ISOLATION AND PARTIAL CHARACTERIZATION OF PROTEUS MIRABILIS PHAGES

A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

---

by

Lawrence A. Falk, Jr.

August 1966

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ABSTRACT

Four phages of Proteus mirabilis were isolated. Three of these phages, P1, P3, and P6 were further characterized on the basis of plaque morphology, host range, serology, adsorption, latent period, burst sizes, thermal inactivation and pH stability.

On the basis of serology, P1, P3, and P6 were considered closely related and are considered to be one group.

Host range, plaque size, serology, and thermal stability served to differentiate the three phages from one another.

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INTRODUCTION TO BACTERIOPHAGES

Bacteriophages were first isolated by F. W. Twort in 1915 (England) and independently by F. d'Herelle in 1917 (Paris). Twort's original isolate was a staphylococcus phage, while d'Herelle's was a shigella lytic agent (cited from Adams, 1959).

Since the original discovery of staphylococcal and shigella phages, bacteriophages have been isolated against innumerable organisms. To cite a few examples: Pseudomonas (Hoff and Drake, 1960; Dickinson, 1948 Feary et al, 1964), Photobacterium (Spencer, 1960), Brucella (Stineberg and Braun, 1959; McDuff, 1962), Mycobacteria (Takeya et al, 1959), Streptomyces (Alexander and McCoy, 1956), Escherichia coli (Burnet, 1933; Delbrück, 1945), Staphylococcus (Schultz et al, 1929; Rountree, 1955; Krueger, 1931) Lactobacillus (de Klerk et al, 1963), Klebsiella (Humphries, 1948; Park, 1956), Bacillus (Romig and Brodetsky, 1961; Buck et al, 1963; Friedmann and Cowles, 1953), Streptococcus (Brock et al, 1963; Shrew, 1949; Cherry and Watson, 1949), Corynebacteria (Groman and Lockart, 1953), Shigella (Adams, 1955)

Serratia (Wassermann and Seligmann, 1953; Adams, 1954) Pasteurella (Lazarus and Gunnison, 1947), and Proteus (Coetzee, 1958).

Much of the early work was consecrated trying to prove or disprove certain theories concerning the nature of bacteriophages (Asheshov, 1924).

Within the last two decades our knowledge has increased considerably concerning the nature of bacteriophages, namely: (1) their gross structure and chemical composition, (2) the infective process and, (3) their physio-chemical characteristics.

The architecture of bacteriophages has been resolved easily by development of the electron microscope and the improvement of techniques for use of this instrument, such as, shadow casting, thin sectioning of biological materials, and negative staining. Most bacteriophages observed have shown a limited array of morphology. The most common type is that similar to the T set of coli phages, a polyhedral head with a tail extending from the head a varying distance, depending upon the specific phage (Hook et al, 1946). However, spherical froms (Loeb and Zinder, 1961; Hall et al, 1959; Sinsheimer, 1959) and rod shaped phages (Salivar et al, 1964) are known.

All external parts of the phage, head, tail, tail fibers, and base plate are composed of protein.

Within the head is contained the nucleic acid, which for many years was considered to be DNA for all phages. However, several RNA containing phages have been isolated (Loeb and Zinder, 1961). If the nucleic acid is DNA, it may occur in a single-stranded state as with ØX174 phage of E. coli (Mayor and Hall, 1961; Sinsheimer, 1959b), although double-stranded DNA is the more common type.

Lipids and carbohydrates may occur in minute amounts, but the two major constituents are the protein and the nucleic acid (Hook et al, 1946; Taylor, 1946).

In the free state, isolated from the host, bacterial viruses have no metabolic activity (Ajl, 1950) or reproductive mechanism, being strict obligate parasites.

The infective process follows three separate steps: (1) adsorpiton of the phage to the bacterium and penetration of the phage nucleic acid into the host, (2) intracellular replication of phage progeny, and finally (3) lysis of the host, liberating phage particles. Each of the above processes is quite distinct and may be stopped by physical or chemical treatment to allow a more detailed study.

Adsorption is accomplished by two separate steps (Puck, 1953). The first is said to be reversible, while the second is irreversible. During the former there is an attachment of the phage particle to the host cell. This is accomplished by juxtaposition of phage receptor sites, located at the distal end of the phage tail, to the receptor sites on the cell. This is presumed to occur by electrovalent bonding between a positively and negatively charged chemical group of the cell and phage (Puck, 1953). The second step, irreversible, is iniatiated by contraction of the phage tail sheath and the insertion of the core through the cell integument (Strouthamer et al, 1963). The core presents a hollow cylinder through which the nucleic acid of the phage head may drain into the cell cytoplasm.

The failure or success of phage adsorption depends upon three factors: (1) composition of the medium in which adsorption takes place (2) the nature of the host cell, and (3) the nature of the phage particle.

For many phage-cell relationships, there is a requirement for specific ions to be present in the reaction medium before adsorption is complete.

Staphylococci phages will adsorb to their hosts, but unless calcium is present, no penetration of the phage nucleic acid occurs (Rountree, 1955). T2 bacteriophage (Shepherd and Woodend, 1951) and streptococcal phage (Cherry and Watson, 1949) both require sodium chloride before adsorption is complete. Brock et al (1963) observed that the addition of streptomycin to the medium inhibits injection of streptococcus phage DNA, although the particles will be adsorbed to the cell. On the other hand, if streptomycin is added to the medium after the particles have become fixed to the cell, the nucleic acid will be inactivated while being transferred from phage to host.

Tryptophan is required in the adsorption medium by T4. However, tryptophan is not needed for plaque development once a few cells have become infected in a liquid medium (Anderson, 1948). Once T4 has adsorbed, the cells internal medium (plaque assay) can supply the necessary tryptophan for propagation of the phage in the plaque. The requirement for tryptophan in T4, T6, and some mutants of T2 has been related directly to the action of tryptophan upon the tail fibers of the phages (Brenner et al, 1962).

In the absence of tryptophan, the tail fibers are attached to the sheath of the phage tail. On this basis, it has been assumed, the tail fibers have located on them the receptor sites for adsorption to the host. In the presence of tryptophan, Kellenberger, et al, (1965) have observed the release of the tail fibers, making them free for adsorption. Brenner et al, (1965) substituted cadmium cyanide for tryptophan and found this agent to be as effective as tryptophan in promoting adsorption. Kozloff et al, (1957) showed that cadmium cyanide causes contraction of the sheath, freeing the tail fibers, and making the receptor sites (located on the fibers) free for reaction with the host.

Sagik, (1954) demonstrated that some phage lysates of T2 are capable of exerting a reversible inactivation on phage particles, prohibiting adsorption. However, the inactivation could be reversed by diluting the lysate in distilled water, heating, or treating with dilute anti-phage serum. The inactivating substance(s) originated from the host cell, for the degree of inactivation was found to be proportional to the cell concentration in which the lysate was prepared.

The host also influences adsorption of phage particles. Garen and Puck, (1951), when reacting T1 phage with E. coli mutants, found the second step, irreversible, was not occurring. T1 would adsorb to the cell, but no penetration of the nucleic acid occurred, as the virus could be eluted from the cells and maintain infectivity for other cells.

Luria, (1945) found that mutations in the cell would often cause a change in the receptor sites, which would prevent adsorption.

The receptor sites of phage particles undergo change through mutation to such an extent that adsorption is not possible or is very slow. Kellenberger et al, (1965) found when lethal mutants of T4 were produced, their failure to adsorb and be infective was due to a morphological change in the particle. This change in morphology was the absence of tail fibers, which would impose strict limitation on the adsorbing ability of this particular phage.

Of the factors which influence adsorption, medium, host and phage, the medium is the one factor which has influence in a variety of phage-host relations. The host and phage changes, particularly mutations, are more limited except in a few instances, several of which are cited above.

The scheme of bacteriophage adsorption to bacteria presented here is a generalized one for those phages which have a morphology similar to the T phage of Escherichia coli. These phages have a structure designed for the mechanism of adsorption; the presence of a tail with a base plate and/or tail fibers located on the distal end of the phage tail. Spherical and rod shaped phages, on the other hand, must have a different mechanism of adsorption, since there is no specific structure yet identified with receptor sites.

Once the phage nucleic acid has entered the host cell, several alternatives for its future exist, depending upon the phage and host used. An unusual condition is one of lysogeny, first described by d'Herelle. In this state, the phage nucleic acid becomes closely bound to that of the host. This association will be continued for a variable time without propagation of daughter phage, but rather replication of the viral genome along with that of the host genome. To alter this equilibrium of association, so that phage progeny are produced, is known as induction. Induction is usually accomplished by ultraviolet irradiation of the lysogenized cells. Spontaneous induction will occur within a low percentage of any lysogenized cell population.

Other alternatives are: killing of the host without phage development (Gross, 1954), degradation of phage nucleic acid and excretion into the medium without killing the host (Dussoix and Arber, 1962; Lederberg, 1957). The most common condition will be one of virulence where new phage progeny are produced.

Infection with a virulent phage follows a well ordered course of events. During the first stage, the eclipse period, no infectious phage can be isolated from the cell. The eclipse period is usually about half the entire infectious cycle. During the latter half of the infection, infective phage can be recovered. The time interval between adsorption and liberation of new phage is defined as the latent period.

With the use of certain techniques the eclipse period of infection can be studied. Sercarz (1966) applied the system of labeled antibody with fluorescent dyes for studying the early development of T4 phage. The latent period of T4 is between 20-25 minutes. The earliest detection of viral protein occurred at the 7th minute after intiation of infection as demonstrated using this method.

Doermann (1952) first demonstrated the point in the latent period when complete particles could be recovered. Doermann (1953) and Anderson and Doermann (1952) further showed that the infective particles during the latent period increased in the cell at a constant rate. The virus isolated after the eclipse was not equal to that of the average burst, but progressed to equal the burst size.

The latent period of most phages falls within the generation period of the host. Though no specific correlation has ever been made, the generation time of the host gives a presumptive estimate as to the probable latent period of the infecting phage. However, there are phage-host relations where the latent period of the phage far exceeds the generation time of the host (Dobek, 1965). Latent periods of different phages are extended over a wide range, averaging 20-30 minutes for most phages of Enterobacteriaceae, while some bacillus phages have latent periods of 180 minutes (Brodetsky and Romig, 1965).

The latent period may be varied by several factors. Doermann (1948) did a thorough study of T2, T4, and T6 mutants with regard to their latent period. Hershey (1946) first described these mutants

on the basis of plaque morphology and ascribed the symbol r^+ to those plques which were turbid and r to those producing quite clear plaques. Through the experiments of Doermann, the r^+ character was found to be due to lysis inhibition. In this phenomenon, if an infected cell becomes infected again (by the same phage or a closely related one) before the end of the latent period, the second phage will off set the replication of the first phage. The result will be a prolonging of the latent period, or as defined, lysis inhibition. The latent period in this condition may be extended for several hours and the burst size is usually larger.

The physiological condition of the host also influences the latent period and burst size. Delbrück (1940) compared the latent period of a coli phage using rapidly dividing cells and a 24 hour culture as the hosts. The latent period for the former was 17 minutes, but 30 minutes in the latter. The burst size from the older host was approximately half that from the younger cells. On the other hand, Salivar et al, (1964) found that M-13 coli phage burst sizes were higher in older and more slowly metabolizing cells.

Bacteriophages, being composed of approximately 50 per cent protein, are like other proteins, subject to the action of heat. A bacteriophage in infective form may not be physio-chemically in its ultimate stable state. The molecules of the phage protein subunits may be so arranged favoring function rather than chemical stability or structural stability.

