Since both stock and fresh mouse strains of 30 pneumococcus types were included in the study the failure to obtain a phage can not be attributed to the lack of a sensitive strain. The large number of passages for each sewage filtrate with the pneumococci would seem to eliminate any chance of losing a weak pneumophage present in sewage on account of insufficient enrichment.

It is rather difficult to conceive how a pneumophage can be isolated from the sputum of pneumonia patients, as reported by Rodigina (1938), yet not be found in hospital sewage collected during the pneumonia season. Assuming that Rodigina actually obtained a lytic principle from

sputum necessitates the additional assumption that this phage, unlike the usual races, can not survive in sewage. The frequent inhibition and pseudo-lysis of pneumococcus by sewage filtrates may be explained: (1) on the basis of the well-known autolysis of this species, which is stimulated in the presence of certain chemical agents, such as bile and saponin, or (2) on the possibility of weak action by the streptococcus phages (see part V of this study). In some instances, the inhibiting agent was transmissible in low dilutions for a few passages; however, on diminution in concentration by dilution the effect disappeared. This could be due either to loss of the chemical agent or to "starving" out of streptococcus phages.

The isolation from sewage of principles lytic for a wide range of pathogens, both intestinal and otherwise, which stimulated this work, attests to the significance of the results regardless of the failure to find a pneumophage.

Table I

A Search for Pneumococcus and Streptococcus Phages in Evansville Marine Hospital Sewage

Serial Passage	Lysis of mixed cultures after incubation				Inhibition of Pneumococci by filtrate				Lysis[5] of streptococci by filtrate			
	A+751[1]	B+563[2]	C+594[3]	D+693[4]	A+751	B+563	C+594	D+693	A+751	B+563	C+594	D+693
I	–[6]	–	–	–	not tested					not tested		
II	–	–	–	–	not tested					not tested		
III	–	–	–	–	+[7]	–	+	+	+	–	+	+
IV	–	–	–	–	not tested					not tested		
V	–	–	–	–	+	–	–	–	+	–	+	+

1 designates types I – VIII of pneumococci and strep. 751
2 designates types IX – XVI of pneumococci and strep. 563
3 designates types XVII – XXIII of pneumococci and strep. 594
4 designates types XXIV – XXXII of pneumococci and strep. 693

5 i.e. transmissible
6 – designates no evidence of lysis
7 + designates less turbidity than in broth control

Table II

Incidence of Four Serological Types of Streptococcus Phage in Hospital Sewage

Phage	Incidence in samples examined in percentages
A[1]	57
B	43
C	57
D	100

1 Phages A, B, C and D were isolated by means of sensitive streptococcus cultures 751, 563, 594 and 693 respectively.

Table III

Pneumococcus Inhibiting Agents and Streptococcus Bacteriophages Isolated from City and Hospital Sewage during the Pneumonia Season.

Source of sewage	Date Received	No. of sewage filtrate passages with mixed cultures[1]	Pneumococcus "inhibiting agent"	Streptococcus phages			
				A/751[2]	B/563	C/594	D/693
1. Wash. D.C. City pumping station	12-4-39	6	0[3]	not studied			
2. Wash. D.C. City pumping station	1-3-40	6	IV[4]	not studied			
3. Cinn., O. Street Wash, domestic & industrial waste	1-17-40	6	0	not studied			
4. Balt., Md. U. S. Marine Hospital	2-5-40	7	III	III	III	III	III
5. Boston, Mass. U.S. Marine Hosp.	2-9-40	6	III	VI	VI	0	II
6. Wash. D. C. Columbia Hosp.	2-14-40	6	III	0	0	III	III
7. Evans. Ind. U.S. Marine Hospital	2-21-40	6	III	III	0	III	III
8. N. Y. City U.S. Marine Hospital	3-20-40	5	III	0	0	0	III
9. Galves., Texas pooled: U.S. Marine Hosp., John Sealy Hosp., St. Mary's Infirmary.	3-22-40	5	II	V	V	III	I
10. Memphis, Tenn. U.S. Marine Hosp.	3-28-40	4	IV	0	0	0	IV

1. i.e. of pneumococci and streptococci
2. A/751 (See previous table)
3. 0 signifies that no lytic agent was found in the sewage filtrate.
4. The Roman numeral indicates the number of filtrate passages made with mixed culture in the sewage medium before lytic or inhibiting activity appeared.

Table IV

Occurrence of Pneumococcus Inhibiting Agents for 30
Types of this Species in Sewage Filtrates

Pneumococcus type	Filtrates causing "inhibition" of the pneumococcus	No. of times isolated	No. of times transmitted
I	Ev[1] III_A[2] NT[3]	1	0
II	Ev III_A T_7	1	7
III	Balt III_D NT; VII_A NT Ev III_A NT; V_A NT NY III_D NT Gal. II_D NT; V_A NT	7	0
IV	Ev III_A NT	1	0
V	Balt III_A NT; Ev III_A NT	2	0
VI	Ev III_A NT	1	0
VII	Ev III_A NT	1	0
VIII	Bos. VI_A NT; Ev III_A T_3	2	3
IX	Bos. VI_B NT	1	0
X	None	0	0
XI	Balt. III_A NT	1	0
XII	Balt III_A T_2; III_B NT; III_D NT; $IV_A T_4$; Balt IV_D T_2; Bos. III_A NT; III_B NT; III_C NT; Bos. III_D NT; Col III_C NT; Ev III_A NT; III_C NT; NY III_B NT; III_D NT; V_D NT; Gal II_D NT; III_D NT; V_A NT.	18	4
XIII	None	0	0

1. For explanation of Ev., Balt., etc see table III
2. Roman numerals designate number of passages made with mixed culture; letter indicates one of four groups of mixed cultures
3. T plus figure indicates number of times transmissible
4. NT signifies not transmissible

Table IV (continued)

Pneumococcus	Filtrates causing "inhibition" of the pneumococcus	No. of times isolated	No. of times transmitted
XIV	Bos. VI_BNT	1	0
XV	Bos. VI_BNT	1	0
XVI	None	0	0
XVII	None	0	0
XVIII	None	0	0
XIX	Bos. VI_CNT	1	0
XX	None	0	0
XXI	NY III_CNT; Gal. III_CNT	2	0
XXII	Wash. IV_CNT; Bos. VI_CNT	2	0
XXIII	Bos. VI_CNT; NY III_CNT; Gal. III_CNT	3	0
XXIV	Balt. IV_DNT; Gal. III_DNT; Mem. IV_DNT	3	0
XXV	Balt IV_DNT	1	0
XXVII	None	0	0
XXVIII	Mem. IV_DT_2	1	2
XXIX	Bos. VI_DNT; Gal. III_DNT	2	0
XXXI	Bos. III_DNT; Col. V_DNT; Gal. V_DNT	3	0
XXXII	None	0	0

IV

THE ISOLATION AND SEROLOGICAL IDENTIFICATION OF A NEW TYPE OF STREPTOCOCCUS BACTERIOPHAGE

Four serological types of streptococcus phage have been described by Evans (1934 b) and designated A, B, C and D; the reactions of the hemolytic streptococci to these phages classed them into phagological groups which generally correspond with groups formed on other bases. It appeared that another type of phage, actively lysing a given sub-group of streptococci, might aid in classifying that group.[1] Hence several samples of hospital sewage were examined for new phage types and one was isolated. It also lysed the pneumococcus in the nascent state.

