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CHARACTERIZATION OF STAPHYLOCOCCUS BACTERIOPHAGES

DIFFERENTIAL CHARACTERIZATION AND SELECTION OF STAPHYLOCOCCUS BACTERIOPHAGES

by

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A Thesis

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I. INTRODUCTION AND PURPOSE

Bacteriophages, regarded today as viruses parasitic on bacteria, are characterized by a number of well-defined properties, one of these being the specificity exhibited by some varieties of phages, not only for a single species of bacteria, but also for certain strains or types of that single species. A practical application of this property has been the use of bacteriophage for the identification or phage typing of certain strains of a given species.

Fisk (1942) introduced a method of phage typing of staphylococci which has been successfully used for tracing sources of infection in epidemic outbreaks of staphylococcal enterotoxin food-poisoning and of staphylococcal infections of the skin in babies in maternity hospitals. Although this method is relatively simple and rapid, many strains of staphylococci are resistant to the action of the available phages and the absolute specificity of the phage types is questionable.

The purpose of this work is to investigate the growth and multiplication requirements and the stability of the staphylococcus bacteriophages now available, and to use anti-bacteriophage sera (prepared by immunizing rabbits with selected phage strains) as a means for recognition of specificity.

If the phages could be divided into a few representative groups, it is hoped that by a careful selection of these phages, a simpler method of phage typing applicable in every laboratory may be developed.

II. REVIEW OF THE LITERATURE

A. HISTORICAL OUTLINE:

The original observations on transmissible bacterial autolysis were made by Twort in 1915. While engaged in a search for vaccine virus, he observed that some cultures of a staphylococcus from vaccine lymph developed "glassy-looking", transparent areas in the midst of the normal opaque growth and that a small amount of this vitreous material when transferred to successive cultures of the staphylococcus would induce the same change. Bacteria-free filtrates from the affected cultures, even when highly diluted, were also active in causing this effect in fresh growth of the organism. Twort was undecided as to the nature of the filterable agent but he supposed, without expressing any conclusive opinion, it might be some kind of a parasitic organism which destroyed the bacteria, or probably a substance derived from the bacteria themselves, since it occasionally reappeared in glassy cultures months after subculture.

Two years later, d'Herelle (1917) observed that the filtrate of the fecal discharges of a patient recovering from bacillary dysentery rapidly lysed a young broth culture of the causative organism and that this type of lysis was transmissible in series. Throughout his extensive studies on the subject, (d'Herelle 1921, 1926, 1930) he considered the agent responsible for the lytic effect to be a submicroscopic living organism to which he gave the name "bacteriophage", which implied that the submicroscopic organism was a parasite upon bacteria.

There can be no doubt that the findings of Twort and of d'Herelle are examples of the same process though d'Herelle has strenuously opposed this view; hence the lytic action has come to be known as the "Twort-d'Herelle phenomenon", and the name bacteriophage, originally applied to the lytic agent by d'Herelle, is now generally used, usually in the abbreviated form "phage".

Following its discovery the bacteriophage aroused great interest and the hope of practical application in the treatment of bacterial infections sustained investigation for a period. When the hopes in this direction did not materialize, interest in the agent subsided, until around 1930 it was revived by the recognition of the similarity of the bacteriophages with the viruses which grow in the cells of plants and animals. The size of different phages was estimated by filtration (Elford and Andrewes 1932), and confirmed more recently by the electron microscope (Luria, Delbruck, and Anderson 1943); the neutralization of phage by specific antiserum was extensively studied by Andrewes and Elford (1933a, b), Burnet, Keogh, and Lush (1937), Kalmanson, Hershey, and Bronfenbrenner (1942), Delbruck (1945), and others; the host specificity of a large group of phages was studied, and the receptor spots on the bacterial cell, to which the phage is specifically absorbed, were found to be identical with a bacterial antigen (Burnet 1934, Levine and Frisch 1934, Burnet and Lush 1935, Boumtree 1947b). Extensive investigations were carried out on certain groups of phages, in particular on the staphylococcus phages by Northrop (1939), Fisk (1942, 1944), Wilson and Atkinson (1945), Williams Smith (1948a, b), salmonella and

coli-dysentery phages by Burnet (1934), Delbruck (1942, 1945), Delbruck and Luria (1942), and others, cholera phages by Asheshov et al (1933), and typhoid phages by Craigie and Yen (1938), Craigie (1946).

In recent years there has been an increasing realization of the value of bacteriophages as materials for basic studies. Because of their non-pathogenicity, the ease and economy of their production, the availability of a rapid, simple, accurate method of assay, their capacity for growth on bacteria cultured in a synthetic medium, they are being selected as models in the study of the nature of virus reproduction, in the study of the phenomenon of virus interference, and as test agents in the search for possible chemotherapeutic substances against virus diseases. They are also being used in the study of the process of acquired resistance which bears a relation to the unsolved problem of virus immunity, as an aid in the classification of closely related groups or species of bacteria, or for differentiation of strains of a given species, in the study of bacterial metabolism, and in the study of viral and bacterial genetics which may lead to the solution of genetic problems which other studies have not yet resolved.

These agents may thus be regarded as ultramicroscopic technicians whose study may prove the key to several unsolved problems of biology.

B. GENERAL PROPERTIES OF BACTERIOPHAGES:

1) Nature:

Ever since the discovery of the bacteriophage by Twort and d'Herelle, speculation has arisen regarding the exact origin and nature

of the agent responsible for the lysis of growing bacteria. The claim by d'Herelle that the bacteriophage is an ultramicroscopic filterable organism, a sort of parasite living on bacteria, although greatly upheld by him and his supporters, has been contested by many authors and a number of alternative theories have been put forward.

Kabeshima (1920) interpreted the bacteriolytic phenomenon as being due to ferment action. He suggested that the phage was a catalytic agent originating in the mucous membranes of the intestinal canal (or in the leucocytes), which activated a pro-ferment normally present in the bacteria. Once liberated in an active form, the ferment induced enzyme digestion of the organisms.

Bordet (see Bordet and Guica 1920, Bordet 1923, 1925) regarded the lytic agent as a bacterial enzyme produced as "the result of an inherited vitiation of bacterial metabolism". According to his theory, the phenomenon is a true autolysis resulting from a disturbance of the normal equilibrium between the assimilative and metabolic activities of the bacterial cell. This autolysis liberates substances (bacterial enzyme) which communicate to susceptible but unaffected cells an exaggerated tendency to produce descendants which are liable to similar autolysis, the process, once started, being transmissible in series.

Other hypotheses related the phenomenon to the genetics of the bacterial cell. Thus Wollman's "gene theory" (1925, 1927) regarded the bacteriophages as the substantial bearers of hereditary qualities, while Bail's "splitter" doctrine (1925) identified them with "splitter" fragments of the essential hereditary determinants (chromosomes or

genes?) of the bacterium. These fragments when liberated were supposed to be adsorbed only to bacteria having similar units in their hereditary determinants and so affect these units that their normal balance of anabolic-catabolic control was displaced toward an entirely disruptive action on the organism.

Hadley (1927) postulated that the phage was a genelike substance representing a "filterable stage in the cyclogeny of the bacteria", a view which closely connected phage with the variation and mutation of bacteria.

The concept of phage as enzymes has been strongly advocated by a number of investigators. Northrop (1939) demonstrated the similarity in the production of an extra-cellular gelatinase and of phage from a lysogenic strain of Bacillus megatherium and concluded that a formal analogy exists between the production of phage and the formation of enzymes. Similar views were adopted by Kalmanson and Bronfenbrenner (1939) and Krueger and Scribner (1941).

A great deal is now known of the physical and constitutional properties of the phages, the study of which has brought considerable evidence that they possess most of the properties commonly ascribed to the animal and plant viruses. The electron microscope has been of the greatest use in determining their sizes and rather complicated shapes. The morphology of certain phages active against Escherichia coli has been fully described by various workers (Luria and Anderson 1942, Luria, Delbruck, and Anderson 1943, Delbruck 1946); they have been shown to be either sperm-shaped bodies or spherical structures without tails. Luria et al (1943) have shown that 3 out of 4 Escherichia coli phages they

studied had an opaque "head" consisting of a pattern of granules about 80 millimicrons in diameter and a less opaque "tail" about 120 millimicrons long. Some beautifully clear micrographs of purified coliphage T7 have shown it to be essentially spherical with a diameter of 51 millimicrons, with a bilobar arrangement of internal structure (Kerby et al 1949). A phage attacking Streptococcus lactis has been found to have a head 70 millimicrons in diameter and a tail about 150 millimicrons long and 30 millimicrons in diameter (Parmalee, Carr and Nelson 1949). Either round particles or tadpole-shaped structures with varying degrees of internal differentiation have been described for staphylococcus (Luria et al 1943), and pyocyaneus phages (Schultz et al 1948).

In constitution phages are also similar to plant and animal viruses, being complexes consisting of protein, nucleic acid and lipid (Taylor 1946, Cohen and Anderson 1946, Hook et al 1946, Kerby et al 1949).

Like other viruses they are resistant and sensitive to the same physical and chemical agents. Phage inactivation by shaking (Campbell-Renton 1942), drying and freezing (Campbell-Renton 1941), ultraviolet light (Baker and Hanavutty 1929, Campbell-Renton 1937, and others), methylene blue (Schultz and Krueger 1928), lecithin (Levin and Lominski 1936), cholesterol, cephalin, bacterial and non-bacterial phospholipins (Williams, Sandholzer, and Berry 1940) has been reported, although different strains of phage, like different species of bacteria, differ from one another in their resistance to various physical and chemical agents.

Phages are also similar to other viruses in that they are specifically adapted to certain host cells which they may destroy or in which they

may continue to exist in a latent state.

In view of the numerous similarities between bacteriophages and other viruses, the present-day concept is that phages are to be classified with the animal and plant viruses as "bacterial viruses". They are to be regarded as "a group of filterable infectious agents of complex morphological and genetic structure which are capable of reproduction only in specific bacterial hosts".

ii) Distribution in Nature:

Bacteriophages are widely distributed in nature. As shown by Twort and by d'Herelle, they may be obtained from filtrates of faeces, from sewage, or from practically any material that has been subjected to faecal pollution, such as soil samples, surface waters, etc. They have also been isolated from exudates, diseased tissues, and filtrates of ground-up flies or roaches. These bacteria-free filtrates may be found to be active against dysentery, coliform, typhoid, or other bacteria, and may contain several strains of phage which can be separated and propagated indefinitely in cultures of susceptible organisms.

Bacteriophages are obligate parasites and, as such, are dependent upon bacterial host cells for their existence. Consequently wherever particular species of bacteria occur in nature, there are likely to be found phages to which these bacteria are sensitive. Therefore to obtain a bacteriophage active against a given strain of bacteria, one has to go to the natural habitat of the bacterial strain. For instance phage active against an intestinal pathogen such as the dysentery bacillus will be most easily isolated from horse and fowl faeces, or from the stool of patients who have

recovered from such an infection; phage active against any strain of Escherichia coli will fairly regularly be found in filtrates of stool or sewage; phage active against streptococci or staphylococci will often be isolated from the infected tissues.

iii) Bacteriophage Activity:

a) Clearing of Bacterial Cultures:

If a few drops of bacteriologically sterile filtrate containing bacteriophage are added to a young, actively growing broth culture of a susceptible organism, the culture will be found after a few hours incubation, to have become nearly or entirely clear and the live susceptible organisms in it are either reduced in number or entirely absent. This clearing may be partial or apparently complete, but usually on prolonged incubation, renewed bacterial growth occurs. This secondary bacterial growth, leading to a secondary clouding of the medium, is due to the occurrence in a population of sensitive bacteria, of mutant or variant bacteria which are resistant to the action of the particular phage concerned, though they may be susceptible to the action of other phages (Demerec and Fano 1945). According to Delbruck (1946) only a few hundred such resistant bacteria are probably present in a culture containing a billion sensitive organisms.

If a lysed culture, of the type mentioned above, is filtered while it is fairly clear through a Seitz, Berkefeld, or collodion filter of suitable porosity, and a drop of the bacteria-free filtrate obtained is added to another young broth culture of the susceptible organism, a similar clearing will be induced and filtrates from it will also induce the clearing

phenomenon in other cultures of the same organism. With dissolution of bacteria the lytic potency of the filtrate increases with each transfer as shown by the fact that the clearing occurs much more rapidly and completely after a few transfers than at first. The above procedure is a rough outline of the method commonly used in the laboratory for the propagation of phage when high titer phage stocks are needed.

b) Plaque Formation:

The activity of bacteriophage can also be shown when the susceptible organisms are grown on an agar surface. If the surface of a dry agar plate is inoculated with a few drops of a susceptible organism so as to give a confluent growth, and a fairly active phage-containing filtrate is inoculated at one point on the surface, the activity of the bacteriophage will be indicated, after incubation at 37°C for 6 to 24 hours, by a clear area free from bacterial growth at the point on the plate where the phage was inoculated. Higher dilutions of the filtrate will show irregular "worm-eaten" areas of normal growth in the cleared area, and at a certain dilution (up to 10^{-8} or 10^{-9} in some instances), depending on various conditions, the uniform layer of normal growth will be seen to be interrupted by discrete, well-separated clear areas, each of them circular, or roughly circular, where no growth will be evident. These clear areas were described as "taches vierges" or "plages" by d'Herelle, while in most English descriptions they are called "plaques", in German "locher". The number of plaques gives a rough indication of the number of phage-bearing particles in the original filtrate, a linear relation existing between the degree of dilution and the number of plaques. Isolated bacterial

colonies may show irregularities in their margins, as though pieces had been eaten out of the edges. These have been described as "moth-eaten" colonies.

The plaques produced by phage in the bacterial layer may be regarded as its 'colonies' consisting of billions of particles of bacteriophage which has destroyed the bacteria around it. The appearance of isolated plaques varies to some extent with the cultural conditions. When these are kept constant the size of the plaques, the nature of the edge and the appearance of the zone of bacterial growth immediately around the plaque tends to be fairly constant for any one phage growing on the same bacterial culture. According to Asheshov (1924, 1933) bacteriophage plaque morphology must be studied in the same way as the morphology of bacterial colonies is studied, and just as the size and morphology of colonies on agar may aid in the identification of bacterial species, the size and character of plaques are of value in the identification and classification of bacteriophages.

It has been shown by Elford and Andrewes (1932) that, provided the cultural conditions are kept constant and the same bacterial host is used, the size of the plaque varies inversely with the particle size of the phage producing it, the large plaques resulting from the greater diffusibility of the smaller phage particles liberated from the lysed bacterial host.

iv) The Mechanism of Phage Growth:

The rigorous experiments of Delbruck and his collaborators, summarized in various reviews (Ellis and Delbruck 1939, Delbruck and Luria 1942, Delbruck 1945, 1946) have demonstrated the existence of three well-defined phases, namely, adsorption, latent period of intracellular reproduction, and finally, release of newly-formed phage.

a) Adsorption:

It is generally accepted that the first step in the interaction of a bacteriophage and a sensitive host cell is the adsorption of the phage to the host cell. The rate of adsorption depends on a number of factors such as concentrations of phage and cell, the physiological state of the host, the temperature, and the concentration of electrolytes.

Delbruck (1940a, b) found that, in a mixture of phages and bacteria, the fraction of phages remaining free decreases exponentially with time and most rapid adsorption is attained onto bacteria during their logarithmic phase of growth in a favourable medium.

Barry and Goebel (1951) observed that the ability of Phase II Shigella sonnei to adsorb coliphages T3, T4, and T7 could be altered by treating the bacteria with physical and chemical agents.

Fong and Krueger (1949) reported that electrolytes in proper proportions expedite adsorption, but that excessive amounts may hinder the process, the inhibitory effect being greater at higher temperatures. Salt concentrations of about one per cent seem to be optimal. The importance of electrolytes, at certain critical concentrations, in promoting the attachment of phage to host cell, has recently been noted by

Puck and his associates (1951). They reported that, by the proper addition of salts, the rate of attachment of T1 to Escherichia coli (strain B) can be adjusted to any desired value up to the maximum limit set by the diffusion rate of the phage. Their experimental results led them to believe that the union of phage to its host cell consists, for the first step at least, of an electrostatic attachment between points of opposite polarity distributed in complementary fashion over the two surfaces. The role of inorganic ions is visualized as simple addition to specific sites on the two bodies, thereby furnishing the charge distribution necessary for phage-cell attachment.

It has often been assumed that adsorption is highly specific and occurs only with bacteria susceptible to the lytic action of the phage (d'Herelle 1926, Burnet 1929a, b, Krueger 1931, Delbruck 1942). However numerous investigators have reported adsorption of phage upon strains of bacteria that were incapable of supporting their multiplication. Applemans (1922) recorded adsorption by both susceptible and resistant bacteria of the same strain. Bakieten and co-workers (1936-7-8) found that a number of unrelated organisms such as enterococci and certain members of the Bacillus group would adsorb staphylococcus phages. Henry and Henry (1946) obtained resistant variants of a strain of Staphylococcus pyogenes which adsorbed phage as readily as the sensitive parent strain, and Craigie (1946) pointed out that various mutants of type 11 Vi phage were as readily adsorbed by phage-resistant Vi strains of Salmonella typhosa as by the sensitive strain.