When viruses, like proteins, are exposed to heat for a period of time, it is logical to expect certain changes will occur within the molecules. Molecules exposed to moderate temperatures, 60-70 C, will assume a configuration of the highest stability for the particular protein in question. This will be achieved by vibrations of atoms, by stretching and by shifting of chemical bonds (Pollard, 1953). The result is a much more stable physical structure. For biologically active materials, such as bacteriophages, the resultant stability may cause alteration in infectivity. Increased stability will have altered the function of the changed molecules, which is related to infectivity.

D'Herelle first observed the heat resistance and sensitivity of bacteriophages. His observation was: no phages were rendered uninfective at temperatures

below 65 C, while no phages observed were infective after heating at 75 C (cited from Krueger, 1932). Later, Nanavutty studied quantitatively the effect of heat on shigella and coli phages (1930). Nanavutty observed inactivation to proceed logarithmically until 90-99 per cent of the phages were inactivated. Inactivation no longer continued beyond this degree. His theory was, some of the phages were heat resistant. Krueger (1932) opposed this explanation. His experiments with staphylococci phages showed a continued logarithmic inactivation, indicating that all members of the population were equally sensitive. (Krueger was a firm critic of the plaque assay method and developed his own quantitative method. The work of d'Herelle and Nanavutty were both done by the plaque assay method (Krueger, 1930)).

Thermal inactivation studies with numerous phages have indicated, that indeed, a proportion of the population is more heat resistant than the majority. In most instances, heat resistance is a phenotypic expression, while genotypically it remains heat sensitive. This has been demonstrated by Adams and Lark (1950) and Fischer (1950). The former used T5

phage and picked heat resistant plaques, passed these, and tested again for thermal stability. The majority were heat labile.

Thermal inactivation is similar to adsorption in respect to the effect the medium has on inactivation or adsorption. Heat inactivation always proceeds at a much faster rate when conducted in saline or lower concentrations of sodium chloride as compared with inactivation in nutrient broth (Adams and Lark, 1950; Adams, 1949; Nanavutty, 1930).

Lark and Adams (1953) investigated this observation further, using T5 coli phage again. When T5 was inactivated in the presence of 0.1 M NaCl, inactivation was quite rapid. However, when cations were added, such as calcium or magnesium, no inactivation occurred. From this data the conclusion was drawn that cations reacted with the heat labile portion of the phage (specifically that portion which governs infectivity) to form a stable complex, less sensitive to heat.

Further observations were made with the inactivated particles using the electron microscope. Numerous "ghosts" were found (a "ghost" is a phage with no nucleic acid in the head, making the head

less electron dense) as opposed to an untreated control. High concentrations of heat inactivated phage no longer showed a Tyndall effect, but the rise in optical density at 260 mu was significant over an untreated sample. This indicated that the loss of infectivity was associated with a loss of nucleic acid into the medium.

Lark and Adams correlated these results with the stability of T5 in salt solutions (Adams, 1949), and on this basis, concluded that the inactivation site of T5 was at the distal tip of the tail, where adsorption also occurs. This conclusion was based on the fact adsorption does not occur without calcium being present. The role of calcium is probably activation of an enzyme at the tip of the tail. When cations are present, magnesium or even calcium, a stable complex will be formed with the enzyme site, rendering it more stable.

Therefore it would not be unreasonable to assume that during thermal inactivation, changes occur within the protein molecules of the phage coat, making it structurally and chemically a more stable entity. But, these changes occur at positions which control infectivity, thereby reducing infectivity.

Various schemes have been proposed for the identification, classification and grouping of bacteriophages. This is simplified to a degree by the limited host range found amoung a group of bacteriophages. The coli phages, T series, are limited mainly to the various strains of E. coli, although a few of the phages have a host range which extends to other members of Enterobacteriaceae, such as Salmonella, Shigella, and Serratia (Adams, 1952; Wasserman and Seligmann, 1953).

Other characteristics which serve to identify a phage are plaque morphology, average burst size, latent period and thermal inactivation constants. However, all of these are influenced by a range of conditions, such that these are not always reliable determinants.

Burnet (1933) found serological classification for his set of coli bacteriophages presented a definitive method of separating these phages into groups. Although antigenic differences were observed within a group, no intermediates were found which would connect groups. This concept was considered correct until a more intensive study of phage mutants was made (Reed and Eisenstark, 1962).

Mutants, though being similar to the wild type in many respects, will often demonstrate a resistance to neutralization by wild type antiserum, due to specific changes in the phage protein coat.

Reactions of anti-phage serum will show it to be a relatively heterogenous mixture of antibodies capable of different functions. Schultz et al, (1929) reported no compliment fixing (CF) activity or precipitation of phage protein with an anti-staphylococcus phage serum. On the other hand Burnet (1933) demonstrated the agglunitating ability of antiserum for coli phages when the former was mixed with phage suspensions. Schultz's failure to get precipitation and CF activity was a result of low titer anti-phage serum.

Lanni and Lanni, (1953), proposed the segregation of antigen theory to account for multiple reactions. Evidence was clearly presented, demonstrating the presence of two distinct antigenic regions on T2 phage. The head contained an antigen which reacted with the antiserum to cause agglutination and CF. In contrast, neutralizing antibodies were found to react with the phage tail. When phage "donuts" (phage particles which have not been fully developed, lacking fine structures of tail and nucleic acid) were absorbed

with antiserum, the antiserum was still capable of neutralizing complete phage particles.

Fodor and Adams (1955) observed that distantly related phages at low concentrations were not readily neutralized by heterologous antiserum. However, infectivity was greatly diminished if a higher concentration of phage was used (10^6 to 10^8 particles). The increased inactivation was due to the presence of a large amount of agglutinating antibodies and a low concentration of neutralizing antibody. This higher concentration of phage led to more collisions among phage particles so aggregates were rapidly formed.

The above was sufficient evidence to warrent a re-examination of the "per centage law", (Andrews and Elford, 1933). This hypothesis stated that a given dilution of serum would inactivate a certain percentage of phage, regardless of the initial phage concentration. Kalmanson et al, (1942) were more reserving in their conclusion that neutralization rates did not vary with phage concentrations.

Hershey et al, (1943a, 1943b) did quantitative studies with phage-anti-phage serum reactions. A single particle of coli phage could accomodate

170 molecules of antibody when the latter was in excess. A phage particle was found to be neutralized by 2 molecules and agglutinated by 3 molecules of antibody.

The influence of salt and pH on neutralization has been a debatable question. Hershey (1941) found that neutralization rates were not influenced by pH or electrolytes. In contrast Cann and Clark (1955) obtained 7 fractions of anti-phage serum by electrophoretic separation and analyzed the neutralizing ability of each with electrolyte and pH variables. Results indicated that the antibody molecules of the different fractions were different physical entities and the effect of electrolytes and pH was not on the reaction but rather components of the fractions.

Other variables also influence the phage-antiserum reaction. Tanami and Mujajima, (1956) prepared stocks of T4 in 4 different strains of E. coli. Neutralization rates were determined on each stock with one antiserum. The plot of the curves of neutralization varied slightly, but enough to suggest that the different hosts had induced modifications to the phage, affecting antigenic structure.

Jerne and Perlo, (1956) detected a difference in early and late serum of immunized animals with

phage. Early serums showed less tendency for a reversible reaction, while late serums demonstrated a reversibility which may account for 1-10 per cent survivors in neutralization tests.

The reversibility of neutralization has likewise been recorded by Andrews and Elford (1933). The reaction of neutralization was considered to progress in two steps. The first step, reversible, was quite rapid, while the second step was irreversible. Incomplete neutralization may occur in the first step, and reactivation of the particles to infectivity is brought about by dilution.

When susceptible bacteria and phage are mixed together, the phage are susceptible to inactivation until the nucleic acid has been injected. The virus is still sensitive to neutralization after adsorption before injection has been completed (Reiter, 1963). From these observations, another mechanism must be in operation, for the adsorption sites should be fixed to the cell wall and not free for antibody binding and subsequent neutralization. Once infection has been actively set up, the particles are no longer neutralizable until lysis liberates phage progeny (Delbrück, 1945).

I-B

INTRODUCTION TO PROTEUS PHAGES

Examination of the literature shows that phages for the genus Proteus have not been extensively studied within the last two decades, even though d'Herelle was one of the first to isolate a Proteus phage (ca. 1926) (cited from Coetzee, 1958).

Coetzee, has been the most productive investigator of proteus phages. He isolated a series of 31 virulent phages from sewage for Proteus mirabilis and Proteus vulgaris (Which he groups in one species, Proteus hauseri) (Coetzee, 1958). Of these 31 phages he chose 14 for more thorough intensive study. This collection of phages could be placed within 7 serological groups, and, if grouped according to plaque morphology, 8 groups.

On the basis of plaque size, the range was 0.5 mm to 3.0 mm in diameter. The average burst sizes ranged from 50 to 260 phage per cell. Latent periods were distributed over a range of 17 to 53 minutes. At least half of the phages, 7 out of 14, were relatively heat sensitive, approximately 90 per cent being inactivated at 60 C in 20 minutes. Three of

the 14 were quite heat labile, 99.9 per cent being inactivated, while the remaining phages were intermediate in sensitivity.

For K value determinations of phage-anti-sera relations, homologous phage-homologous serum and heterologous cross neutralizations were tested. The K values for homologous neutralizations ranged from 45 to 1,135, heterologous neutralization, 2-460.

Electron micrographs of purified phage preparations showed all phages to have a morphology similar to that of the T phages.

Later, Coetzee and Sacks (1959) used 23 of the original 31 hosts for phage phage isolation (31 phages were originally isolated) to study lysogeny. Three methods were employed for phage induction: (1) growing each strain in broth cultures for 10 days and assaying the supernatant, (2) ultraviolet irradiation, and (3) growing all combination of pairs in broth culture for 10 days. For indicator strains, 49 different strains of Proteus were used. Of the 23 strains tested for lysogeny, 12 proved to be lysogenic. By analysis of results, each lysogenic strain appeared to have a preferred method of induction. All strains induced by method (3) were mirabilis and 4 out of 5 induced by method (2) were vulgaris.

With the isolation of the 12 temperate phages, Coetzee and Sacks (1960) endeavoured to demonstrate transduction of genetic material between P. mirabilis and P. vulgaris. These attempts were not fruitful, only intrastrain transduction could be deomonstrated among strains of P. mirabilis. From the 12 temperate phages, 2 showed transduction of streptomycin resistance, from a lysogenic streptomycin resistant mutant (donor) to a streptomycin sensitive strain (recepient). The transduced organisms were not biochemically different from the sensitive strains, although they had acquired phage resistance.

Further investigations were continued with streptomycin transduction (Coetzee and Sacks, 1960). Of 11 temperate phages tested, 5 were capable of intrastrain transduction of the streptomycin resistance marker. Coetzee had hoped to isolate an auxotroph so two genetic markers could be used in these experiments, but all attempts were negative.

When the lysogenic transduced strains were tested for suscepbility to homologous phage, they were found to be resistant, while the non-lysogenic transductant strains were able to support growth of homologous phage.