Experimental Procedures

A. Methods of Isolation

Strains: It was impossible to foretell which strains would be susceptible to a yet unisolated phage or phages. Therefore, three strains of streptococci representing each of two sub-groups based on sugar fermentations were employed in the isolation of the new phage type. The National Institute of Health designations for the strains were:

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[1]Dr. Alice Evans selected strains of streptococci representing two sub-groups with this fact in mind. All investigations relating to reactions of the streptococci with the new phage type will be included in future publications from her laboratory. It is with reactions of this phage toward the pneumococci that Part V of this thesis is concerned.

Nos. 846, 1268, 1387, 657, 985 and 990.

Sewage Samples: Five of the samples of hospital sewage--those obtained from the Baltimore Marine Hospital, the Boston Marine Hospital, the Columbia Hospital for Women, the Evansville Marine Hospital and the New York Marine Hospital--were examined for phage.

Multiple Inoculation Technique: (See Tables V and VI.) One drop of each of the six young cultures was pooled in 10 ml. of neopeptone broth, a drop of which was planted into sterile sewage medium made up of 8 ml. of a sewage filtrate, 8 ml. of double strength neopeptone broth and 4 ml. of sewage culture filtrate. This latter filtrate was obtained by planting unfiltered sewage in sterile broth, incubating overnight at 37° C. and filtering through a Berkefeld filter. A drop of the mixed inoculum planted in 10 ml. of double strength broth plus 10 ml. of distilled water served as the culture control.

The cultures were incubated overnight at 37° C. The following morning the sewage medium culture was examined for evidence of lysis by comparing its turbidity with that of the broth culture control. The sewage medium culture was filtered and the filtrate was enriched a second time with the mixed, diluted culture of streptococci in the sewage medium if lysis had not occurred, i.e., 4 ml. of this fresh filtrate was added to 16 ml. of sewage medium and the mixture was planted with a drop of diluted mixed suspension

of streptococci. This process of alternate enrichment and filtration was continued until lysis of the mixed culture appeared or until the enriched filtrate had been serially passed 5 times. All third-passage filtrates were routinely tested for phage for the individual strains regardless of whether or not lysis of the mixed culture had occurred.

Testing of Mixed Sewage Culture for Phage Content: It has been explained (part III) that the range of lytic activity of phage is widened in the presence of growing, sensitive cells. Hence, it was not known whether a given mixed culture showing lysis contained a phage which, in the filtered state would lyse more than one of the strains. For the determination of phage content of the filtrate, it was tested for lysis of each strain as follows: To 4.5 ml. of neopeptone broth 0.5 ml. of filtered lysate and 1 drop of young streptococcus culture were added; 1 drop of young culture in plain broth represented the control. The tests for lysis were incubated at 37° C. and read frequently during the day. When a culture was cleared the tube was immediately stored in the refrigerator for further study.

B. Combined Method of Purifying and Increasing the Potency of Freshly Isolated Races[1] of Phage.

The new phage races were purified by a series of titrations which both "starved" and diluted out all other contaminating races which might have been present. For instance, phage D/693, a very widespread sewage type, may have been contaminating a new race, but when it was not enriched with culture 693 and was constantly diluted in the titration series, it disappeared. Likewise, while isolating a pure type of phage by titration methods, the phage in question was strengthened by continuous passage with its homologous strain. (See Table VII.) For example, the first enriched filtrate obtained from a mixed culture in the Baltimore Hospital sewage medium lysed strain 985 in nine hours. This lysate was diluted with 20 ml. of sterile broth, filtered and titrated with its homologous organism (strain 985) as follows: Tenfold dilutions ranging from 10^{-1} through 10^{-8} were made in 9 ml. quantities of sterile neopeptone broth. (Separate pipettes were used for each dilution in all tests.) A drop of young culture of 985 was planted in each dilution and in a broth control. Usually the series was incubated at 37° C. and examined frequently during the day, stored in the cold room over-

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[1]The term "race" applied to phage is analogous to the term "strain" with reference to bacteria. Each phage isolated is a new race irrespective of the serological type.

night and reexamined for lysis the following morning. The titer of the phage was regarded as the highest dilution producing lysis of its homologous organism; the lysate resulting from the highest active dilution of phage was filtered for the second titration, the whole dilution process being repeated at least five times to insure elimination of all contaminating phages and to increase the potency (titer) of the type-pure phage.

C. Identification of the New Streptococcus Phage Type by Cross Neutralization Tests.

Neutralization tests with the untyped races of phage and antisera for phages A, B, C, D and D_2[1;2]: The purpose of these tests was to identify races of phage (freshly isolated from sewage) with one of the recognized types or to differentiate them as one or more new serological types. (See Table VIII.) Each type antiserum and a normal serum control was diluted 1:100 and 1:200 in neopeptone broth, and the untyped phage was diluted so that 0.1 ml. contained approximately 100 phage particles.[3] One-tenth ml. of

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[1] D_2 is a phage isolated by Doctor Evans (1935) that lyses streptococcus 693, but differs antigenically from phage D.

[2] The sera had been prepared and used by Doctor Evans (1934 b).

[3] To compute this dilution, the titer of the phage was determined, i.e., if it was 10^{-6}, then 10^{-5} contained approximately 10 particles per ml., 10^{-4} contained 100 particles per ml., 10^{-3} contained 1000 particles per ml. or 100 per 0.1 ml.

diluted phage was added to the serum dilutions and to a tube of sterile broth. Phages A, B, C, D and D_2 were added in similar concentrations to dilutions of homologous antiserum in order to control the specificity of the sera in the given dilutions Phage-culture controls were made in normal serum similarly diluted to 1:100 and 1:200.

After the phage-serum mixtures had stood overnight in the cold room, each tube of mixture and a tube of sterile broth were planted with a drop of young homologous streptococcus and incubated at 37° C. Readings were made only after the phage-culture controls were lysed in the normal serum dilutions and were inhibited in the respective antiserum dilutions. The data in Table VIII show that Evansville phage 985 and Baltimore phage 985 represent one or more new serological types, since neither was neutralized by the stock antisera.

Production of Antibacteriophagic Serum for a New Type of Streptococcus Phage: An antilysin specific for Evansville phage 985 was prepared by injecting a rabbit intravenously into the marginal ear vein with filtered phage-lysed cultures. A total quantity of 55 ml. of potent phage was injected over a six-weeks period, starting with 2 ml. doses and gradually increasing the amount to 10 ml. doses. The rabbit was bled from the ear a week

after the last injection[1]. The serum was drawn off and preserved with merthiolate (1:10,000). The rabbit was bled again from the ear a week later without further inoculations.