In a series of papers (Burnet 1927, 1929a, b, Burnet and McKie

1929, 1930), the thesis has been put forward that susceptibility to lysis by phage was more or less directly related to the antigenic structure of the bacteria concerned. Phage specificity like antibody specificity was primarily determined by the ability of phage particles to be specifically adsorbed to certain antigenic constituents on the bacterial surface. The relationship between antigenic structure and phage sensitiveness has been recorded in a number of bacterial species; salmonella, dysentery, cholera, and staphylococcal phages, have been shown to react specifically with a specific surface polysaccharide (Burnet, Keogh, and Lush 1937, Delbruck 1946, Freeman 1957, White 1936); with certain phages such as typhoid Vi phages, adsorption is specifically related to the presence of Vi antigen.

The possibility that a specific polysaccharide antigen of the bacterial surface is concerned in the specific adsorption of phage is further suggested by the observation of Levine and Frisch (1934) that certain bacterial extracts, prepared from bacteria of the salmonella and shigella groups, could specifically inhibit the lysis of the homologous organisms by phage. Similar observations were recorded by Gough and Burnet (1934) who found that the phage-inactivating agent (P.I.A.), present in bacterial extracts, consists mainly of the specific polysaccharide somatic antigens.

Phages are active antigens, and it has been shown that the specific antibodies unite directly with the phage surface (Andrewes and Elford 1933a, b, Burnet 1933c, d). To explain the experimental facts of inactivation of phage by both antiserum and phage-inactivating agent, it

was postulated by Andrewes and Elford (1933b) and Burnet (1934) that the phage surface consists of a mosaic of two kinds of active groups or receptors - A and B - the A-receptors determining the antigenicity and serological specificity of the phage and capable of being inactivated by specific antiserum, the B-receptors serving as the specific point of attachment to susceptible bacteria and capable of being inactivated by extracts of susceptible bacteria which contain phage-inactivating agents in a suitably active form. The phage-inactivating agent combines with the phage and blocks the B-receptors which have an affinity to the bacterium.

On the basis of the above theory, adsorption may be regarded as an interaction of complementary chemical groupings on the surface of the phage particle and the surface of the bacteria, in a manner similar to the combination of antigen and antibody.

Recent observations however suggest that the mechanism of phage adsorption by its host cell may require more than the direct interaction of complementary groupings on phage and host cell. Anderson (1945b) observed that coliphages T4 and T6 were not adsorbed on the host cells in a synthetic ammonium lactate (F) medium, but that the activity of these phages was greatly enhanced by the addition of L-tryptophane at concentrations as low as 14 micrograms per ml. The bacteriophages T1 and T7 were found similarly to require certain amino-acids such as isoleucine, norleucine, and methionine for adsorption (Anderson 1948).

These substances, which have the power of promoting adsorption of phages on their host cells, were called "adsorption cofactors". The

inactivity of D-tryptophane and many derivatives of L-tryptophane in which the L-amino-acid grouping has been tampered with, suggests a specificity in cofactor phenomena comparable to that observed in enzyme systems.

It has been suggested by Delbruck (1948) that adsorption of phage on its host cell may actually occur in two steps, involving first a physical adsorption that is static in nature, secondly the "invasion" of the host cell, meaning a chemical reaction, presumably an enzymatically controlled process, by which the physically adsorbed particle effects an entry into the host cell. Cofactors may be involved in the second stage of the reaction and act, either as a cement substance in the specific combination between phage and host receptive spots, or as a sort of coenzyme in whose presence the phage particles, during their chance encounters with the host cells, are able to become attached to them and begin their parasitic activity.

b) Latent Period of Intracellular Bacteriophage Multiplication:

Following adsorption of the bacteriophage to the bacterial host cell, there is a latent period during which phage multiplication occurs inside the bacterial cell before dissolution or lysis occurs. During this period, multiplication proceeds behind the cloak of the cell wall, and the result is not revealed until the cell lyses.

The number of phage particles formed by each bacterial cell during the latent period has been determined by the use of two methods. The first method, devised by Burnet (1929), consists essentially in diluting a suspension of infected bacteria to the point that small samples

of the dilution contain no more than one infected bacterium. These aliquot samples are incubated until lysis occurs, then each sample is plated to determine the phage yield of the single infected bacterium. In this way it is possible to follow accurately the first stage of phage multiplication and to calculate the yield of phage formed by a single bacterium.

The other method, originally used by Ellis and Delbruck (1939), is regarded as one of the most important contribution to phage research in recent years. It involves the mixing of bacteria and phage in nutrient medium at high concentration for a short period to ensure rapid adsorption of the phage on the host cell, and then dilution of the mixture several thousand-fold, so that any phage released will not be further adsorbed. Periodical titrations are then carried out on the diluted mixture. It is found that for each phage, under constant conditions, there is a definite, accurately reproducible period, the "constant period", during which no change in plaque count occurs, followed by a "rise period" during which the plaque count increases sharply due to liberation of phage from bacteria infected during the initial adsorption period. After this the plaque count levels off and becomes constant. By this method, one step in the growth of phage is isolated, namely the step of the liberation of phage from the bacteria infected during a short initial adsorption period.

By means of "one-step" growth experiments, Delbruck and Luria (1942) were able to determine quantitatively the two important elements which characterize the life-cycle of phage in susceptible host, namely latent period and burst size. By knowing how many bacteria were originally

added but not adsorbed, the average yield of phage per bacterium, i.e. the burst size, was computed. In all cases this number was found to vary enormously from a few to around 1000 (Delbruck 1945b).

Following adsorption of phage to the host cell, certain reactions are set in motion resulting in experimentally determinable changes within the phage-infected host cells. Thus the respiratory rate of the infected cell is not impaired but it also does not increase, as it would in the uninfected bacterium (Cohen and Anderson 1946). The ability on the part of the bacterium to form adaptive enzymes has also been lost (Monod and Wollman 1947). The infected cell no longer undergoes cellular division and cannot be made to do so by any known means (Cohen and Anderson 1946, Luria and Delbruck 1942).

The failure of the infected cell to divide however does not appear to interfere with the process of phage multiplication. Krueger and Fong (1937), Scribner and Krueger (1937) could, under well-defined experimental conditions, inhibit bacterial growth and still get phage production to continue at a rapid rate. Northrop (1939) reported a large increase in phage around pH 5.5, although no increase in the host cells (Bacillus megatherium) occurred. Price (1947) found that phage will increase a thousand-fold in Staphylococcus muscae cultures whose multiplication had been inhibited by penicillin. Spizizen (1943) reported phage multiplication in Escherichia coli in glycine anhydride, a medium which does not support bacterial division. The question has been answered more clearly by the finding that Escherichia coli rendered incapable of multiplication by ultraviolet, or X-irradiation, or mustard gas, still support phage multiplication (Anderson 1948, Rouyer and Latarjet 1946, Herriott and Price 1948).

Krueger and Baldwin (1937), Northrop (1939), and others, have claimed that phage synthesis was the autocatalytic conversion of preformed cellular protein (phage precursor or prophage) into phage. If this were the case, the number of steps in the process of synthesizing active phage from inactive precursor would be expected to be very small. On the contrary, numerous investigators have shown that a great many steps are essential in the synthesis of phage from simple compounds in the external environment of the host cell.

Cohen (1948) and Kozloff and Putnam (1950) have shown by isotope methods that the greater part of the phosphate in the desoxybonucleic acid of the T series of phages comes from the medium and not from bacterial nucleic acid. Using the same method, Kozloff et al (1950) have found that the medium is also the source of the major part of phage nitrogen, only a small portion of the bacterial nitrogen being utilized in the synthesis of phage protein and nucleic acid.

Spizizen (1943) attempted to connect phage growth with specific nutrient sources by transferring infected cells into solution containing single metabolites. The results showed that compounds such as 4C-dicarboxylic acids, various coenzymes and phosphorylated compounds, play definite roles in the metabolic reactions of the cell which make phage multiplication possible.

Another approach to the problem of intracellular phage multiplication has involved the use of antimetabolites and respiratory inhibiters. Spizizen (1943) reported the inhibition of phage multiplication by very low concentrations of cyanide, iodoacetate, arsenite, 2,4-dinitrophenol, and p-aminophenol. The sulfonamides also inhibited phage multi-

plication but the inhibition was relieved by the addition of p-amino-benzoic acid (PABA). Fitzgerald and Lea (1946) observed that inhibition of the multiplication of a coliphage by 2-amino-9-(p-aminophenyl)-acridinium chloride could be relieved by ribonucleic acid, suggesting that some cellular mechanism involving nucleic acid or a related substance is essential to phage reproduction. Cohen and Fowler (1947) have reported a detailed study of the inhibitory action of 5-methyl tryptophane on T2 synthesis. 5-methyl tryptophane (5MT) has been shown to be a specific competitor of tryptophane in protein synthesis.

From these studies it is evident that phage multiplication is dependent on certain specific cellular reactions and does not occur if the respiration of the infected cells is blocked in one or another manner.

c) Lysis and Phage Liberation:

As d'Herelle (1926) had early suggested, and as quantitative studies have since shown (Delbruck 1942), the infection of a bacterium, after a latent period characteristic of each phage, is followed by lysis of the bacterium with liberation of a large number of new phage particles. Under conditions of one-step growth, the release is manifested by a rapid rise in the plaque count until all the originally infected bacteria have liberated their share of newly-formed phage particles. Massive lysis of a bacterial culture with subsequent liberation of phage seems to occur when the number of phage particles is greatly in excess of the number of bacteria. The threshold value differs in different phage-bacterium systems.

Within certain limits the amount of phage particles liberated by one bacterium (burst size) is independent of the number of phage particles originally adsorbed. It appears that if more than one particle is adsorbed,

only one of them actively induces the production of more phage. When a large number of phage particles are adsorbed to one bacterium, the cell will be attacked from without, and will be lysed in a few minutes. In such an instance there may be no increase in the amount of phage and all the adsorbed phage may be lost. The cell begins to swell up at one end, gradually all parts swell and the cell assumes an irregular shape and slowly fades out (Delbruck 1940b, 1942). Delbruck (1940b) refers to lysis under these conditions as "lysis from without" since lysis occurs without phage growth, as opposed to the usual process when lysis follows the multiplication of phage within the cell - "lysis from within". Lysis from within occurs after the adsorption of one or a few particles, but not until the phage has multiplied to the threshold value. Delbruck (1945) postulates that when phage and bacteria are mixed so that there is an excess of phage over bacteria, only one particle enters the cell and that the cell membrane then becomes impermeable to other particles of the same phage. This "exclusion" is also seen when excess of two immunologically unrelated phages is added to a bacterial culture susceptible to both. The phage liberated from each bacterial cell is of one kind only, the first phage to enter exerting an exclusion effect on the others. The effect appears to be similar to the interference phenomenon observed with animal and plant viruses, the first example of which was described by McKinney in 1929.

The occurrence of phage lysis can be observed in mass cultures by the clearing of the turbidity or it can be observed with individual cells under the microscope. Direct microscopic observation usually shows

swelling of the bacterial cells in the early stages of the process (Hetler and Bronfenbrenner 1932, Delbruck 1940b). The swollen cell may disintegrate explosively giving rise to a cloud of granular debris, or the outline of the cell may fade out quickly and the cell may disappear without the liberation of the granular cloud. Photomicrographic moving pictures of lysing cells, taken by Bronfenbrenner, Muckenfuss, and Hetler (1927) and Bayne-Jones and Sandholzer (1933), have shown that the cell sometimes swells up and sometimes fades out without any change of shape. It appears that phage lysis occurs in several different ways, according to the nature of the phage and bacterium involved.

Electron micrographic studies of Escherichia coli undergoing phage lysis (Luria, Delbruck, and Anderson 1943) have shown the adsorption of the phage particles on the host and after the predicted time, the lysis of the host with liberation of a large number of new phage particles. Quantitatively, there was agreement between the number of particles visible on the micrographs and the number predicted on the basis of growth experiments for which plaque count assays had been made. The micrographs revealed that the phage particles were liberated from the interior of the bacterial cell, for they were not visible on its surface up to the moment of lysis. There was no indication however in which part of the bacterium the phages were produced, whether in the deep interior of the cell or close to the inner surface of the cell wall. In cases of multiple infection, the infecting particles of phage, or at least the majority of them, seem not to enter the cell but to remain attached to the outside of the bacterial cell wall. Along with the phage particles, the lysing cells also shed protoplasmic material of uniform granular structure. These particles were

liberated from the cell in great abundance and seemed to constitute the bulk of its protoplasm. In a later stage of lysis, "ghost cells", that is, empty cell walls from which all content had been liberated, were seen surrounded by phage particles and protoplasmic granules. Holes of various sizes were visible in these bacterial cell walls.

The mechanism of the actual lysis of infected bacteria is not understood and very little is yet known of the nature of the materials present in the lysate. A number of workers have recorded observations which seem to indicate that, in the process of lysis by phage, one or several lytic enzymes secreted either by phage or by bacteria undergoing lysis, are involved. d'Herelle (1926) was the first to postulate the liberation of a lytic enzyme by phage at the time of lysis. He reported that a resistant strain of Escherichia coli would undergo complete or almost complete lysis in an environment containing phage and a susceptible strain of Escherichia coli. He stated that the cause of the dissolution of the resistant strain, under these conditions, was the elaboration of an enzyme - a lysin - by the bacteriophage. This lysin, he claimed, was produced during the multiplication of the phage at the expense of the susceptible strain of bacteria. Similar observations were reported by Rakieten (1933), by Evans (1940), and by Wahl and Jos.se-Bicchot (1949) with resistant strains of staphylococci and streptococci.

The observations of Sertic (1929) and Sertic and Boulgakov (1939) also present evidence in favor of the view that a lytic enzyme is secreted by phage. Investigating Escherichia coli phage, Sertic described a sort of "halo", that is, a lysed zone free of the transmissible lytic principle, surrounding the plaques clarified by phage. The non-transmis-

sible active agent in this zone was considered to be a lysin. It could be obtained by glycerol extraction as well as by ultrafiltration of the phage.

The Wollmans (1932, 1933) observed that in the presence of a growing sensitive strain, phage may lyse not only dead bacteria of the sensitive strain, but also resistant living bacteria. They regarded lysis under these conditions as being due indirectly to the bacteriophage which releases specific enzymes from the bacteria undergoing lysis. Pirie (1939) separated an enzyme from normal Escherichia coli cells which attacked bacterial cellular polysaccharides from homologous heat-killed cells. She was unable to demonstrate such an enzyme in concentrated phage preparations. Her results favor the view of the Wollmans.

Delbruck (1942) expressed the opinion that the phenomena described above are probably manifestations of the autolytic enzymes of the bacterial cells, which are liberated when the host cell is lysed by the bacteriophage. At any rate the mechanism by which resistant strains of bacteria are lysed, in mixed cultures with sensitive strains and their bacteriophages, is still obscure. Furthermore the presence of an enzyme which decapsulates both living and killed cells of Klebsiella pneumoniae has been reported to be present in a phage lysate (Humphries 1948).

v) Antigenicity of Bacteriophage:

The fact that a bacteriophage could function as a specific antigen was first established by Bordet and Guiea (1921). They showed that following repeated injections of lysed culture, the serum of the rabbit could inactivate the homologous phage. They also found that this

inactivating power was not present in antibacterial sera produced by immunization with the susceptible bacterial culture.

The reaction between phages and specific antibody has been demonstrated in several ways: a) by inhibition of phage lysis, i.e. neutralization; b) by agglutination of phage particles; and c) by the agglutination of phage-coated bacteria by antiphage sera. There is evidence that the phage-antibody reactions are essentially similar to other antigen-antibody systems. When a suspension of phage is mixed with specific antiserum, the phage particles are rapidly "inactivated", that is, they lose the power to attack bacteria and to multiply. This inactivation is due to the union of the phage particles with the antibody (Delbruck 1945). The presence of antiphage serum strongly inhibits adsorption of the phage on the bacterial cell. According to Burnet et al (1937) residual phage particles not inactivated completely by the serum are adsorbed onto the bacteria at the usual rate and multiply as if the serum were not present. Already adsorbed or intracellular phages appear to be completely insusceptible to the action of even strong specific antiphage serum, a protective effect of the cell which is seen with other viruses. Every infected cell treated with antiphage serum during the latent period of phage growth is lysed at the expected time and liberates its normal yield of phage (Delbruck 1945).