Resistance of the lysogenic streptomycin resistant mutants to homologous phage was found to be in failure of the phage to adsorb. Lysogenic conversion within these cells was causing restriction of adsorption. This line of thinking was further pursued (Coetzee, 1961) with two lysogenic streptomycin resistant transductants. These cells were immune to homologous phage adsorption and their antigenic structure had been altered as evidenced by a positive Dienes phenonemon (Dienes, 1946). (When a line of demarcation is formed between two swarming strains of Proteus on a plate, a positive Dienes phenonemon is indicated. The line of demarcation, the periphery of each swarm not making contact with one another, is thought to occur as a result of the repulsion of strains which differ). ($L_y$sogenic conversion is a relatively new phage induced character. This condition is brought about by a prophage which in some way alters the genome of the lysogenic host, possibly causing new phenotypic expression. Normally, lysogenic strains will adsorb homologous phage, but no infection will be set up. Rather, the genetic material will be gradually diluted into the medium. On the other hand, strains which show lysogenic conversion will usually fail to adsorb homologous phage).

One of the lysogenic phages, capable of streptomycin resistance transduction, could also transduce within P. mirabilis the swarming phenomenon to a non-swarmer(Coetzee, 1963).

Coetzee then published a series of reports seeking to demonstrate the relatedness of the Providence group with the Proteus group by the interreactions of proteus phages with Providence strains and vice versa, (1963a; 1963b; 1963c).

Bloss (1963; 1964) has investigated the interreactions of proteus phages with spheroplasts and L-forms of P. mirabilis. In the first attempts (1963), a P. mirabilis phage, designated g, was used. Spheroplasts and L-forms proved resistant to this phage. Later, (1964), a lysogenic proteus phage was isolated, 1578. This phage was capable of adsorbing and being propagated in spheroplasts but not L-forms.

Dobek (1965) has studied the characteristics of 17 temperate phages. These studies were directed toward establishing a phage typing system for Proteus mirabilis.

The characterizations of these temperate phages were based on the following: (1) reproductive cycle, (2) spontaneous induction, (3) ultraviolet irradiation,

(4) thermal inactivation, (5) stability during cold storage, (6) plaque morphology, (7) physical structure, and (8) nucleic acid type.

On the basis of serology, the 17 temperate phages were placed into one of 12 groups. Latent periods and average burst sizes were 48-58 minutes and 71-268 phages per cell, respectively. The phages exhibited a wide range of thermal sensitivity. The titer of one phage was reduced 2 logs in 1 minute at 60 C, while the most resistant was reduced less than 1 log in 120 minutes.

The stability of infectivity of the phages at 4 C for 60 days was variable, the lowest percentage surviving being 3, while the most stable showed 88 per cent infectivity. Plaque sizes ranged from 0.25 mm to 2.0 mm in diameter, all having turbid centers with defined or irregular margins.

Two of the phages examined contained DNA as the nucleic acid type. Morphology, as ascertained by electron miscroscopy, showed all phages being similar in structure, having hexagonal heads with long flexible tails.

Statement of Research Problem:

The introduction to bacteriophages adequately demonstrates the variety of phages which are known, as well as conditions effecting their stability and characters (burst size, latent period, and plaque morphology, to name but a few).

The work which has been done with proteus bacteriophages is quite dimunitive when compared to the investigations with coli, bacillus and staphylococci phages.

The genus Proteus has been the object of extensive research in this laboratory for the past few years. A collection of proteus phages could compliment this work to such an extent that the research might be broadened.

Therefore, it was the purpose of this investigation to establish a collection of proteus phages and characterize them. The criteria to be used for characterization will be plaque morphology, host range, serology, latent period, burst size and thermal and pH stability.

II

MATERIALS AND METHODS

Bacteria.

Proteus mirabilis (Pm) phase B (Belyavin, 1951) was the bacterial host on which the bacteriophage to be described was originally isolated. The host commonly used will be designated Pm-2. Several other strains of Proteus mirabilis were used: two streptomycin resistant mutants, kindly supplied by N. Prashad, University of Houston. These mutants, designated Pm-3 and Pm-5 were transformed by treating streptomycin sensitive cells with cell free washes of Proteus mirabilis and plated on minimal agar to which 1000 ug of streptomycin/ml. had been added (Prashad, personal communication). Pm-4, a streptomycin sensitive strain, was also supplied by N. Prashad.

Cultures of the genera Escherichia coli, Klebsiella, Bacillus, Salmonella and Protues mirabilis phase A were tested for sensitivity to the phage. Forty-five cultures of Proteus, isolated from urine or fecal material, were kindly supplied by H. Brown, Ben Taub Hospital, Houston. These strains were classified on biochemical reactions according to the method given in Bergey's Manual.

For determining sensitivity to the phage, sufficient broth culture cells were added to soft agar to give a lawn of bacteria upon incubation. After solidification of the soft agar, 10^8 plaque forming units (PFU) were spotted on each plate.

All bacterial cultures were maintained on 2 per cent nutrient agar slopes at 4 C and transferred monthly. The streptomycin resistant strains, Pm-3 and Pm-5, were periodically streaked on streptomycin minimal agar (1000 ug streptomycin/ml.) to make certain the mutants remained stable.

Bacterial concentrations were determined by viable plate counts and/or turbidity readings compared to a standard curve prepared, using a Klett-Summerson photoelectric colorimeter, filter 66.

Bacteriophage.

Isolation: The phage was originally isolated from raw sewage by H. Lichtenstein in this laboratory. Plaque variants were picked and plaque purified three times before stock lysates were prepared.

Phage lysates: Phage lysates were prepared by three methods as described below:

(1) Soft agar method as described by Swanstrom and Adams (1951) with some modifications.

To 0.5 ml. of fresh host cells, 0.1 ml. of phage (2000 PFU) was added. Approximately 20 to 30 samples were prepared in this manner and poured with 2.5 ml. of soft agar onto nutrient agar plates. The plates were incubated for 12-18 hours at 37 C. To each plate, 4.0 ml. of nutrient broth was added. The plates were left at room temperature for 2-4 hours. The nutrient broth was poured from the plates into a beaker and the soft agar scraped into this same beaker. This mixture was left standing for 2-4 hours with occasional shaking. The soft agar was removed by passing the mixture through several layers of cheesecloth. The filtrate was centrifuged at 5,000 rpm for 20 minutes to sediment bacteria and small particles of agar. The supernatant was filtered through a sintered glass filter. This final filtrate was dispensed into sterile screw cap vials and stored at 4 C.

(2) Differential centrifugation: 100 ml. of lysate prepared as previously described in method (1), but which had not been filtered through a sintered glass filter, was centrifuged at 15,000 rpm, 4 C for 60 minutes. The supernatant was discarded and the pellets were allowed to drain dry. To each pellet, 5.0 ml. of saline was added. The mixtures were pooled, and

centrifuged for 20 minutes at 5,000 rpm to remove insoluble matter. This was followed by another cycle of fast and low speed centrifugation. The final supernatant was decanted into a sterile vial.

(3) Broth culture lysis: To 100 ml. of early log cells (2×10^8/ml.) 1.0 ml. of phage was added (10^9 PFU). Incubation on a rotary shaker at 37 C was continued until the culture cleared, usually within 90 minutes. The lysate was centrifuged at 5,000 rpm, then filtered through a sintered glass filter and dispensed into sterile vials.

Plaque morphology: Petri plates were prepared with 25 ml. of 1.5 per cent nutrient agar, these were dried for 24 hours at 45 C. Fifty to 100 PFU of each phage were plated with host cells and incubated for 6 hours at 37 C; measurements were then made. In some instances, incubation was continued for an additional 6 hours.

Assay Procedures: All assay procedures were done by the soft agar overlay method, as described by Adams (1959).

Media.

Bacteria were grown in nutrient broth. Plaque assay plating media consisted of 1.5 per cent nutrient agar basal layers, and 0.75 per cent nutrient agar for

overlays. Soft agar overlays were prepared with Difco purified agar. In several experiments, brain heart infusion broth was used. "C" medium (Roberts et al, 1957), supplemented with 20 ug of gelatin/ml. was used in one set of experiments. Nutrient agar with 0.5 per cent sodium chloride was used in experiments where indicated. All dilutions of the phage were done in nutrient broth.

Preparation of Antiserum.

Antiserum was prepared by injecting a rabbit intraperitoneally with phage at concentrations ranging from 1-3 x 10^{12} PFU/ml, twice a week for a period of three weeks. One week from the last injection, the rabbit was bled from the heart and given another injection (1.0 ml.). One week later the rabbit was again bled by cardiac puncture. All collected blood was left at room temperature for one hour, then centrifuged at low speed. The clear serum supernatant was inactivated at 56 C for 30 minutes, dispensed into sterile vials and stored at -20 C.

For rapid detection of antibody to the phage, the serum was tested by the ring precipitation test at appropriate dilutions of the serum and phage.

To determine the K value of the serum, the phage neutralization test was used, as described by Adams, (1959). To 0.9 ml. of a 1/1000 dilution of the serum

pre-warmed nutrient broth, 10^7 PFU were added in 0.1 ml. amounts. At 5 minute intervals, 0.1 ml. amounts were removed, rapidly diluted in cold nutrient broth and plated out in duplicate with host cells.

Adsorption.

For determing the rate of phage adsorption to host cells, early log phase cells were mixed with sufficient phage to give a multiplicity of infection (MOI) of .1 to 1 phage per cell. At given time intervals, samples were removed and several drops of chloroform added. This was mixed well, and after the debris had settled, assayed for free unadsorbed phage. An alternate method consisted of sedimentation of the bacteria by centrifugation after the adsorption period and then assay of the supernatant for free phage.

Latent Period and Average Burst Size.

The latent period and average burst size were determined by the method described by Adams (1959). Early log phase cells were infected with a MOI of .1 to 1 and were reacted for 10 minutes at 37 C. From this mixture a 1/100 dilution was made in an appropriate dilution of phage antiserum which would neutralize 99 per cent of free phage in 5 minutes.

From the neutralization tube, further dilutions were made so the infective center (virus infected cells) concentration gave rise to 20-100 plaques per plate when plated out (usually these terminal dilutions were 10^{-5} and 10^{-6} from the original adsorption tube). Samples from these final dilutions were removed at various time intervals, added to host cells and plated out.

The latent period was also determined by observing turbidity changes in an infected culture by making periodic readings on a Klett-Summerson photoelectric colorimeter, filter 66.

Single Burst.

Single burst experiments were also done according to the method of Adams (1959), with some modification. From the neutralization tube described above, dilutions were made to a point where it was calculated there would be approximately 1 infected cell per ml. From this dilution, 0.1 ml. or 0.5 ml. samples were dispensed into tubes; these were allowed to incubate for 2 hours at 37 C to insure all cells had burst. Host cells were added and the contents poured by the soft agar method. In each experiment, 20-40 plates were used.

Thermal Inactivation.

To 0.9 ml. of nutrient broth, saline or nutrient broth pH 7.4, pre-heated to 60 C, 0.1 ml. of phage (10^7 PFU) was added. At 5 minute intervals, 0.1 ml. samples were withdrawn and added to 0.9 ml. of cold nutrient broth. The phage was further diluted and duplicate samples assayed for survivors.

Effect of pH.

Nutrient broth was adjusted to various pH values ranging from 5-10 by addition of 1N HCl or 1N NaOH. The broth was then autoclaved. To 0.9 ml. of broth at these pH values, 0.1 ml. of phage (10^7 PFU) was added. This mixture was maintained at 37 C for 60 minutes and phage survivors determined by standard assay procedures.

III

RESULTS

Bacteriophage.

Isolation: The lysate which was received from Lichtenstein, originated from one plaque he had picked and passed (personal communication). Upon receipt of this lysate it was passed several time and a plaque picked, and purified three times, and a stock prepared. This phage was designated as the Parent strain (Pal); the original lysate was coded with the month and year of receipt (11-64).