Neutralization Tests with Phages A, B, C, D, D_2 and "Baltimore Phage 985", and Antiphage Serum for the New Type:[2] The purpose of these tests was (1) to demonstrate the antigenic specificity of the new type, (2) to ascertain the serological specificity and titer of the antiphage serum prepared with this type and (3) to determine whether the Baltimore race was antigenically similar to the race used in preparation of the serum. (See Table IX.) Normal and antiphage sera were diluted 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} in neopeptone broth (7 tubes for each dilution). Phages A, B, C, D, D_2 and the two newly isolated races were diluted so that 0.1 ml. quantities contained approximately 100 phage particles. Each was planted in the diluted normal and immune serums. After the phage-serum mixtures were incubated for 15 hours at 37° C., a drop of homologous culture was planted in each tube and in sterile broth. Both 12° C. and 37° C. were satisfactory temperatures for allowing the phage-serum mixtures to react.

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[1]After the ear was shaved and cleansed with alcohol, a small cut was made in the marginal ear vein near the tip and 25 ml. were collected in a wide-mouthed tube.

[2]designated Evansville phage 985.

The tests were incubated at 37° C. until the phage-culture mixtures in the normal serum dilutions were cleared and the broth control cultures were turbid; readings were then made.

Results

Two of the five hospital sewage samples (obtained from Baltimore, Boston, Washington, Evansville and New York) contained phage lytic for streptococcus No. 985 representing a certain sub-group of streptococci. Phages specific for strains 846, 1268, 1387, 657 and 990 were not present in the sewage samples examined.

Table V indicates that the Baltimore sewage sample contained a lytic principle sufficiently active in the first enrichment passage to lyse strain 985. The filtrate containing the phage was passed twice again with the other five streptococcus strains; 985 was omitted in order that any other races of phage present in the sewage filtrate might be enriched by one or more of the streptococcus strains making up the mixed culture. No phage was isolated for these strains.

A second race of phage active for strain 985 was isolated from Evansville Hospital sewage. (See Table VI.) Evidence of lysis of the mixed enrichment culture did not appear until the third filtrate passage; then it was only slight as compared with the first filtrate passage of the Baltimore sample. However, a second race of phage was

isolated specific for 985, and enrichment passages were made twice again with all the streptococcus strains with the exception of 985. Again phage isolations for these strains were unsuccessful.

Table VII shows that the titer of "Baltimore phage 985", only 10^{-3} when first isolated from the sewage filtrate, gradually increased in potency to 10^{-6}, the maximum titer obtained. The freshly isolated "Evansville phage 985" possessed a titer of 10^{-2} which was gradually extended by the same titration methods to a maximum of 10^{-6}.

Though the two races of phage cleared homologous cultures of 985 rapidly and distinctly, secondary growth developed in approximately four hours after complete lysis had occurred. As a result, overnight incubation of lytic tests with these phages was not feasible.

Both races of phage were demonstrable by development of plaques on glucose agar seeded with sensitive strain 985.

The cross neutralization tests with the Baltimore and the Evansville races of phage 985 and antisera of stock phages of the various types revealed antigenic differences. The results of these tests, summarized in Table VIII, show that antiphages A, B, C, D and D_2 contained no specific antibodies capable of inhibiting lytic action (by neutralization) of the two 985 phages. This signified a serologically distinct race or races of lytic principle.

It was then necessary to determine whether these new races, proven to be antigenically different from the stock phages, belonged to the same type. Antilytic serum specific for "Evansville phage 985" was prepared in a rabbit.

Data on cross neutralization tests with antilytic serum for "Evansville phage 985" are presented in Table IX. The results show that: (1) Evansville and Baltimore phages 985 belonged to the same phage type; antiserum prepared with the Evansville phage neutralized the Baltimore race in the same dilutions; (2) since the antiphage contained no neutralizing antibodies for phages A, B, C, D or D_2, its homologous phage and likewise the Baltimore phage belong to a fifth serologically distinct type; and (3) the antiphage was potent, capable of inhibiting lysis in a 10^{-5} dilution.

The serological type of the Evansville and Baltimore phages was designated E.

Discussion

It was noteworthy that the new phage E was successfully isolated from two sewage samples which also contained several other types previously described by Evans (1934 b). The sample of Baltimore Hospital sewage filtrate yielded 5 different serological types of streptococcus lytic principle, including the new type E; the sample of Evansville Hospital Sewage filtrate contained 4 types--E and A, C and D. The results indicate the relative ease with which

streptococcus bacterophage can be demonstrated in sewage filtrates.

Successful isolation of a lytic principle for any species from sewage filtrate depends upon the use of a sensitive strain; however, there is no characteristic which distinguishes a phage-susceptible strain. The presence of a single sensitive strain, No. 985, provided the necessary substrate suited to bacteriophagy

Sensitivity of the pneumococci to phage E, in the filtered and nascent state, was investigated in part V.

Conclusive points of evidence that the two phage races which lysed 985 belonged to a new serological type were presented in the following order: (1) Strain 985 was not lysed by any of the four known types of streptococcus phage, and it was sensitive to phages present in Baltimore and Evansville sewage; (2) the new phages were not neutralized by any of the antisera specific for the stock phages; (3) highly potent antiserum produced against the Evansville race neutralized the Baltimore race, but none of the stock types.

Table V

Isolation of a New Streptococcus Phage Type from Baltimore Marine Hospital Sewage

Passage no. of filtrate enriched with mixed culture	Appearance of mixed culture after incubation in sewage medium	Lytic action of filtered culture on individual strains
1	lysed[2]	Strain 985 effectively lysed; lysate filtered and purified with homologons strain[3]
2[1]	Turbid	None
3	Turbid	None

1. Passages with the mixed cultures were continued with the possibility of isolating a phage specific for others of the mixed group (Strain 985 omitted).
2. Although there was only a phage for 985 present, the "nascent" phage lysed the resistant strains.
3. This race of phage was designated "Baltimore phage 985" until serologically typed.

Table VI

Isolation of a New Streptococcus Phage Type from Evansville Marine Hospital Sewage

Passage No. of filtrate enriched with mixed culture	Appearance of mixed culture after incubation in sewage medium	Lytic action of filtered culture on individual strains
1	turbid	none
2	turbid	not tested
3	slightly lysed	strain 985 effectively lysed; lysate filtered and purified.[2]
4[1]	turbid	none
5	turbid	none

1. Passages with the mixed cultures were continued with the possibility of isolating a phage specific for others of the mixed group (Strain 985 omitted).
2. This phage designated as "Evansville phage 985" until serologically typed.