The union between phage and antiserum is not reversible (Hershey 1943), but the phage is not damaged. Kalmanson and Bronfenbrenner (1943) have recovered active phage by digestion of the phage-antibody complex with papain. Andrewes and Elford (1933a) have demonstrated that neutralization of phage by antiserum follows a "percentage

law"; they showed, with three coliphages and corresponding antisera, that a given concentration of antiserum acting for a given period of time will neutralize a constant percentage of phage irrespective of the concentration of phage over a wide range. This behaviour has been confirmed by Burnet, Keogh, and Lush (1937), and by Kalmansen and Bronfenbrenner (1942) and is generally believed to indicate that the particles are inactivated individually and not by aggregation. The results suggest also the heterogeneous nature of a phage population, a certain percentage of the particles being less readily neutralized by antiserum than others.

Experiments by Burnet (1933e) have shown that small plaque large particle phages can be specifically aggregated by homologous high titre antiphage serum if the concentration of phage exceeds 10 billions particles per ml. In another experiment (Burnet, Keogh, and Lush 1937) it was shown that formalin-killed bacteria heavily coated with phage are agglutinated by the homologous antiphage serum but not by the heterologous serum. This indicates that the phage is still able to react with antiserum when adsorbed to a killed bacterium.

Serological specificity is a highly constant character of a given strain of a bacteriophage (Andrewes and Elford 1933a) and is independent of the host on which it is grown. The serological classification of phages has proved to be the only significant one (Burnet et al 1937, Delbruck 1946). Cross-reactions occur with related phages and their antisera, and by studying these, it is possible to arrange a large number of phages in serological groups. Burnet (1933a) has recognized 11 different serological groups of phages acting on Escherichia coli alone. Serological studies by Delbruck (1946) and Hershey (1946a) on the members of

the T system of phages of Escherichia coli, strain B (Demerec and Fano 1945), have shown the seven types called T1, T2, ----- T7 to fall into 4 unrelated serological groups as follows: T1; T2, T4, T6; T3, T7; T5. Antiserum against a phage belonging to any one of these groups has no detectable action on phages in any of the other groups. The relationships within groups are exemplified by the cross-reactions among the even-numbered phages. Phages which belong to the same serological group are closely related as far as size, shape, and other morphological characters are concerned. Thus, T2, T4, and T6 are tadpole-shaped and morphologically identical; T3 and T7 are spherical, about 45 millimicrons in diameter and they do not have tails; T1 and T5 have tails but the heads are spherical and greatly differ in size (Delbruck 1946).

The nature of the phage inactivation by immune serum has been discussed by Andrewes and Elford (1933b) on the basis of the hypothesis that the phage surface is a mosaic of two entities, one A.C. (antigenic component), responsible for the serological specificity and the other B.C. (bacterial component), serving as a specific point of attachment to susceptible bacteria and determining the "resistance group" of the phage. With the union of antibody to the former of the two entities a progressive blocking of the second set occurs, eventually rendering it impossible for the phage to make specific contact with and to lyse the susceptible bacteria. Burnet (1934) has made use of this hypothesis to explain phage inactivation by P.I.A. of bacterial extracts, in which case it is the mosaic component directed toward the bacterial surface (B.C.) which is affected.

C) THE STAPHYLOCOCCUS BACTERIOPHAGES:

i) Distribution in Nature and Methods of Isolation:

As mentioned previously, a bacteriophage should be looked for in the natural habitat of its host. Therefore, a bacteriophage specific for one or more strains of staphylococcus can be isolated from nasal passages, the skin, or from acute staphylococcus infections such as carbuncles, furuncles, and various abscesses of man and animal.

d'Herelle (1921) reported the isolation of a staphylococcus bacteriophage from the pus of an infected finger. Eight drops of the pus were put into 20 ml. of broth, incubated 24 hours, and filtered. The filtrate was active on albus but not on aureus strains of staphylococci although both strains were present in the pus.

Callow (1922) demonstrated the presence of a phage active on Staphylococcus pyogenes in the pus of a series of 16 staphylococcus infections. Sterile swabs containing pus from the boils were streaked directly on plain and blood-agar plates. Growths were examined on the next day and the individual colonies minutely searched for irregularities suggesting lytic changes. Only in two cases direct streaks from pus yielded irregular colonies, in all others the lytic principle was obtained after the following manipulations: Pus swabs were placed each in 10 ml. of broth (pH 8.0), and incubated at 37°C for 4 hours. The tubes were then placed in the icebox overnight, centrifuged and filtered through a Berkefeld filter. Each filtrate was tested against the autogenous strain, i.e. against a strain derived from a normal colony of the same boil. 0.1 ml. of an 18 hour broth culture of a Staphylococcus pyogenes strain which had been recently isolated from a boil was added to 2 ml. of broth to which

0.5 ml. of the suspected lytic filtrate had been added. The mixture was incubated at 37°C for 4 hours. Streaks of the mixture were made to plain agar plates at 2-hr. and at 4-hr. intervals. Lytic action was recognized by characteristic moth-eaten areas in the streaks, by irregular colonies with edge showing evidence of lytic changes, and by colonies with mottled surfaces. In the absence of any evidence of lytic action in the first generation, the experiment was carried through a second or a third generation, and so forth. By this technique, Gallow demonstrated that in two of the 16 staphylococcus infections the lytic principle was active against the autogenous strain as well as against other staphylococcus strains; in 6 others it proved lytic for one or more heterologous strains, but not for the homologous strain, in spite of repeated tests.

Other sources of staphylococcus bacteriophages are the so-called "lysogenic" strains of staphylococci. Such strains are contaminated with latent phages which can be detected by Fisk's cross-testing technique (1942). The method consists in streaking on agar plates over a small area, a loopful of a broth culture of the staphylococcus strain to be examined for the presence of latent phage, and in spotting the same inoculum or other strains at points along this streak. In this way a heavily inoculated spot superimposed on a less heavily inoculated streak is obtained. After an incubation period of 6-8 hours at 37°C and at room temperature overnight, phage action is revealed next morning by the formation of plaques which vary in size and number. From these plaques a phage can be isolated and propagated on susceptible strains.

By following the aforementioned procedure, Rountree (1947a) isolated 5 different lysogenic strains and the appropriate susceptible

propagating strains from a series of 40 cultures of Staphylococcus pyogenes which had been isolated from various pathological conditions, nasal mucosa and scrapings of scurf.

Staphylococcus bacteriophages have also been found in sources other than infected tissues. Northrop (1938) studied a staphylococcus bacteriophage which had been isolated from house flies by Glaser (1924) and by Shope (1927).

The isolation of phages for members of the coli-dysentery group of organisms by conventional phage technique has been shown to be a relatively simple matter. However, application of these methods to demonstrate staphylococcus phages has led to less frequent success. According to Fisk (1942) the common inactivation of staphylococcus phages, possibly by adsorption, by tissue cells and various microorganisms including susceptible and resistant staphylococci, probably play a part in the difficulties encountered in their isolation.

ii) Physical and Chemical Properties

a) Morphology and Size:

The little research that has been done on the morphology of staphylococcus bacteriophages has shown it to be somewhat similar to that of the better known coli-dysentery phages. Elford and Andrewes (1932) estimated the particle size of a staphylococcus phage Au_2 by means of graded collodion membranes and found it to be of the same size as the largest particle of coli-dysentery phages, namely 50-75 millimicrons. Judging from the plaque size Burnet and Lush (1935) reasoned that all the 'aureus' phages under study should be of approximately this particle size.

Northrop (1938) has made extensive studies with purified preparations of staphylococcus phage. From the rate of sedimentation in the ultracentrifuge, a value of 60-90 millimicrons (molecular weight $3x10^8$) was obtained, in fair agreement with that obtained by Luria, Delbruck, and Andersen (1943) by means of the electron microscope (100 millimicrons).

Smiles and co-workers (1948) have also used the electron microscope to examine concentrated preparations of phages from filtrates of lysed cultures of the Oxford "H" strain of Staphylococcus pyogenes. This bacteriophage was found to have a round head opaque at 50 kV. electrons and a relatively long slender tail. Measurements of the head indicated a diameter 50-60 millimicrons, and a tail length of 200-250 millimicrons. The presence of considerable excess of 'free tails' and the association of phage with masses of 'fibrillar' material, particular in the early stages of purification, led them in doubt as to whether the tail was an essential part of the phage's morphology.

b) Chemical Composition:

The chemical composition of a staphylococcus phage has been reported by Northrop (1938). Following the chemical procedures previously employed for the purification of enzymes, he isolated from staphylococcal lysates a macromolecular nucleoprotein having the activity of bacteriophage and possessing a chemical composition quite similar to that reported for the coli phages. Detailed studies of the isolated nucleoprotein showed that it was homogeneous in the ultracentrifuge and had a sedimentation constant corresponding to a molecular weight of about 300,000,000. It was very unstable and was denatured by acidity greater

than pH 5.0, by temperatures over 50°C for 5 minutes, and by glycerine, alcohol, and acetone. The loss in activity by heat, acid, and chymotrypsin digestion was found to be roughly proportional to the amount of denatured protein formed under these conditions. An analysis of the phage preparations disclosed 14.1, 5.0, and 1.5 per cent of nitrogen, phosphorus, and carbohydrate respectively.

c) Growth Requirements:

It is well known that media which are adequate for bacterial multiplication do not necessarily support phage multiplication. Price (1947) found that in staphylococcal systems, nicotinamide had to be added to the medium to support phage multiplication. Rountree (1949b) reported that for some phages belonging to serological group A, although a rise in phage count occurred after 100-110 minutes incubation, no clear-cut evidence of burst could be obtained unless 0.002 milligram per ml. tryptophane was added to Todd-Hewitt broth (1932). Work in progress with phages belonging to serological group B and group C indicated that these phages require the addition to the broth of factors present in the vitamin B complex and of unknown factors that were removed from the broth during its sterilization by heat.

A number of investigators have found that many staphylococcus phages are difficult to grow in broth (Burnet and Lush 1935, Fisk 1942, Rountree 1949b). Burnet and Lush (1935), in their study of staphylococcus phage, reported that the aureus phages which they investigated fell into three groups, which they called "strong", "weak", and "intermediate". The strong phages multiplied well in broth (pH 8.0) at 37°C

giving complete lysis of susceptible strains and providing filtrates (Seitz) with a titre of around 10^9 particles per ml. The intermediate phage could be grown in broth at 37°C provided care was taken to use only a slight bacterial inoculum. The weak phages, on the other hand, failed to show any appreciable multiplication in broth under any conditions of pH or temperature tried. They produced good lysis on one per cent nutrient agar at 28°C and stock filtrates could be obtained from cellophane agar plates.

Fisk (1942) found that his phages resembled Burnet and Lush's weak phages in their inability to produce lysis regularly in broth cultures. The phages examined by Rountree (1947a) did not fall into any of the categories defined by Burnet and Lush but resembled the intermediate phage in their growth temperature requirements.

Complete clearing of broth cultures infected with staphylococcus phages is more difficult to obtain than with the coli-dysentery phages. This is probably due to the longer latent period and smaller burst size obtained with staphylococcus bacteriophages. For instance, Rountree (1949b) found that the liberation of some phages of serological group A from infected staphylococci occurred after a latent period of 55-70 minutes, a generation time which is long compared with that of Escherichia coli phages or of other staphylococcus phages with a generation time of 35 minutes. The average burst size determined for one phage proved to be 35. Merling-Eisenberg (1941), using the ultramicroscopic method, found that the number of phage bodies originating from one lysed staphylococcus was usually 2 and never more than 4. Therefore it is obvious why complete clearing of broth

cultures infected with these phages is rarely seen; the growth of the staphylococcus quickly outstrips that of the phage unless the dose of added phage is a large one.

The lower number of phage bodies obtained from a staphylococcus also seems to suggest an explanation for the lower titre of staphylococcus phage as compared with Escherichia coli phage. The average titres attained with staphylococcus phages appear to be in the neighborhood of 10^{-5} to 10^{-6} (McIntosh and Selbie 1940). Merling-Eisenberg (1938) reported that under identical conditions, the titre of Escherichia coli phage seemed to be about 10^{-8} to 10^{-9} and that the number of phage particles liberated from one Escherichia coli varied from 150 to 350.

d) Resistance to Physical and Chemical Agents:

It has been shown by Burnet (1933b) that useful differentiation amongst dysentery phages can be effected by certain biochemical tests, for instance, susceptibility to methylene blue, ability to effect lysis on citrate agar, and susceptibility to inactivation by strong urea solution. Using the same technique, Burnet and Lush (1935) applied these tests to the staphylococcus bacteriophages. Preliminary tests with 1:50,000 methylene blue showed that all the strong aureus phages were completely inactivated after 2 minute's exposure on the bench to bright diffuse sunlight. Similar observations with methylene blue had been recorded by Schultz and Krueger (1928) with one staphylococcus phage. When the phages were exposed to strong urea solution (27 per cent) for one hour at 37°C, all the aureus phages were completely inactivated.

The results obtained with citrate agar showed that the strong phages were not inhibited by 1.5 per cent sodium citrate while the intermediate and weak phages were inhibited by 1 per cent and 0.25 per cent sodium citrate respectively.

Similar results were obtained by Rountree (1947a). She found that 3 out of 5 phages isolated from lysogenic cultures of staphylococci were inhibited by 0.25 per cent sodium citrate whereas the other 2 phages were not inhibited by a concentration of 1.5 per cent sodium citrate. Thus, by citrate sensitivity tests, she could divide her phages into citrate sensitive and citrate resistant phages and, on this basis, could classify them as weak and strong.

Krueger and Baldwin (1935) recorded the inactivation of a staphylococcus phage by safranine, the inactivation effect being partly photodynamic. Levin and Lominski (1936) found that various lecithins slightly inhibited a phage active against certain strains of staphylococci. Later, in the same year, they reported that cholesterol had a similar action.

The staphylococcus bacteriophages are much more inactivated by heat than the great majority of the coli-dysentery phages. Certain of Burnet and Lush's phages, when first isolated were inactivated even at 37°C but were found to be strongly protected against heat inactivation by serum, by dead bacteria, and by certain bacterial extracts (Burnet and Lush 1935). Rountree (1949b) studied the effect of temperature on a series of staphylococcus bacteriophages by exposing them at 49°C for 1 hour. With two exceptions, in which the survival rate was 90-100 per

cent, inactivation of the phages occurred. Most of the phages belonging to serological group A were devoid of activity after heating at this temperature, but 20-30 per cent of 3 phages in this group were viable at the end of one hour. It was suggested that heat treatment of lysates or of cultures of staphylococci is of no value in freeing such material from staphylococci since the phages are inactivated at lower temperatures than are the bacteria.

A number of workers have carried out experiments to determine what action, if any, penicillin has on staphylococcus bacteriophages. Meter and Clark (1944) reported that penicillin in a concentration of 2000 units per ml. did not affect the lytic activity of a staphylococcus phage for a susceptible strain of Staphylococcus pyogenes when the phage was exposed to its action for 18 hours at 37°C. Similarly Jones (1945) stated that a filtrate of a staphylococcus phage was unaffected by an exposure of 1000 units of penicillin per ml. for 24 hours at 37°C. Elford (1948) showed that penicillin in concentrations up to 100 units per ml. in broth or synthetic medium, and in one test up to 400 units per ml. in broth, was without significant effect on staphylococcus K bacteriophage after 30 hours incubation at 37°C. Rountree (1947a) also reported that penicillin and penicillinase had no demonstrable effect on filtrates of 5 staphylococcus phages derived from lysogenic strains of staphylococci. Lysis by penicillin of the strains carrying phage failed to release the phage into the surrounding medium.

Various strains of staphylococcus phages vary in their stability. Rountree (1949b) reported that certain of the phages she used for

typing could be maintained in the refrigerator for long periods of time without any loss in activity, while others showed a marked fall in titre on testing a few weeks after preparation. The unstable phages proved to be those which had been found more sensitive to a variety of agents. Thus she found that one of the unstable phages (42D) lost 95 per cent activity after 90 minutes at 37°C in one-quarter strength Ringer's solution. The instability of certain staphylococcus phages makes it necessary constantly to check the titre of the phages used for typing.

e) Plaque Size:

The staphylococcus bacteriophages produce relatively small plaques, and on reasonably firm agar, there is often great difficulty in counting them. For any given phage it is found that there is a fairly wide range of plaque size. Burnet and Lush (1935) in their study of the staphylococcus phages, found that all aureus phages produce small sharp-edged plaques ranging 0.1-1.0 millimeter in diameter on 1 per cent agar at 22°C. According to Bountree (1949b) the plaque size varies inversely with the concentration of agar in the medium. It also varies with the brand of agar used. In her investigation a medium containing 1.25 per cent of shredded agar was used, with added peptone, Yeastrel, and Fildes's peptic digest of blood, and the plaques grown overnight at 37°C. This medium provided satisfactory conditions for the maximum development of plaque size. Among serological group A phages, the mean plaque size varied from 0.33 to 0.7 millimeter, the range of individual diameters being 0.1 - 1.0 millimeter. Among the group B phages there were some which produced plaques up to 1.7 millimeter in diameter. One

phage (X_2) produced characteristic large plaques, which were overgrown by secondary bacterial growth, giving a hazy appearance after overnight incubation.

iii) Bacteriophage Adsorption by Staphylococci:

Staphylococcus phages are adsorbed on all strains of Staphylococcus pyogenes, irrespective of whether they lyse these strains or not. Thus, Burnet and Lush (1935) found that the phages which they designated as "strong" were readily adsorbed by all aureus strains including those which failed to allow multiplication in broth. The "intermediate" phage behaved like the strong phage, being adsorbed almost equally well by all aureus strains irrespective of whether they were susceptible or insusceptible to its action.