In the course of experiments with Pal, the plaque morphology was observed to be quite heterogenous, suggesting the existence of more than one plaque variant. Several plaque purification procedures were again employed and new stocks prepared. This second preparation of stocks showed the same heterogeneity. However, on the basis of extensive plaque purification, the stocks were considered pure.

Subsequent work with Pal showed that mutations were occurring in the population. From a stock prepared 3-66, and designated by this number, four plaques were picked. This is given in Figure 1, as well as the prior history.

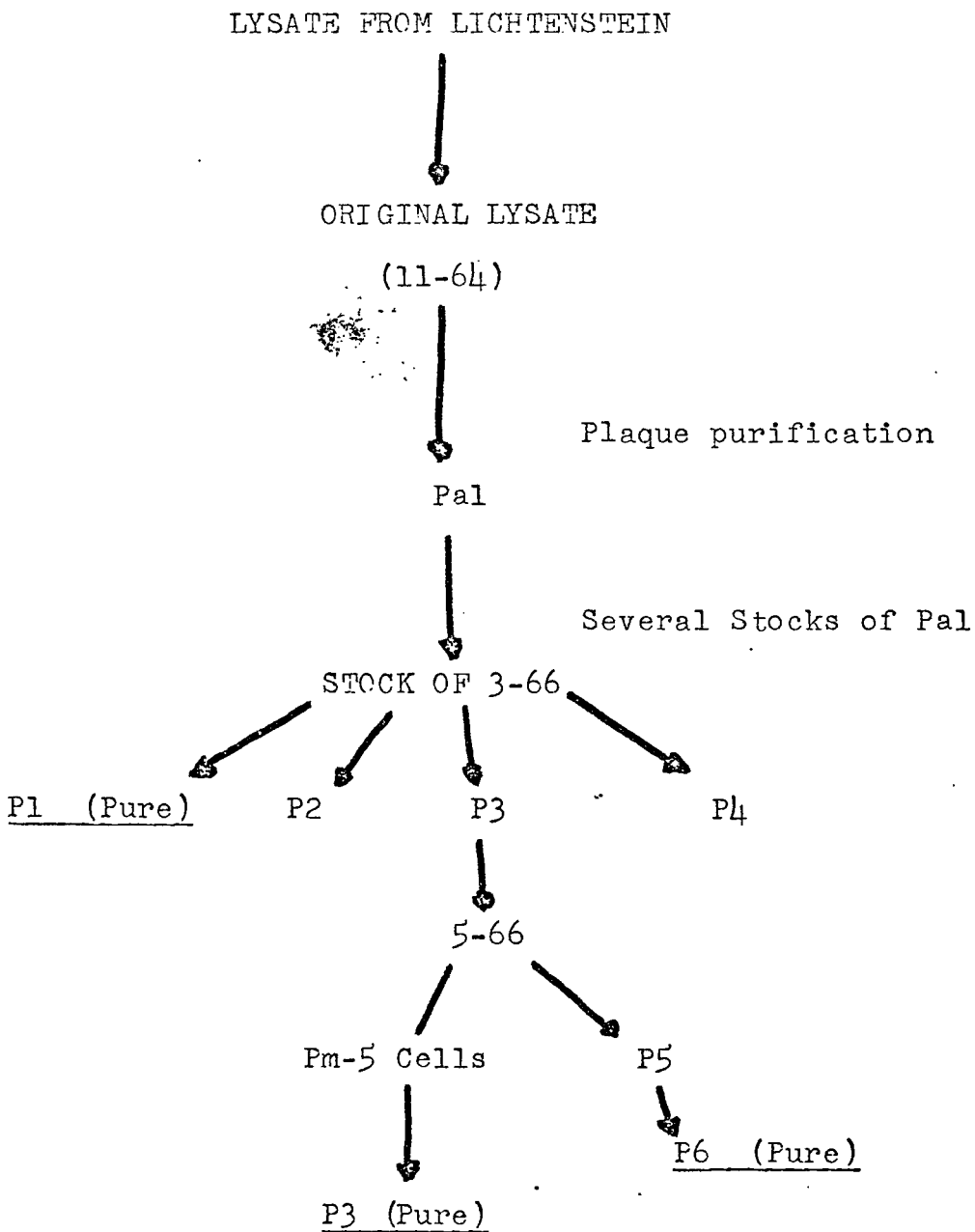

FIGURE 1

ORIGIN OF PROTEUS PHAGE PLAQUE VARIANTS

The first plaque picked was recorded as P1. P1 was characterized by having well defined edges and an extremely clear center. This plaque was quite distinct from the rest of the population.

The second and fourth plaques picked, P2 and P4, appeared to be intermediates of P1 and the third plaque picked, P3, described below. On further purification of P2 and P4, they so closely resembled P3, they were considered the same.

P3, in contrast to P1, was turbid on viewing it with reflected light. When the plaque edges of P1 and P3 overlapped slightly, the difference in the two was quite apparent.

The plaque purified lysate of P3 began to show plaques resembling those of P1. In additions, another plaque variant was occurring in small numbers. This plaque was distinguishable from P1 and P3 on the basis of size (0.5 mm in diameter) and the absence of a halo. This variant was designated P5 (Figure 1), plaque purified and stocks prepared.

The stock of P5 also had a proportion of the plaques which were similar to P1, as well as another plaque type. This plaque type was designated P6.

Passage of P6, gave a population of homogenous plaques. Therefore, P1 and P6 stocks were considered plaque pure, while P3 and P5 stocks still gave plaques of P1 and P6.

Further attempts were made to plaque purify P3 and P5. Plaques were picked immediately on becoming visible, usually within 2-3 hours after incubation. These attemps were not successful.

Streptomycin resistant cells were then received. All of the stocks, P1, P3, P5 and P6 were assayed on Pm-2 and Pm-4, streptomycin sensitive cells, and on Pm-3 and Pm-5, streptomycin resistant cells.

These results are given in Table I. When all four phages were plaqued on Pm-2, there was no reduction of stock titer. However, when P1 was plaqued on Pm-3 and Pm-5, the plaque titer was reduced more than 99 per cent. When P3 was plaqued on all four cell lines, the only reduction occurred when Pm-3 and Pm-5 were used as hosts. This was interpreted to mean that the reduction of P3 was due to a failure of P1, present in P3 stocks, to plaque efficiently on Pm-3 and Pm-5. It will be noted (Table I) that the reduction of P3 on Pm-3 and Pm-5 is about the same.

TABLE I

LYSATE TITERS OF P1, P3, P5, P6 AND P3* ON PROTEUS MIRABILIS HOSTS

HOST	P1	P3	P5	P6	P3*
Pm-2	5.4×10^9	7.1×10^8	1.8×10^9	2.3×10^9	4.0×10^9
Pm-3	1.0×10^6	3.0×10^8	4.6×10^7	0	3.8×10^9
Pm-4	3.3×10^9	7.4×10^8	1.8×10^9	1.7×10^9	4.2×10^9
Pm-5	3.0×10^5	4.6×10^8	1.6×10^6	0	3.1×10^9

* Titer of P3 after stock prepared on Pm-5 cells.

The titer of P5 also dropped on Pm-3 and Pm-5 cells. P6 was totally insensitive to Pm-3 and Pm-5, while it gave normal titers on Pm-2 and Pm-4. (When concentrated suspensions of P6, 10^{10} PFU, were plated with Pm-3 or Pm-5 cells, no plaques were formed).

From this data, it was logical to prepare stocks of P3 on Pm-5 cells. When this stock was again assayed on all four cells, there was no drop in titer as is indicated in the last column of Table I.

The original lysate (11-64) and Pal (3-66) were assayed on Pm-2, Pm-4 and Pm-5. These results are presented in Table II. When the original lysate was plated on Pm-5, the plaque titer was reduced 96 per cent, compared with its plaque titer on Pm-2 and Pm-4. It will also be noted that the titer of 3-66 stock is relatively constant on all three cell lines.

Plaque morphology: The plaque morphology for each phage was quite distinct, especially when differential host cells were used for plating. Given in Table III are the characters and the sizes of the plaques produced by each phage after 6 hours incubation at 37 C.

TABLE II

TITER OF ORIGINAL LYSATE AND PA1

ON *PROTEUS MIRABILIS* HOSTS

VIRUS STOCK	Pm-2	Pm-4	Pm-5
11-64 (Original Lysate)	7.3×10^7	7.6×10^7	2.7×10^6
3-66 (Parent Stock)	2.3×10^{10}	2.5×10^{10}	2.9×10^{10}

TABLE III

PLAQUE MORPHOLOGY OF P1, P3, P5, AND P6

ON PROTEUS MIRABILIS HOSTS

HOST	P1	P3	P5	P6
Pm-2	3 mm clear	2mm turbid	0.5 mm clear	1 mm clear
Pm-3	1.5 mm clear	1 mm clear	0.5 mm clear	-
Pm-4	3 mm clear	2 mm turbid	1 mm clear	1 mm clear
Pm-5	0.5 mm clear	2 mm clear	1 mm clear	-

When Pm-2 was the host for all four phages, P1, P5 and P6 gave quite clear plaques, while P3 formed turbid plaques after 6 hours. After 6 hours these plaques were pinpoint in size with a broad band of altered cell growth surrounding the plaque. Upon further incubation for 6 hours, P3 plaques would become clear to resemble those of the other three phages. When Pm-3 and Pm-5 were used for plaquing, all the phages except P6 produced clear plaques.

Each phage produced a plaque of a characteristic size, as recorded in Table III. The largest plaque consistently formed was by P1, when plated on Pm-2 or Pm-4 cells. However, the plaque size was reduced when P1 was plated on Pm-3 or Pm-5 cells. The smallest plaque was produced by P5, whose maximal size was only 1.0 mm in diameter. P3 and P6 had intermediate plaque sizes of 2.0 mm and 1.0 mm respectively.

All of the phages cause halo formation surrounding their plaques. However, halos appear to depend upon various conditions. During the first 6-10 hours of incubation (sometimes longer), halos did not generally appear around the plaques. Continued incubation would bring halos, but not always. If plates were left at

room temperature for several days, halos would be formed and attain widths twice the diameter of the plaque. Several attempts were made to isolate phage from the halos by picking the periphery of a halo with a needle and plating it out. No phage was isolated by this method.

Under ideal conditions, especiall those defined in materials and methods, P1, P3, P5, and P6 could be distinguished readily from one another by plaque morphology alone. However, other conditions were found to influence the character and development of the plaques.

High concentrations of phage, 300-400 PFU per plate, always gave reduced size, while 50-60 plaques gave normal plaque sizes. Varying the volume and age of the basal agar layer had a markedinfluence on plaque morphology. Basal layers exceeding 25-35 ml. of agar gave smaller plaques for all four phages. When the agar was more than 5-6 days old, the plaque size was reduced appreciably. Plaque sizes were always diminished when cell concentrations exceeded 2-5 x 10^8/ml.

Phage Lysates: All three methods of preparing lysates, (1) confluent lysis , (2) differential centrifugation, and (3) broth culture lysis, were equally

efficient in yielding high titered stocks. Method (1) generally yielded lysates with titers of 10^{10} - 10^{11} plaque forming units/ml. With method (3), lysate titers were predominately 10^{10} PFU/ml. The second method, differential centrifugation, invariably gave small volumes of 10^{12} PFU/ml. This concentrate had a blue opaque color and showed a very strong Tyndall effect.

Lysates are very stable at 4 C for long periods of time as compared with some proteus phages (Dobek, 1965). After a 17 month period of storage at 4 C, one stock had retained 43 per cent of its original infectivity.