Table VII

Purification and Increase of the Lytic Potency of a Freshly Isolated Race of Phage[1]

No. of titrations	Lysis[2] in phage dilutions of:							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1	+	+[3]	±	−	−	−	−	−
2	+	+	+	+[3]	−	−	−	−
3	+	+	+	+	+[3]	−	−	−
4	+	+	+	+	+[3]	−	−	−
5	+	+	+	+	+	+[4]	±	−

1. Designated as "Baltimore phage 985" until serologically typed
2. i.e. of the homologous strain 985.
3. The dilution selected for the next titration
4. Final titer of this phage was 10^{-6}

Table VIII

Cross Neutralization Tests on "Baltimore phage 985" and "Evansville phage 985" with Antisera of all Stock Streptococcus Phage Types

Phage	Culture	Dilutions of rabbit sera											
		Normal[1]		Antiphage A		Antiphage B		Antiphage C		Antiphage D		Antiphage D_2	
		1:100	1:200	1:100	1:200	1:100	1:200	1:100	1:200	1:100	1:200	1:100	1:200
"Evansville 985"	985	+[2]	+	+	+	+	+	+	+	+	+	+	+
"Baltimore 985"	985	+	+	+	+	+	+	+	+	+	+	+	+
A	751	+	+	-[3]	-	+	+	+	+	+	+	+	+
B	563	+	+	+	+	-	-	+	+	+	+	+	+
C	594	+	+	+	+	+	+	-	-	+	+	+	+
D	693	+	+	+	+	+	+	+	+	-	-	+[4]	+
D_2	693	+	+	+	+	+	+	+	+	-[5]	-	-	-

1. Phage - culture controls in normal serum of same dilutions
2. + designates lysis which, in turn, signifies that there were no neutralizing antibodies in the serum
3. - designates no lysis (turbid as broth culture control) which, in turn, signifies that there were neutralizing antibodies in the serum.
4. antigenic difference between phages D and D_2
5. antigenic similarity between phages D and D_2

Table IX

Cross Neutralisation Tests of Antiphage E Serum with Phages A, B, C, D, D_2 and "Baltimore phage 985"

Phage	Culture	Dilutions of rabbit sera							
		Normal				Antiphage E			
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
E	985	+[1]	+	+	+	-[2]	-	-	-
Baltimore phage 985	985	+	+	+	+	-	-	-	-
A	751	+	+	+	+	±[3]	+	+	+
B	563	+	+	+	+	±[3]	+	+	+
C	594	+	+	+	+	+	+	+	+
D	693	+	+	+	+	+	+	+	+
D_2	693	+	+	+	+	+	+	+	+

1. + designates lysis which, in turn, signifies that there were no neutralising antibodies in the serum
2. - designates no lysis (turbid as broth culture control) which, in turn, signifies that there were neutralising antibodies in the serum
3. zone phenomenon

V

A STUDY OF THE LYTIC ACTION OF FILTERED AND NASCENT STREPTOCOCCUS PHAGES ON THE PNEUMOCOCCI

In part III above sewage filtrates were described which inhibited the pneumococcus, but the principle of which was not transmissible. Since these same filtrates were shown to contain streptococcus phages, it was reasoned that possibly the lytic action of these heterologous phages was responsible for the dissolving of the pneumococci; that although the phages were capable of lysing the pneumococcus, they could not propagate on this species and thus would "starve" for lack of a sensitive streptococcus substrate. In order to determine the effect of heterologous phage on the pneumococcus, the lytic activity of streptococcus, staphylococcus, and coli phages was investigated for the pneumococcus.

In addition, it was observed that when a streptococcus phage attacked a sensitive streptococcus of the mixed pneumococcus streptococcus suspension in the sewage medium, the pneumococci often appeared to be lysed. This indicated an enhanced lytic potency of the streptococcus phage in the presence of its sensitive organism. Therefore, the lytic action on the pneumococci was determined for the following types of nascent phage: five types of streptococcus phage, one type of staphylococcus phage, and one type of coli phage. Those showing a positive dissolving action

were studied in greater detail.

Experimental Procedures and Results

Lytic Action of Heterologous Phage Filtrates on the Pneumococci: Pneumococcus types I through XXXII (exclusive of XXVI and XXX which are antigenically similar to VI and XV, respectively) were tested for sensitivity to streptococcus phage types A, B, C, D and E, to a staphylococcus phage and to a coli phage. To avoid unnecessary repetition future reference to "phage" will apply to the lytic filtrate; "nascent phage" will refer to the phage in the presence of sensitive bacteria. For each strain of pneumococcus four tubes containing 4.5 ml. of neopeptone broth were planted with enough 10-hour 1 per cent blood broth culture[1] to produce a definite turbidity. Thus with a definite turbidity initially present, there could be no error in mistaking inhibition of pneumococcus growth for lysis. The lytic activity of a given race of phage was determined by planting three of the tubes with phage filtrate in dilutions of 10^{-1}, 10^{-3} and 10^{-5}, respectively[2], and a fourth tube without phage served as the broth culture control. Similar dilutions of phage planted with strepto-

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[1]In planting with the blood broth culture, the blood cells which settled to the bottom of the tube were carefully avoided.

[2]Three dilutions were included because of the possibility of a zone phenomenon.

cocci alone made up the phage controls. Altogether, for each phage tested, there were 120 tubes containing broth suspensions of the 30 pneumococcus types. The tubes, incubated at 37° C., were read for lysis at frequent intervals.

None of the 30 pneumococcus strains was sensitive to the lytic action of any of the 5 streptococcus, Staph. aureus or Es. coli phage filtrates in any dilution. (See Table X.) The 10^{-1} dilution of the streptococcus, aureus and coli phages stimulated the growth of many of the strains of pneumococci, as determined by comparing with the turbidity of the broth culture controls. The stimulation occurred during the first four to six hours, and might be attributed to the presence of metabolic products of the lysed streptococci.

Lytic Action of Heterologous Nascent Phages on the Pneumococci: Four tubes containing 4.5 ml. of neopeptone broth and planted with enough 10-hour blood broth culture of each pneumococcus to produce a definite turbidity, were incubated for one to two hours to allow the pneumococci to begin active multiplication in the new medium[1]. After this period the lytic action of the nascent phage was tested by first adding to three of the tubes filtered phage to make dilutions of 10^{-1}, 10^{-3} and 10^{-5}; the three tubes

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[1]In order that lysis readings could be made on turbid, actively growing cultures of pneumococci.

were then planted with a drop of sensitive streptococcus, so that each tube contained pneumococci and phage in the presence of its sensitive organism. The pneumococci in the remaining fourth tube were heat-killed to serve as the turbidity control. For phage controls similar dilutions of phage were planted with sensitive streptococci alone. For each nascent phage tested, 120 tubes containing broth suspensions of the 30 pneumococcus types were required. The tests, incubated at 37° C., were examined for lysis at frequent intervals. Lysis was recorded as positive when a pneumococcus culture, in the presence of nascent phage, became less turbid than its heated turbidity control.

Table XI indicates that nascent streptococcus phages A, B and C possessed no lytic activity for any of the 30 pneumococcus strains. The nascent staphylococcus and coli phages were also ineffective. That the lytic power of these phages for the homologous strain was unimpaired by the presence of pneumococci was demonstrated by streaking on blood agar. For example, after suitable incubation of a suspension of pneumococci in the presence of nascent phage C, all streptococci originally present in the mixture were found to be lysed and only actively growing pneumococci remained to grow on the blood agar.

Nascent streptococcus phages D and E lysed all 30 of the pneumococcus strains. The results are presented in Tables XII and XIII. Lysis always occurred in the 10^{-3} and 10^{-5} dilutions; it was usually irregular and less distinct

in the 10^{-1} dilution. Pneumococci were more clearly and distinctly lysed by nascent phage D. Lysis generally appeared first in the 10^{-3} dilution and shortly afterwards in the 10^{-5} dilution, though in some instances there was a simultaneous clearing of the pneumococci in these two dilutions or the 10^{-5} dilution cleared first. Nascent E phage cleared the 30 pneumococcus strains in the 10^{-3} and 10^{-5} dilutions. As shown in Table XIII lysis could not be determined after 8 hours incubation because of secondary growth of the streptococcus.