Rakieten and his co-workers (1936, 1937, 1938) in a series of studies on the adsorption of staphylococcus phages confirmed this non-specificity and also found that a number of unrelated organisms such as enterococci and certain members of the Bacillus group would adsorb staphylococcus phages. It was suggested that some portion of the antigenic constitution of these organisms resembles that of susceptible staphylococci, and that it is this factor which is responsible for their staphylococcus-phage-adsorbing quality. The adsorption of staphylococcus phages by organisms other than staphylococci therefore reveals evidence of heterogenetic antigenic relations between staphylococci and otherwise totally unrelated organisms (Rakieten et al 1936).

The adsorbability of the staphylococcus phages studied by Rountree (1947b) seems to be correlated with the coagulase production of the host cells. Phage adsorption tests using heat-killed cultures of

staphylococci were made with 3 groups of phages: (a) those which lysed coagulase-positive staphylococci of human origin, (b) those which lysed coagulase-positive staphylococci of animal origin, and (c) those which lysed coagulase-negative staphylococci of human origin. The phages of class (a) and (b) were inactivated and presumably adsorbed on heat-killed coagulase-positive cultures whether they were able to lyse the intact cells from such cultures or not. On the other hand, there were no observable reactions between coagulase-positive and coagulase-negative phages and cultures. The presence of 0.5 per cent sodium citrate inhibited multiplication of phage in living susceptible cultures but did not inhibit adsorption. Viscid mutants of staphylococci were resistant to lysis by phages lysing the parent strains, but still adsorbed them.

As mentioned previously, the studies of Burnet (1929 - 1934) have demonstrated that adsorption of phages which attack bacteria of the enteric group is largely determined by the nature of the surface antigens of the bacterial cell. This concept has been strengthened by the discovery by Levine and Frisch (1933, 1934) that extracts prepared from susceptible bacteria have the power of inactivating phage. Staphylococcus phages have been found by a number of investigators to behave in a similar manner toward extracts derived from the bacterial cells. Rakieten et al (1936), working with staphylococci and staphylococcus phages, could prepare watery extracts of bacteria which inhibited the action of phage. These extracts were heat-stable, being active after autoclaving, but were deprived of phage-inactivating ability by filtration through Chamberland L3 and L5 candles, and Berkefeld and Seitz filters. The phage-inactivating property of the extracts was lost on precipitation with homologous anti-bacterial serum. It

was found that the ability of a given extract to inactivate a bacteriophage was largely dependent on the susceptibility of the culture furnishing the extract, the most potent extracts being derived from highly susceptible bacteria. They were unable to demonstrate phage inactivation by extracts from resistant staphylococci.

Rountree (1947b) prepared lysates from a number of coagulase-positive human strains and examined their phage inactivating power. The results showed that lysates from strains of coagulase-positive human staphylococci inactivated all the phages of this group, but that there was no inactivation by these lysates of the phage lysing the coagulase-negative human staphylococcus.

Various methods of chemical fractionation of the lysates have been carried out in an attempt to separate the component responsible for phage inactivation. Burnet and Lush (1935) reported that staphylococcus phage is specifically bound to an antigenic complex from which the polysaccharide haptene can be derived. Working with one of their phages, Freeman (1937) found that precipitation of nucleoprotein from extracts prepared by grinding staphylococci in a ball mill removed the phage-inactivating activity of the extract. By tryptic digestion of this nucleoprotein complex, polysaccharide material was obtained which possessed phage-inactivating power.

On the other hand, Rakieten and Tiffany (1938) found that protein fractions from whole cultures of staphylococci possessed phage inactivating activity. Phage adsorption tests with intact cells, with lysates, and with fractions of the lysates, performed by Rountree (1947b),

suggested that nucleoprotein may be involved in the adsorption of phage by staphylococci. By the chemical fractionation of a number of lysates, a protein fraction was isolated which, when tested for phage-inactivating activity, was as active as the original lysate. It was found that coagulase-positive staphylococci of human origin, whether phage susceptible or not, all possess a common nucleoprotein component situated at the surface of the cells on which phage adsorption may take place. No work was done on the nature of the phage-inactivating agent of the coagulase-negative strains, but since no cross adsorption occurred with these strains and the coagulase-positive phages, it would appear that the specific nucleoprotein of the coagulase-positive staphylococci is absent from the coagulase-negative strains.

Rountree (1947a) studied the adsorption of phage by penicillin-treated staphylococci. She found that staphylococci after 3 hours' contact with penicillin, but not lysed by it, adsorbed phage as readily as normal staphylococci. On the other hand, staphylococci completely lysed by penicillin failed to adsorb phage. It appears that the disruption of the staphylococcal cell, as a result of penicillin action, destroys the surface molecular configuration which is concerned in the phage adsorption.

iv) <u>The Phenomenon of Lysogenicity in Staphylococci</u>:

Destruction of the bacterial host cell is not an essential characteristic of bacteriophage. As mentioned previously, some bacterial cultures are known to carry phages with them which fail to lyse an appreciable proportion of the cells in the culture. Such "carrier" strains are resistant to the phage carried; they harbor the phage and secrete it, but show

no pathological symptoms. These strains have been called "lysogenic", since filtrates of cultures of them will contain the phage which can be demonstrated by its capacity to produce lysis and plaques when allowed to act on other sensitive or "indicator" strains. The indicator strains usually belong to the same species of bacteria as the carrier strain. They are often useful in the detection of lysogenic strains.

This stable association of a bacteriophage with a bacterial strain, known as "lysogenesis", was first reported by Lisbonne and Carrere (1922) from a strain of Escherichia coli. Since then, the phenomenon has been commonly encountered among laboratory cultures of bacteria, and possibly more so in staphylococci. Fisk (1942) investigated the lysogenicity of strains of Staphylococcus pyogenes recovered from various human lesions. By cross-testing the strains on agar, 19 out of 43 coagulase-positive strains were found to be phage carriers; none of these phages was lytic for any of the 40 coagulase-negative strains tested. None of the coagulase-negative strains yielded a phage lytic for any of the coagulase-positive strains. Using Fisk's method, Wilson and Atkinson (1945) found 25 lysogenic cultures among 46 staphylococcus strains examined. Seven different phages were obtained from these 25 cultures. Rountree (1947a) examined a series of 40 strains of Staphylococcus pyogenes obtained from a variety of pathogenic conditions and found 5 of them to be lysogenic. Similarly, Williams Smith (1948a, b) examined 22 strains used by Wilson and Atkinson as propagating strains for their typing phages and found 17 of them to be lysogenic.

Rountree (1949a) has recently made an extensive study of the

phenomenon of lysogenicity in the staphylococci. The phenomenon is of wide occurrence in these organisms for she found that 27 out of 30 coagulase-positive strains studied were lysogenic. Lysogenicity appears to be a permanent feature of these strains for every bacterial cell was apparently carrying phage, and treatment with heat, with specific anti-phage serum, or cultivation in broth containing sodium citrate, to which certain of the phages are sensitive, did not make the cells non-lysogenic. Rountree (1949) postulates an intracellular method of passage of the phage in lysogenic strains, each daughter cell receiving at least one phage particle at each division.

There are contradictory reports in the literature on the presence of free phage in filtrates of broth cultures of staphylococci. Callow (1922, 1927) reported that she could obtain phage by the filtration of staphylococcus cultures. Burnet and Lush (1936) described a lysogenic mutant of a white staphylococcus which liberated bacteriophage in young and old cultures. These observations were not confirmed by Fisk (1942) nor by Williams Smith (1948b) who failed to demonstrate the presence of phage in filtrates of staphylococcus cultures known to be lysogenic.

That a small proportion of the cells in lysogenic cultures of staphylococci do release free phage into the surrounding medium was demonstrated by Rountree (1949a). She obtained free phage in filtrates of young broth cultures of 25 of the strains studied. Of seven cultures chosen for more extensive studies, one produced 3 different but serologically related phages, while another produced 5 different phages belonging to 3 different

serological groups.

The failure of the previous workers to demonstrate free phage in filtrates of lysogenic cultures may be ascribed to such factors as re-adsorption of the free phages to other bacterial cells in the cultures, or to the state of the phage-bacterium relationship at the time of filtration; any disturbance in this relationship, though not sufficient to produce visible changes in the bacterial culture, might result in a filtrate that contains phage (Williams Smith 1948b).

The nature of the host-phage relationship in lysogenesis is not well understood but it appears that the association of phage and lysogenic cell is very intimate. The host can support its own growth while harbering many strains of phage. Attempts by many workers to liberate phage by artificial disruption of lysogenic bacteria by means of lysozyme, penicillin, infection with an unrelated phage, or by mechanical grinding, have been unsuccessful until recently Lwoff and co-workers (1950, 1951) claim to have obtained positive results by irradiation of the lysogenic cells with a small dose of ultraviolet light, or by exposure to certain compounds containing sulfhydryl groups.

It has been found by Rountree (1949a) that only a small proportion of cells in each lysogenic strain liberates phage. The usual proportion appears to be of the order of 1 cell in every 1000. No examples have been found in which every cell in a strain releases phage. The mechanism of the release is at present unknown but liberation by lysis is the most probable method of release of phage from lysogenic cells (Rountree 1949a). Bertani (1951) suggested that phage is carried by lysogenic bacteria in a 'prophage' state. He believes that liberation may be caused

by maturation of all or several of the prophages, so that each prophage gives origin to one mature infective phage particle; or it may be the consequence of a 'change' in the state of one of the prophages which multiplies and gives origin to a clone of mature and infective phage particles.

The available evidence suggests that the lysogenic bacteria, or more accurately, the phages they harbor, are the origin and continuing source of all phages in nature. Rountree (1949a) is of the opinion that the phenomena of lysogenicity as exhibited by the staphylococci may throw some light on the problems associated with the mode of reproduction of latent viruses generally, whether in bacterial or animal cells.

v) Serology of Staphylococcus Bacteriophages:

As shown by Burnet (1933a), Asheshov (1933), and Craigie (1940), anti-bacteriophage sera have been a valuable tool for the definition of the relationships of various races of bacteriophages.

The only serological investigations of staphylococcus phages are those of Burnet and Lush (1935) and Rountree (1949b). Burnet and Lush (1935) examined a series of 13 staphylococcus phages and by serological and other methods distinguished 6 groups among them. As with coli-dysentery phages, it was found that all the staphylococcus phages of a given serological type reacted uniformly with regard to photodynamic inactivation, power to grow on citrated media, and inactivation by strong urea solution. A number of experiments were made to determine whether phage-antiphage reactions could be demonstrated by macroscopic aggregation tests such as were used for one group of dysentery phages (Burnet 1933c, e). It

was found that no agglutination of phage particles could be detected in the presence of antiphage serum, probably because they were unable to obtain sufficiently high-titre phage filtrates. However, with serological group 1 and 2 it was readily demonstrated that phage-coated staphylococci could be agglutinated by antiphage serum from which all antibacterial agglutinins had been removed.

By the use of anti-bacteriophage sera prepared in rabbits, Rountree (1949b) divided 39 staphylococcus phages into 6 serological groups which, for purposes of convenience, were designated A, B, C, D, E, and F. The serological characteristics of the phages were associated with other properties such as stability and the range of organisms lysed. Thus, the first group (A) comprised phages lysing coagulase-positive staphylococci of human origin. They were stable at 20°C but inactivated at 49°C. They multiplied in broth cultures containing sufficient tryptophane but rarely produced clearing of such cultures. The second group (B) lysed both bovine and human coagulase-positive staphylococci. They were markedly sensitive to heat and required growth factors present in the vitamin B complex. The third group (C) comprised phages of ovine origin which were antigenically related to group B phages and also resembled them in their growth requirements. The differences, however, were great enough to justify their separation as a distinct serological group. Group D comprised phage K, which lysed both coagulase-positive and negative staphylococci and was antigenically related only to phage W which belonged to group E and lysed only some coagulase-negative staphylococci. Group F was related in its general characters with the phages of group A.

Many strains of staphylococci are lysogenic, and consequently, lytic filtrates may contain contaminating phages which manifest themselves during adaptation of phage from one strain of staphylococcus to another. According to Rountree, adequate serological characterization of the phages, whether they are to be used for phage typing of staphylococci or for observations on the fundamental problems of phage-bacterium relationships, is therefore necessary.

vi) The Use of Staphylococcus Bacteriophages in Epidemiological Investigations:

Many investigators have noted that phages often demonstrate a selective action for certain strains of organism within a species. Craigie and Yen (1938) noted the specificity of V form typhoid phages for cultures possessing Vi antigen. When one such phage was grown on typhoid strains of different origin, strains of phage were obtained which were found to have developed a high degree of specificity for the particular strain on which they had been propagated. By means of a series of phages prepared in this way, they were able to divide cultures of Salmonella typhosa into 18 types according to their reaction to a critical dilution of the adapted phage. The evidence obtained from a study of the origin of the strains revealed a high degree of correlation between the phage type and the epidemic source.

A method of phage typing has also been applied to the differentiation of strains of Staphylococcus pyogenes and has shown its usefulness in epidemiological studies. Fisk (1942) described a technique by which the latent carrier state could be revealed in Staphylococcus pyogenes, and different strains of this organism distinguished from one another. By cross-testing cultures of staphylococcus on agar, 44 per cent of 43 strains were

found to be latent phage carriers, and from these lysogenic strains, 24 different phages were isolated. By testing the susceptibility of 95 strains of Staphylococcus pyogenes to 27 different phages he was able to distribute 44 of the 95 cultures among 37 different groups.

In a further paper Fisk and Mordvin (1944) studied a series of 78 culture strains of Staphylococcus pyogenes isolated from 30 patients to compare certain culture characteristics and alpha hemotoxin production with phage susceptibility. It was found that strains of the same phage type sometimes differed in hemolytic properties and chromogenesis and not infrequently varied in toxogenicity. Most of the cultures isolated from related sources were found to be of the same phage type but examples were given in which strains of dissimilar phage type were isolated from the same patient.

Wilson and Atkinson (1945) extended Fisk's work and obtained 7 different phages from 25 lysogenic cultures among 46 staphylococcal strains examined. Eleven more phage strains were obtained by growing one of these in the presence of later strains of Staphylococcus pyogenes, a variant of the phage being developed in a manner similar to that used by Craigie and Yen (1938) for the phage typing of typhoid bacilli. Of 1340 strains of Staphylococcus pyogenes, the last 460 were tested with the 18 phages. Of these 60.4 per cent were successfully typed, 22.6 per cent were acted on by phage but not typed, and 17 per cent proved insensitive to the 18 phages. The observations of Williams Smith (1948a, b) suggest that some strains of staphylococci are resistant to typing because they carry phage.

The method of phage typing has been successfully used for tracing

sources of infection in outbreaks of staphylococcal enterotoxin food-poisoning, in staphylococcal infections of skin in babies and breast abcesses in mothers in maternity hospitals, and in staphylococcal infections of wounds and burns. In outbreaks of staphylococcal enterotoxin food-poisoning, when the infective organism has been isolated from the foodstuff, the typing of staphylococci by the bacteriophage method has been invaluable in tracing the infective organism to the nose and hands of a particular food-handler.

III. MATERIALS AND METHODS

BACTERIOPHAGE PREPARATIONS:

When this work was started the following 'crude' phage suspensions and corresponding propagating strains of Staphylococcus pyogenes were available:

(a) Wilson and Atkinson series: phage 3A/284, 3B/211, 51/145, 6/3, 47/36, 47A/761, 47C/1163, 3C/1339, 7/4, 42B/1163, 42C/1307, 42E/1670, 44A/873, 47B/987, 52/144.

Of this series the last 8 phages mentioned were not used in the work here reported.

(b) Fisk's series: phage 47/44, 74/76, 534/533, 534/535, 543/533, 4/10, 48/62, 535/535, 544/542.

Of this series, the last 4 phages mentioned were not used in the work here reported.

(c) A series of 'adapted phages' which had been developed in this laboratory prior to the beginning of this work: phage 3A/44, 3A/62, 3A/144, 3A/145, 3A/533, 3A/535, 3A/542, 36/3, 47/3, 145/62, 145/535, 145/542, 542/76, 543/62, 543/144, 543/535, 543/542.