Assay Procedures: P1 and P6 gave quite linear plaque counts by the plaque assay method. P3, on the other hand, will not give as linear counts as the former two, particularly when P3 is assayed free. When infected cells of P3 are plated out, the counts were as linear as P1 or P6 counts.

Early experiments with Pal gave extremely variable results on plaquing. Counts varied so much that it was difficult to perform quantitative work from one experiment to the other.

The additions of 0.5 per cent sodium chloride to the plating media was contributing to this defect. Table IV presents results of parallel experiments with the addition and omission of 0.5 per cent NaCl to basal and soft overlay agar.

As will be noted from Table IV, the highest plaque titers were obtained when no NaCl had been added to the basal or soft agar layers. These conditions were chosen as standards, giving 100 per cent plating efficiency. The per cent reduction in plaque titer increases as the NaCl is increased in the environment, reaching a low when NaCl is present in both layers of agar. The composition of the broth culture in which assay cells were grown had no effect on plaque reduction.

On the basis of these results, sodium chloride was omitted from all growth media and plating media.

Host Range.

The host range of P1, P3 and P6 are given in Table V, as was determined by the spot test. None of the phages were active on any bacterial group other than Proteus. Both phase A and phase B strains used for phage sensitivity testing were from our

TABLE IV

EFFECT OF SODIUM CHLORIDE ON

PLAQUE TITER OF PA1

COMPOSITION OF BASAL LAYER AND OVERLAY	TITER	PER CENT REDUCTION
Cells Grown in Nutrient Broth without Addition of Salt:		
Basal Free Top Free	2.8×10^9	0
Basal Free Top Salt	1.4×10^9	50
Basal Salt Top Free	4.0×10^8	71
Basal Salt Top Salt	3.0×10^8	79
Cells Grown in Nutrient Broth with 0.5 per cent Sodium Chloride		
Basal Free Top Free	3.5×10^9	0
Basal Free Top Salt	1.4×10^9	60
Basal Salt Top Free	7.0×10^8	80
Basal Salt Top Salt	4.0×10^8	89

TABLE V

HOST RANGE OF P1, P3 AND P6

ORGANISM	NUMBER TESTED	ORIGIN	P1	P3	P6
E. coli	2	University of Houston	-	-	-
Salmonella	2	"	-	-	-
Klebsiella	1	"	-	-	-
Bacillus	3	"	-	-	-
P. mirabilis phase A	2	"	-	-	-
P. mirabilis	40	Ben Taub Hospital	4	4	4
P. vulgaris	5	"	-	-	-

laboratory. Only phase B was found to support phage replication.

Of the 45 *Proteus* isolates from clinical specimens, 4 showed clearing by the spot test. These four cultures were all classified *Proteus mirabilis* on the basis of positive urea and dextrose, and negative indole and maltose tests. In contrast, 5 of the 41 remaining insensitive strains, were found to be *Proteus vulgaris* on the basis of positive urea, indole, maltose and dextrose tests.

One of the 4 sensitive strains, isolate #8, in reacting with P3, did not give a completely clear zone of lysis, as did P6 and P1 on this host. Instead, there was a hazy growth of cells in the center of the opaque clearing. This indicated that perhaps these cells had been lysogenized by P3. A loopful of cells was removed from the middle of the lysis area and streaked out for isolation. From this plate, a second passage of the cells was made. A broth culture of the cells was made and these cells were tested again. The lysing zone was completely clear. Theoretically, had a lysogenic condition been established, these cells would have been immune to P3 super-infection, and no

lysis would have occurred.

The phase of all 4 sensitive isolates was determined by spotting a loopful of broth culture cells on nutrient agar plates, supplimented with 0.5 per cent NaCl, and incubating for 12 hours at 37 C. Three of the strains were phase A type as indicated by profuse swarming in concentric rings within 12 hours. The fourth strain did not swarm. Two of the swarmers were different as demonstrated by a line of demarcation formed between them when swarming together on the same plate (Dienes, 1946).

Preparation of Antiserum and Serum Neutralization:

Antiserum was prepared only against P1, for the purity of these stocks was more certain than those of P3 and P5, which were heterogenous as mentioned previously.

After 5 injections of 1-3 x 10^{12} PFU/ml. (total of 6 ml. injected), a high titered antiserum was obtained. When 10^{12} PFU of P1 or P6 was layered over an undilute or 1/10 dilution of antiserum, a visible ring of precipitation was formed at the interface. The precipitation, using undilute antigen and antibody was a diffuse one, extending away from the interface in either direction. The precipitate formed using dilute antiserum and antigen (10^{11} PFU)

gave a very sharp ring at the interface.

The results of neutralization experiments are given in Table VI. In these experiments, a 1/1000 dilution of the serum was used and 10^7 PFU of each phage. All experiments were carried out at 37 C. The data of Table VI is logarithimically plotted against time in Figure 2. As will be noted from Table VI, P1 is neutralized 3 logs in 15 minutes by anti-P1 serum, whereas P3 and P6 are not as sensitive to neutralization by heterologous serum, being neutralized 2 logs and 1 log, respectively, in 15 minutes.

When surviving phage are plotted logarithimically against time after neutralization, the neutralization rate of each phage was found to be quite constant, as seen by the linear curves, Figure 2.

From the data found in Table VI, it was possible to compute the serum K value for each phage. These K values are given in Table VII. The K values for P1 and P3 are 460 each, while the K value for P6 was only 152.

TABLE VI

ANTISERUM NEUTRALIZATION OF P1, P3, AND P6 WITH 1/1000 DILUTION OF P1 ANTISERUM

PHAGE	TIME MINUTES	PHAGE TITER	PER CENT NEUTRALIZATION
1	0	1.9×10^7	0
	5	1.5×10^6	92
	10	2.5×10^5	99
	15	4.5×10^4	99
3	0	4.4×10^7	0
	5	5.1×10^6	88
	10	2.1×10^6	95
	15	3.8×10^5	99
6	0	1.8×10^7	0
	5	8.0×10^6	56
	10	3.8×10^6	78
	15	1.4×10^6	92

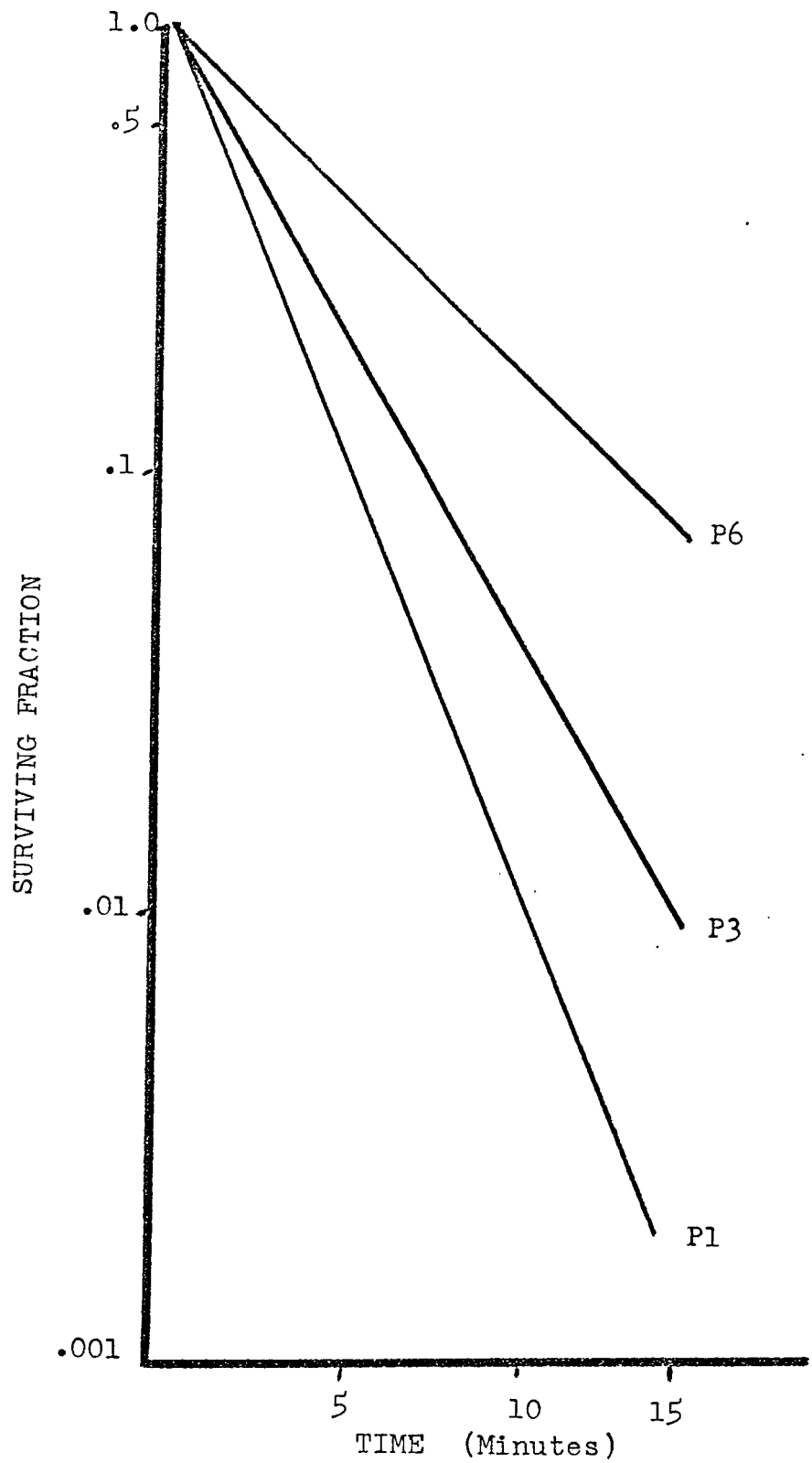

FIGURE 2

ANTISERUM NEUTRALIZATION OF P1, P3 AND P6

TABLE VII

ANTISERUM K VALUES FOR P1, P3 AND P6

PHAGE	K VALUE	TIME-MINUTES
1	460	5
3	460	5
6	152	10

Computed from the following formula:

$$K = 2.3 \ D/t \ \times \log \ p_o/p$$

Where:

D = Final dilution of antiserum

t = Time in minutes

Po = Phage assay at zero time

p = Phage assay at t minutes

Adsorption, Latent Period, Average and Single Burst Sizes:

For determing percentage of phage adsorption to the hosts in a given time period, the two methods described in materials and methods were equally efficient. The results of such an experiment, where both methods were employed, using P1, are given in Table VIII.

The multiplicity of infection as determined by viable cell counts of the host and plaque assay of virus input, was 0.58 phage per cell. After a 10 minute adsorption period, the free virus remaining after treating a sample of the adsorption mixture with chloroform, was 6 per cent of the original phage input. With the alternate method, the titer of the supernatant, after centrifugation of a sample of the adsorption mixture, was 2 per cent of the original virus input. As will be noted from Table VIII, some free virus was sedimented with the pellet of cells. But the two figures, 6 and 2 per cent, are in close agreement.