<u>Relative Proportions of Pneumococci and Streptococci preceding, during and after Lysis of the Pneumococcus by Nascent Phage</u>: When nascent heterologous phages were found capable of lysing the pneumococci, the action was studied in detail by streaking a loopful of culture on blood agar at frequent intervals before, during and after lysis of the pneumococcus. The conditions necessary for lysis of the pneumococci were determined by the following technique: the 10^{-1}, 10^{-3} and 10^{-5} dilutions of nascent phage D were planted in broth containing turbid suspensions of type I pneumococci, and at 0, 1, 2, 3, 4, 5½, 24 and 48 hours of incubation at various temperatures (see Table XIV) the tests were read for lysis and immediately afterwards streaked on blood agar to determine the relative numbers of pneumococci and streptococci[1] at any

[1] i.e., by the development of alpha and beta hemolytic colonies, respectively.

given period during the lytic process. Controls of pure pneumococcus and streptococcus broth cultures and of the phage-streptococcus mixture were also streaked simultaneously.

Tables XIV and XV point out the relative proportions of pneumococci and streptococci before, during and after lysis of the pneumococcus by nascent phages D and E. A detailed interpretation of the results is included in the tables. The experiments demonstrated that lysis of the pneumococci occurred only after initial lysis of the sensitive streptococcus. The secondary clouding was due to the multiplication of the sensitive streptococcus.

<u>Lysis of Killed Pneumococci by Nascent Streptococcus Phages</u>: Lysis of killed pneumococci was tested by the same method employed in testing the lysis of the living pneumococci: 10^{-1}, 10^{-3} and 10^{-5} dilutions of phage were planted in slightly clouded suspensions of heat-killed pneumococci plus a drop of sensitive streptococcus culture. Lysis was recorded as positive when the killed suspension was less turbid than the control suspension without nascent phage.

Killed pneumococci were lysed in the 10^{-1}, 10^{-3} and 10^{-5} dilutions of nascent phages D and E, but it was noted that lysis was not as complete as with living cells. In contrast with lysis of the living pneumococci, clearing first occurred in the lowest phage dilutions. (See Table XVI.

Tests for Absorption of Heterologous Phage Filtrates by the Pneumococcus: Tests for absorption or destruction of heterologous lytic filtrates by the pneumococcus were made by planting phage, diluted 10^{-1}, 10^{-3} and 10^{-5} in suspensions of pneumococci of approximately the same turbidity as that employed in the lytic tests. After six hours incubation, the tubes were stored in the cold room for two days and then centrifugated at high speed for 1½ hours to remove the cells. The supernaatant fluids were titrated in tenfold broth dilutions to determine the decrease in phage titer as compared with phage control tubes to which pneumococci had not been added.

Table XVII indicates that there was no absorption of either phage D or E by the pneumococci under the conditions of the previously described lytic tests. Thus pneumococcus absorption of the streptococcus phage preliminary to the lysis of the sensitive strain appeared not to be a factor in the secondary lysis of the pneumococcus.

Titration of Nascent D Phage with the Pneumococcus:

To determine the range of dilution in which nascent D phage would lyse type I pneumococcus, 1.0 ml. amounts of a 10-hour blood broth culture were planted in 12 tubes containing 9.0 ml. of neopeptone broth and incubated for one hour at 37° C. To each of 10 of the tubes containing phage D in dilutions of 10^{-1} through 10^{-10} a drop of sensitive streptococcus culture was added. One tube to which

merthiolate (1:10,000) was added served as an initial turbidity control; the remaining tube served as the pneumococcus broth culture control. A similar series of tubes was planted with phage D and a drop of the streptococcus culture in order to determine the titer of the lytic filtrate. The tubes, incubated at 37° C., were read at frequent intervals.

The results in Table XVIII show that the pneumococci were lysed by nascent D phage in every dilution which the streptococci were lysed by the filtrate.

Lytic Action of Nascent D Phage on Pneumococci of Varying Turbidities: The supernatant fluid was aspirated from twelve 10-hour blood broth cultures of type I pneumococcus and was centrifugated for one hour. The packed cells were emulsified in 5.0 ml. of neopeptone broth. This stock suspension was used for preparing suspensions with turbidities equivalent to 75, 100, 200 and 400 p.p.m. of the silica standard in 4.5 ml. of broth. Four tubes of each containing 4.5 ml. of broth were prepared for each concentration. Three tubes of each series were planted with phage D[1] in 10^{-2}, 10^{-4} and 10^{-6} dilutions plus a drop of sensitive streptococcus culture. To preserve controls with the initial turbidity, merthiolate was added to remaining tube of each series. Phage controls of the same dilutions without pneumococci were made for comparative readings.

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[1]The titer was 10^{-6}.

The pneumococci were lysed by two or more dilutions of nascent D phage irrespective of the initial turbidity. (See Table XIX.) However, it was noted that lysis progressed to the definitely cleared stage only with the concentration of 75 p.p.m. or less concentrations as shown in tests not included in the table. The degree of lysis of the pneumococci decreased with increasing concentrations, although it progressed beyond the initial turbidity control in each series.

Discussion

Preliminary to the testing of the lytic action of the various heterologous phage filtrates on the pneumococci, the methods of other workers were considered. Clark and Clark (1927) included only the three "fixed" types in testing the action of a streptococcus phage; Balsamelli (1935) studied only four types. Since the 30 types used in this study were a mixture of mouse and stock cultures, the possibility of including sensitive strains appeared to be greater. Brainard and Noble (1935) included both stock and recently isolated strains in an attempt to adapt certain types of pneumococci to a streptococcus phage, but in this case the workers limited themselves to the study of only one streptococcus phage. The results obtained in part V of this paper clearly indicate that neither fresh mouse passage strains nor stock strains of any of the 30 types of pneumococci were lysed by the five different streptococcus phage

filtrates. Confusion due to the zone phenomenon was avoided by the use of three phage dilutions. The high species specificity of streptococcus phages was thereby demonstrated. The possibility of obtaining a pneumophage by the action of streptococcus phage on the pneumococci seems to have been thoroughly investigated. The fact that no dilution of phage filtrates A, B, C, D or E lysed any of the pneumococci indicated that these phages were not the non-transmissible pneumococcus inhibiting agents found in the sewage filtrates as described in part III. The coli and staphylococcus phage filtrates were also ineffective lytic agents for the pneumococcus--an anticipated result since the phages of the more closely related streptococcus group were incapable of dissolving the pneumococci. Brainard and Noble (1935) described a dysentery phage and Balsamelli (1935) a "strepto-staphylococcus" phage which were inactive toward the pneumococcus. The growth stimulatory effect on the pneumococci of heterologous phage filtrates in 10^{-1} dilutions appeared to be due to the presence of metabolic products of the lysed streptococci which offered additional growth substances of some nature.