The numbers by which these phages are designated correspond to the number of the lysogenic strain of staphylococcus from which the phage was derived followed by the number of the strain upon which it was propagated, i.e. phage 543/542 was derived from lysogenic strain 543 and was propagated upon strain 542.

(d) One phage W isolated at Winnipeg from a strain of Staphylococcus

pyogenes referred to as strain W.

(e) A series of 32 phages obtained through the courtesy of Dr. Igor N. Asheshev, New York Botanical Gardens. Their nomenclature is as follows: phage 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, A, B, C, D, S3K. The original sources of this group of phages are varied but none have been used in practical phage typing of Staphylococcus pyogenes. All these phages lyse and have been propagated on a strain of staphylococcus designated strain 11.

As most of these phage preparations were in an impure state, and of low titre, their purification and propagation were desirable before any further work could be carried out.

PURIFICATION OF 'CRUDE' PHAGE SUSPENSIONS:

The 'crude' phage suspensions were purified by the technique described by Wilson and Atkinson (1945) with a few slight modifications. A loopful of the crude filtrates and 4 drops of a 3 hour broth culture of the susceptible strain were plated out over the surface of a peptone agar plate which had been freed of excess moisture by storage in the incubator at 37°C for 2-3 hours with the lid partly removed. This plate was incubated for 6-8 hours at 37°C and then kept at room temperature overnight. A single plaque, with the underlying agar and a little of the surrounding intact culture, was picked off next morning with a sterile needle and plated on peptone agar with 4 drops of the susceptible strain. This plate was again incubated for 6-8 hours at 37°C and at room temperature overnight. A fresh plaque was picked off next morning and the process repeated three times. Five ml. of broth were poured over the sur-

face of the agar plate containing the last plating, left for 20 minutes, collected and centrifuged. The phage in the supernatant fluid was then propagated.

PROPAGATION OF PHAGE:

The strains of staphylococci used for propagation were maintained on peptone agar slants at room temperature, transfers being made every two weeks.

Six drops of the purified phage preparation and 4 drops of a 3 hour broth culture of the propagating strain were added to 10 ml. of tryptose phosphate broth. The mixture was incubated at 37°C until complete lysis of the bacteria had taken place (usually 4-6 hours). More culture was then added and incubation continued until lysis again occurred. This process was repeated several times. Usually cultures were left overnight at room temperature but if it was not practicable to continue serial passage on the following day they were transferred to the refrigerator. Next morning, if secondary growth appeared, the material was filtered through a Zsigmondy membrane (Zsigmondy and Bachmann 1918) and 6 more drops of the original purified phage preparation were added to the filtrate, after which propagation was continued. Lysis would sometimes take place three times in 8 hours and a potent filtrate could be obtained in a short time. The number of passages required to obtain a sufficiently high degree of lytic ability varied usually from 4 to 12. The final phage cultures were filtered through a Zsigmondy membrane and the filtrates stored in the refrigerator in sterile screw-capped tubes without preservative.

DETERMINATION OF 'CRITICAL TEST DILUTION':

Peptone agar plates were dried at 37°C for 2-3 hours with the lid partly removed. The phage preparations were diluted 1/10 to 1/1,000,000 with tryptose phosphate broth. Four drops of a 3 hour tryptose phosphate broth culture of the susceptible strain were spread evenly over the surface of a plate. When the agar had adsorbed the fluid which had been applied (optimum 5 to 10 minutes), a standard loopful of each dilution of the phage preparations was 'spotted' on to the culture. Precaution was taken to avoid actual contact of the loop with the inoculated area as much as possible. When the drops had been adsorbed, the plates were then incubated at 37°C for 6 hours, left at room temperature overnight, and read next morning. The scheme of notation used was as follows:

Confluent lysis with no secondary growth ------ CL

Confluent lysis with secondary growth --------- LSG

Numerous semi-confluent plaques ---------- +++

Discrete plaques ---------- ++

Less than 20 plaques ---------- +

Lesser degrees of lysis, or no lysis ---------- —

The highest dilution of phage preparation producing confluent lysis when plated on agar with the susceptible strain was regarded as the 'critical test dilution'.

DETERMINATION OF PHAGE CONCENTRATIONS (plaque counts):

The number of active phage particles per milliliter of lytic filtrate was determined by two methods, namely the "loop" method, and the

"agar-layer" method. Both methods depend on the ability of a given bacteriophage to produce plaques when varying dilutions of a lytic filtrate are plated on agar with the susceptible strain of bacteria. These two methods are described below.

(1) 'LOOP' METHOD:-

a) Principle of the Method:

This method was first described by Asheshov et al (1933). Standard wire loops are used to transfer definite volumes of bacteriophage suspension to two dilution tubes, one of which contains measured amounts of sterile broth and the other, measured amounts of a corresponding bacterial suspensions. A measured drop of fluid from the final dilution tube is spread on one quadrant of a peptone agar plate by means of one of these loops. By plating a range of dilutions and then counting the number of plaques that develop from the most suitable dilution, the number of active phage particles that were present in the original phage suspension can be calculated.

b) Requirements:

i) A standard 'small loop' made of 0.3 mm thick platinum wire, the inside diameter being 1.5 mm.

ii) A standard 'big loop' made of 0.5 mm thick platinum wire, the inside diameter being 3 mm.

iii) Two plugged Pyrex glass tubes 18 X 150 mm for diluting the phage suspension that is being titrated. The first dilution tube contains 10 ml. of broth whereas the second contains 10 ml. of an emulsion of corresponding bacteria made by adding 1 ml. of a 3 hour broth culture to 9 ml.

of broth.

iv) 10 X 100 mm peptone agar plates.

c) Calibration of Wire Loops:

The average amount of fluid taken by the two loops was ascertained by the following procedure: To calibrate the 'big loop', a tube containing a few milliliters of broth was accurately weighed and then the loop was used to remove a standard drop. The tube and contents were weighed again and the difference in weights gave the weight of broth in the drop. The procedure was repeated ten times and the average volume of fluid per loopful was calculated. The loop was taken out of the liquid flat, with a smooth jerk, so that an almost hemispherical drop formed in the loop. To calibrate the 'small loop' the same procedure was followed, except that 10 small loopfuls were removed for each weighing. The 'small loop' after immersion in the liquid was slowly taken out of it, not flat but sidewise, so that only a thin film was formed in the opening of the loop.

The 'big loop' and 'small loop' were found to remove 0.018 ml. and 8.5×10^{-4} ml. of broth per loopful respectively.

d) Procedure for Routine Counting:

The following scale of dilutions was used:

1. 'Small of the small loop' dilution - 'ssl': - One small loop of the filtrate was introduced in the first dilution tube containing 10 ml. of broth. After thorough shaking, a small loop from this tube was transferred into the second dilution tube containing 10 ml. of bacterial emulsion and a big loop of that was plated on a quadrant of an agar plate.

2. 'Small of the big loop' dilution - 'sbl': - One big loop of the filtrate was introduced into the first tube, a small loop from it into the second, and again a big loop from the last plated.

3. 'Big of the big loop' dilution - 'bbl': - A big loop of the filtrate into the first tube, a big loop from it into the second and a big loop of the last was plated.

4. 'Small loop' dilution - 'sl': - A small loop into the second tube, and a big loop was plated direct from it.

5. 'Big loop' dilution - 'bl': - A big loop of the filtrate into the first tube, and the same amount plated from it.

6. If very few phage particles were believed to be present in a filtrate, two more dilutions were used: 'one c.c.':- One ml. of the filtrate into 10 ml. of bacterial emulsion and a big loop plated from it; and 'direct' - 'd':- When a big loop of the filtrate was spread direct on agar and then covered with one big loop of emulsion.

e) Calculations:

To calculate the original concentrations, the plaque counts per quadrant were multiplied by the appropriate dilution factors. Using the small loop that holds 8.5×10^{-4} ml. and the big loop that holds 0.018 ml., the counts were multiplied by the following factors:

For 'ssl' dilution, by 7.8×10^{9}

For 'sbl' dilution, by 3.65×10^{7}

For 'sl' dilution, by 6.54×10^{5}

For 'bbl' dilution, by 1.7×10^{5}

For 'bl' dilution, by 3.1×10^{4}

For '1 c.c.' dilution, by 5.6×10^{2}

For 'd' dilution, by 5.6×10^{1}

f) Comments:

Although this method is simple and logical, it was abandoned early in this work for the following reasons:

1. As in most cases the approximate titre of the phage preparations was unknown, too much guess-work was involved in judging the correct dilution and too many dilutions had to be plated to obtain a suitable number of well-isolated, discrete plaques.

2. The plaques which developed were not uniformly distributed on the surface of the plates, and consequently, were difficult to count.

3. In cases where replicate platings of a dilution were made, the plaque counts were variable; this is probably due to the irregularity in the volume of fluid removed by the loops.

(2) AGAR-LAYER METHOD:

a) Principle of the Method:

This method seems to have been first described by Gratia (1936). The host bacteria and phage particles are mixed in a small volume of warm 0.7 per cent tryptose phosphate agar and the mixture is poured over the surface of an ordinary 1.5 per cent peptone agar plate and allowed to harden to form a thin layer. The bacteria grow as tiny subsurface colonies in this layer and are nourished by the deep layer of 1.5 per cent nutrient agar which is used as foundation. The plaques appear as clear holes in the opaque layer of bacterial growth.

b) Procedure:

The soft 0.7 per cent tryptose phosphate agar was melted in a boiling water bath and cooled in a 46°C water bath. It was then transferred with a warmed pipette in 2.5 ml. amounts to warmed test-tubes in a

46°C water bath. One drop from a Pasteur pipette of a 3 hour tryptose phosphate broth culture of the susceptible bacteria was added to each tube of soft agar. Then one ml. of the phage suspension, diluted to give a readable number of plaques (50 to 500), was pipetted into the tubes of soft 0.7 per cent tryptose phosphate agar and the entire content of the tube was poured over the surface of a sterile solidified 1.5 per cent peptone agar plate. The plate was rocked gently to mix the bacteria and phage particles and set aside to harden at room temperature. Both the 1.5 per cent agar and the soft agar layer were allowed to harden with the plates resting on a level surface, to insure uniform distribution of the phage plaques over the plate surface.

Because in this method plaques form late during the life of the culture, growth must be induced to continue as long as possible. Therefore the plates were incubated for 18-24 hours at 37°C and read the following morning.

c) Comments:

By this method, the host bacteria and phage particles are uniformly distributed over the surface of the plate. Since fixation of the phage by bacterial cells occurs almost entirely after the plate is poured, the low concentration (0.7 per cent) of agar in the seeded layer permits more rapid diffusion of the phage particles. The plaque count is strictly proportional to the dilution used.

CULTURE MEDIA:

For propagation of the phages and for experimental purposes the following media have been used:

a) Beef heart infusion broth (Difco).

b) Beef heart infusion broth (Difco) with 0.2 per cent glucose added.

c) Bacto tryptose phosphate broth (Difco).

d) Peptone broth (McGill).

e) Peptone agar (McGill).

f) Bacto nutrient broth (Difco).

These are standard media which are used routinely in the Department of Bacteriology and Immunology at McGill University.

In addition Fildes' synthetic medium (Fildes et al 1936, 1937) has served in some experiments described further. The exact composition and preparation of the medium is as follows:

Nutrient	Quantity	Nutrient	Quantity
KH_2PO_4	4.5 gm.	l-oxyproline	0.08 gm.
Water	550 ml.	s-aspartic acid	0.18 gm.
N/1 NaOH	26 ml.	d-glutamic acid	0.09 gm.
s-alanine	0.12 gm.	s-phenylalanine	0.08 gm.
s-valine	0.15 gm.	l-tyrosine	0.05 gm.
s-leucine	0.17 gm.	l-arginine HCl	0.05 gm.
s-glycine	0.05 gm.	l-histidine HCl	0.05 gm.
l-proline	0.07 gm.	s-lysine HCl	0.09 gm.

The amino acids were dissolved, the pH brought to 7.4, and the volume adjusted to 600 ml. with distilled water. This basal mixture was distributed into tubes in 6 ml. volumes and autoclaved for 20 minutes at 120°C. To each of these tubes the following compounds were added separately:-

Ferrous ammonium sulfate, M/500 in M/50 HCl (autoclaved)	0.25 ml.
$MgSO_4 . 7H_2O$, M/60 (autoclaved)	0.1 ml.
$NaNO_3$, M/5 (autoclaved)	0.1 ml.
s-aneurin (Vit B_1), M/2000 in M/1000 HCl (autoclaved 10 min. at 100°C., diluted 1/100)	0.2 ml.
s-nicotinamide, M/200 (autoclaved, diluted 1/10)	0.2 ml.
s-methionine, M/100 (filtered)	0.2 ml.
l-tryptophane, M/1000 (autoclaved)	0.1 ml.
l-cystine, M/100 in M/10 HCl (filtered)	0.2 ml.
NaOH, M/5 (autoclaved)	0.1 ml.
Glucose, M/2 (filtered	0.25 ml.
Water	2.3 ml.

- denotes a substance of synthetic origin.

The medium was tested for sterility by incubation at 37°C for 48 hours and stored in the refrigerator until used.

BECKMAN pH METER:

This instrument was employed for the determination of the pH of all aqueous solutions. The instrument was standardized every time it was used by the use of Beckman buffer solution at pH 7.0.

BROWN'S TUBES:

These tubes containing suspensions of standardized opacity as described by Brown and Kirwan (1915) were used for the estimation of the number of organisms in bacterial suspensions.

PIPETTES:

Whenever possible, Kimble-Exax pipettes (T.D.) were used for the preparation of dilutions and quantitative transfers of lytic filtrates. A new pipette was used for each dilution.

IV. EXPERIMENTAL WORK

The staphylococcus bacteriophages used in this study have been purified and propagated on their homologous cultures by the methods described under 'Materials and Methods'.

EXPERIMENT 1: Evaluation of Different Culture Media for the Growth of Staphylococcus Bacteriophages.

This experiment was performed in an attempt to find a culture medium, preferably a synthetic medium, which would provide constant cultural conditions for the growth of staphylococcus phages in all further work. Phage 543/533 was grown in different culture media, including Fildes' synthetic medium (prepared as described under 'Materials and Methods'), in order to find the medium most suitable for phage multiplication.

Method of Conduction:

Six drops of a lytic filtrate of phage 543/533 were added to 10 ml. of each of the following nutrient media: beef heart infusion broth, 0.2% glucose-beef heart infusion broth, and Fildes' synthetic medium. Each of these suspensions was then inoculated with four drops of a 3 hour peptone broth culture of the susceptible strain of staphylococcus. The mixtures were incubated at 37°C for 6 hours and were then filtered through a Zsigmondy membrane. Various dilutions of each filtrate were prepared with the corresponding medium by Asheshov's loop method and a standard loopful of each dilution was layered over a quadrant of an agar plate previously seeded with the susceptible culture. The plates were incubated at 37°C for 6 hours, left at room temperature overnight, and read the following morning. The yield of phage in each medium

TABLE I.

Dilution of filtrates	Beef heart infusion broth		0.2% glucose-beef heart infusion broth		Fildes' synthetic medium	
	No. of plaques	No. of particles per ml.	No. of plaques	No. of particles per ml.	No. of plaques	No. of particles per ml.
"d"	CL•	—	CL	—	0	—
"one c.c."	LSG✱	—	LSG	—	0	—
"bl"	+++●	—	+++	—	0	—
"bbl"	16	2.7×10^6	25	4.3×10^6	0	—
"sl"	0	—	3	1.96×10^6	0	—
"sbl"	0	—	0	—	0	—
"ssl"	0	—	0	—	0	—

• = confluent lysis; ✱ = confluent lysis with secondary growth; ● = numerous semi-confluent plaques.

was determined by multiplying the plaque counts by the appropriate dilution factors. The results are summarized in Table I.

Results:

The results obtained were not very satisfactory. Only with a few dilutions could a number of well-isolated, discrete plaques be obtained, and furthermore, the number of plaques that developed was not proportional to the dilution that was plated. For these reasons, Asheshov's loop method for preparing dilutions and plating of bacteriophage was abandoned.

However it appeared from the results obtained that Fildes' synthetic medium is unsuitable for the growth at least of phage 545/533 since no plaque developed from the dilutions which were plated.

EXPERIMENT 2: The Multiplication of Staphylococcus Bacteriophages in Fildes' Synthetic Medium and in Peptone Broth.

Since Fildes' synthetic medium does not provide optimal cultural conditions for the growth of phage 545/533 (as shown by the preceding experiment), other phages were tested and the yield of phage in this medium was compared with that in peptone broth. Unsatisfactory results were obtained in the preceding experiment by the use of Asheshov's loop method for determining phage concentration. Therefore, in this experiment, the phage concentration was estimated by determining the 'critical test dilution' of the phages, a method which gives a rough indication of the number of particles in a phage suspension.

TABLE II.