When the viable cell input, infective centers, and viable cell counts after infection are analyzed, quite a difference is apparent. The infective center count represents 53 per cent of the phage input, while 94 per cent of the phage was adsorbed. And yet

TABLE VIII

ADSORPTION AND INFECTIVE CENTERS OF P1

Viable cell input:	9.0×10^8/ml.
Virus input:	4.7×10^8/ml.
Free virus after 10 minute adsorption:	
Treating sample with chloroform:	2.9×10^7/ml.
Centrifugation: titration of supernatant:	1.2×10^7/ml.
Free virus in cell pellet:	8.6×10^6/ml.
Infective centers:	2.5×10^8/ml.
Viable cells after infection:	2.3×10^8/ml.
From the above data the following were calculated:	
Multiplicity of Infection:	0.58 phage/cell
Per cent adsorption:	
Chloroform treatment:	94 per cent
Centrifugation of cells:	98 per cent
Infective centers:	53 per cent of virus input

approximately 50 per cent of the original viable cells remain unaccounted for, they do not appear as viable cells after infection or infective centers.

On the basis of equal efficiency of the two methods for determining percentage adsorption, the chloroform method was chosen for all adsorption experiments, on the basis of its technical simplicity.

Table IX gives representative experiments for determining percentage adsorption, using P1, P3 and P6. As was mentioned above, and is further illustrated in Table IX, P1 characteristically has a high adsorption rate, but this percentage does not equal that of the infective centers. On the other hand, P3 and P6 yield infective center concentrations which approximate the percentage adsorption.

Since P6 does not plaque on Pm-5 cells, it was thought the reason might be failure of this phage to adsorb to these cells. In a parallel experiment, where the percentage adsorption of P6 on Pm-2 was compared to adsorption on Pm-5, surprisingly the percentage adsorption on Pm-5 and Pm-2 was 40 per cent after 10 minutes. This percentage further increased to 60 per cent after 20 minutes on both cells. However,

TABLE IX

ADSORPTION AND INFECTIVE CENTERS OF P1, P3 AND P6

PHAGE	PER CENT ADSORPTION	PER CENT INFECTIVE CENTERS
1	90	50
	91	75
	96 (92)*	40 (53)*
3	57	33
	40	36
	50 (49)*	28 (32)*
6	53	35
	58	42
	57 (56)*	43 (40)*

* Average of three experiments.

after 30 minutes the free phage in the Pm-2 cell tube had showed a burst at 30 minutes, while the free virus in the Pm-5 tube was 40 per cent of the original input, indicating adsorption had leveled off from the 20 minute interval.

The latent periods of P1, P3, and P6 were the same as determined by one step growth curves and turbidity changes of infected broth cultures. These results are given in Table X. When nutrient broth was the medium in which the one step growth experiment was conducted, the latent periods were approximately 24-26 minutes for each phage. However, if brain heart infusion broth or "C" medium was used, the latent period was reduced to 17-21 minutes for P1 and P6.

Figure 3 shows the results of determining the latent period of P1 by the one step growth method and turbidity changes of an infected broth culture of cells. The third curve represents turbidity changes of a P6 infected culture. The broth culture of P1 begins to show lysis at 22 minutes, while the rise in plaque titer begins to occur at 24 minutes. The broth culture of P6 indicates lysis to begin at 24 minutes.

TABLE X

LATENT PERIODS OF P1, P3 AND P6

PHAGE	MEDIUM	NUMBER OF EXPERIMENTS	METHOD	RANGE IN MINUTES
1	Nutrient Broth	5	One Step	24-26
	Nutrient Broth	2	Turbidity Change	22-26
	Brain Heart Infusion	2	One Step	17-21
	"C" Medium	1	One Step	17-19
3	Nutrient Broth	3	One Step	24-26
6	Nutrient Broth	2	One Step	24-26
	Brain Heart Infusion	2	One Step	17-21
	Nutrient Broth	1	Turbidity Change	24-26

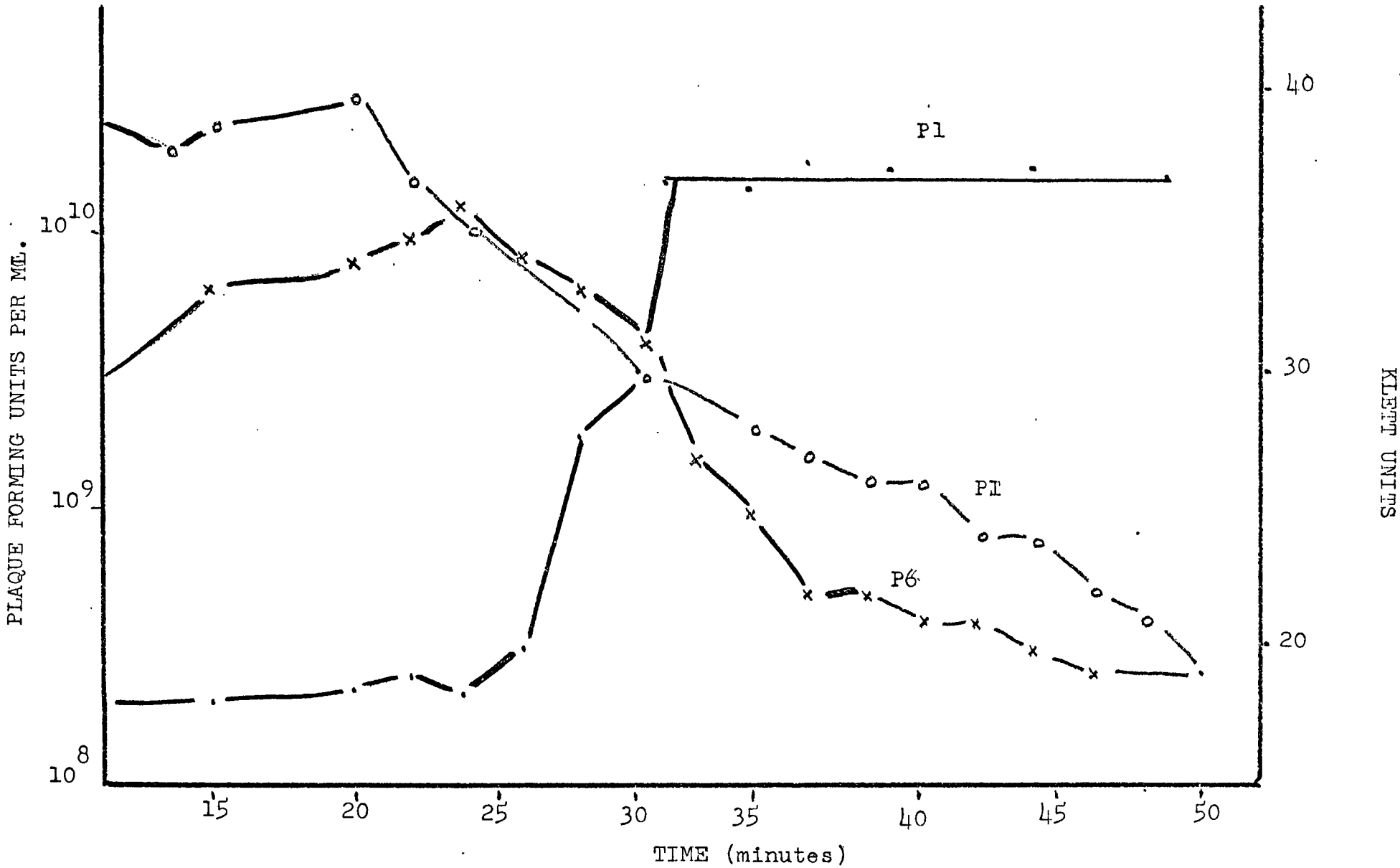

FIGURE 3

LATENT PERIOD OF P1 AND P6

When late log phase cells were used, the latent period for the phages was extended to 30-40 minutes.

The results of several average burst and single burst experiments for each phage are represented in Table XI. All three phages had burst sizes approximately the same. The range of burst sizes as determined by single burst experiments was quite narrow. P1 had a range of 31-96 phage per cell, P3, 12-91, and P6, 8-99 new progeny per cell.

Thermal Inactivation at 60 C:

Figures 4, 5, and 6 give the results of thermal inactivation of P1, P3 and P6 conducted in nutrient borth, pH 7.4, nutrient broth (pH unadjusted) and saline, respectively.

As shown in Figure 4, P6 is much more sensitive to inactivation at 60 C than are P3 and P6. Comparision of Figure 4 with Figure 5 (nutrient broth with unadjusted pH) demonstrates the effect of heat and ionic conditions on inactivation. In the latter, the phages are more thermal stable, but with combined heat and ionic conditions, they are less stable.

All three phages were readily inactivated when inactivation was carried out in saline, Figure 6.

TABLE XI

AVERAGE AND SINGLE BURSTS OF P1, P3 AND P6

PHAGE	METHOD	NUMBER OF EXPERIMENTS	RANGE OF AVERAGES	AVERAGE
1	Single Burst	4	55-70	63
	Average Burst	4	44-66	54
3	Single Burst	3	76-73	75
	Average Burst	2	53-59	56
6	Single Burst	3	31-78	58
	Average Burst	3	55-63	57