The enhanced potency of a phage in the presence of its sensitive organism was evidenced by the dissolving of pneumococcus suspensions by two different streptococcus phages in the nascent state. These two phages were "activated" to the extent of lysing a species, although related, quite distinct from the streptococci for which the

lytic filtrates were highly specific. Since phages A, B and C in the presence of the homologous streptococci did not possess the property of lysing the pneumococci, it seemed possible that the pneumococcus might inhibit lysis of the streptococcus. If this occurred, there could be no secondary lysis of the pneumococci because there had been no initial lysis of the sensitive streptococcus. Yet experiments previously mentioned demonstrated that the homologous streptococci for phages A, B and C were lysed in a turbid pneumococcus suspension. Therefore, the various streptococcus phage types, in the presence of the homologous streptococci, differed in their capacity for lysing pneumococci.

No one of the nascent streptococcus phages A, B and C, staphylococcus phage or coli phage was "activated" or enhanced to the point of dissolving pneumococci. In connection with this, Rakieten (1933) reported that his staphylococcus "lysin" produced by the action of phage on sensitive staphylococci did not dissolve pneumococci.

On studying the relative number of pneumococci and streptococci in cultures preceding, during and after lysis of the former by nascent streptococcus phage, the findings clearly showed that the dissolution of pneumococci took place only after initial lysis of the homologous streptococcus. As previously noted, lysis of the pneumococci in the 10^{-1} dilution of nascent phage was slower and less distinct than in the 10^{-3} and 10^{-5} dilutions, a result probably

explained on the basis of the zone phenomenon.

Rakieten (1938) observed that pneumococcus types I, II and III had no demonstrable absorbing activity toward staphylococcus or enterococcus phage and he had previously reported the inactivity of nascent staphylococcus phage toward the pneumococcus (1933). In the present work, experiments have been presented which indicate that pneumococci neither absorbed nor destroyed a streptococcus phage which, in the nascent state, was capable of lysing this species. Therefore, in these experiments phage absorption by the pneumococci was not a preliminary process in their secondary lysis.

Clearing of the pneumococcus suspension was independent of the dilution of phage provided a trace of sensitive streptococci was present. However, no concentration of nascent phage completely dissolved pneumococcus suspensions more turbid than 75 p.p.m.

In the present study, no premise is made as to the identity of the agent capable of lysing the pneumococcus which is produced during the lysis of sensitive streptococci. In the early course of this work, the "agent" was found to be non-transmissible, a result which logically invites consideration of a chemical concept of dissolution, since it has been pointed out by several workers that lysis of the pneumococcus, a species which readily undergoes autolysis in broth culture, is produced or accelerated by several chemical agents. That pneumococci are soluble in

bile was shown by Neufeld as early as 1900. Downie, Stent and White (1931) reported that of the chemical substances which they tested, saponin and the sodium salts of many of the bile-acids produced "lysis". These observations suggest the production of a dissolving substance during the lysis of sensitive streptococci which is possibly similar in action to chemical agents such as bile; yet it must be emphasized that lysis by nascent phage occurs in a complex biological system in which the nature of the action of two of the components, phage and sensitive bacteria, is still incompletely explained. Add to this already complex combination a third constituent in the form of a living heterologous species, and the explanation of the results secured is obscure. If this proposed explanation is maintained, then it must be assumed that only in some streptococcus phage-lysing combinations is there produced a chemical entity having the ability to lyse the pneumococcus. It is interesting, too, that only streptococcus nascent phages possessed this property, an observation which suggests a conceivable relationship between the two species.

Table X

Lytic Inactivity of Heterologous Phage Filtrates for the Pneumococci

Pneumococcus types	Lytic action of heterogous phage filtrates in dilutions of 10^{-1}, 10^{-3} and 10^{-5}						
	A[1]	B	C	D	E	aureus	coli
I thru XXXII	0[2]	0	0	0	0	0	0

1. A, B, C, D and E are streptococcus phages
2. O designates no lytic activity

Table XI

Lytic Inactivity of Some Nascent[1] Heterologous Phages for the Pneumococci

Pneumococcus types	Lytic action of heterologous phage filtrates in dilutions of 10^{-1}, 10^{-3}, and 10^{-5}				
	A[2]	B	C	aureus	coli
I thru XXXII	0[3]	0	0	0	0

1. "Nascent" refers to lytic filtrate in the presence of a drop of sensitive streptococci
2. A, B, and C are streptococcus phages
3. O designates no lytic activity

Table XII

Lytic Action of Nascent[1] Phage D/693 on Pneumococci of Thirty Types

Type	3 hours at 37° C.			3 hrs. at 37° C.; then overnight in cold room			8 hrs. at 37° C.; then 24 hrs. at room temp.		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
I	−[2]	+[4]	−	−	+	+	+	±	−
II	±[3]	+	−	±	+	+	±	+	−
III	−	+	±	−	+	+	−	+	−
IV	−	+	−	−	+	+	±	+	±
V	−	+	−	−	+	+	±	+	−
VI	−	+	−	−	+	+	+	+	−
VII	−	+	−	−	+	+	±	−	−
VIII	−	+	−	−	+	+	±	+	±
IX	−	+	−	−	+	+	−	±	−
X	±	+	±	−	+	+	±	+	−
XI	±	+	±	−	+	+	±	+	−
XII	±	+	±	−	+	+	±	±	+
XIII	±	+	±	−	+	+	±	±	±
XIV	−	+	−	−	+	±	±	±	−
XV	−	+	−	−	+	+	+	−	±
XVI	−	+	−	−	+	+	+	±	+
XVII	−	+	−	−	+	+	±	−	±
XVIII	±	+	+	−	+	+	±	−	±
XIX	−	+	±	−	+	+	±	−	±
XX	−	+	±	−	+	+	±	+	−
XXI	−	+	±	−	+	+	−	±	±
XXII	−	+	+	−	+	+	±	+	±
XXIII	−	+	±	−	+	+	±	+	±
XXIV	−	+	±	−	+	+	±	+	−
XXV	−	+	−	−	+	+	±	−	+
XXVII	−	+	±	−	+	+	+	±	±
XXVIII	±	+	+	±	+	+	−	±	−
XXIX	−	+	±	−	+	+	±	±	±
XXXI	−	+	+	−	+	+	−	±	±
XXXII	−	+	+	−	+	+	−	+	±
693	+	+	±	+	+	+	+	±	±

[1] "Nascent" refers to lytic filtrate in the presence of a drop of sensitive streptococci.
[2] − designates no lysis (like broth culture control).
[3] ± designates partial lysis (less turbid than broth culture control).
[4] + designates complete or almost complete lysis (less turbid than turbidity control).