Phage No.	Medium	Dilutions of Supernatant Phage				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
3A/284	Peptone broth	CL	CL	CL	LSG	+++
	Fildes' medium	CL	LSG	+++	+	—
47/36	Peptone broth	CL	CL	CL	LSG	+++
	Fildes' medium	CL	LSG	++	—	—
534/535	Peptone broth	CL	CL	CL	LSG	++
	Fildes' medium	LSG	+++	+	—	—
47C/1163	Peptone broth	CL	CL	CL	LSG	+++
	Fildes' medium	+++	++	+	—	—

Method of Conduction:

Phage 3A/284, 47/36, 543/533, and 47C/1163 were used in this experiment. A 1/100 dilution of each phage was prepared in Fildes' synthetic medium and in peptone broth. 0.1 ml. of a 3 hour peptone broth culture of the homologous strain was added to 10 ml. volumes of each of these phage suspensions. The mixtures were incubated at 37°C for 4 hours and left at room temperature overnight. Next morning each mixture was centrifuged and the 'critical test dilution' of the phage in the supernatant fluid was determined by the method described under 'Materials and Methods'.

Results:

The results are shown in Table II, where it may be seen that the critical test dilution of each phage grown in Fildes' synthetic medium is about one hundredfold less than in peptone broth. For instance, the critical test dilution of phage 3A/284 grown in peptone broth is 10^{-4} compared to 10^{-2} when grown in Fildes' synthetic medium. Similarly, the critical test dilution of phage 543/533 grown in peptone broth is 10^{-4}, whereas in Fildes' synthetic medium, it is less than 10^{-2}. This indicates that Fildes' synthetic medium, although adequate for bacterial multiplication, does not necessarily support phage multiplication.

EXPERIMENT 5: The Effect of pH on the Multiplication of Staphylococcus Bacteriophages.

In the preceding experiments, the phages were grown in Fildes' synthetic medium at pH 7.4, which might not have been the optimum pH for the growth of these phages. This experiment was undertaken to ascertain whether alteration in the pH of Fildes' synthetic medium would provide more favourable conditions for phage multiplication.

Method of Conduction:

Ten ml. of 1/10 dilutions of phage 3A/284, 47/36, and 47C/1163 were prepared in Fildes' synthetic medium and in Bacto nutrient broth at pH 7.4, 7.6, and 7.8. In addition, 10 ml. of a 1/10 dilution of each phage was prepared in peptone broth at pH 7.2. Each of these suspensions was then inoculated with 0.5 ml. of a 3 hour peptone broth culture of the susceptible strain. The mixtures were incubated at 37°C for 6 hours and then left at room temperature overnight. The next morning the mixtures were centrifuged and the critical test dilution of the phage in the supernatant fluid was determined. Table III shows the lytic reactions obtained.

Results:

In the preceding experiment it was found that the phages used grew favourably in peptone broth at pH 7.2. Therefore, it might be of interest to compare the critical test dilution of each phage grown in Fildes' synthetic medium and in Bacto nutrient broth at different pH values with that obtained when the phages were grown in peptone broth at pH 7.2.

It can be seen from Table III that the critical test dilutions of phage 3A/284, 47/36, and 47C/1163 when grown in peptone broth are 10^{-5}, 10^{-5}, and 10^{-4} respectively. In Bacto nutrient broth at pH 7.8, the critical test dilution of phage 3A/284 is 10^{-4}, therefore slightly lower than in peptone broth. At pH 7.4 and 7.6 in the same medium, the critical test dilution of phage 3A/284 is still much lower. Therefore phage 3A/284 grows best in Bacto nutrient broth at pH 7.8.

Similarly phage 47/36 grows better in Bacto nutrient broth at

TABLE III.

Effect of pH on phage multiplication

Phage No.	Medium	pH	Dilution of Supernatant Phage				
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
3A/284	Pept. br.*	7.2	CL	CL	CL	CL	LSG
	Fildes'‡	7.4	LSG	+++	—	—	—
	Fildes'	7.6	CL	LSG	+++	++	—
	Fildes'	7.8	—	—	—	—	—
	Bact.n.br.+	7.4	LSG	LSG	+++	++	—
	Bact.n.br.	7.6	CL	CL	LSG	LSG	+++
	Bact.n.br.	7.8	CL	CL	CL	LSG	LSG
47/36	Pept. br.	7.2	CL	CL	CL	CL	LSG
	Fildes'	7.4	LSG	+++	+	—	—
	Fildes'	7.6	LSG	+++	+	—	—
	Fildes'	7.8	LSG	+++	+	—	—
	Bact.n.b.	7.4	CL	CL	LSG	+++	++
	Bact.n.b.	7.6	CL	CL	LSG	LSG	+++
	Bact.n.b.	7.8	CL	CL	CL	CL	+++
47C/1163	Pept. br.	7.2	CL	CL	CL	LSG	++
	Fildes'	7.4	+	—	—	—	—
	Fildes'	7.6	—	—	—	—	—
	Fildes'	7.8	—	—	—	—	—
	Bact.n.b.	7.4	LSG	LSG	+++	+	—
	Bact.n.b.	7.6	CL	LSG	+++	+	—
	Bact.n.b.	7.8	CL	LSG	+++	++	+

* peptone broth; ‡ Fildes' synthetic medium; + Bacto nutrient broth.

pH 7.8 since its critical test dilution when grown in this medium at pH 7.8 is the same as that in peptone broth. On the other hand, the critical test dilution of phage 47C/1163 grown in Bacto nutrient broth at all the pH values tested is considerably lower than in peptone broth at pH 7.2.

Within the range of pH tested, Fildes' synthetic medium proved unsuitable for the growth of these phages. Why this medium tested at different pH values with various phages does not enhance phage multiplication has not been elucidated, and still awaits future investigation. There is a possibility that there is a substance absent, or present in so small an amount in this medium, that phage formation stops when this compound is depleted. Consequently, Fildes' synthetic medium was not used in further work.

EXPERIMENT 4: The Rate of Adsorption of a Staphylococcus Bacteriophage on its Homologous Strain and on an Heterologous Strain.

This experiment was done as a preliminary to determine the period of incubation required for complete adsorption of a staphylococcus phage on its homologous strain and on an heterologous strain, to serve as basis for further experiments of 'selective' adsorption.

a) The Rate of Adsorption of Phage 47A/761 on Homologous Strain 761.

Method of Conduction:

A young culture of strain 761, grown at 37°C for 3 hours in tryptose phosphate broth, was standardized by Brown's opacity method to contain approximately 6.0×10^8 bacteria per milliliter. A 5.5 ml. aliquot of this suspension was immediately added to an equal amount of a lytic filtrate of phage 47A/761 containing 6.2×10^6 lytic particles per milliliter.

The mixture was rapidly mixed and incubated in a 37°C water bath. Samples of the adsorption mixture were removed at known time intervals (Table IV) and each was divided into two portions. One portion was filtered through a Zsigmondy membrane to remove the bacteria and adsorbed phage, the filtrate diluted 1/10,000 with tryptose phosphate broth, and an aliquot of this dilution accurately assayed for residual free phage by the agar-layer method (described under 'Materials and Methods'). The other portion was centrifuged at 4°C for 15 minutes to sediment the bacteria and adsorbed phage, the cell sediment washed twice in sterile saline and re-suspended in a volume of broth equal to the original amount before centrifugation. An aliquot of a 1/10,000 dilution of this suspension was then accurately assayed for adsorbed phage by the agar-layer technique.

The cell concentration in the adsorption mixture was approximately a hundredfold greater than that of the phage to give infection of each bacterial cell by no more than one phage particle. This method gives directly at the same moment the number of both the free and the adsorbed phage particles in the adsorption tube, the sum of which should equal at any time the total phage input in the adsorption mixture.

Results:

Table IV summarizes the results obtained. It shows that the great majority of the phages (83.3%) have been adsorbed on to the bacterial cells within 30 minutes. The large increase in the amount of phage in the adsorption mixture at 2 hours is presumably due to the release of new phage from bacteria infected earlier. The titre before mixing the phage and culture is twice the value in the adsorption mixture, owing to dilution of the phage with the added culture. This is taken into account in the calculation of the

TABLE IV.

Showing the progress of adsorption of phage 47A/761 on strain 761 at 37°C. Initial no. of phage in adsorption mixture, 3.1 X 10^6 lytic particles per ml.

Time after addition of culture	Plaque count of unadsorbed phage	Titre of unadsorbed phage (a)	Plaque count of adsorbed phage	Titre of adsorbed phage (b)	Sum of (a) and (b)	Percentage adsorption
5 min.	245	2.45 X 10^6	118	1.18 X 10^6	3.63 X 10^6	20.9
10 "	143	1.43 X 10^6	172	1.72 X 10^6	3.15 X 10^6	53.9
20 "	91	0.91 X 10^6	240	2.40 X 10^6	3.31 X 10^6	70.7
30 "	52	0.52 X 10^6	304	3.04 X 10^6	3.56 X 10^6	83.3
2 hours	greater than 6000	greater than 60 X 10^6	—	—	—	—

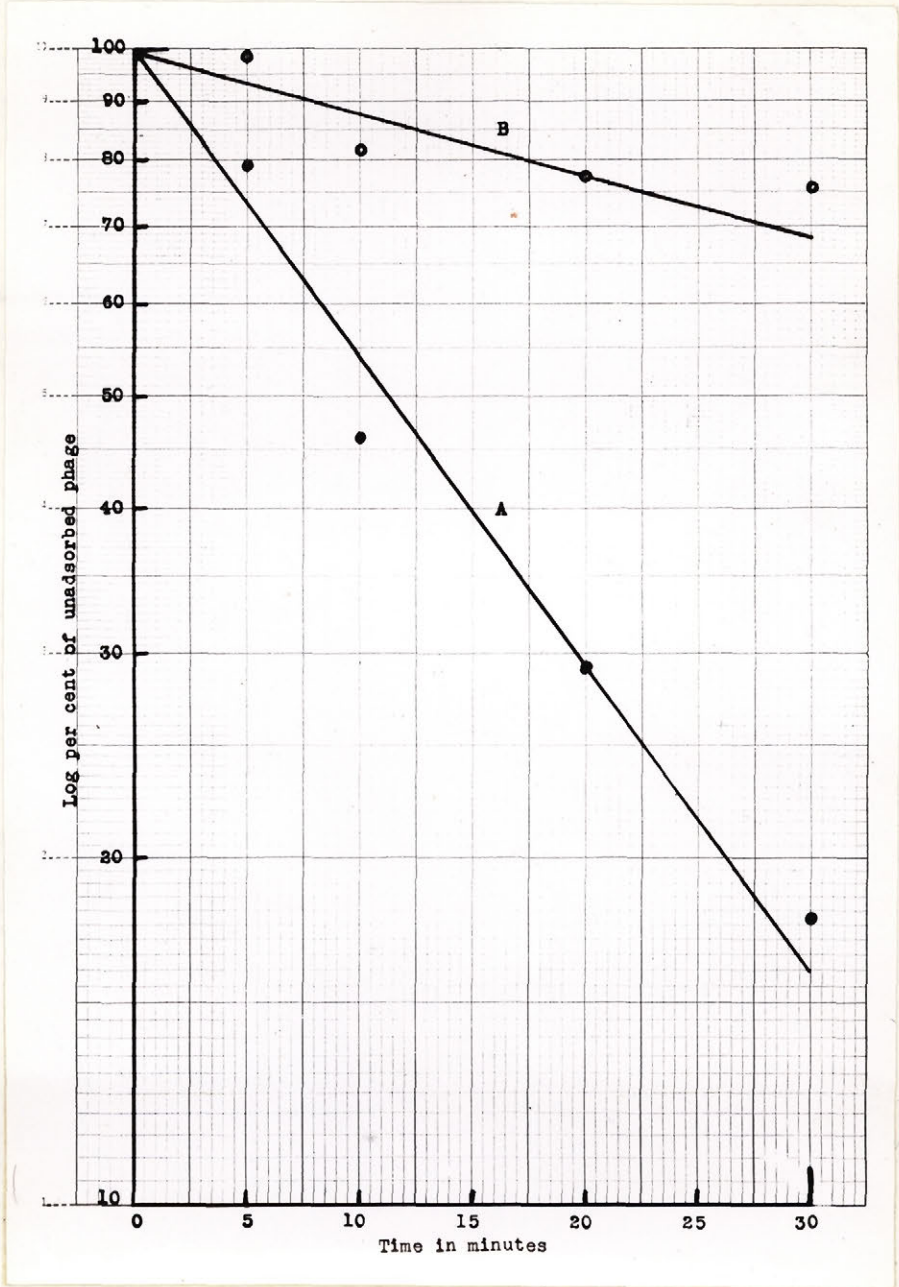

Fig. I showing the logarithmic adsorption of phage 47A/761 on its homologous strain 761 (curve A) and on heterologous strain 44 (curve B).

percentage adsorption.

As expected, the sum of the titre of unadsorbed phage and that of adsorbed phage at any moment equals, within the limits of experimental error, the total phage input in the adsorption mixture. This indicates that no loss of phage occurred when the samples of the phage-bacterium mixtures were filtered through the Zsigmondy membrane.

Fig. I shows the curve obtained (curve A) when the percentage of unadsorbed phage is plotted against the time. The linear relation indicates that adsorption of phage 47A/761 on its homologous culture at 37°C in tryptose phosphate broth proceeds at a logarithmic rate.

b) The Rate of Adsorption of Phage 47A/761 on Heterologous Strain 44.

Method of Conduction:

The first step in this experiment was to determine whether strain 44 was susceptible to lysis by phage 47A/761. This was carried out as follows: A few drops of a tryptose phosphate broth culture of strain 44 were spread evenly on the surface of a peptone agar plate. When these had dried, a few drops of an undiluted lytic filtrate of phage 47A/761 were spotted on the surface of the culture. The plate was incubated at 37°C for 6 hours, left at room temperature overnight, and examined the following morning for any evidence of phage action in the form of plaques or rings of inhibition of growth, but with entirely negative results.

With the knowledge that strain 44 is completely insusceptible to the lytic action of phage 47A/761, the progress of adsorption of this phage on strain 44 was followed by this procedure: A 3 hour tryptose phosphate broth culture of strain 44 was standardized by Brown's opacity method to

contain 5.2 X 10^7 bacteria per ml. and 4 ml. of the suspension were immediately added to an equal amount of a lytic filtrate of phage 47A/761 containing 7.16 X 10^5 lytic particles per milliliter. The mixture was incubated at 37°C, and at known time intervals (Table V) samples were removed and filtered through a Zsigmondy membrane to remove the bacteria and adsorbed phage. The filtrate of each sample was diluted 1/1000 with tryptose phosphate broth and an aliquot plated on culture 761 by the agar-layer method.

Results:

Table V shows that only 24.6% of the phages have been adsorbed within an incubation period of 30 minutes. Adsorption of phage 47A/761 on strain 44 proceeds very slowly as shown by the low percentage (30.8%) of phages adsorbed even after 2 hour incubation at 37°C and by the slope of curve B in Fig. I.

Discussion and Summary of Experiment 4:

The progress of adsorption of phage 47A/761 on its homologous strain 761 and on heterologous strain 44 has been followed by the method already described. The chief point of interest which emerges from the results obtained is that adsorption proceeds at a logarithmic rate on both the susceptible and the resistant strain, but at a much slower rate on the resistant strain.

At the end of 2 hour incubation, new phages were liberated by the susceptible strain, whereas in the case of the resistant strain no release could be observed, the adsorbed phages being inactivated.

The results indicate that at least 45 minute incubation at 37°C is required for complete adsorption of phage 47A/761 on its homologous

TABLE V.

Showing the progress of adsorption of phage 47A/761 on strain 44 at 37°C. Initial no. of phage in adsorption mixture, 3.58×10^5 lytic particles per ml.

Time after addition of culture	Plaque count of unadsorbed phage	Titre of unadsorbed phage	Percentage adsorption
5 min.	355	3.55×10^5	0.9
10 "	292	2.92×10^5	18.5
20 "	276	2.76×10^5	22.9
30 "	270	2.70×10^5	24.6
2 hours	248	2.48×10^5	30.8

strain, at least under the conditions of this experiment.

EXPERIMENT 5: The Effect of Time, Temperature, and Medium on the Stability of Staphylococcus Bacteriophages.

It is well known that most staphylococcus bacteriophages, unlike coli and dysentery phages, are very sensitive to a variety of agents. Suspensions of these phages often show a marked decrease in titre a few weeks after preparation. It is the purpose of this experiment to describe the effect of several conditions of storage on the stability of staphylococcus bacteriophages.