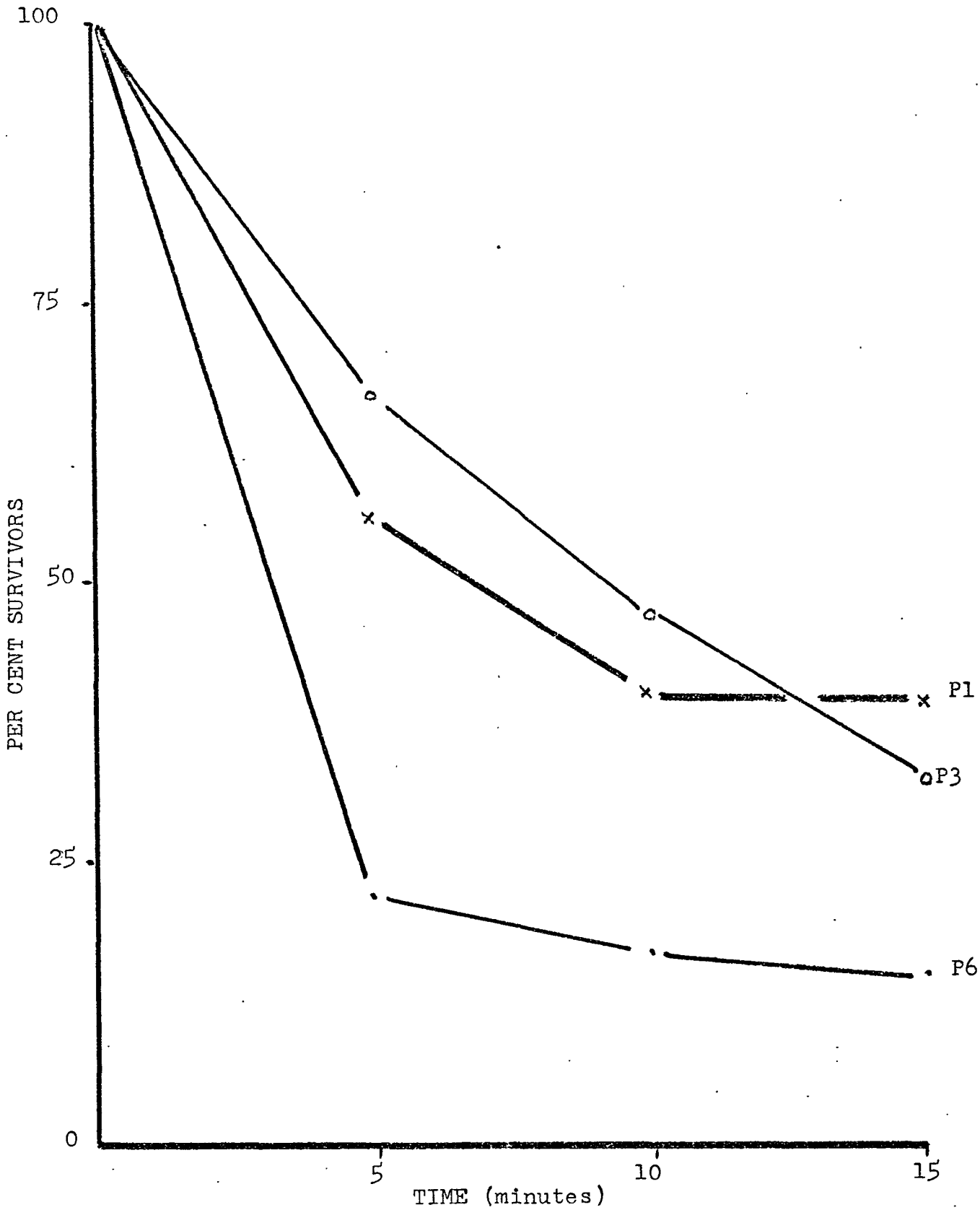

FIGURE 4

THERMAL INACTIVATION P1, P3 AND P6, 60 C, pH 7.4

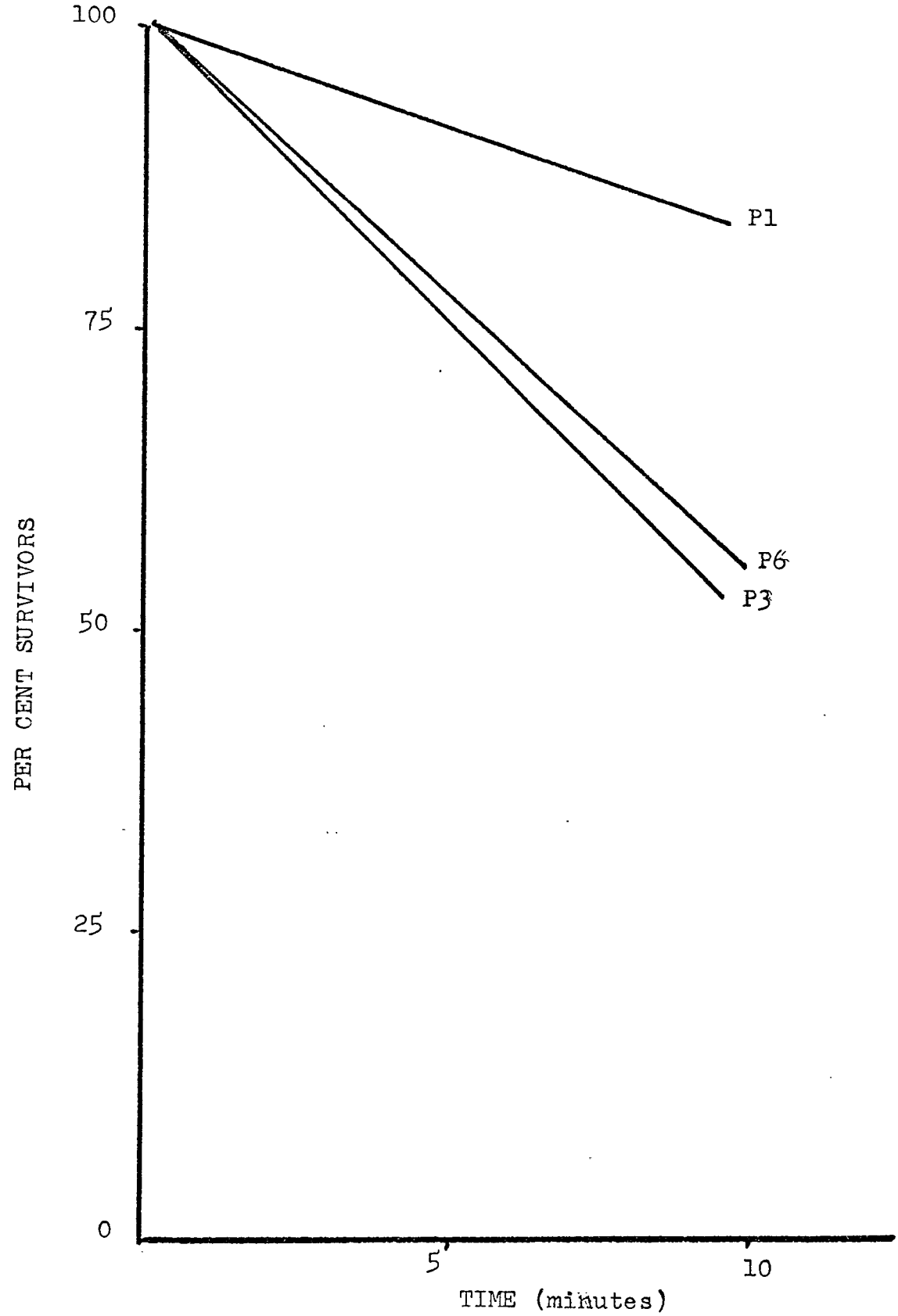

FIGURE 5

THERMAL INACTIVATION P1, P3 AND P6, 60 C

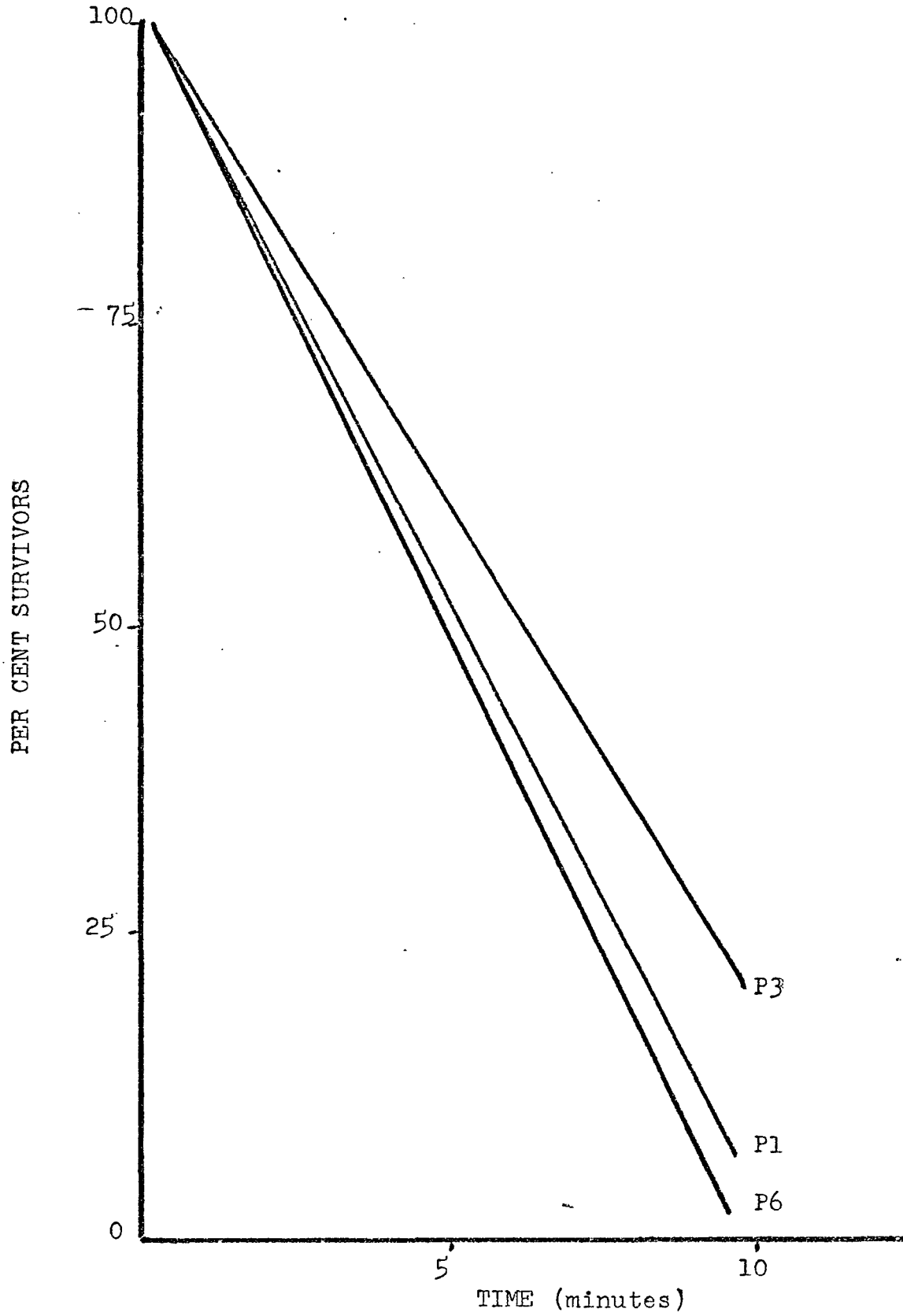

FIGURE 6

THERMAL INACTIVATION 60 C, SALINE

Effect of pH:

When 10^7 PFU of P1, P3, and P6 were inoculated into nutrient broth of pH ranges 5-10, and incubated for 1 hour at 37 C, no appreciable decrease in phage titer was noted at any of the above pH ranges. On this basis, it was concluded that the phages were stable over a wide pH range.

IV

DISCUSSION AND SUMMARY

From the detailed origin of P1, P3, P5 and P6 (Figure 1), it appears these four phages originated from a genetically unstable phage. The original lysate received (11-64) had supposedly originated from a single plaque. Upon receipt of this lysate, it was further plaque purified three times before stocks were prepared. Although the plaque sizes of this stock showed a wide range, this was probably attributable to the slow adsorption of the phage, a phenomenon observed by other workers (de Klerk, 1963). This stock became the object of investigation until 3-66. During this interval several new stocks were prepared.

Doermann (1953) experienced the same difficulty in obtaining genetically stable stocks of T4. If late plaques were picked (24 hours old) the stock would be genetically heterogenous. However, if young plaques were picked, the stock would be homogenous. The age of the host culture on which the picked plaques were passed also influenced the genetic stability. Furthermore, Freifelder (1966) has demonstrated heterogenous phage types within single plaques of T4 and T7.

From the data given in Tables I and II, it is possible to understand this mutation somewhat better. On the basis of host range, Table I, we may say P1 and P5 are restricted (R+) on Pm-5, while P3 is not restricted (R^-) and P6 is 100 per cent restricted (R++). The stocks of 11-64 (original lysate) show an R+ character, while Pal (3-66) is not restricted (R^-), when assayed on Pm-5.

Figure 7 shows a schematic representation of the origin of the phages based on this host range scheme. On this basis, the original lysate was composed mainly of R+ type phages. But, on plaque purification from this lysate, an R^- variant was picked, from which mutations arose giving 2 R+ plaque types, 1 R++ variant, and 1 R^- phage.

The complete restriction of P6 on Pm-5 is not due to failure of adsorption, for P6 adsorbs as well to Pm-5 as it does to its regular host, Pm-2.

The fate of P6 on Pm-5 may be determined by one of several alternatives. The phage adsorbs to the host and kills the cells, making productive infection impossible. This did not seem to be feasible, for control plates and plates inoculated with 10^{10} PFU of P6 showed the same amount of host growth in 4 hours.