Table XIII

Lytic Action of Nascent[1] Phage E/985 on Pneumococci of Thirty Types

Type	2 hrs. at 37° C.			4 hrs. at 37° C.			8 hrs. at 37° C.			Then 24 additional hrs. at room temp.		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
I	−[2]	−	+[4]	−	+	±	±	−	+	−	−	−
II	±[3]	±	±	±	+	±	±	±	+	−	−	−
III	±	−	±	±	+	±	±	±	+	−	−	−
IV	−	−	−	±	+	±	+	+	+	−	−	−
V	−	−	−	±	+	±	±	±	+	−	−	−
VI	−	−	−	−	+	±	+	+	+	−	−	−
VII	±	±	−	−	+	−	−	−	+	−	−	−
VIII	−	−	−	−	+	±	±	±	+	−	−	−
IX	±	±	−	−	+	±	±	±	+	−	−	−
X	−	−	−	−	+	±	±	±	+	−	−	−
XI	−	−	−	−	+	±	+	+	+	−	−	−
XII	−	−	−	−	+	±	−	+	±	−	−	−
XIII	±	−	−	−	+	±	±	−	+	−	−	−
XIV	−	−	−	−	+	−	−	+	±	−	−	−
XV	±	−	−	−	+	±	±	+	+	−	−	−
XVI	−	−	−	−	+	±	±	±	+	−	−	−
XVII	−	−	−	−	+	±	±	+	+	−	−	−
XVIII	±	−	−	−	+	±	±	±	+	−	−	−
XIX	±	−	−	−	+	±	±	+	+	−	−	−
XX	−	−	−	−	+	±	±	+	+	−	−	−
XXI	−	−	−	−	+	±	±	±	+	−	−	−
XXII	−	−	−	−	+	±	+	+	+	−	−	−
XXIII	±	±	±	−	+	±	±	±	+	−	−	−
XXIV	−	−	−	−	+	±	+	+	+	−	−	−
XXV	−	−	−	−	+	−	+	±	+	−	−	−
XXVII	−	−	−	−	+	−	+	±	+	−	−	−
XXVIII	±	−	−	−	+	±	−	−	+	−	−	−
XXIX	±	−	−	−	+	±	+	+	+	−	−	−
XXXI	±?	−	−	−	+	±	−	−	+	−	−	−
XXXII	−	−	−	−	−	−	+	+	+	−	−	−
985	+	−	−	+	+	±	±	±	+	−	−	−

1. See Table XII for definition.
2. − designates no lysis (like broth culture control).
3. ± designates partial lysis (less turbid than broth culture control).
4. + designates complete or almost complete lysis (less turbid than turbidity control).

Table XIV

Relative Numbers of Pneumococci and Streptococci preceding, during and after Lysis of Type I Pneumococcus by Nascent[1] Phage D/693

Hours of Incubation	Dilutions of nascent[1] phage D/693.											
	10^{-1}				10^{-3}				10^{-5}			
	Control		Pneumococcus added		Control		Pneumococcus added		Control		Pneumococcus added	
	Turbidity of broth	Colonies on blood agar	Turbidity of broth	Colonies on blood agar	Turbidity of broth	Colonies on blood agar	Turbidity of broth	Colonies on blood agar	Turbidity of broth	Colonies on blood agar	Turbidity of broth	Colonies on blood agar
0	-[4]	None (phage had been absorbed by str.[2]; lysis occurred on plate)	-	Numerous pn.[3]; some str.	-	Numerous (absorption not as complete as in 10^{-1}	-	Numerous pn.; more str. than in 10^{-1} dil. (fewer phage particles)	-	More numerous than in 10^{-3} dil. (less absorption)	-	Numerous pn.; more str. than in 10^{-3} dil. (less absorption)
1	-	None (see 0 hrs.)	-	Numerous pn.; one str. (most of the str. had absorbed phage; lysis occurred on the plate.)	-	Fewer, phage-eaten	-	Numerous pn.; fewer str. than at o hr.	-	Numerous	-	Numerous pn.; les str. than at 0 hr (absorption was progressing)
2	+[6]	None (lysis had occurred in the broth	-	All pn. (str. had been lysed by phage)	-	None (absorption complete; lysis had occurred on plate	-	Numerous pn.; more str. than at 1 hr. (stimulation?)	-	Phage-eaten	-	Numerous pn.; more str. than at 1 hr. (stimulatio n?)
3	+	None	-	All pn.	+	None (lysis complete)	±[5]	Few pn.; no str. (str. had been lysed)	-	None (absorption complete; lysis had occurred on plate)	±	Fewer pn. and str. than a t 2 hr.
4	+	None	-	All pn.	+	None	±	No. pn.; few str.	+	None (lysis complete)	±	None (too few viable cells to be picked up in loop)
5 1/2	+	2	-	All pn.	+	3	+	None (too few viable cells to be picked up in loop)	+	1	+	None
Then, overnight in cold room	+	Few	-	All pn.	+	Few	+	Few str.	+	Few	+	Few str.
Then, 24 hrs. at room temp.	+	None	±	Pn.; a few str.	+	None	+	None	+	Few	+	Few str.
Then, 24 more hrs. at room temp.	+	None	+	No. pn.; a few str.	+	None	+	2 str.	+	Few	+	Several str.

1. See Table XII for definition
2. Str. designates streptococcus
3. Pn. designates pneumococcus
4. - designates no lysis (like broth culture control)
5. ± designates partial lysis (less turbid than broth culture control)
6. + designates complete or almost complete lysis (less turbid than turbidity control).

Table XV

Relative Numbers of Pneumococci and Streptococci preceding, during and after Lysis of Type I Pneumococcus by Nascent Phage E/985[1].

Hours of Incubation	Dilutions of nascent phage E/985. 10^{-1} Control: Turbidity of broth	Colonies on blood agar	10^{-1} Pneumococcus added: Turbidity of broth	Colonies on blood agar	10^{-3} Control: Turbidity of broth	Colonies on blood agar	10^{-3} Pneumococcus added: Turbidity of broth	Colonies on blood agar	10^{-5} Control: Turbidity of broth	Colonies on blood agar	10^{-5} Pneumococcus added: Turbidity of broth	Colonies on blood agar
0	-	Phage-eaten	-	Pn. and Str.	-	Numerous as broth culture control (no immediate absorption as with E/693).	-	Numerous str.; a few pn.(str. not yet acted upon by the phage were so numerous that they had inhibited the growth of pn. on the plate)	-	Numerous as broth culture control (no immediate absorption as with E/693.	-	Str.so numerous that growth of pn. on agar was inhibited
1	-	Phage-eaten	-	Numerous pn.; a few str.(str. had absorbed phage)	-	Numerous; some phage-eaten (beginning absorption of phage)	-	Numerous str.; pn. were appearing (phage absorption was progressing)	-	Numerous; some phage-eaten (absorption of phage was beginning)	-	Numerous str .; pn. were appearing (phage a bsorption was beginning.
2	+	Few and phage-eaten	-	All pn. (str. had been lysed)	-	Numerous and phage-eaten	-	Equal number of pn. and str.(lysis of str.was beginning)	-	Numerous and phage-eaten	-	Equal numbers of str. and pn.
3	+	Few and phage-eaten	-	All pn.	+	Phage-eaten	-	Almost all pn.(str. had been lysed)	-	Numerous and phage-eaten	-	Almost all p n.(str. had been lys ed)
4 1/2	+	Several (phage-resistant cells were developing)	-	Many pn.; a few phage resistant str.	+	Numerous as broth culture control (phage-resistant cells were developing	+	Few pn.; numerous resistant str.	±	Numerous and phage-eaten	±	Almost all str.
5 1/2	±	Numerous (secondary growth)	-	Numerous pn.; a few str.	±	Numerous as broth culture control (secondary growth)	+	Numerous str.; no pn.	±	Numerous and phage-eaten	±	Almost all str.
Then, overnight in cold room	±	Numerous as broth culture control	±	Numerous str.(secondary growth had concealed complete lysis of pn.)	-	Numerous as broth culture control	±	No. pn.; numerous str.(secondary growth of the str.)	+	Numerous resistant cells)	+	No. pn. numerous resistant str.
10	-	Numerous as broth culture control	-	Numerous str.	-	Numerous as broth culture control	-	Num rous str.	±	Numerous (secondary growth	±	Numerous str. (secondary growth of the str.