Method of Conduction:

Lytic filtrates of phages 3A/284, 47A/761, and 543/533 were used in this experiment. The phage concentration of each filtrate was determined by the agar-layer technique. A sample of each filtrate was mixed with an equal amount of each of the following media: peptone broth, 50% glycerine, and tryptose phosphate broth. A 1/100 dilution of each phage in sterile distilled water was also prepared. Two ml. samples of each preparation were stored at the following temperatures: -30°C in the deep-freezer, +4°C in the refrigerator, and at room temperature. In addition, 2 ml. samples of each phage in peptone broth and tryptose phosphate broth were lyophilized in the Edwards' Centrifugal Freeze Dryer. An aliquot of each of these experimental preparations was then titrated by the agar-layer method at the following time intervals: phage 3A/284 and 543/533 every 3 weeks during a period of 15 weeks; phage 47A/761 every $3\frac{1}{2}$ weeks during a period of $15\frac{1}{2}$ weeks. The phage concentration of each preparation under the conditions of storage mentioned above was determined by multiplying the plaque counts by the appropriate dilution factors. The results are summarized in Table VI.

TABLE VI

The Effect of Time, Medium, and Temperature on Phage Concentration

Phage No.	Medium	Environment	Phage Concentration (No. particles/ml. Initial No. particles per ml.	3 weeks	3.5 weeks	6 weeks	6.5 weeks	9 weeks	9.5 weeks	12 weeks	12.5 weeks	15 weeks	15.5 weeks
543/533	peptone broth	-30°C	6.5×10^8	2.4×10^8		2.4×10^8		2.56×10^8		2.5×10^8		2.46×10^8	
543/533	peptone broth	+4°C	6.5×10^8	1.14×10^8		1.2×10^8		1.3×10^8		1.34×10^8		1.24×10^8	
543/533	peptone broth	room t°	6.5×10^8	4.0×10^6		8.0×10^5		3.2×10^6		—		—	
543/533	peptone broth	lyophilized	6.5×10^8	7.2×10^7		7.2×10^7		7.0×10^7		9.0×10^7		8.76×10^7	
543/533	50% glycerine	-30°C	6.5×10^8	1.8×10^8		1.4×10^8		1.88×10^8		1.98×10^8		1.74×10^8	
543/533	50% glycerine	+4°C	6.5×10^8	1.3×10^8		6.8×10^7		7.0×10^7		6.4×10^7		6.0×10^7	
543/533	50% glycerine	room t°	6.5×10^8	2.8×10^7		2.0×10^7		4.0×10^6		6.0×10^6		5.4×10^6	
543/533	distilled water	-30°C	6.5×10^8	1.2×10^8		9.8×10^7		1.2×10^8		1.1×10^8		1.12×10^8	
543/533	distilled water	+4°C	6.5×10^8	9.0×10^7		7.0×10^7		5.4×10^7		5.6×10^7		5.1×10^7	
543/533	distilled water	room t°	6.5×10^8	5.1×10^7		3.3×10^7		3.1×10^7		3.3×10^7		—	
543/533	tryptose phosphate	-30°C	6.5×10^8	2.6×10^8		2.6×10^8		2.62×10^8		2.7×10^8		2.68×10^8	
543/533	tryptose phosphate	+4°C	6.5×10^8	1.5×10^8		1.1×10^8		1.04×10^8		1.06×10^8		1.0×10^8	
543/533	tryptose phosphate	room t°	6.5×10^8	3.4×10^6		1.2×10^6		5.6×10^5		—		—	
543/533	tryptose phosphate	lyophilized	6.5×10^8	6.0×10^7		5.6×10^7		5.9×10^7		7.3×10^7		8.04×10^7	
3A/284	peptone broth	-30°C	9.0×10^7	2.8×10^7		2.1×10^7		2.12×10^7		2.24×10^7		2.76×10^7	
3A/284	peptone broth	+4°C	9.0×10^7	5.0×10^7		2.52×10^7		3.16×10^7		3.28×10^7		3.52×10^7	
3A/284	peptone broth	room t°	9.0×10^7	1.3×10^7		1.0×10^7		1.2×10^7		—		—	
3A/284	peptone broth	lyophilized	9.0×10^7	1.14×10^7		8.6×10^6		9.2×10^6		1.38×10^7		—	
3A/284	50% glycerine	-30°C	9.0×10^7	3.38×10^7		3.16×10^7		3.7×10^7		3.6×10^7		3.48×10^7	
3A/284	50% glycerine	+4°C	9.0×10^7	2.1×10^7		1.86×10^7		1.7×10^7		1.96×10^7		1.8×10^7	
3A/284	50% glycerine	room t°	9.0×10^7	1.12×10^7		8.0×10^6		7.9×10^5		4.6×10^5		6.0×10^3	
3A/284	distilled water	-30°C	9.0×10^7	7.1×10^6		5.5×10^6		5.4×10^6		6.4×10^6		6.9×10^6	
3A/284	distilled water	+4°C	9.0×10^7	2.9×10^6		2.6×10^6		2.3×10^6		2.6×10^6		3.0×10^6	
3A/284	distilled water	room t°	9.0×10^7	7.0×10^5		6.2×10^5		6.3×10^5		—		—	
3A/284	tryptose phosphate	-30°C	9.0×10^7	3.4×10^7		2.9×10^7		3.1×10^7		3.1×10^7		3.16×10^7	
3A/284	tryptose phosphate	+4°C	9.0×10^7	3.92×10^7		3.6×10^7		3.4×10^7		4.0×10^7		4.6×10^7	
3A/284	tryptose phosphate	room t°	9.0×10^7	4.0×10^1		0		—		—		—	
3A/284	tryptose phosphate	lyophilized	9.0×10^7	9.6×10^6		9.4×10^6		9.6×10^6		1.32×10^7		1.38×10^7	
47A/761	peptone broth	-30°C	15×10^8		2.4×10^8		2.5×10^8		1.82×10^8		2.56×10^8		2.50×10^8
47A/761	peptone broth	+4°C	15×10^8		2.8×10^8		2.76×10^8		1.92×10^8		2.78×10^8		2.86×10^8
47A/761	peptone broth	room t°	15×10^8		1.4×10^6		1.8×10^2		—		—		—
47A/761	peptone broth	lyophilized	15×10^8		1.5×10^8		8.0×10^7		9.0×10^7		1.76×10^8		1.18×10^8
47A/761	50% glycerine	-30°C	15×10^8		2.0×10^8		2.34×10^8		2.1×10^8		2.3×10^8		2.36×10^8
47A/761	50% glycerine	+4°C	15×10^8		1.2×10^8		1.4×10^8		7.2×10^7		6.8×10^7		7.4×10^7
47A/761	50% glycerine	room t°	15×10^8		6.0×10^5		8.4×10^2		0		—		—
47A/761	distilled water	-30°C	15×10^8		1.1×10^8		1.0×10^8		8.7×10^7		1.18×10^8		1.15×10^8
47A/761	distilled water	+4°C	15×10^8		1.0×10^8		1.0×10^8		8.4×10^7		1.11×10^8		1.16×10^8
47A/761	distilled water	room t°	15×10^8		4.0×10^2		0		—		—		—
47A/761	tryptose phosphate	-30°C	15×10^8		2.2×10^8		2.1×10^8		1.74×10^8		2.32×10^8		2.24×10^8
47A/761	tryptose phosphate	+4°C	15×10^8		3.4×10^8		3.2×10^8		1.96×10^8		3.46×10^8		3.26×10^8
47A/761	tryptose phosphate	room t°	15×10^8		1.0×10^4		0		—		—		—
47A/761	tryptose phosphate	lyophilized	15×10^8		1.4×10^8		7.8×10^7		9.8×10^7		1.04×10^8		9.4×10^7

Results:

The results of this experiment may be evaluated by comparing the phage concentration of each preparation at the time intervals mentioned above with the initial phage concentration. From Table VI it is evident that a loss of approximately 75% of the phages occurs in all preparations during the first three weeks, after which the phage concentration remains more or less constant.

Most phages are very unstable and become inactivated rapidly at room temperature but retain their activity for a longer period of time at lower temperatures. On the average they are more stable at -30°C in the deep-freezer than at +4°C in the refrigerator. Thus the concentration of phage 543/533 in the media used is twice as great at -30°C than at +4°C. The concentration of phage 34/884 in both peptone broth and tryptose phosphate broth is about equal, but in glycerine and in distilled water the concentration is twice as great at -30°C than at +4°C. The concentration of phage 47A/761 in peptone broth, distilled water, and tryptose phosphate broth is the same at -30°C and at +4°C, but in glycerine its concentration is about twice as great at -30°C than at +4°C.

In the lyophilized state, the phages are about equally stable when suspended in peptone broth and in tryptose phosphate broth. However the phage concentration is lower than in the preparations which were stored at -30°C and +4°C.

Fig. 2 shows the type of curves obtained when the phage concentration in a number of preparations is plotted against the time. These curves illustrate the rapid inactivation of phage at room temperature, the decrease in phage concentration during the first three weeks and stabilization thereafter.

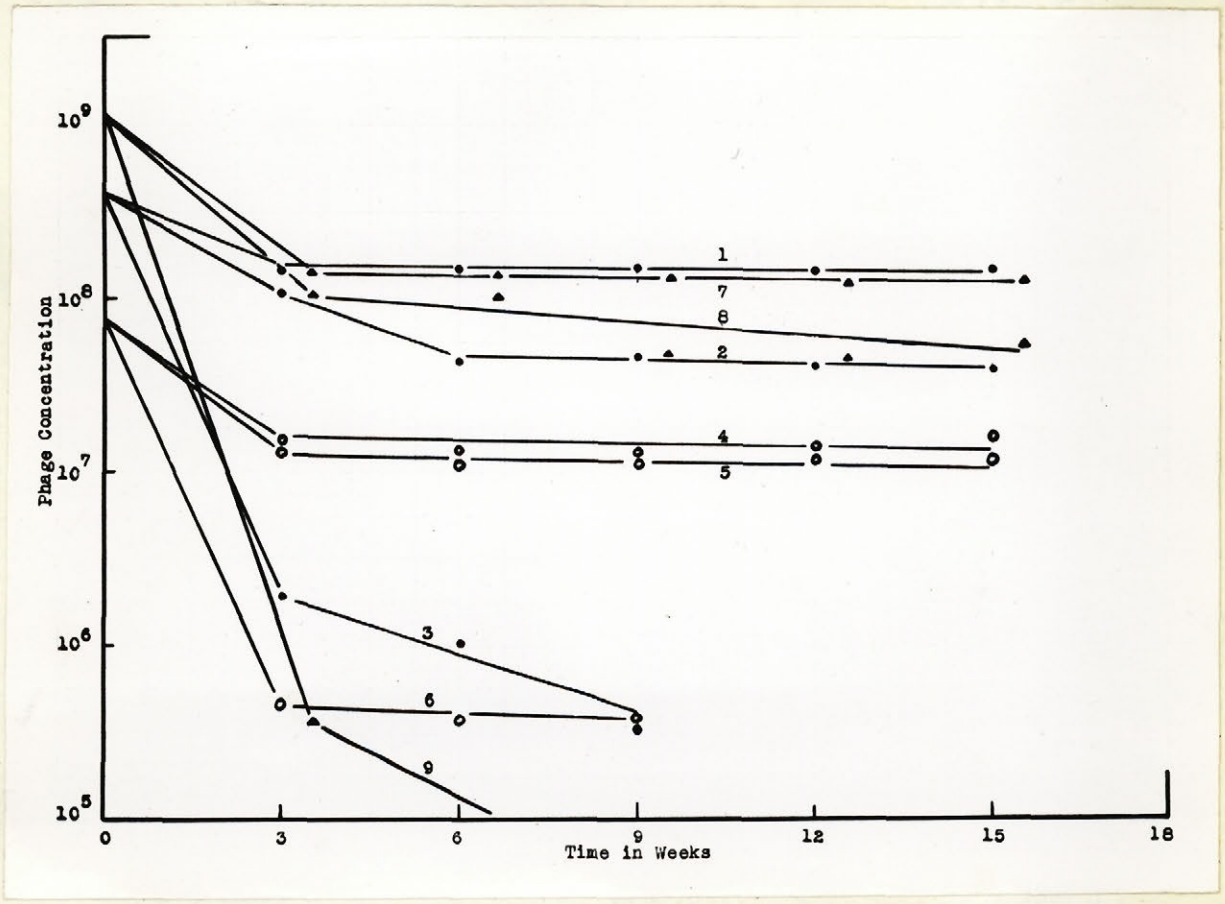

Fig. 2 The effect of time, medium, and temperature on phage concentration.

Curve 1. Phage 543/533 in peptone broth at -30°C.

Curve 2. Phage 543/533 in 50% glycerine at +4°C.

Curve 3. Phage 543/533 in tryptose phosphate broth at room temperature.

Curve 4. Phage 3A/284 in peptone broth at -30°C.

Curve 5. Phage 3A/284 in 50% glycerine at +4°C.

Curve 6. Phage 3A/284 in distilled water at room temperature.

Curve 7. Phage 47A/761 in peptone broth at -30°C.

Curve 8. Phage 47A/761 in 50% glycerine at +4°C.

Curve 9. Phage 47A/761 in 50% glycerine at room temperature.

Summary of Experiment 5:

The results of this experiment indicate that a loss of approximately 75% of the phages occurs during the first three weeks of storage. Maximum stability seems to be achieved at a temperature of -30°C in the deep-freezer. The phages were on the average equally stable in all media tested, provided they were stored at low temperatures.

EXPERIMENT 6: Detection of "Latent Phage" in Cultures of Propagating Strains.

As shown by Rountree (1949a), many strains of staphylococci are lysogenic and filtrates of cultures of these strains often contain phages which can be demonstrated by the use of sensitive or 'indicator' strains. According to Williams Smith (1948a, b), many strains of staphylococci are resistant to the lytic action of bacteriophages because a large proportion of these strains are lysogenic.

The phages carried by the lysogenic staphylococci which are used in the propagation of stock phages occur as contaminants in stock filtrates. Therefore to obtain stock phages which are free from these contaminant phages, it is advisable to use propagating strains of staphylococci which are known to be non-lysogenic. An attempt was made in this investigation to detect the presence of 'latent phages' in cultures of the twenty-four propagating strains of staphylococci available.

Method of Conduction:

Lysogenic strains were detected by Fisk's cross-culture method (1942). Four hour and 24 hour broth cultures of the propagating strains grown in tryptose phosphate broth at 37°C were examined. Four drops of the culture to be examined were spread evenly over the surface of a peptone

agar plate, and after the plate had dried, the remaining cultures were 'spotted' upon it. For the sake of convenience the bottom of the plate was divided by a glass-writing pencil into twenty-four one-half inch squares, so that the twenty-four propagating strains could be spotted upon any one strain on one plate. When the fluid which had been applied had dried sufficiently to permit the plates of being safely moved, they were incubated at 37°C for 6-8 hours and left at room temperature overnight. The next morning they were examined for any evidence of phage action in the form of plaques or rings of inhibition of growth around the edge of the spotted culture. The growth of any pair of strains showing evidence of phage action was scraped off the agar and suspended in one ml. of broth. This was centrifuged and the supernatant fluid was tested against both strains to find out which was the lysogenic and which the susceptible strain.

Results:

Among the twenty-four propagating strains of staphylococci examined by this method, only three were found to be lysogenic, namely strain 4, 10, and 36. Strain 4 liberated free phages which could be detected in the 24 hour but not in the 4 hour broth culture. Strain 3 was found to be the indicator strain sensitive to the phages carried by strain 4.

Strain 10 liberated free phages which could be detected in the 4 hour but not in the 24 hour broth culture, while free phages were detected in both the 4 hour and the 24 hour broth cultures of strain 36. The indicator strains sensitive to the phages carried by strain 10 and 36 were found to be strain 535 and 3 respectively.

EXPERIMENT 7: The Serological Differentiation of Staphylococcus Bacteriophages.

Preparation of Anti-bacteriophage Sera:

Antisera against phage 3A/284, 47A/761, and 543/533 were obtained by injecting high titre, filtered broth lysates of these strains subcutaneously into rabbits. Three animals (1), (2), and (3) were injected with each of these strains of phage to allow for individual variation in immunological response and to provide insurance against loss of animals through sickness or accidental death. One rabbit (3) from each group was lost during the course of immunization. Two injections of 5 ml. per week for 3 weeks were made followed by a test bleeding 7 days after the last injection. The test bleeding was made by slitting the marginal ear vein and collecting about 5 ml. of blood. The serum was then titrated for antiphage activity, and if the titre was unsatisfactory, a second course of injections was given. When the sera were sufficiently potent for use, the rabbits were bled by cardiac puncture 7 days after the last injection and 35-50 ml. of blood was removed and allowed to clot at room temperature for 1-2 hours. After centrifugation the serum was decanted, centrifuged again if necessary to remove residual red cells, heated for 1/2 hour in a 56 C water bath to destroy complement, and stored in sterile screw-capped tubes in the refrigerator.

Antisera to phages 13 and 30 (Asheshov's series) were prepared in a similar manner but in this case the usual course of injection of the phage lysates was 0.5, 1.0, 1.5, and 2.0 ml. injected intravenously at 5-day intervals, the rabbits being bled 7-9 days after the last injection. Two rabbits (1) and (2) were immunized with each of these two phages.