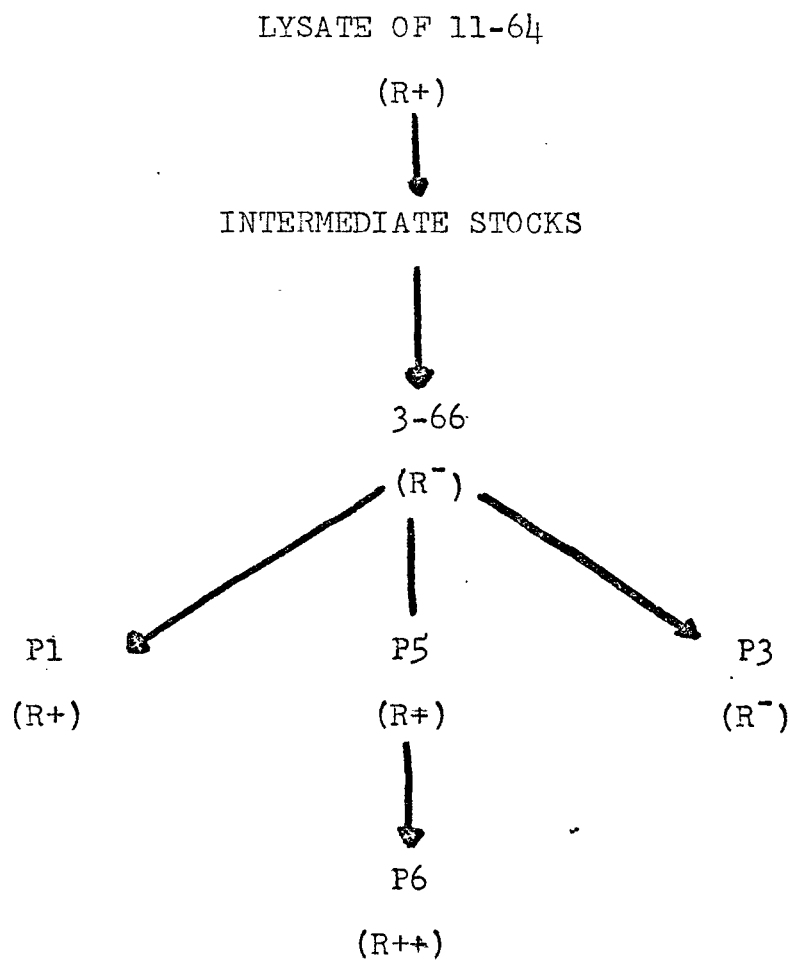

FIGURE 7

ORIGIN OF PHAGES BASED ON HOST RANGE

The possibility of lysogeny is unacceptable. P6 is not a temperate phage, as it characteristically forms quite clear plaques. However, if it did lysogenize Pm-5 cells, a small fraction of the cells would be lysed by a few phages reverting to the virulent form, thus giving rise to plaques (Dickinson, 1954).

Two alternatives remain: some step in the adsorption process is not being completed. It is possible this is the second step, the injection of nucleic acid. And finally, should the nucleic acid be injected, it may be degraded by the cell and excreted into the medium (Dussioux, 1962). More detailed experiments are needed before an accurate explanation can be proposed to explain this total restriction.

The plaque morphology of each phage (Table III) was quite distinct under ideal plaquing conditions, (materials and methods). The fact that P3 gives rise to turbid plaques on Pm-2 after the first few hours of incubation has also been described by Coetzee (1958) for many of the proteus phages he studied. However, when P3 is plaqued on Pm-5, the plaques are quite clear.

All of the phages are capable of producing halos. On the other hand, P1 and P6 generally produced halos more frequently than was observed

for P3 or P5. P3, when plaqued on Pm-5, rarily ever produced halos around its plaques.

Halo formation is generally conceded to be due to a diffusable enzyme released by the infected cells, and which is controlled by the phage genotype (Park, 1956). Park, studying 3 variants of klebsiella phages, found the halos formed after different periods of incubation for each phage. In a subsequent paper, Adams and Park (1956) concluded that the enzyme yield was under control of both phage and host.

These points were well demonstrated with P1, P3 and P5. Pm-2 cells readily produce halos with all the phages, whereas P3 on Pm-5 does not. This could be interpreted to mean that modifications in this host reduced halo forming ability, although the phage genotype still possessed this character.

The early observations that the inclusion of sodium chloride to basal and soft agar layers reduces plaque titers may be explained by two facts. These experiments were conducted with Pal (parent stock), which is a slow adsorbing phage (30-40 per cent of input in 10 minutes). Also, sodium chloride, present in a semi-solid medium will induce some Proteus strains to swarm (Annear, 1964; Schnierson, 1961; Kopper, 1962). Even those which

are considered to be of the phase B type, non-swarmers (Belyavin, 1951), will sometimes show an atypical swarming when spotted on medium containing sodium chloride. Also, the addition of sodium chloride to a broth culture of cells increased its growth rate, as compared to a culture, sodium chloride deficient. On this basis, it was concluded that the host cells plated on agar containing sodium chloride would be induced to stationary phase of growth at a much faster rate.

The slow adsorbing phage would not have sufficient time to initiate infection productively, for a majority of the cells would already have reached stationary phase. Once cells have attained stationary phase virus production in the plaque ceases (Anderson, 1948). Whereas, when the plating agar is deficient in sodium chloride, cell growth is slower, allowing for slow adsorbing phage to produce plaques before the cells attain stationary phase.

Of the bacterial strains tested for host range, (Table V), only members of the Proteus group were susceptible to the three phages. The sensitive Proteus strains were all P. mirabilis, as determined by biochemical tests. Coetzee (1963) reported the ability of some proteus phages to productively

infect strains of E. coli, Shigella, Salmonella, Serratia and Providence. Dobek (1965) reports no attempts to test proteus temperate phages against other genera of bacteria. Other specific enterobactericeae phages have been reported to lyse members of other genera (Wasserman and Seligmann, 1953; Adams, 1952; Adams and Wade, 1954).

The failure to show extra-genera infection may be accounted for by the small number of these organisms tested and not a specific specificity of the phage for Proteus. Had a large number of strains been tested, it is possible some strains might have been susceptible.

On the basis of serum neutralization, P1, P3 and P6 are antigenically closely related. P1 and P3 are more closely related than P6 to P1 or P3. When 10^7 PFU of P1 and P3 were incubated with a 1/1000 dilution of anti-P1 serum for 15 minutes, P1 was reduced 3 logs, P3 2 logs, while the same concentration of P6 was reduced only 1 log.

The antigenic similarity of P1 and P3 is further substantiated by anti-P1 serum, having K values identical for both phages, 460. Whereas, the serum K value for P6 was only 152, demonstrating antigenic similarity but not as markedly.

The antiserum, besides possessing a neutralizing antibody, also has an agglutinating type antibody, as was demonstrated by precipitate formation with high phage concentrations. Whether this agglutinating type antibody is associated with the head (Lanni and Lanni, 1953), as in most phage systems, has yet to be determined.

The latent period, adsorpiton and burst sizes of the phages were typical for phages active against members of Enterobacteriaceae.

The failure of P1 infective centers to equal the percentage of adsorbed phage may be interpreted to mean that abortive infection is occurring. Abortive infection has been observed in bacillus phage (Brodetsky and Romig, 1965) and coli phages (Gross, 1954). Abortive infection is thought to occur when a proportion of a phage population is defective and not capable of causing an infection. P3 and P6, on the other hand, do not show abortive infection to any appreciable degree.

The adsorption rates of P3 and P6 are lower, compared with P1 rates. Whether adsorption could be enhanced by the addition of calcium or tryptophan (Anderson, 1948; Brenner et al, 1962; Lark and Adams, 1953) was not determined.

Certainly the absence of sodium chloride in the medium cannot account for the low adsorption, for the early work with Pal was conducted in medium supplimented with 0.5 per cent sodium chloride.

The phages of Coetzee (1958) had no requirement for cations, as was demonstrated by the ability of adsorption to progress normally in the presence of the chelating agent, sodium citrate. Neither Coetzee (1958) nor Dobek (1965) give any information as to the adsorption of their proteus phages.

The latent period (Table X) for P1, P3 and P6 were the same, 24-26 minutes, when nutrient broth was the growth medium. This latent period falls within the generation time of the host, compared with 10 of Coetzee's phages with latent periods longer than the generation time of the host. All of Dobek's phages had latent periods longer than those of the host.

The latent period was found to be influenced by the medium in which the infection occurred, Table X. When brain heart infusion broth or "C" medium was used, the latent period for P1 and P6 was reduced to 17-21 minutes. Phage P3 was not tested in this media. This may be explained by the faster growth rate of the host cells.

The growth rate of Pm-2 was determined in brain heart infusion and nutrient broths. The rate in the former was found to be faster than in nutrient broth. Delbrück (1945) observed the latent period of coli phages to be reduced, when rapidly dividing cells were employed, compared with a 24 hour aerated culture of cells.

Very little variation was observed among the three phages with regard to average or single burst sizes (Table XI). The burst sizes are within the low range for bacteriophages, as some phages have larger burst sizes (Adams, 1951). However, Coetzee (1958) described two phages of Proteus with burst sizes of 50 and 70. The average burst of P1 did not show a decrease in size, when the experiment was conducted in "C" medium.

The deletion of sodium chloride had no effect on the burst size. Burst size experiments performed in brain heart infusion broth (containing 0.5 per cent sodium chloride) demonstrated no increase in the average burst size. Brodetsky and Romig (1965) reported that the deletion of calcium from bacillus phage growth medium reduced the average burst size.

This observation that sodium chloride does not affect the average burst size would further support the assumption that additional adsorption co-factors are not required by P1, P3 and P6.

Phages P1, P3, and P6 were relatively heat stable. Without adjusting the ionic environment, none of the phages were inactivated more than 50 per cent, or half a log, in 10 minutes. P1 was most stable in this condition, only being inactivated about 15 per cent of the original input.

However, when there was a combined effect of heat and ionic conditions, inactivation proceeded more rapidly. Under these conditions, P6 was very sensitive to heat, 1 log being inactivated in 15 minutes. All of the phages were thermal sensitive when the inactivating medium was saline (Figure 6).

From Figure 4, the inactivation curves are linear for the first 5 minutes, especially for P6. Inactivation of P3 continues at a nearly approximately constant rate for 15 minutes, while P1 plateaus after 10 minutes. The continued thermal inactivation of P6 beyond 5 minutes is insignificant. This suggests the existence of heat resistant phages within the population of P1 and P6.

Coetzee (1958) interprets the work of Alexander and McCoy (1956) to imply that the combined effect of heat and ionic conditions will give first order inactivation curves. Coetzee's inactivation curves for 14 proteus phages inactivated at 60 C, pH 7.4, are quite linear. However, examination of the work of Alexander and McCoy reveals first order inactivation at pH 7.0 and not pH 9.0. The inactivation curves at pH 9 were not linear and showed the existence of heat resistant phages.

Summary.

Four bacteriophages of Proteus mirabilis were isolated. Three of these four, P1, P3 and P6, originated by mutations from the parent strain, Pal. Phages P1, P3 and P6 were purified, and the difference in host range (especially P6 om Pm-5) attested to their plaque purity.

The phages may be readily distinguished from one another by plaque morphology, provided ideal plaquing conditions are used.

High titered stocks of the phages were easily obtained and showed a high degree of stability, when stored at 4 C for long periods.

Phages P1, P3 and P6 had similar latent periods and burst sizes. The latent period could be reduced by use of enriched or minimal media.

The phages are related antigenically, as was demonstrated by serological neutralization, although P6 is not as similar antigenically as are P1 and P3.

The three phages, differing antigenically, also showed slight distinction in thermal stability. All variants were stable over a pH range of 5-10.

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