1 See Table XIV for legend.

Table XVI

Lysis of Killed Pneumococci by Nascent[1] Streptococcus Phages.

Phage D/693

Type	2 hrs. at 37° C.			3 hrs. at 37° C.			5 hrs. at 37° C.; then overnight in cold room		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
I	±[3]	-[2]	-	+[4]	+	-	+	+	+
II	±	-	-	+	+	-	+	+	+
III	±	-	-	+	+	-	+	+	+
IV	±	-	-	+	+	-	+	+	+
V	±	-	-	+	-	-	+	+	+
693	+	-	-	+	+	-	+	+	+

Phage E/985

Type	2 hrs. at 37° C.			3 hrs. at 37° C.			5 hrs. at 37° C.; then overnight in cold room		
	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}	10^{-1}	10^{-3}	10^{-5}
I	±	-	-	±	±	-	+	+	+
II	±	-	-	±	±	-	+	+	+
III	±	-	-	+	±	-	+	+	+
IV	+	-	-	+	±	-	+	+	+
V	+	-	-	+	+	-	+	+	+
693	+	±	-	+	+	-	+	+	+

1. See table XII for definition
2. - designates no lysis (like broth culture control).
3. ± designates partial lysis (less turbid than broth culture control).
4. + designates complete or almost complete lysis (less turbid than turbidity control).

Table XVII

Test for Absorption of Streptococcus Phages by Type I Pneumococcus.

Initial phage dilution + pneumococci	Phage D(after "absorption")					Phage E (after "absorption")					Phage controls (no pneumococci)				
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10^{-1}	+[1]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-3}			+	+	+			+	+	+			+	+	+
10^{-5}					+					+					+

[1]Designates complete lysis.

Table XVIII

Titration of Nascent D/693 Phage with the Pneumococcus

Dilution of Phage	3 1/2 hrs. at 37° C.		5 hrs. at 37° C.		Then, overnight in cold room	
	Control	pneumo added	Control	pneumo added	Control	pneumo added
10^{-1}	+[3]	-[1]	+	-	+	±
10^{-2}	+	-	+	±	+	+
10^{-3}	+	±	+	+	+	+
10^{-4}	±[2]	-	+	+	+	+
10^{-5}	-	-	±	+	+	+
10^{-6}	-	-	+	+	+	+
10^{-7}	-	-	-	-	+	+
10^{-8}	-	-	-	-	-	-
10^{-9}	-	-	-	-	-	-
10^{-10}	-	-	-	-	-	-

1. - Designates no lysis (like broth culture control)
2. ± Designates partial lysis (less turbid than broth culture control)
3. + Designates complete or almost complete lysis (less turbid than turbidity control).

Table XIX

Lytic Action of Nascent D/693 Phage on Pneumococci of Varying Turbidities.

Turbidity of Pneumo. Cultures (ppm).	3 1/2 hrs. at 37° C.			Then, overnight in cold room			Then, 6 add. hrs. at 37° C.			Then, 2 days in cold room		
	10^{-2}	10^{-4}	10^{-6}	10^{-2}	10^{-4}	10^{-6}	10^{-2}	10^{-4}	10^{-6}	10^{-2}	10^{-4}	10^{-6}
75	+ [3]	+	– [1]	+	+	+	–	+	+	± [2]	–	+
100	–	+ [4]	–	–	+	+	–	+	+	±	±	±
200	–	+	+	–	+	+	–	+	+	+	–	–
450	–	+	–	–	+	+	–	+	+	–	–	–
Control: 693 only	+	+	–	+	+	+	+	+	+	–	+	+

1. – Designates no lysis (like broth culture control)
2. ± Designates partial lysis (less turbid than broth culture control)
3. + Designates complete or almost complete lysis (less turbid than turbidity control)
4. Lysis was only crystal clear with 75 ppm of pneumococci or less. Lysis progressed at all concentrations of pneumococci beyond the initial turbidity control, but not completely.

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VI

SUMMARY AND CONCLUSIONS

Ten samples of city and hospital sewage collected from geographically-well-separated cities during the pneumonia season were examined for pneumococcus phage and for the four known serological types of streptococcus lytic principle. A minimum of 230 culture filtrates, specifically enriched with multiple cultures of pneumococci and streptococci, were either read for lysis or tested for lytic activity on the individual strains employed in the enrichment mixtures.

The various types of streptococcus phage, differentiated by Evans (1934b), and readily isolated from hospital sewage by the technique employed, were distributed as follows: Phages A and C, in 57 per cent of samples; phage B, in 43 per cent; and phage D, in 100 per cent. From these results it was concluded that hospital sewage samples differ in streptococcus phage content, a fact which must be considered in the search for any given streptococcus race of lytic principle.

Notwithstanding the ready isolation of streptococcus phages, which served to check the efficacy of the technical methods used in searching for pneumophages, the latter was not isolated from the enriched sewage filtrates. The large number of filtrates tested with stock and mouse strains of 30 pneumococcus types suggests that pneumophage, if existent, can not survive in sewage, or that the isolation

technique was not satisfactory.

The appearance of pneumococcus inhibiting agents in specifically enriched sewage filtrates was tentatively explained on the basis of autolysis, which is stimulated in the presence of certain chemical substances. The effect of the inhibiting agents, found in sewage, occasionally "transmissible" in low dilutions for a few passages, was lost on continued diminution of concentration.

A new serological type of streptococcus phage, hitherto unreported, was isolated from 2 out of 5 samples of hospital sewage. It was purified and identified serologically by cross neutralization tests with stock antilytic sera and antilysin prepared against the new type. Since the four stock phages had been designated A, B, C and D, the new phage was given the designation "E".

A study of the sensitivity of the 30 types of pneumococci to the lytic action of 5 streptococcus phages, one staphylococcus phage and one coli phage in the filtered and nascent state was made. None of the stock or mouse pneumococcus strains was sensitive to any of the filtered principles, a result which demonstrates the high species specificity of the streptococcus phages and which indicates the impossibility of securing a pneumophage by the action of streptococcus phages on pneumococci. Nascent phages A, B, C, coli and staphylococcus were also inert against the pneumococci.

A detailed study was made of nascent phages D and E

which dissolved broth suspensions of the 30 pneumococcus strains. Among the observations made were: (1) Lysis of pneumococci by nascent phage occurred only after initial lysis of the sensitive streptococcus: (2) killed pneumococcus suspensions were also cleared by nascent phages D and E, but not so completely as living cultures; (3) with the concentrations of pneumococci employed in the tests, heterologous phage absorption was not a preliminary step in the secondary lysis of the pneumococci; (4) pneumococci were lysed to the same dilution of nascent streptococcus phage as were the sensitive streptococcus cells to filtered principle; (5) complete or almost complete lysis of pneumococci by nascent phage occurred only when the concentration of the pneumococcus was 75 p.p.m. or less; (6) a theoretical discussion of the lysis of pneumococci by some nascent streptococcus phages is presented.

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