All operations in bleeding the animals and preparing the sera were performed with precautions to avoid microbial contamination.

Titration of Antiphage Sera:

The antisera were titrated against their homologous phages by the following procedure: Dilutions of the antisera (Table VII) were prepared in tryptose phosphate broth and 0.9 ml. of each dilution was added to 0.1 ml. of the homologous phage so diluted in broth that on plating one ml. of the final mixture, 50-500 plaques would develop in the absence of any neutralization. The phage-serum mixtures were incubated in a 37°C water bath for 2 hours after which 0.1 ml. samples of each mixture were added to 9.9 ml. of tryptose phosphate broth to prevent further inactivation of the phages. One ml. of each of the final mixtures was plated on the appropriate propagating strain of staphylococcus by the agar-layer method. The titre of each antiserum was expressed as the reciprocal of that dilution which gave approximately 80 per cent reduction in the plaque count as compared with that given by controls consisting of phage diluted with tryptose phosphate broth.

Table VII shows the increase in plaque counts with increasing dilutions of antiserum, and the titres of the antisera which demonstrate the variation in the immunological response of rabbits immunized in the same way with the same phage. Thus the titre of antiserum against phage 3A/284 (1) is 300 while that against phage 3A/284 (2) is only 100.

The titres of antisera against phage 13 and 30, prepared by intravenous injections, range between 2000 and 6000, and against phage 3A/284, 47A/761, and 543/533, prepared by subcutaneous injections, between 100 and 400.

Phage Neutralization Tests:

Antisera against phage 3A/284 (2), 47A/761 (1), 543/533 (2), 13 (2), and 30 (2) were used for the phage neutralization tests. All the

TABLE VII.

Titration of Antiphage Sera Against Homologous Phages

Rabbit No.	Antiserum against phage	Plaque counts with increasing dilutions of antiserum								
		1/20	1/100	1/200	1/300	1/400	1/500	1/1000	Control	Titer‡ of antiserum
1757	3A/284 (1)	0	7	12	26	31	38	64	91	300
1759	3A/284 (2)	0	12	38	44	49	55	57	62	100
1760	47A/761 (1)	0	15	72	93	99	103	118	242	190
1761	47A/761 (2)	3	26	92	107	127	325	338	565	400
1763	543/533 (1)	0	2	42	65	112	113	182	275	300
1768	543/533 (2)	2	17	93	117	169	124	182	234	160

Titration of Antiphage Sera Against Homologous Phages

Rabbit No.	Antiserum against phage	Plaque counts with increasing dilutions of antiserum									
		1/1000	1/2000	1/4000	1/6000	1/8000	1/10,000	1/100,000	1/1,000,000	Control	Titer of antiserum
1785	13 (1)	8	37	54	99	106	166	185	212	204	2000
1786	13 (2)	0	0	21	43	82	105	214	215	204	6000
1787	30 (1)	0	16	80	194	253	249	254	286	292	4000
1788	30 (2)	0	0	16	52	126	132	196	192	198	6000

‡ The titre of each antiserum is expressed as the reciprocal of that dilution which gives approximately 80 per cent reduction in the plaque count as compared with that given by controls consisting of phage diluted with tryptose phosphate broth.

phages from Fisk's series except phage 74/76, Wilson and Atkinson's series except phage 47C/1163, the series of 'adapted' phages except phage 542/76, and the 32 phages from Asheshov's series were tested with these antisera. Phage 74/76, 47C/1163, and 542/76 could not be used in these tests because high titre filtrates of these phages could not be obtained.

The following procedure was used for the phage neutralization tests: To 0.9 ml. of antiserum diluted to titre with tryptose phosphate broth was added 0.1 ml. of a lytic filtrate of the phage to be tested, so diluted that on plating 1 ml. of the final mixture, a readable number of plaques would develop in the absence of any neutralization. A control consisting of the same amount of phage diluted with 0.9 ml. of tryptose phosphate broth was also prepared. The mixtures were incubated in a 37°C water-bath for 2 hours after which a 0.1 ml. aliquot of each mixture was added to tubes containing 9.9 ml. of tryptose phosphate broth to prevent further neutralization of the phages. One ml. of the final mixtures was then titrated by the agar-layer method and the plaque counts were used to calculate the percentage of phages neutralized by the antiserum. The results of these tests are recorded in Tables VIII, IX, and X.

The results show that it is possible to divide the phages into 3 serological groups, which for purposes of convenience, are designated A, B, and C.

Group A contains phages 3A/284, 3A/542, 3A/144, 3A/535, 3A/533, 3A/145, 3A/44, 3B/211, 3A/62, 47A/761, 47/36, 47/44, 47/3, 543/533, 534/535, 543/535, 543/144, 543/62, 543/542, 534/533, W, 145/535, 6/3, 145/62, 51/145, 56/3, and 145/542. All these phages belong to Fisk's series, Wilson and Atkinson's series, and the series of 'adapted' phages. They are all neutral-

ized by antisera against phage 3A/284, 47A/761, and 543/533 (Table VIII), but not by antisera against phage 13 and 30 (Table IX). It is not known why all these phages should cross-react with antisera against phage 3A/284, 47A/761, and 543/533. It is felt that further investigation is required to see whether these phages are serologically alike. The fact that none of these phages are neutralized by antisera against phage 13 and 30 indicates a certain degree of relatedness between these phages.

Group B contains phages 2, 3, 8, 9, 11, 12, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 28, 29, 30, A, B, C, D, and S3K. These phages are neutralized by antiserum against phage 30 but not by antiserum against phage 13, as shown in Table X.

Group C comprises phages 4, 5, 6, 10, 13, and 16, which are neutralized by antiserum against phage 13 but not by antiserum against phage 30 (Table X). That the phages of Groups B and C are different serologically from those of Group A is indicated by the fact that phage 13 and 30 are not neutralized by antisera against phage 3A/284, 47A/761, and 543/533.

As shown in Table X, phage 1 and 27 are neutralized by antisera against phage 13 and 30. Phage 1 appears to be fairly closely related to the phages of Group C since a higher percentage is neutralized by antiserum against phage 13 than by antiserum against phage 30. On the other hand, the opposite is true for phage 27, a higher percentage being neutralized by antiserum against phage 30 than by antiserum against phage 13, indicating that this phage is closely related to those of Group B. These observations suggest that phage 1 and 27 are not identical but are closely related and it is questionable whether the differences between these phages

TABLE VIII.
Neutralization tests with antisera against phage 3A/284, 47A/761, and 543/533

Phages tested	Antiserum against phage								
	3A/284			47A/761			543/533		
	Plaque counts		Percent neutralization	Plaque counts		Percent neutralization	Plaque counts		Percent neutralization
	Neutralization tube (phage + antiserum)	Control tube (phage + broth)		Neutralization tube (phage + antiserum)	Control tube (phage + broth)		Neutralization tube (phage + antiserum)	Control tube (phage + broth)	
3A/284				8	73	89.1	12	73	83.6
3A/542	25	139	82.0	8	46	82.7	6	46	87.0
3A/144	16	94	83.0	131	>750	>82.0	224	>750	>70.0
3A/535	13	62	79.1	8	36	77.8	10	36	72.3
3A/533	48	218	78.0	23	134	82.9	35	134	73.9
3A/145	38	327	88.4	37	92	59.5	39	92	57.7
3A/44	41	239	82.9	24	164	85.4	20	164	87.8
3B/211	10	82	88.0	58	307	81.2	84	307	72.7
3A/62	18	71	75.0	17	49	65.3	16	49	67.4
47A/761	3	31	90.4				2	31	93.6
47/36	21	157	86.7	6	27	77.8	49	157	68.8
47/44	96	>1000	>90.0	18	119	85.0	176	>1000	>80.0
47/3	0	158	100.	26	131	80.0	3	158	98.2
543/533	10	53	81.2	7	53	86.8			
534/535	38	>1200	>90.0	51	>1200	>90.0	24	102	76.5
543/535	14	>1000	>95.0	75	>1000	>95.0	0	119	100.
543/144	28	>500	>95.0	30	>500	>95.0	30	154	80.6
543/62	76	228	66.7	19	228	91.7	48	243	80.3
543/542	14	>1000	>95.0	113	>1000	>90.0	29	108	73.2
534/533	76	258	70.6	110	258	57.4	132	298	55.8
W	18	224	92.0	52	188	72.4	27	133	79.7
145/535	6	>800	100.	8	>800	100.	7	>800	100.
6/3	0	128	100	0	174	100	0	175	100
145/62	46	206	77.7	63	248	75.0	68	212	68.0
51/145	7	81	91.4	13	74	82.5	24	76	68.5
36/3	2	>1000	100.	7	>1000	100.	9	>1000	100.
145/542	0	>500	100.	6	>500	100.	8	>500	100.
13	>800	>800	0	>800	>800	0	>800	>800	0
30	>800	>800	0	>800	>800	0	>800	>800	0

TABLE IX.

Neutralization tests with antisera against phage 13 and 30

Phages tested	Antiserum against phage					
	13			30		
	Plaque counts		Percent neutralization	Plaque counts		Percent neutralization
	Neutralization tube (phage + antiserum)	Control tube (phage + broth)		Neutralization tube (phage + antiserum)	Control tube (phage + broth)	
3A/284	233	256	0	242	256	0
3A/542	135	134	0	136	134	0
3A/144	193	196	0	183	196	0
3A/535	129	131	0	126	131	0
3A/533	193	184	0	186	182	0
3A/145	236	232	0	228	232	0
3A/44	277	281	0	268	281	0
3A/62	143	152	0	154	152	0
3B/211	171	180	0	183	180	0
47A/761	163	156	0	158	156	0
47/36	151	154	0	146	154	0
543/533	166	174	0	163	174	0
543/542	298	292	0	281	292	0
W	172	181	0	174	181	0
145/535	> 600	> 600	0	> 600	> 600	0
6/3	> 500	> 500	0	> 500	> 500	0
145/62	171	165	0	151	160	0
51/145	312	308	0	294	308	0
36/3	189	198	0	192	198	0
145/542	212	218	0	205	218	0

TABLE X.
Neutralization tests with antisera against phage 13 and 30

Phages tested	Antiserum against phage					
	13			30		
	Plaque counts		Percent neutralization	Plaque counts		Percent neutralization
	Neutralization tube (phage + antiserum)	Control tube (phage + broth)		Neutralization tube (phage + antiserum)	Control tube (phage + broth)	
1	20	229	91.3	62	229	73.0
2	138	135	0	20	135	85.2
3	> 500	> 500	0	48	> 500	90.0
4	96	413	76.8	403	413	0
5	51	194	73.7	204	194	0
6	72	> 300	> 75.0	> 300	> 300	0
8	318	329	0	55	329	83.3
9	106	101	0	9	98	90.9
10	16	183	91.3	184	183	0
11	249	238	0	37	238	84.5
12	396	382	0	171	382	55.3
13				123	138	0
16	59	> 800	> 90.0	> 800	> 800	0
17	237	241	0	61	241	74.7
18	119	123	0	26	123	78.9
19	240	239	0	50	239	79.1
20	380	390	0	30	390	92.4
21	> 400	> 400	0	35	> 400	> 90.0
22	71	78	0	14	78	82.1
23	> 450	> 450	0	31	> 450	> 90.0
24	273	286	0	64	286	77.7
25	138	142	0	5	142	96.5
26	82	93	0	8	93	91.4
27	72	122	41.0	32	122	73.8
28	277	284	0	50	284	82.5
29	145	147	0	24	147	83.7
30	212	219	0			
A	173	192	0	17	192	91.2
B	72	76	0	13	76	82.9
C	90	100	0	16	110	85.5
D	117	128	0	7	128	94.6
S3K	184	188	0	18	188	90.5

and those of Groups B and C are great enough to justify their separation into different serological groups.

V. DISCUSSION

One of the main difficulties encountered in the beginning of this work was to find a suitable method for determining phage concentration. Asheshov's loop method was tried but was abandoned during the course of the work for reasons mentioned previously. It was found that more satisfactory results could be obtained by the agar-layer method which is in general use by practically all workers. When only an estimate of the number of phage particles in a phage suspension was required, determination of the critical test dilution of the phages yielded satisfactory results.

In carrying out experiments on the growth requirements for phage formation, it is desirable to grow the host organisms in chemically defined media. Since Fildes' synthetic medium is adequate for the growth of staphylococci, it was thought that it would also support phage multiplication. The medium was therefore tested at different pH values with a number of phages but was found unsuitable for the growth of these phages. The author feels that further work is required to determine whether the addition of various substances (growth factors, etc.) to this medium would result in a greater yield of phage.

As a preliminary to further experiments of 'selective' adsorption, the period of incubation required for complete adsorption of phage 47A/761 on its homologous strain 761 and on heterologous strain 44 was determined. It was found that at least 45 minute incubation is required for complete adsorption of phage 47A/761 on its homologous strain 761. Similar results were obtained by Rountree (1947b) with phage 51/145 and culture 145 where complete adsorption occurred within 30 minutes of incubation. Adsorption

of phage 47A/761 on heterologous strain 44 occurs at a much lower rate as shown by the fact that only 30.8% of the phages were adsorbed after 2 hour incubation at 37°C. Puck et al (1951) found that coli phage T_1 is adsorbed on a broth-grown culture of Escherichia coli at a rate of about 80% in 5 minutes at 37°C. This adsorption rate is extremely rapid as compared with the rate of adsorption of staphylococcus phages on their host cells.

A study of the effect of time, temperature, and medium on the stability of staphylococcus bacteriophages revealed that a loss of approximately 75% of the phages occurred during the first three weeks of storage. Maximum stability was achieved when the phages were stored at -30°C in the deep-freezer. The cause of the drop in phage titre during the first three weeks of storage is at present unknown and has not been investigated, but it may be due to phage-inactivating agents present in the phage lysates. As mentioned previously, Burnet and Lush (1935) and Freeman (1937) found evidence that polysaccharide material from cultures of staphylococci possessed phage-inactivating power. On the other hand, Rountree (1947b) could not demonstrate any activity in the preparation of polysaccharide studied but obtained evidence that nucleoprotein was concerned in the phage inactivation. It is conceivable that the phage-inactivating agents (polysaccharide or nucleoprotein?) present in the phage lysates exhaust their activity during the first three weeks after which the phage titre becomes stable.

The lysogenicity of 24 propagating strains of staphylococci was investigated by the method described by Fisk (1943). Only 3 strains, namely strain 4, 10, and 36 were found to be lysogenic. It is advisable to discard

the phages which have been propagated on these strains, as preparations of these phages probably contain phages derived from the lysogenic strains.

By the use of antiphage sera prepared by immunizing rabbits with selected phage strains, 57 staphylococcus phages were divided into 3 serological groups. Using this method, Burnet and Lush (1935) examined a series of 15 staphylococcus phages and distinguished 6 groups among them. It was found that all the phages of a given type reacted uniformly with regard to photodynamic inactivation, power to grow on citrated media, and inactivation by strong urea solution. Similarly, Rountree (1949b) distinguished 6 serological groups among 39 staphylococcus phages examined. There was correlation between the serological groups and other characters of the phages such as stability, range of organisms lysed, plaque size, etc. It is felt that further work along similar lines is required for adequate characterization of the staphylococcus bacteriophages at our disposal.

VI. SUMMARY

The following is a resume of the results of this investigation.

1. Unsatisfactory results were obtained with Asheshov's loop method for determining phage concentrations.

2. A synthetic medium, namely Fildes' synthetic medium, was tested at different pH values with a number of phages in the hope that it might provide constant cultural conditions for the growth of phage in further experiments. However, within the range of pH tested, the medium was found unsuitable for the growth of these phages.

3. Although Fildes' synthetic medium is adequate for bacterial multiplication, it does not necessarily support phage multiplication.

4. Peptone broth at pH 7.2 and Bacto nutrient broth at pH 7.8 were found suitable for the growth of staphylococcus bacteriophages.

5. In a preliminary experiment in order to determine a suitable time period of incubation to serve as basis for further experiments of 'selective' adsorption, it was found that at least 45 minute incubation at 37°C is required for complete adsorption of a staphylococcus bacteriophage on its homologous strain. Adsorption on an heterologous strain occurs at a much slower rate.

6. The effect of time, temperature, and medium on the stability of staphylococcus bacteriophages was studied. The results showed that a loss of approximately 75% of the phages occurs during the first 3 weeks of storage. The phages were on the average equally stable in all media tested provided they were stored at low temperatures. Maximum stability was achieved at -30°C in the deep-freezer.

7. The lysogenicity of 24 propagating strains of staphylococci was investigated by the method developed by Fisk (1942). Only 3 strains were found to be lysogenic.

8. By the use of antiphage sera prepared by immunizing rabbits with selected phage strains, 57 staphylococcus phages were divided into 3 serological groups. Two other phages were found to be related to two of these groups.

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